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Ethics approval and consent to participate	This study was conducted according to the guidelines of the experimental procedures and approved by the Animal Ethics Committee of Shangqiu Normal University (2020-1012).

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Abstract

Two experiments were conducted in this research. Experiment 1 investigated the spatial expression characteristics of calcium (Ca) and phosphorus (P) transporters in the duodenum, jejunum, and ileum of 21-day-old broilers provided with adequate nutrient feed. Experiment 2 evaluated the effects of dietary vitamin D_3 (VD_3) concentration (0, 125, 250, 500, 1000, and 2000 IU/kg) on growth performance, bone development, and gene expression levels of intestinal Ca and P transporters in 1–21-day-old broilers provided with the negative control diet without supplemental VD_3. Results in experiment 1 showed that the mRNA levels of calcium-binding protein 28-kDa (CaBP-D28k), sodium-calcium exchanger 1 (NCX1), plasma membrane calcium ATPase 1b (PMCA1b), and IIb sodium-phosphate cotransporter (NaPi-IIb) were the highest in the broiler duodenum. By contrast, the mRNA levels of inorganic phosphate transporter 1 (PiT-1) and 2 (PiT-2) were the highest in the ileum. Results in experiment 2 showed that adding 125 IU/kg VD_3 increased body weight gain (BWG), feed intake (FI), bone weight, and percentage and weight of Ca and P in the tibia and femur of 1–21-day-old broilers compared with the negative control diet ($p < 0.05$). The rise in dietary VD_3 levels from 125 to 1000 IU/kg further increased the BWG, FI, and weights of the bone, ash, Ca, and P ($p < 0.05$). No difference in growth rate and leg bone quality was noted in the broilers provided with 1000 and 2000 IU/kg VD_3 ($p > 0.05$). Supplementation with 125–2000 IU/kg VD_3 increased the mRNA abundances of intestinal Ca and P transporters to varying degrees. The mRNA level of CaBP-D28k increased by 536, 1161, and 28 folds in the duodenum, jejunum, and ileum, respectively, after adding 1000 IU/kg VD_3. The mRNA levels of other Ca and P transporters (PMCA1b, NCX1, NaPi-IIb, PiT-1, and PiT-2) increased by 0.57–1.74 folds by adding 1000–2000 IU/kg VD_3. These data suggest that intestinal Ca and P transporters are mainly expressed in the duodenum of broilers. Moreover, the addition of VD_3 stimulates the two mineral transporter transcription in broiler intestines.

Keywords: Vitamin D_3, Broiler chicken, CaBP-D28k, PMCA1b, NaPi-IIb, PiT-1

INTRODUCTION

Vitamin D_3 (cholecalciferol, VD_3) is a nutrient for animal growth, health, and bone development. Its deficiency damages bone quality, while the addition of VD_3 improves growth performance and blood calcium (Ca) and phosphorus (P) homeostasis in broiler chickens [1]. The VD_3 requirement of 1–21-day-old broiler chickens estimated by NRC (1994) is 200 IU/kg [2]. The recommended dietary VD_3 level for broilers in China is 1000 IU/kg [3]. Research has shown that 1000–2000 IU/kg VD_3 is needed to support the growth performance and leg bone mineralization of broilers [4]. Thus, the requirement of modern broilers for VD_3 may be higher than that recommended by NRC (1994) [2]. The primary function of VD_3 is to regulate the absorption of Ca and P in animal intestines. Ca and P are the two mineral elements that are added most in feed and needed most in animal body. Research in mammals has shown that the response of intestinal Ca and P absorption of rats to vitamin D correlates with the gene expression of Ca and P transporters [5,6].

Calcium is absorbed through active transcellular transport and passive transport in animal enterocytes [7]. The transcellular transport of Ca involves three procedures: Ca enters intestinal cells, moves to the basolateral membrane, and extrudes into the blood [7]. The first procedure is intestinal Ca transport, which relies on transient receptor potential channels (TRPV6 and TRPV5) [7]. The second procedure is the movement of Ca in the cytoplasm, which depends on two Ca-binding proteins (i.e., CaBP-D28k and CaBP-D9k) [8]. The final procedure is the extrusion of Ca, which is performed through sodium-calcium exchanger 1 (NCX1) and plasma membrane calcium ATPase 1b (PMCA1b) [8]. CaBP-D28k and CaBP-D9k exist in the intestines of poultry and mammals, respectively. Intestinal TRPV6, CaBP-D28k, and PMCA1b have been cloned in laying hens [9,10]. There is no report of TRPV6 in broilers [11].

Phosphate transport from the brush border membrane of intestinal enterocytes into the cytoplasm is an active absorption process. Three P transporters, namely, IIb sodium-phosphate cotransporter (NaPi-IIb), inorganic P transporter 1 (PiT-1), and inorganic P transporter 2 (PiT-2), are involved in active P absorption in animal intestines [8]. The mRNA abundances of NaPi-IIb, PiT-1, and PiT-2 have been detected in the apical membrane of rat intestinal cells [12,13]. NaPi-IIb undertakes the majority of P absorption in mouse intestines, whereas, PiT-1 and PiT-2 assume a minor function in total P uptake [14]. These three P transporters have been cloned in broiler intestines [15].

The active absorption of Ca and P in the intestines of broilers relies on the existence of Ca and P

transporters. The capacity of P absorption and gene expression abundance of NaPi-IIb in the duodenum are higher than those in the jejunum and ileum of broiler chicks [16]. The difference in protein abundance of duodenal, jejunal, and ileal CaBP-D28k has been noted in laying hens [17]. By contrast, the spatial expression characteristics of CaBP-D28k, PMCA1b, NCX1, PiT-1, and PiT-2 in the three intestinal segments of broilers have not been reported.

The injection of 1,25-dihydroxycholecalciferol ($1,25\text{-}(OH)_2\text{-}D_3$) increases blood Ca and P concentrations and improves Ca and P absorption by upregulating the protein expression of CaBP-D9k and the mRNA abundance of NaPi-IIb in rat intestines [6,18]. Meanwhile, the relationship between dietary VD_3 dosage and the expression of Ca and P transporter genes in broiler intestines has not been clarified.

Thus, two experiments were conducted in this research. First, the differences in gene expression levels of Ca and P transporters in the duodenum, jejunum, and ileum were explored. Second, the response of the mRNA abundances of intestinal Ca and P transporters to dietary VD_3 concentrations in broiler chickens was examined.

MATERIALS AND METHODS

The animal experiment procedures in this research were implemented in accordance with the guidelines of the Animal Ethics Committee of Shangqiu Normal University (2020-1012).

