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The Safety Evaluation of Novel Bio-based Calcium D-pantothenate Obtained from Recombinant *Escherichia coli* K12 on Growth Performance and Health Status of Broiler Chickens

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ABSTRACT

The production of traditional synthesized calcium D-pantothenate (D-PA) is accompanied with chemical pollution, therefore, the eco-friendly bio-fermentation technology has received widespread attention. In order to verify the safety of a novel D-PA product produced by genetically engineered bacteria (*Escherichia coli* K12), the authors of the current study investigated the influence of adding D-PA to the diet on growth performance and health status of broiler chickens. A total of 192 day-old healthy Arbor Acres broiler chickens with similar weight (43.21 ± 0.12 g) were randomly divided into 4 treatments with 6 replicates and 8 broiler chickens in each replicate (male and female in half). The *Escherichia coli* K12 was genetically engineered for the production of D-PA. The control group was fed with the basal diet containing 20 mg/kg synthesized D-PA (CT group). The treatments were supplemented with 20 (TCaP1 group, recommended dose group), 100 (TCaP5 group, 5-fold-dose group), and 200 (TCaP10 group, 10-fold-dose group) mg/kg bio-based D-PA product, respectively. The experiment lasted for 42 days and the growth performance and health status of broiler chickens were determined. The results indicated that the addition of 5- and 10-fold doses of bio-based D-PA could increase the average daily weight gain during 22-42 days of age and decrease the feed conversion rate during 22-42 and 1-42 days of age of broilers. There were some differences in white blood cell count, intermediate cell absolute value (MID) count, absolute granulocyte count, absolute lymphocyte count, granulocyte percentage, mean corpuscular volume, red blood cell distribution width-standard deviation, mean platelet volume and serum phosphorus and total bilirubin in different groups, compared with the CT group. Histological observations of the liver, spleen, pancreas, and small intestines showed that the tissue structures of various organs of the broiler chickens fed with the bio-based D-PA were clear, and no abnormal changes such as inflammatory cell infiltration and fibrous tissue hyperplasia were observed in all groups. In summary, dietary supplementation of bio-based D-PA was safe within the 10-fold- dose (200mg/kg) to broiler chickens during 1-42 days.

Keywords: Biological safety evaluation, Broiler chicken, Calcium D-pantothenate, Growth performance, Vitamin B_5

INTRODUCTION

D-calcium pantothenate (D-PA), a white or slightly yellow crystalline powder, is mainly used in feed or the pharmaceutical industry. Pantothenic acid, the precursor of coenzyme A and acyl-carrier protein, is an important water-soluble vitamin which involved in animal growth and development and plays a crucial role in cellular metabolism and the oxidation of fats, carbohydrates, and proteins (Eggersdorfer et al., 2012; Tang et al., 2020a; 2020b). For poultry, dietary supplementation of D-PA can help improve the growth performance, feather quality and decrease the dermatosis and mortality (Hegsted et al., 1949; Beer et al., 1963). Although pantothenic acid is widely distributed in foods and can be synthesized by the intestinal microbiota, it's still necessary to supply

additionally in the feed to satisfy the need of fast-growing animals (Wang et al., 2016). Deficiency of pantothenic acid in animals leads to reduced nutrient utilization, weakened immune system, and various diseases, and it can also reduce the body's tolerance of stresses through hormone synthesis (Tang et al., 2021).

The commercial production of D-PA has currently always relied on developed chemical synthesis routes. There exists highly toxic raw materials and cyanide-containing wastewater pollution in the classical chemical process (Acevedo-Rocha et al., 2019). As people are paying more and more attention to environmental protection and energy shortages, researchers are looking for more environmentally friendly and sustainable methods of the production of D-PA (Leonardi and Jackowski, 2007; Zou et al., 2021). The microbial fermentation method uses natural renewable resources to realize the environmentally friendly and sustainable production of D-PA, which could be the replacement of chemical synthesis routes (Huser et al., 2005; Zhang et al., 2019). Although the report of the toxicity of the traditional synthesized D-PA has not been found, the safety of bio-based D-PA remains unknown, therefore, this experiment investigated the nutritional effects and safety of D-PA produced by biological synthesis methods using genetically engineered bacteria on broiler chickens.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China) and performed in accordance with the guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS (approval no.202321, date : 2023.02.16).

Experimental design and treatments

A total of 192 1-day-old healthy Arbor Acres broilers obtained from a local hatchery (Hebei Luanping Huadu Food Co., Ltd, china) with similar weight (43.21 ± 0.12 g) were randomly assigned to 4 treatment groups with 6 replicates per treatment group and 8 chicks per replicate (half hens and half roosters). The experiment was conducted at the experimental farm of IAS-CAAS. The control group was fed with a corn-soybean meal basal diet containing 20 mg/kg of syntheized D-PA (CT group), and the treatments were supplemented with 20 mg/kg (TCaP1 group, recommended dose group, Announcement No. 2625 of the Ministry of Agriculture of the People's Republic of China, 2018), 100 mg/kg (TCaP5 group, 5-fold dose group) or 200 mg/kg (TCaP10 group, 10-fold dose group) bio-based D-PA product, respectively. The experiment lasted for 42 days.

Birds and diets

The bio-based D-PA product (99%) obtained from recombinant *Escherichia coli* K12 (PT06) was provided by Heilongjiang NHU Biotechnology Co., Ltd (China), and there were no genetically engineered bacteria existing in the final D-PA product. The engineered bacteria of recombinant *Escherichia coli* K12 was prepared for the production of D-PA product. This microbial fermentation method uses natural renewable resources - glucose and the recombinant *Escherichia coli* K12 to produce D-PA. The pyruvate and keto-isovalerate are the key precursors of D-PA biosynthesis.

The standard starter diet (1 to 21 days of age) and grower-finisher diet (22-42 days of age) were formulated according to the NRC recommendations (1994) mainly using corn, soybean meal, and the premix. The details of the experimental diets are presented in Table 1. The birds were raised according to the management regulations of Arbor Acres broilers (Aviagen, 2018). The broiler chickens were kept in thermostatically controlled, stainless cages coated with plastic (100 × 50 × 45 cm) and equipped with fiberglass feeders and waterers. Feed and tap water were available *ad libitum*. The temperature in the house was controlled at 33 - 35°C from days 1 - 5, which was gradually reduced every week until 26°C. The house was cleaned and disinfected before the entry of broiler chickens. The broiler chickens were vaccinated against Newcastle disease, infectious bronchitis, avian influenza, and infectious bursal disease vaccines (produced by ShanDong HuaHong Biological Engineering CO.,LTD., china) at 7 days of age.

Sample collection and determination

Broilers were weighed on days 1, 21, and 42 of the experiment. Feed consumption and mortality data were recorded at the end of the experiment. Growth performance indexes such as average daily feed intake (ADFI), average daily weight gain (ADG), feed conversion rate (F/G), and mortality were calculated based on these measurements.

In the study, blood samples were collected from the wing vein broiler chickens on day 42 for testing various blood physiological indexes after 8 hours of fasting. In this

regard, 2 mL whole blood samples were preserved using an EDTA anticoagulant tube to test the blood physiological indexes, including white blood cell count (WBC), granulocyte absolute value (GRA), percentage of granulocyte (GRA%), absolute lymphocyte count (LYM), absolute intermediate cell value (MID), absolute lymphocyte percentage (LYM%), intermediate cell percentage (MID%), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width-standard deviation (RDW-SD), red blood cell distribution width-coefficient of variation (RDW-CV), platelet count (PLT), platelet crit (PCT), mean platelet volume (MPV), platelet distribution width (PDW) with a TEK-II mini automatic animal blood analyzer (Tecom, China). Another 10 mL blood was collected in a vacuum blood collection tube and placed for 4 h at room temperature. The blood sample was then centrifuged to obtain the serum at 3.500 r/min for 10 min and was stored at -80°C in eppendorf tubes. The serum calcium, phosphorus, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-gamma-glutamyl transpeptidasee, urea nitrogen, creatinine, total bilirubin, lysozyme and serum superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, malondialdehyde, total antioxidant capacity and hydroxy free radical scavenging activity were determined by automatic biochemical analyzer (Hitachi 7600, Japan) using the assay kits (Nanjing Jiancheng Bioengineering Institute, china). Serum immunity parameters (total protein, albumin, globulin, albumin/globulin, immunoglobulin G, immunoglobulin M, immunoglobulin A, complement 3, complement 4, interleukin-1β, tumor necrosis factor α, lysozyme) were determined by enzyme-linked immunoassay using microplate reader (Thermo Multiskan MK3, Finland). Serum hormones (thyroid hormones T3, thyroid hormones T4, insulin) were determined by automatic radioimmunoassay counter (Zonkia GC-2010, China).