Animals, Diets, and Management

Experiment 1

Experiment 1 investigated the spatial expression characteristics of Ca and P transporters in the duodenum, jejunum, and ileum of 21-day-old broilers. One-day-old Arbor Acres broiler chickens (70, male) were grouped into five repetitions of 14 broilers per repetition. Broilers were provided with an adequate nutrient diet (Table 1)[3]. On day 21, total 10 broilers (2 chicks per repetition) were randomly selected and euthanized by cervical dislocation. The duodenum (after the gizzard), jejunum (proximal to Meckel's diverticulum), and ileum (proximal to the ileocecal junction) were isolated. Intestinal mucosal samples from the three segments were scraped with a glass slide, collected in a centrifuge tube, immediately put into liquid nitrogen, and stored in –80 ℃ refrigerator.

Experiment 2

Experiment 2 evaluated the effects of dietary vitamin D_3 concentration on growth performance, bone development, and gene expression levels of intestinal Ca and P transporters in 1–21-day-old broilers. One-day-old Arbor Acres broiler chickens (420, male) were randomly grouped into six treatments. Each treatment contained five repetitions with 14 broilers per repetition. Dietary VD_3 levels were 0, 125, 250, 500, 1000, and 2000 IU/kg. The negative control diet contained 10.0 g/kg Ca and 4.5 g/kg non-phytate P and did not contain supplemental VD_3 (Table 1) [2].

The VD_3 crystal was supplied by Tianhecheng Biological Technology Co., Ltd. (Jiaxing, Zhejiang, China). The VD_3 solution was prepared in accordance with previous research [19]. After weighing, the crystalline VD_3 was dissolved in ethanol. Propylene glycol was used to dilute the VD_3 solution. The concentration of the VD_3 solution was 45.3 μg/mL (1812 IU/mL) as measured by high-performance liquid chromatography. The VD_3 solution was supplemented in broiler chicken feed with a pipette.

The broilers were housed in pens (width 140 cm, depth 70 cm, and height 35 cm). The broilers were fed with mash feed *ad libitum* and provided with 23 h of lighting on days 1–3 and 18 h of lighting on days 4–21. The temperature of the room was kept at 32 ℃ on days 1–3, 30 ℃ on days 4–7, and 28 ℃ on days 8–21.

Sample collection

The broilers were weighed by pen at 1 and 21 days of age. The feed intake (FI) per day of the broilers was weighed. The total FI was calculated from 1 to 21 days of age. Body weight gain (BWG) was the difference between the body weight (BW) of the broilers at 21 and 1 day of age. Feed conversion ratio (FCR) was the ratio of the FI to the BWG. The dead broilers during the experiment were weighed and recorded. On day 21, 10 broilers per treatment (2 broilers per repetition) were chosen and euthanized by cervical dislocation. The small intestine was exposed after the broilers were euthanized. Intestinal mucosal samples from the duodenum (after the gizzard), jejunum (proximal to Meckel's diverticulum), and ileum (proximal to the ileocecal junction) were scraped with a glass slide and collected in a centrifuge tube. Then, the mucosal samples were immediately put into liquid nitrogen and stored in –80 ℃ refrigerator.

The two leg bones (tibia and femur) were removed, stored, and pre-treated [20]. Bone weight and length were measured after the bone was dried at 105 ℃ for 24 h. The ash weights of the tibia and femur

were measured after the samples were ashing in a muffle furnace (Selecta, Barcelona, Spain) at 650 ℃ for 48 h. Bone ash, Ca, and P percentages are the ratios of their weight to the bone weight. The Ca contents in the diet and leg bones were analyzed by the AOAC method [21]. Total P contents were measured using the photometric method [22].

RNA extraction and real-time PCR

Total RNA extraction from the intestinal mucosal samples was implemented by RNAiso Plus Kit on the basis of the recommendation of the manufacturer (Takara Biotechnology Co., Ltd., Dalian, Liaoning, China). RNA concentration was analyzed by spectrophotometry.

The cDNA was reverse-transcribed from RNA using PrimeScript™ RT Reagent Kit based on the instructions of the manufacturer (Takara Biotechnology Co., Ltd., Dalian, Liaoning, China).

Real-time PCR analysis was conducted for the Ca and P transporter gene expression (i.e., CaBP-D28k, PMCA1b, NCX1, NaPi-IIb, PiT-1, and PiT-2). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. PCR primers (Table 2) were synthesized in Shanghai Sangon Biotech Co., Ltd. Gene expression was performed using the TB Green™ Premix EX Taq™ II Kit (Takara Biotechnology Co., Ltd., Dalian, Liaoning, China) and the Roche Lightcycler® 480 Real-time PCR System (Risch, Switzerland). The PCR products were verified by melting curve analysis based on the following conditions: 95 ℃ for 60 s, 40 cycles of 95 ℃ for 10 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. The mRNA abundances of the target genes relative to that of the GAPDH gene were calculated by the $2^{-\Delta\Delta Ct}$ method [23]. The average ΔCt value from the negative control diet was used as the calibrator of each gene.

Statistical analysis

Repetition pens were used as experimental units. Data analysis was conducted by one-way ANOVA in SAS 9.0 software [24]. Orthogonal polynomials contrast analysis was used to evaluate the linear and quadratic effects of dietary VD_3 concentration on growth performance, bone development, and gene expression levels of intestinal Ca and P transporters. Figures were produced with GraphPad Prism software. Means were compared by Tukey's test. The significance level was set at $P < 0.05$.

RESULTS

Experiment 1

The highest mRNA abundances of the three Ca transporters (CaBP-D28k, PMCA1b, and NCX1) were expressed in the broiler duodenum (Figures 1a, 1b, and 1c). For P transporters, the mRNA abundance of NaPi-IIb in the duodenum was higher than those in the two other intestinal segments ($p < 0.05$, Figure 1d). By contrast, the mRNA abundances of PiT-1 and PiT-2 were the lowest in the duodenum and highest in the ileum (Figure 1e and 1f).

Experiment 2

Growth performance

The addition of VD_3 increased the growth rate of 1–21-day-old broilers (Table 3). The broilers provided with 125 IU/kg VD_3 had higher BW, BWG, and FI, and lower FCR than those supplied with the negative control diet ($p < 0.05$). Increasing the VD_3 level from 125 to 1000 IU/kg enhanced the BWG and FI ($p < 0.05$). No difference was detected in the BWG, FI, and FCR of the broilers provided with 1000 and 2000 IU/kg VD_3 ($p > 0.05$). Dietary VD_3 levels did not affect the mortality of broilers ($p > 0.05$).