After blood sampling 2 broiler chickens from each replicate were dissected (Tang et al., 2021) and the liver, spleen, pancreas, and small intestines were quickly separated, weighted and immersed in 4% paraformaldehyde solution for the determination of organ indexes and observation of organ development and histopathological changes under light microscope (10x, Zhanjing, China). The abnormal structure of organs, proliferation of Kupffer cells, inflammatory cell infiltration, congestion, and fibrous tissue hyperplasia indexes was used to evaluate the health status of broiler chickens (Al-Sultan and Gameel, 2004; Kumar et al., 2009).

Table 1. Composition and calculated nutrient content of basal diets for broiler chickens during 1-42 days of age

Ingredient	**Grower (1-21 days; g/kg)**	**Finisher (22-42 days; g/kg)**
Corn	53.20	57.10
Soybean meal（43%）	38.20	34.00
Soybean oil	4.15	5.00
Dicalcium phosphate	1.70	1.56
Limestone	1.00	0.80
Salt	0.30	0.30
DL- Methionine	0.24	0.14
L- Lysine, 98%	0.21	0.10
Premix[1]	1.00	1.00
Total	100.00	100.00
Nutrition level[2]		
Metabolism（MJ/kg）	12.49	12.89
Crude protein（%）	21.51	20.00
Calcium（%）	1.00	0.87
Available phosphorous（%）	0.46	0.42
Methionine（%）	0.52	0.41
Lysine（%）	1.17	1.00
Tryptophan（%）	0.22	0.20
Threonine（%）	0.64	0.59

[1] Supplied per kilogram of the diet. Vitamin A, 10,000 IU as vitamin A acetate; vitamin D3, 4,500 IU as cholecalciferol; 65 IU of vitamin E; vitamin K, 3.0 mg as menadione sodium bisulfate; thiamine, 2.5 mg as thiamine mononitrate; riboflavin, 6.5 mg; pyridoxine, 3.2 mg as pyridoxine hydrochloride; vitamin B12, 0.03 mg; pantothenic acid, 18 mg as D-calcium pantothenate; niacin, 60 mg; folic acid, 1.9 mg and biotin, 0.25 mg, copper 7.5mg, ferrous 20 mg, manganese 120mg, iodine 1.25mg, selenium 0.3mg.

Statistical analysis

Data were subjected to one-way ANOVA by using the GLM procedures of SAS 9.4 (SAS Inst., Inc., Cary, NC, USA). Cage served as the experimental unit. When one-way ANOVA showed significant ($p < 0.05$) differences among treatments, treatment means were compared using Duncan's method. Data were expressed as "mean ± standard deviation".

RESULTS

Growth performance

The effect of adding D-PA obtained from microbial fermentation method to the diet on the broiler growth performance is shown in Table 2. There were no differences between the CT group and the treatment groups in terms of ADG, ADFI, F/G, and mortality during 1-21 days of age ($p > 0.05$). During 22-42 days of age, there was an increase in ADG in the TCaP5 and TCaP10 group, and an decrease on F/G in the TCaP5 and TCaP10 group compared with the CT group ($p < 0.05$). During days 1-42, the F/G in the TCaP5 and TCaP10 groups was lower than that of the CT group ($p < 0.05$). No difference was observed in ADFI and mortality during 1-21, 22-42, and 1-42 days of age and ADG during 1-42 days of age ($p > 0.05$).

Organ indexes

The effects of dietary supplementation of bio-based D-PA on the broiler organ indexes are shown in Table 3. There was no difference in the organ indexes of the liver, heart, spleen, pancreas, thymus, bursa of Fabricius, muscular stomach, duodenum, jejunum, ileum, and cecum of broilers in different treatments ($p > 0.05$).

Blood physiological parameters

The effects of dietary supplementation of bio-based D-PA on the blood physiological parameters are shown in Table 4. The white blood cell count (WBC), granulocyte absolute value (GRA) and percentage of granulocyte (GRA%) in the TCaP5 group increased compared with that of the CT group or the TCaP10 group ($p < 0.05$), but the percentage of lymphocyte (LYM%) in the TCaP5 group decreased compared with that of the CT group or the TCaP10 group ($p < 0.05$). Intermediate cell absolute value (MID) in the TCaP5 group, mean erythrocyte volume (MCV) in the TCaP1 group, the standard deviation of red blood cell distribution width (RDW-SD) in the TCaP1 group and the TCaP10 group, and mean platelet volume (MPV) in the TCaP1 group increased compared with the CT group ($p < 0.05$).

Serum biochemistry and antioxidant parameters

The effects of dietary supplementation of bio-based D-PA on the serum biochemistry parameters are shown in Table 5. Serum phosphorus level in the TCaP10 group increased compared with that of the TCaP5 group ($p < 0.05$). Serum total bilirubin in the TCaP5 group increased compared with that of the CT group ($p < 0.05$). The effects of dietary supplementation of bio-based D-PA on the serum antioxidant indicators are shown in Table 6. Dietary supplementation of bio-based D-PA had no influence on the activities of serum superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), the content of malondialdehyde (MDA), total antioxidant capacity (T-AOC), nitric oxide and Hydroxyl radical (OH) ($p > 0.05$).

Serum hormone parameters

The effects of dietary supplementation of bio-based D-PA on the serum hormone parameters are shown in Table 7. Compared with the CT group, the serum T3, T4, and insulin were not affected in the treatment groups ($p > 0.05$).

Serum immunity parameters

The effects of dietary supplementation of bio-based D-PA on the serum immunity indicators of broilers are shown in Table 8. The serum interleukin-1β level in the TCaP1 group was lower compared with the CT group ($p < 0.05$). The serum lysozyme level in the TCaP5 and TCaP10 group was lower compared with the CT and TCaP1 group ($p < 0.05$). Adding D-PA obtained from the microbial fermentation method to the diet had no influence on serum total protein, albumin, globulin, albumin to globulin ratio, IgG, IgM, IgA, complement-3, complement-4, and tumor necrosis factor-α ($p > 0.05$).

Organ histomorphology

As shown in Figure 1, the clear hepatic lobules from all groups, the uniform hepatocytes, regular hepatic cord arrangements, the round single or dual hepatocyte nuclei with regular morphology, and the clear and visible hepatic sinuses were clear. Compared with the control group, the test groups showed no proliferation of Kupffer cells, inflammatory cell infiltration, or fibrous tissue proliferation in the portal region. As shown in Figure 2, the intact splenic capsules, the abundant white pulp, the

clear margin of the splenic corpuscles, and the obvious germinal centers could be seen in all test groups. No congestion was found in the blood sinus of the red pulp, macrophages were visible in the medullary sinuses and marginal areas, and the red and white pulp structures of the splenic tissues were clear. Compared with the control group, no abnormalities were found in the test groups. As shown in Figure 3, in all the test groups, the delimited pancreatic tissues, the ducts, and blood vessels in the connective tissues of the lobules, the clear structure of pancreatic islet was obvious, and there was no inflammatory cell infiltration in the lobular mesenchyma. As shown in Figure 4, in all the test groups, the clear structure of the duodenal mucosa, the healthy and strong villi, the abundant mucosal epithelial goblet cells, the clear chorionic interstitial, intestinal crypt, and intrinsic layer structures, the visible Paneth cells at the bottom of the crypt was clear, and no inflammation was observed.

Table 2. Effects of dietary supplementation of bio-based D-PA on the broiler chickens' growth performance during 1-42 days of age [1]