Bone development

Dietary VD_3 improved leg bone (tibia and femur) quality in 1–21-day-old broilers (Tables 4 and 5). The addition of 125 IU/kg VD_3 increased the weight, length, and percentage and weight of Ca and P in the two leg bones ($p < 0.05$). The rise in dietary VD_3 levels from 125 to 1000 IU/kg increased the weights of the ash, Ca, and P ($p < 0.05$), but it did not influence their contents ($p > 0.05$). No difference in the weight, length, and percentage and weight of ash, Ca, and P of the tibia and femur was noted in the broilers provided with 1000 and 2000 IU/kg VD_3 ($p > 0.05$).

Gene expression levels of intestinal Ca and P transporters

The primary function of CaBP-D28k is to transfer Ca in intestinal cells. Compared with the negative control feed, supplementation with 125 IU/kg VD_3 increased the mRNA abundance of intestinal CaBP-D28k in broilers ($p < 0.05$, Figures 2a, 3a, and 4a). Increasing the VD_3 level from 125 to 1000 IU/kg enhanced the mRNA abundances of jejunal and ileal CaBP-D28k ($p < 0.05$). The mRNA levels of duodenal, jejunal, and ileal CaBP-D28k increased by 536, 1161, and 28 folds, respectively, after adding

1000 IU/kg VD_3. No differences in the mRNA abundances of jenunal and ileal CaBP-D28k were detected between the broilers fed with 1000 and 2000 IU/kg VD_3 ($p > 0.05$).

PMCA1b is responsible for Ca extrusion. Dietary VD_3 levels upregulated the mRNA abundances of duodenal and jejunal PMCA1b ($p < 0.05$, Figures 2b and 3b). The mRNA abundances of PMCA1b in the duodenum of broilers supplied with 250–1000 IU/kg VD_3 and that in the jejunum of broilers supplied with 500 IU/kg VD_3 were higher than those in broilers provided with the negative control feed ($p < 0.05$). Dietary VD_3 did not influence the mRNA abundance of ileal PMCA1b ($p > 0.05$, Figure 4b).

NCX1 is a sodium and Ca exchanger. The broilers fed with 1000 IU/kg VD_3 had higher mRNA abundances of duodenal and jejunal NCX1 than those supplied with the negative control feed ($p < 0.05$, Figures 2c and 3c). Dietary VD_3 levels did not affect the mRNA abundance of ileal NCX1 ($p > 0.05$, Figure 4c).

NaPi-IIb is the main P transporter. Dietary VD_3 enhanced the mRNA abundances of intestinal NaPi-IIb ($p < 0.05$, Figure 2d, 3d, and 4d). The mRNA abundance of NaPi-IIb was the highest in the duodenum of the broilers fed with 250 IU/kg VD_3, in the jejunum of the broilers provided with 125 IU/kg VD_3, and in the ileum of the broilers supplied with 500 IU/kg VD_3 ($p < 0.05$).

PiT-1 is inorganic phosphate transporter 1. VD_3 regulated the mRNA abundances of PiT-1 in the duodenum and jejunum ($p < 0.05$, Figures 2e and 3e). The mRNA abundance of duodenal PiT-1 was enhanced after the addition of 250–1000 IU/kg VD_3 ($p < 0.05$), and that of jejunal PiT-1 was increased by 1000 IU/kg VD_3 ($p < 0.05$). On the contrary, the mRNA abundance of ileal PiT-1 was not affected by dietary VD_3 ($p > 0.05$, Figure 4e).

The function of PiT-2 is similar to that of PiT-1. The mRNA abundances of duodenal and ileal PiT-2 were enhanced by VD_3 ($p < 0.05$, Figures 2f and 4f). The mRNA abundance of PiT-2 was higher in the duodenum of the broilers fed with 250–500 IU/kg VD_3 and in the ileum of the broilers supplied with 1000–2000 IU/kg VD_3 than those of the broilers provided with the negative control feed ($p < 0.05$). The mRNA abundance of jejunal PiT-2 was not affected by VD_3 ($p > 0.05$, Figure 3f).

DISCUSSION

Experiment 1

The duodenum is the first segment of the small intestine, followed by the jejunum and ileum. The mRNA and protein abundances of duodenal CaBP-D28k and PMCA1b are higher than those in the two

other segments in laying hens [10,17,25]. Our results were consistent with those of previous reports. The highest mRNA abundances of CaBP-D28k, PMCA1b, and NCX1 were noted in the duodenum of broilers in this research. Thus, the Ca transporters are mainly expressed in poultry duodenum. These data suggest that the capacity of active Ca absorption declines from the duodenum to ileum of poultry.

NaPi-IIb is the primary P transporter in mouse intestines [14]. The mRNA abundances of jejunal and ileal NaPi-IIb are lower than that of duodenal NaPi-IIb in broilers [16,26]. Similar results were observed in this research, and the highest mRNA abundance of NaPi-IIb existed in the duodenum. Thus, the ability of NaPi-IIb to transport P in the duodenum is higher than that in the jejunum and ileum of poultry. PiT-1 and PiT-2 play a smaller role in intestinal P absorption than NaPi-IIb [14]. The mRNA abundance of duodenal PiT-1 is lower than those of jejunal and ileal PiT-1 in laying hens [10]. The present research showed that the highest mRNA abundances of PiT-1 and PiT-2 were observed in the ileum of broilers. PiT-1 and PiT-2 may promote the P absorption in the distal segment of the small intestine of poultry.

Experiment 2

Growth performance

Dietary VD_3 insufficiency decreases the growth rate of poultry [19]. In this research, the lowest BWG and FI were detected in the broilers provided with the negative control feed. Adding 125 IU/kg VD_3 increased the FI and decreased the FCR of broilers. The BWG was increased with the addition of VD_3 when more nutrients were retained in the body of broilers. Increasing VD_3 concentration from 125 to 1000 IU/kg significantly elevated the BWG. The recommended VD_3 dosage by NRC (1994) is 200 IU/kg [2], which can not meet the requirement of broilers for growth rate. A further increase in VD_3 level from 1000 to 2000 IU/kg did not improve broiler performance in this research. Similar results have been reported [27], in which increasing dietary VD_3 level from 1000 to 7000 IU/kg does not influence the BW of 1–38-day-old broilers. Thus, it's necessary to maintain the VD_3 levels of broilers at 1000–2000 IU/kg.

Bone development

Ingested Ca and P cannot be effectively absorbed in the blood and deposited in bones of broilers when dietary VD_3 is deficient [1]. The lowest Ca and P weights of the leg bones were noted in the broilers provided with the negative control feed in this research. The addition of 125 IU/kg VD_3 increased the leg

bone Ca and P contents. Ash includes Ca, P, and other minerals. The ash percentage and weight were elevated by the addition of VD_3. Our results were in accordance with those reported by previous research [4]. The leg weight and length enhanced with the increase in bone mineral contents after adding 125–1000 IU/kg VD_3. Dietary 200 IU/kg VD_3 given by NRC (1994) [2] can not meet the needs of bone development of broilers. No difference was noted in the bone quality of broilers provided with 1000 and 2000 IU/kg VD_3 in this research. Thus, in order to support the leg bone growth and mineralization, 1000–2000 IU/kg VD_3 should be added to the broiler diets.

Gene expression levels of intestinal Ca and P transporters

CaBP-D28k and CaBP-D9k are observed in poultry and mammal enterocytes, respectively. The present research showed that supplementation with 125–2000 IU/kg VD_3 increased the mRNA abundance of CaBP-D28k in the three intestinal segments of broilers to varying degrees, especially in the jejunum. 1,25-$(OH)_2$-D_3, the final active form of VD_3, increases mRNA abundance of intestinal CaBP-D28k in laying hens [28]. Thus, VD_3 and 1,25-$(OH)_2$-D_3 stimulated *CaBP-D28k* gene transcription in the intestinal cells of poultry and contributed to the increase in leg bone Ca content. In addition, research in mammals has shown the positive effect of 1,25-$(OH)_2$-D_3 on the gene expression of intestinal CaBP-D9k [5].

PMCA1b is expressed in the basolateral membrane. The addition of 1,25-$(OH)_2$-D_3 enhances the mRNA abundance of duodenal PMCA1b in mice [29]. Similar results were noted in this research. The mRNA abundance of PMCA1b in the duodenum of broilers increased after adding 250–1000 IU/kg VD_3. Thus, optimal levels of VD_3 upregulated *PMCA1b* gene expression and promoted Ca extrusion from intestinal cells into the blood. Notably, the addition of 2000 IU/kg VD_3 insignificantly affected intestinal PMCA1b mRNA level compared with the negative control diet.

Similar to PMCA1b, NCX1 also exists in the basolateral membrane. Dietary VD_3 insufficiency leads to a decrease in the mRNA abundance of NCX1 in chick duodenum, which is increased after the injection of 1,25-$(OH)_2$-D_3 [30]. This research showed that adding 1000 IU/kg VD_3 enhanced the mRNA abundance of duodenal and jejunal NCX1 by 0.67–1.74 folds. These data suggest that VD_3 promoted the exchange of Ca and Na in intestinal cells and the blood.

NaPi-IIb, the major P transporter, is found in the apical membrane of rat intestinal cells [12,13]. This research showed that the mRNA abundance of NaPi-IIb in the three intestinal segments was

increased by dietary VD_3 to varying degrees. The effects of the VD_3 on NaPi-IIb gene expression have been observed in broilers [31], in which a high VD_3 dosage upregulates the mRNA abundance of intestinal NaPi-IIb compared with a low VD_3 level. The addition of VD_3 enhances the protein expression level of jejunal NaPi-IIb in broilers fed with P-insufficient feed [15]. Thus, VD_3 stimulated NaPi-IIb gene expression and the active P absorption in broiler intestines.

PiT-1 is expressed in the apical membrane of rat enterocytes [13]. Compared with NaPi-IIb, PiT-1 plays a minor role in phosphate transport into the intestinal epithelial cells of mice [14]. Supplementation of 250–1000 IU/kg VD_3 increased the mRNA abundance of duodenal PiT-1 in this research. The addition of VD_3 also enhanced the protein abundance of jejunal PiT-1 in broilers provided with P-inadequate diets [15]. These data reveal the positive effects of VD_3 on intestinal *PiT-1* gene transcription in broilers.

The gene expression of PiT-2 has been observed in the apical membrane of rodent intestinal cells [12,32]. PiT-2 involves P absorption in mouse intestines upon dietary P restriction [32]. This research showed that the addition of VD_3 increased the mRNA abundances of duodenal and ileal PiT-2 in broilers. Thus, VD_3 upregulated *PiT-2* gene expression and promoted P transport in the apical membrane of broiler intestinal cells.

In conclusion, the highest mRNA abundances of CaBP-D28k, PMCA1b, NCX1, and NaPi-IIb were detected in the duodenum of broilers. On the contrary, the mRNA abundances of PiT-1 and PiT-2 in the duodenum were lower than those in the two other intestinal segments. Adding 125–2000 IU/kg VD_3 improved growth performance and leg bone quality of 1–21-day-old broilers. The mRNA level of CaBP-D28k increased by 536, 1161, and 28 folds in the duodenum, jejunum, and ileum, respectively, after adding 1000 IU/kg VD_3. By contrast, the mRNA abundances of other Ca and P transporters (PMCA1b, NCX1, NaPi-IIb, PiT-1, and PiT-2) increased by 0.57–1.74 folds by supplementation with 1000–2000 IU/kg VD_3.

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Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

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This study was conducted according to the guidelines of the experimental procedures and approved by the Animal Ethics Committee of Shangqiu Normal University (2020-1012).

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Table 1. Ingredient composition of the experimental diet (as fed basis).

Item	Experiment 1	Experiment 2
Ingredient (g/kg)		
Corn	562.0	574.1
Soybean meal (430 g/kg CP)	332.0	320.0
Soybean oil	28.2	24.7
Soy protein powder (650 g/kg CP)	36.8	41.2
Limestone	13.5	12.2
Dicalcium phosphate	19.4	19.3
L-Lysine·HCl (980 g/kg)	1.4	1.8
DL-Methionine (990 g/kg)	1.4	1.4
Trace mineral premix[1]	0.1	0.1
Vitamin premix[2,3]	0.2	0.2
Choline chloride (500 g/kg)	2.0	2.0
Sodium chloride	3.0	3.0
Nutrient composition		
Metabolizable energy (kcal/kg)	2975.4	2973.0
Crude protein (g/kg)	215.1	212.3
Analyzed calcium (g/kg)	10.0	10.1
Analyzed total phosphorus (g/kg)	6.9	7.0
Non-phytate phosphorus (g/kg)	4.5	4.5
Lysine (g/kg)	11.2	11.1
Methionine (g/kg)	5.0	5.1

[1]Trace mineral premix supplied per kg of diet: Fe ($FeSO_4 \cdot 7H_2O$), 80 mg; Mn ($MnSO_4 \cdot H_2O$), 60 mg; Zn ($ZnSO_4 \cdot 7H_2O$), 40 mg; Cu ($CuSO_4 \cdot 5H_2O$), 6 mg; I (KI), 0.35 mg; and Se (Na_2SeO_3), 0.15 mg.

[2]Experiment 1, vitamin premix supplied per kg of diet: vitamin A (retinyl palmitate), 4.4 mg; vitamin D_3, 25 µg (1000 IU); vitamin E (DL-α-tocopheryl acetate), 20 mg; vitamin K_3 (menadione), 0.5 mg; vitamin B_1 (thiamine), 2 mg; vitamin B_2 (riboflavin), 8 mg; vitamin B_6 (pyridoxine), 3.5 mg; vitamin B_{12}, 0.01 mg; biotin, 0.18 mg; folic acid, 0.55 mg; pantothenic acid, 10 mg; and niacin, 35 mg.