	Item	CT	TCaP1	TCaP5	TCaP10	p-value
Day 1-21	ADFI (g/d)	47.34 ± 0.61	46.93 ± 0.24	46.89 ± 0.97	47.19 ± 0.89	0.964
	ADG (g/d)	31.85 ± 1.26	32.60 ± 0.82	33.26 ± 0.49	31.46 ± 1.78	0.689
	F/G	1.50 ± 0.05	1.44 ± 0.03	1.41 ± .02	1.51 ± 0.07	0.352
	Mortality (%)	0	0	0	0	
Day 21	BW (g)	711.88 ± 26.35	727.86 ± 17.24	741.68 ± 10.18	703.75 ± 37.25	0.685
Day 22-42	ADFI (g/d)	142.25 ± 3.60	142.42 ± 3.01	143.69 ± 2.33	141.39 ± 3.02	0.962
	ADG (g/d)	83.05 ± 2.50^{b}	86.71 ± 2.25^{ab}	88.81 ± 0.60^{a}	89.30 ± 0.75^{a}	0.034
	F/G	1.72 ± 0.03^{a}	1.64 ± 0.02^{ab}	1.62± 0.03^{b}	1.58 ± 0.03^{b}	0.019
	Mortality (%)	0	0	0	0	
Day 42	BW (g)	2372.90 ± 74.15	2462.10 ± 54.57	2517.90 ± 11.92	2489.80 ± 41.94	0.244
Day 1-42	ADFI (g/d)	93.03 ± 2.04	92.90 ± 1.52	93.50 ± 1.58	92.53 ± 1.87	0.985
	ADG (g/d)	57.80 ± 2.04	60.47 ± 1.36	61.87 ± 0.30	61.16 ± 1.05	0.194
	F/G	1.64 ± 0.04^{a}	1.58 ± 0.01^{ab}	1.55 ± 0.02^{b}	1.55 ± 0.01^{b}	0.036
	Mortality (%)	0	0	0	0	-

[1] Means within a row with no common superscripts differ ($p < 0.05$), the same as follow. CT: Control group supplemented with 20 mg/kg synthetic calcium D-pantothenate; TCaP1: Treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; TCaP5: Treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; TCaP10: Treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate. ADFI: Average daily feed intake; ADG: Average daily weight gain; F/G: feed-to-gain ratio; D-PA: Calcium D-pantothenate.

Table 3. Effects of dietary supplementation of bio-based D-PA on the organ indexes of broiler chickens during 1-42 days of age

Item	CT	TCaP1	TCaP5	TCaP10	p value
Liver	20.70 ± 1.05	20.77 ± 0.91	21.45 ± 1.11	21.29 ± 0.74	0.928
Heart	4.87 ± 0.12	5.02 ± 0.26	4.91 ± 0.22	5.25 ± 0.22	0.596
Spleen	1.11 ± 0.07	1.03 ± 0.06	1.33 ± 0.16	1.42 ± 0.23	0.232
Pancreas	2.08 ± 0.11	2.11 ± 0.11	2.09 ± 0.13	2.31 ± 0.09	0.410
Thymus	3.12 ± 0.36	3.36 ± 0.36	3.34 ± 0.35	4.00 ± 0.42	0.381
Bursa of Fabricius	2.12 ± 0.15	2.24 ± 0.22	2.21 ± 0.19	2.12 ± 0.19	0.958
Muscular stomach	16.58 ± 0.84	16.53± 1.33	17.46 ± 0.91	16.14 ± 0.79	0.813
Duodenum	7.28 ± 0.35	6.79 ± 0.27	6.41 ± 0.27	7.42 ± 0.39	0.180
Jejunum	14.48 ± 0.51	13.32 ± 0.94	12.4 7± 0.39	12.47 ± 0.39	0.326
Ileum	13.28 ± 0.99	12.00 ± 0.87	11.21 ± 0.82	13.45 ± 0.48	0.338
Cecum	6.20 ± 0.39	7.40 ± 0.87	6.60 ± 0.46	7.02 ± 0.81	0.617

CT: Control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; TCaP1: Treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; TCaP5: Treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; TCaP10: Treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate.

Table 4. Effects of dietary supplementation of bio-based D-PA on the blood physiological parameters of broiler chickens during 1-42 days of age