439 [3]Experiment 2, vitamin premix (without supplemental VD_3) supplied per kg of diet: vitamin A (retinyl
440 palmitate), 4.4 mg; vitamin E (DL-α-tocopheryl acetate), 20 mg; vitamin K_3 (menadione), 0.5 mg;
441 vitamin B_1 (thiamine), 2 mg; vitamin B_2 (riboflavin), 8 mg; vitamin B_6 (pyridoxine), 3.5 mg; vitamin B_{12},
442 0.01 mg; biotin, 0.18 mg; folic acid, 0.55 mg; pantothenic acid, 10 mg; and niacin, 35 mg.

443 **Table 2.** Primer sequences in real-time quantitative PCR.

Gene	Accession number	Orientation	Primer sequence (5′-3′)	Size (bp)
CaBP-D28k	NM_205513.1	Forward	AGATCTGGCACCACTACGAC	187
		Reverse	TGAGCAAGCTCAACGATTCCT	
PMCAlb	NM_001168002.3	Forward	AGCTCAAGATGGTGCAGCTA	165
		Reverse	AACAAACCTGCTTTGCCAATCT	
NCX1	NM_001079473.1	Forward	TCACCTTCTTCTTCTTCCCAATCT	158
		Reverse	GCAACCTTTCCGTCCATCTC	
NaPi-IIb	NM_204474.1	Forward	TCGGTCCGTTCACTCTGTTG	164
		Reverse	GCCACGTTGCCTTTGTGATT	
PiT-1	XM_015297502.1	Forward	GGCTCCGTGCTTCTGG	239
		Reverse	CATTTGACGCCTTTCTGC	
PiT-2	NM_001305398.1	Forward	GCAGCAGATACATCAACTC	153
		Reverse	ATTTCCACTCCACCCTC	
GAPDH	NM_204305.1	Forward	GAACATCATCCCAGCGTCCA	133
		Reverse	ACGGCAGGTCAGGTCAACAA	

444 Abbreviations: CaBP-D28k, calcium-binding protein 28-kDa; PMCA1b, plasma membrane calcium
445 ATPase 1b; NCX1, sodium-calcium exchanger 1; NaPi-IIb, IIb sodium-dependent phosphate
446 cotransporter; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2;
447 GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

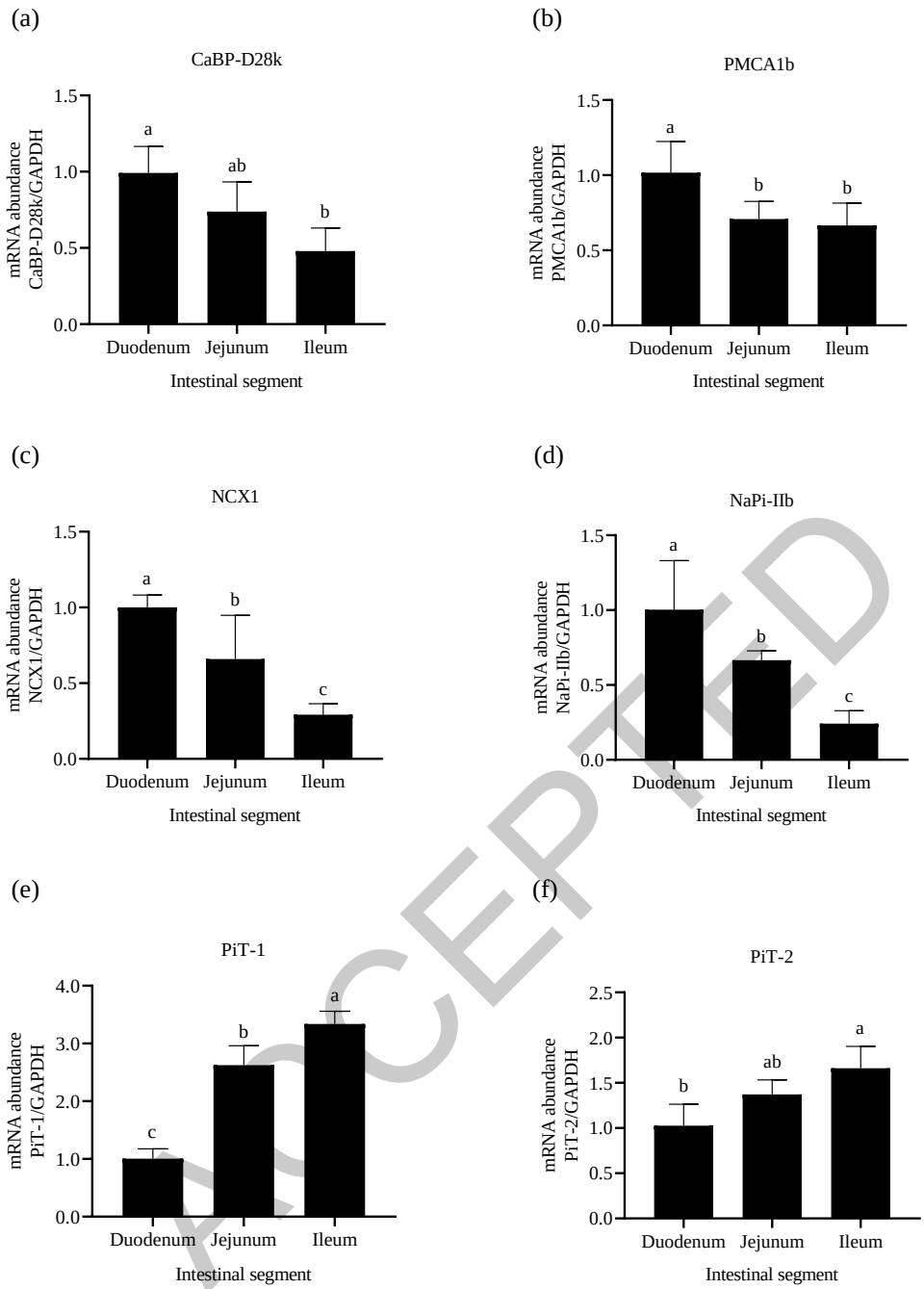

Figure 1. mRNA abundances of calcium and phosphorus transporters in the three intestinal segments (duodenum, jejunum, and ileum) of 21-day-old broilers (experiment 1). a, CaBP-D28k = calcium-binding protein 28-kDa; b, PMCA1b = plasma membrane calcium ATPase 1b; c, NCX1 = sodium-calcium exchanger 1; d, NaPi-IIb = IIb sodium-dependent phosphate cotransporter; e, PiT-1 = inorganic phosphate transporter 1; and f, PiT-2 = inorganic phosphate transporter 2. The values are the means of five repetitions (n = 5) and presented as mean ± SD. [a–c]Values with different superscripts are significantly different ($p < 0.05$).

Table 3. Effects of dietary vitamin D_3 concentration on growth performance of 1–21-day-old broilers (experiment 2) [1].

Vitamin D_3 (IU/kg)	Body weight on day 1 (g/chick)	Body weight on day 21 (g/chick)	Body weight gain (g/chick)	Feed intake (g/chick)	Feed conversion ratio (g/g)	Mortality (%)
0	42.3	452^{c}	410^{c}	678^{c}	1.66^{a}	1.43
125	42.6	756^{b}	713^{b}	974^{b}	1.37^{b}	1.43
250	42.6	805^{ab}	762^{ab}	1037^{ab}	1.36^{b}	4.29
500	42.3	815^{ab}	773^{ab}	1020^{ab}	1.32^{b}	0
1000	42.8	866^{a}	823^{a}	1070^{a}	1.30^{b}	0
2000	42.6	858^{a}	815^{a}	1034^{ab}	1.27^{b}	2.86
SEM	0.16	27	27	25	0.03	0.66
p value						
Linear	0.56	< 0.05	< 0.05	< 0.05	< 0.05	0.92
Quadratic	0.86	< 0.05	< 0.05	< 0.05	< 0.05	0.85

[1]Each value represents the mean of five repetitions (14 broilers per repetition) (n = 5). The negative control feed consisted of 10.0 g/kg calcium and 4.5 g/kg non-phytate phosphorus and did not contain supplemental VD_3. $^{a–c}$Values with different superscripts are significantly different ($p < 0.05$).

Table 4. Effects of dietary vitamin D_3 concentration on tibia development in 21-day-old broilers (experiment 2) [1].

Vitamin D_3 (IU/kg)	Bone weight (g/bone)	Bone length (cm/bone)	Mineral percentage (%)			Mineral weight (g/bone)		
			Ash	Ca	P	Ash	Ca	P
0	0.99^{d}	5.15^{c}	38.3^{b}	13.0^{b}	6.92^{b}	0.37^{d}	0.13^{d}	0.07^{c}
125	1.41^{c}	5.98^{b}	48.3^{a}	17.8^{a}	8.92^{a}	0.67^{c}	0.25^{c}	0.13^{b}
250	1.63^{bc}	6.19^{ab}	49.5^{a}	18.4^{a}	9.56^{a}	0.80^{b}	0.30^{b}	0.16^{a}
500	1.73^{ab}	6.15^{ab}	49.5^{a}	17.8^{a}	9.50^{a}	0.85^{ab}	0.31^{b}	0.16^{a}
1000	1.89^{a}	6.35^{a}	49.3^{a}	18.1^{a}	9.37^{a}	0.93^{a}	0.34^{a}	0.18^{a}
2000	1.89^{a}	6.30^{ab}	48.9^{a}	17.9^{a}	9.19^{a}	0.92^{a}	0.34^{a}	0.17^{a}
SEM	0.06	0.08	0.8	0.4	0.18	0.04	0.01	0.01
p value								
Linear	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Quadratic	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

[1]Each value represents the mean of five repetitions (two broilers per repetition) (n = 5). Ca = calcium; P = phosphorus. $^{a–d}$Values with different superscripts are significantly different ($p < 0.05$).

Table 5. Effects of dietary vitamin D_3 concentration on femur development in 21-day-old broilers (experiment 2) [1].

Vitamin D_3 (IU/kg)	Bone weight (g/bone)	Bone length (cm/bone)	Mineral percentage (%)			Mineral weight (g/bone)		
			Ash	Ca	P	Ash	Ca	P
0	0.81^{c}	3.83^{b}	35.5^{b}	12.8^{b}	6.91^{b}	0.29^{c}	0.10^{b}	0.06^{c}
125	1.22^{b}	4.60^{a}	46.7^{a}	17.5^{a}	8.98^{a}	0.56^{b}	0.21^{a}	0.11^{b}
250	1.33^{ab}	4.66^{a}	45.7^{a}	17.6^{a}	9.19^{a}	0.60^{ab}	0.23^{a}	0.12^{ab}
500	1.33^{ab}	4.69^{a}	47.5^{a}	17.0^{a}	9.15^{a}	0.63^{ab}	0.23^{a}	0.12^{ab}
1000	1.43^{a}	4.79^{a}	46.8^{a}	17.0^{a}	8.97^{a}	0.67^{a}	0.24^{a}	0.13^{a}
2000	1.43^{a}	4.78^{a}	45.9^{a}	17.1^{a}	8.84^{a}	0.66^{a}	0.24^{a}	0.13^{a}
SEM	0.04	0.07	0.8	0.3	0.16	0.03	0.01	0.005
p value								
Linear	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Quadratic	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

[1]Each value represents the mean of five repetitions (two broilers per repetition) (n = 5). Ca = calcium; P = phosphorus. $^{a–c}$Values with different superscripts are significantly different ($p < 0.05$).