Item	CT	TCaP1	TCaP5	TCaP10	*P* value
WBC ($\times10^9$/L)	128.84 ± 2.08^{b}	132.08 ± 1.63^{ab}	134.96 ± 4.35^{a}	129.75 ± 1.78^{b}	0.037
LYM ($\times10^9$/L)	64.59 ± 0.82	63.84 ± 0.74	64.05 ± 0.57	64.73 ± 0.52	0.752
MID ($\times10^9$/L)	18.53 ± 0.33^{b}	18.97 ± 0.30^{ab}	19.58 ± 0.20^{a}	18.79 ± 0.29^{ab}	0.012
GRA ($\times10^9$/L)	45.72 ± 1.88^{b}	49.27 ± 1.18^{ab}	51.33 ± 1.32^{a}	46.23 ± 1.68^{b}	0.043
LYM (%)	50.35 ± 1.00^{a}	48.47 ± 0.58^{ab}	47.62 ± 0.66^{b}	50.13 ± 0.87^{a}	0.030
MID (%)	14.34 ± 0.05	14.32 ± 0.07	14.47 ± 0.05	14.42 ± 0.060	0.260
GRA (%)	35.31 ± 1.00^{b}	37.22 ± 0.57^{ab}	37.92 ± 0.67^{a}	35.45 ± 0.85^{b}	0.033
RBC ($\times10^{12}$/L)	2.01 ± 0.05	1.99 ± 0.03	2.05 ± 0.04	1.98 ± 0.03	0.619
HGB (g/L)	110.42 ± 3.48	112.17 ± 1.61	114.33 ± 1.86	109.67 ± 1.89	0.505
HCT (L/L)	0.18 ± 0.005	0.19 ± 0.003	0.19 ± 0.003	0.18 ± 0.003	0.268
MCV (fL)	90.01 ± 0.79^{b}	93.68 ± 0.59+	91.16 ± 0.80^{b}	90.43 ± 0.69^{b}	0.004
MCH (pg)	54.73 ± 0.51	56.12 ± 0.45	55.64 ± 0.81	55.17 ± 0.64	0.431
MCHC (g/L)	577.75 ± 5.19	569.00 ± 3.25	579.17 ± 4.98	579.25 ± 4.86	0.350
RDW-SD (fL)	27.51 ± 3.29^{b}	34.94 ± 0.54^{a}	30.96 ± 2.64^{ab}	34.88 ± 0.43^{a}	0.019
RDW-CV (%)	23.29 ± 0.60	23.23 ± 0.90	23.29 ± 0.59	23.95 ± 0.40	0.842
PLT ($\times10^9$/L)	31.50 ± 6.17	25.00 ± 2.11	26.42 ± 1.54	26.75 ± 2.07	0.587
PCT (L/L)	0.033 ± 0.006	0.026 ± 0.002	0.028 ± 0.002	0.028 ± 0.002	0.657
MPV (fL)	10.53 ± 0.08^{b}	10.76 ± 0.08^{a}	10.70 ± 0.05^{ab}	10.63 ± 0.05^{ab}	0.017
PDW (fL)	59.23 ± 5.06	70.48 ± 4.67	64.68 ± 4.33	72.59 ± 4.34	0.179

CT: Control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; TCaP1: Treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; TCaP5: Treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; TCaP10 : Treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate; WBC: White blood cell count; LYM: Absolute lymphocyte count; MID: Absolute MID cell count; GRA: Absolute granulocyte count; LYM%: Lymphocyte percentage; MID%: MID cell percentage; GRA%: Granulocyte percentage; RBC: Red blood cell count; HGB: Hemoglobin concentration; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW-SD: Red blood cell distribution width-standard deviation; red blood cell distribution width-coefficient of variation; PLT: Platelet count; PCT: Plateletcrit; MPV: Mean platelet volume; PDW: Platelet distribution width.

Table 5. Effects of dietary supplementation of bio-based D-PA on the serum biochemistry parameters of broiler chickens during 1-42 days of age

Item	CT	TCaP1	TCaP5	TCaP10	*P* value
Ca (mmol/L)	2.68 ± 0.07	2.64 ± 0.06	2.66 ± 0.06	2.58 ± 0.07	0.721
P (mmol/L)	1.79 ± 0.03^{ab}	1.79 ± 0.04^{ab}	1.74 ± 0.03^{b}	1.85 ± 0.03^{a}	0.018
ALT (U/L)	5.27 ± 1.22	2.97 ± 0.47	5.87 ± 0.98	6.12 ± 1.19	0.142
AST (U/L)	469.67 ± 72.29	453.15 ± 56.60	500.10 ± 50.19	601.88 ± 50.87	0.298
ALP (U/L)	125.92 ± 23.44	185.43 ± 43.85	176.28 ± 28.21	104.73 ± 11.35	0.181
GGT (U/L)	18.15 ± 1.32	21.93 ± 1.25	18.55 ± 1.11	17.95 ± 2.39	0.281
BUN (mmol/L)	0.59 ± 0.09	0.49 ± 0.05	0.44 ± 0.20	0.60 ± 0.06	0.318
CREA (μmol/L)	30.18 ± 14.14	24.03 ± 14.26	14.58 ± 5.04	21.05 ± 11.95	0.829
TBiLi (μmol/L)	5.00 ± 0.86^{b}	6.68 ± 0.43^{ab}	7.38 ± 0.38^{a}	6.23 ± 0.73^{ab}	0.015