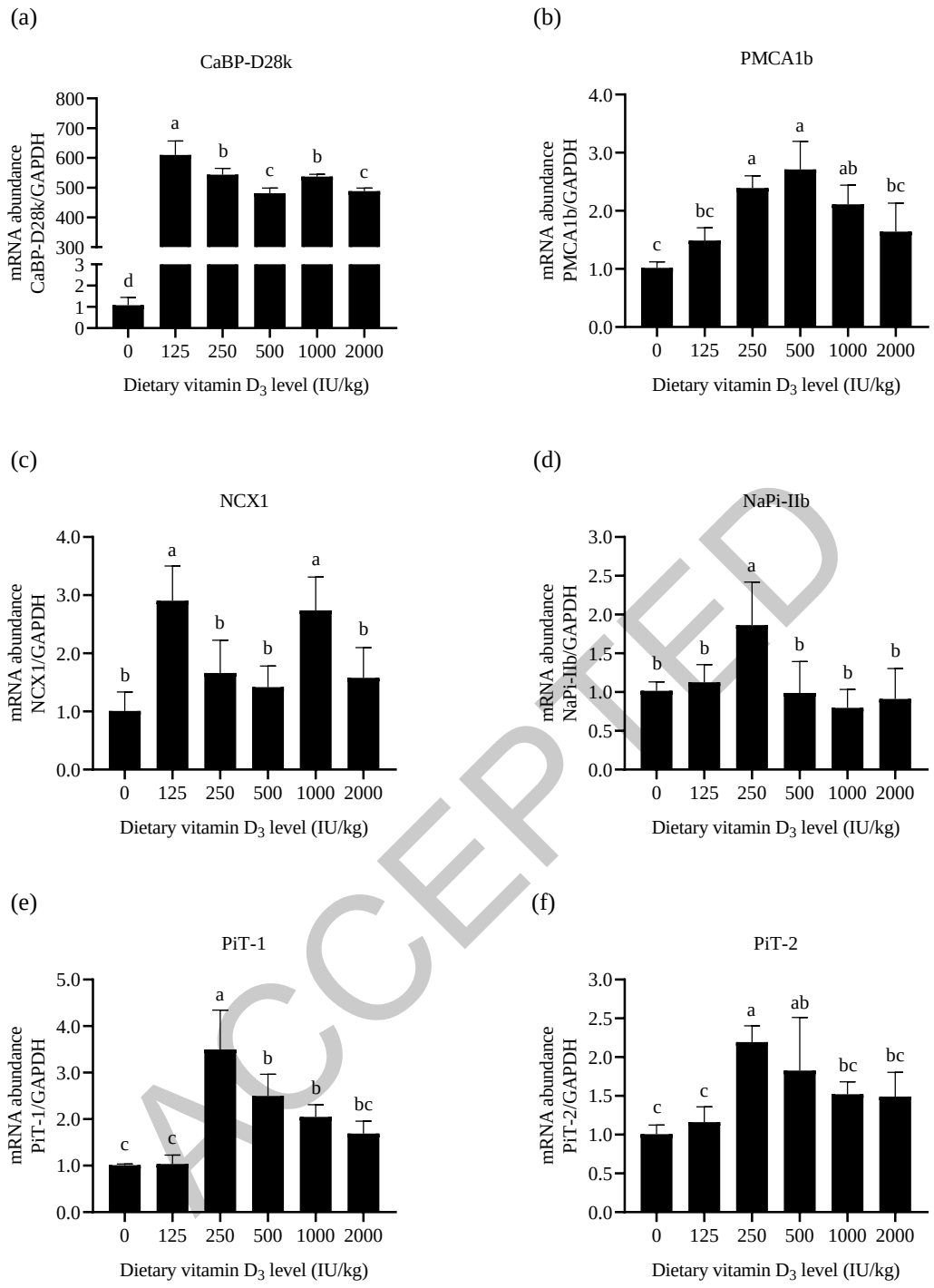

Figure 2. Effects of dietary vitamin D_3 concentration on the mRNA abundances of calcium and phosphorus transporters in the duodenum of 21-day-old broilers (experiment 2). a, CaBP-D28k = calcium-binding protein 28-kDa (Linear $p < 0.05$, Quadratic $p < 0.05$); b, PMCA1b = plasma membrane calcium ATPase 1b (Linear $p < 0.05$, Quadratic $p < 0.05$); c, NCX1 = sodium-calcium exchanger 1 (Linear $p = 0.28$, Quadratic $p < 0.05$); d, NaPi-IIb = IIb sodium-dependent phosphate cotransporter (Linear $p = 0.08$, Quadratic $p < 0.05$); e, PiT-1 = inorganic phosphate transporter 1 (Linear $p < 0.05$,

Quadratic $p < 0.05$); and f, PiT-2 = inorganic phosphate transporter 2 (Linear $p < 0.05$, Quadratic $p < 0.05$). The values are the means of five repetitions (n = 5) and presented as mean ± SD. [a–d]Values with different superscripts are significantly different ($p < 0.05$).

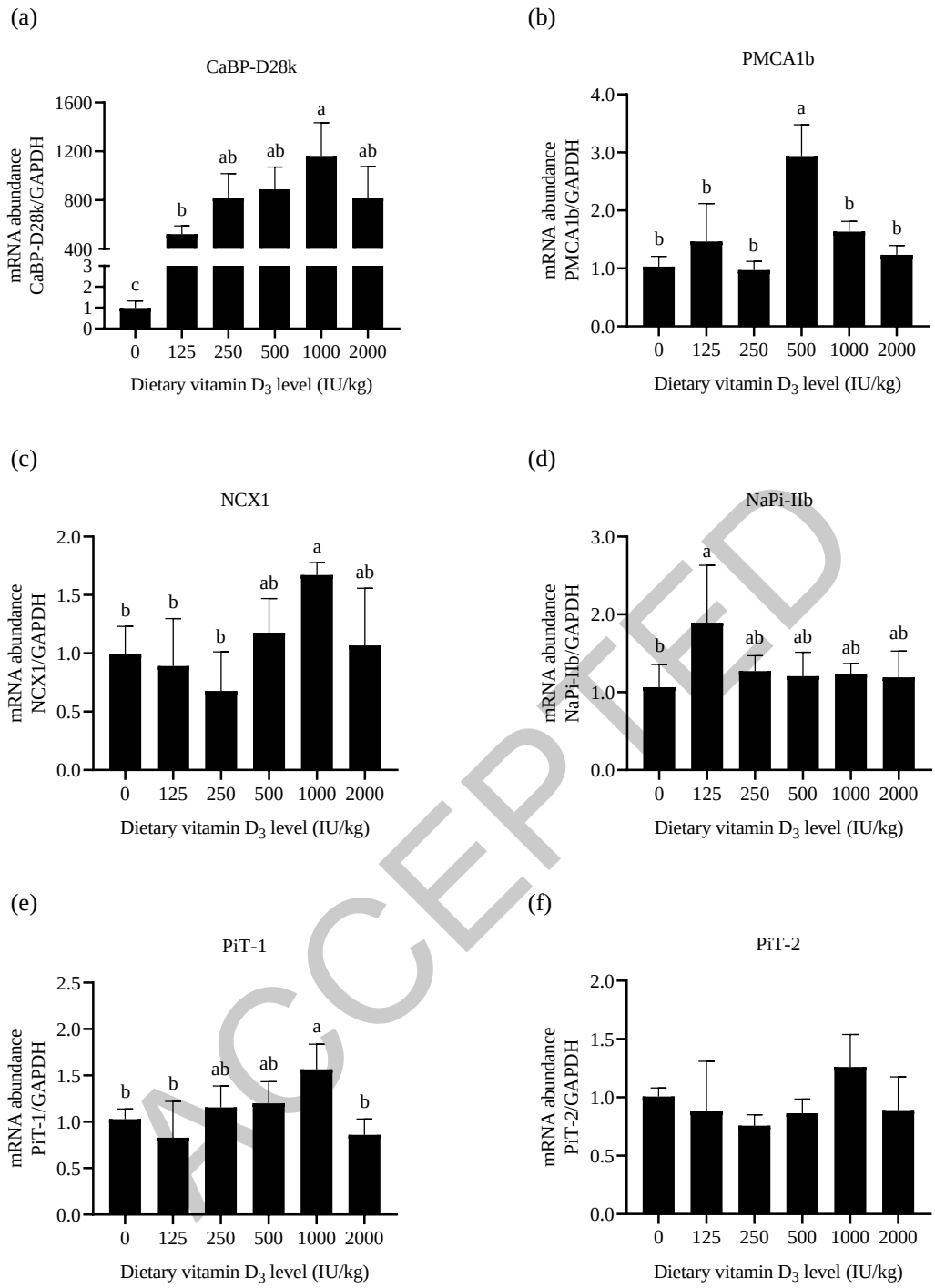

Figure 3. Effects of dietary vitamin D_3 concentration on the mRNA abundances of calcium and phosphorus transporters in the jejunum of 21-day-old broilers (experiment 2). a, CaBP-D28k = calcium-binding protein 28-kDa (Linear $p < 0.05$, Quadratic $p < 0.05$); b, PMCA1b = plasma membrane calcium ATPase 1b (Linear $p < 0.05$, Quadratic $p < 0.05$); c, NCX1 = sodium-calcium exchanger 1 (Linear $p < 0.05$, Quadratic $p = 0.81$); d, NaPi-IIb = IIb sodium-dependent phosphate cotransporter (Linear $p = 0.34$, Quadratic $p = 0.28$); e, PiT-1 = inorganic phosphate transporter 1 (Linear $p = 0.15$, Quadratic $p < 0.05$);

and f, PiT-2 = inorganic phosphate transporter 2 (Linear $p = 0.49$, Quadratic $p = 0.40$). The values are the means of five repetitions (n = 5) and presented as mean ± SD. [a–c]Values with different superscripts are significantly different ($p < 0.05$).

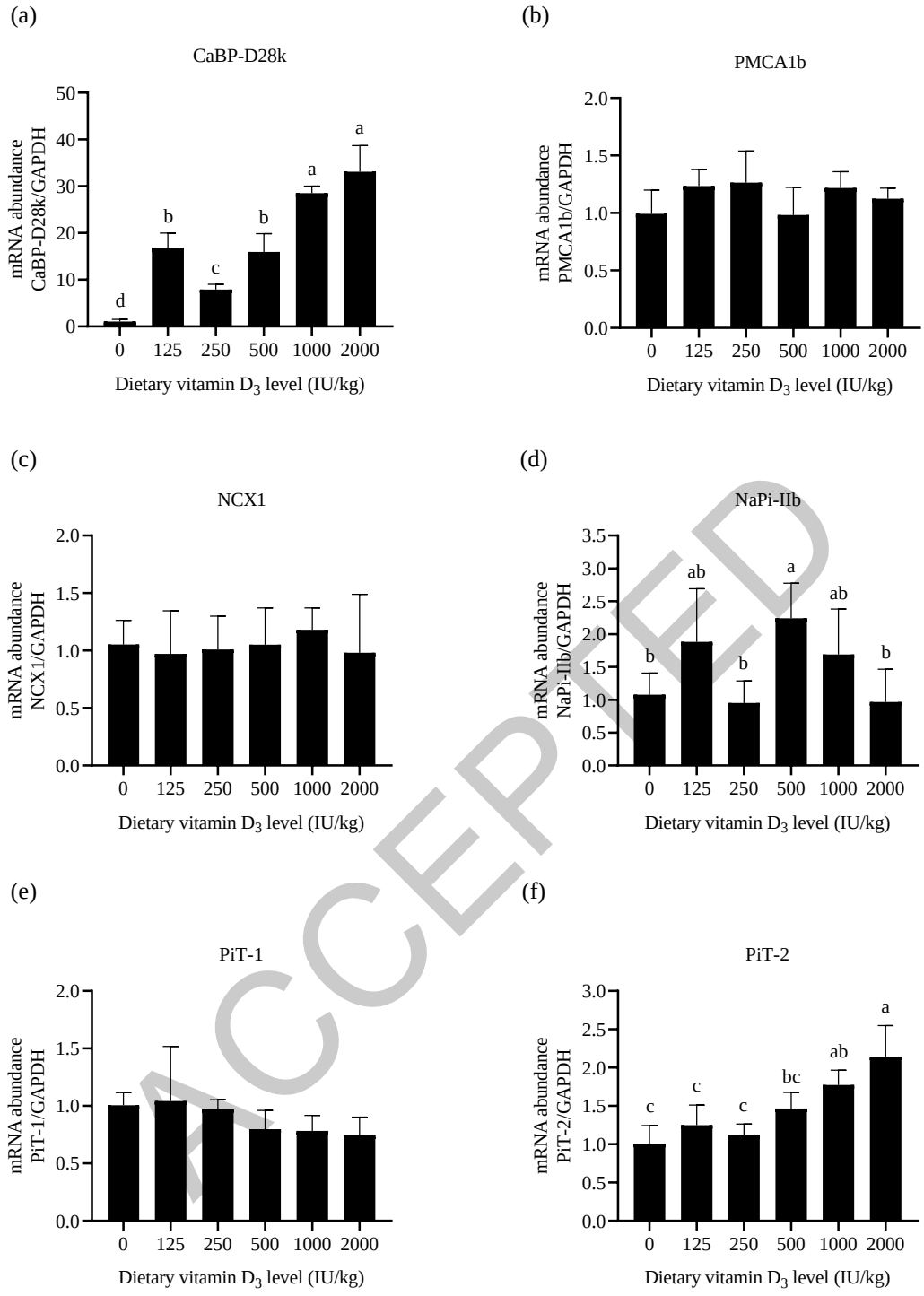

Figure 4. Effects of dietary vitamin D_3 concentration on the mRNA abundances of calcium and phosphorus transporters in the ileum of 21-day-old broilers (experiment 2). a, CaBP-D28k = calcium-binding protein 28-kDa (Linear $p < 0.05$, Quadratic $p < 0.05$); b, PMCA1b = plasma membrane calcium ATPase 1b (Linear $p = 0.66$, Quadratic $p = 0.29$); c, NCX1 = sodium-calcium exchanger 1 (Linear $p = 0.80$, Quadratic $p = 0.87$); d, NaPi-IIb = IIb sodium-dependent phosphate cotransporter (Linear $p = 0.94$, Quadratic $p < 0.05$); e, PiT-1 = inorganic phosphate transporter 1 (Linear $p < 0.05$, Quadratic $p = 0.86$);

516 and f, PiT-2 = inorganic phosphate transporter 2 (Linear $p < 0.05$, Quadratic $p < 0.05$). The values are
517 the means of five repetitions (n = 5) and presented as mean ± SD. [a–d]Values with different superscripts
518 are significantly different ($p < 0.05$).