CT: Control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; TCaP1: Treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; TCaP5: Treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; TCaP10: Treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-gamma-glutamyl transpeptidasee; BUN: Urea nitrogen; CREA: Creatinine; TBiLi: Total bilirubin.

Table 6. The effects of dietary supplementation of bio-based D-PA on the serum antioxidant parameters of Arbor Acres broilers during 1-42 days of age

Item	CT	TCaP1	TCaP5	TCaP10	p value
SOD (U/ml)	134.59 ± 8.68	124.26 ± 9.88	135.59 ± 10.17	108.38 ± 10.52	0.210
CAT (U/ml)	4.63 ± 0.44	5.13 ± 0.17	4.92 ± 0.20	4.85 ± 0.19	0.651
GSH-Px (U/ml)	193.42 ± 5.10	204.35 ± 2.77	195.28 ± 3.87	200.12 ± 4.72	0.281
GR (U/L)	72.35 ± 4.47	61.36 ± 9.60	56.00 ± 4.61	69.67 ± 6.15	0.295
MDA (nmol/ml)	4.00 ± 0.39	4.64 ± 0.44	4.03 ± 0.52	4.88 ± 0.37	0.406
T-AOC (mol/L)	0.59 ± 0.02	0.62 ± 0.03	0.58 ± 0.02	0.57 ± 0.01	0.485
NO (μmol/L)	4.29 ± 0.39	5.08 ± 0.15	4.97 ± 0.26	4.96 ± 0.65	0.514
OH (U/ml)	53.78 ± 1.62	51.64 ± 1.99	48.09 ± 2.29	48.46 ± 2.22	0.189

CT: Control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; TCaP1: Treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; TCaP5: Treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; TCaP10: Treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate; SOD: Superoxide dismutase; CAT: Catalase; GSH-Px: Glutathione peroxidase; GR: Glutathione reductase; MDA: Malondialdehyde; T-AOC: Total antioxidant capacity; OH: Hydroxy free radical scavenging activity.

Table 7. The effects of dietary supplementation of bio-based D-PA on the serum hormone parameters of broiler chickens during 1-42 days of age

Item	CT	TCaP1	TCaP5	TCaP10	p value
T3 (ng/ml)	0.50 ± 0.09	0.62 ± 0.07	0.60 ± 0.12	0.52 ± 0.09	0.736
T4 (ng/ml)	17.88 ± 3.11	20.08 ± 1.66	15.14 ± 1.88	16.87 ± 1.74	0.462
INS (MIμ/L)	8.87 ± 0.94	8.12 ± 0.46	8.86 ± 0.45	9.08 ± 0.84	0.789

CT: Control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; TCaP1: Treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; TCaP5: Treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; TCaP10: Treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate; T3/T4: Thyroid hormones; INS: Insulin.

Table 8. The effects of dietary supplementation of bio-based D-PA on the serum immune parameters of broiler chickens during 1-42 days of age

Item	CT	TCaP1	TcaP5	TcaP10	p value
TP (g/L)	29.58 ± 1.04	29.03 ± 0.83	29.58 ± 0.87	28.68 ± 0.91	0.873
ALB (g/L)	13.45 ± 0.63	13.17 ± 0.17	13.12 ± 0.28	13.40 ± 0.32	0.906
GLB (g/L)	16.13 ± 0.53	15.87 ± 0.79	16.47 ± 0.70	15.28 ± 0.67	0.660
A/G	0.83 ± 0.03	0.84 ± 0.04	0.80 ± 0.03	0.88 ± 0.03	0.373
IgG (g/L)	8.51 ± 0.59	7.02 ± 0.75	7.75 ± 0.53	7.31 ± 0.62	0.385
IgM (g/L)	1.06 ± 0.07	1.05 ± 0.06	1.06 ± 0.04	1.08 ± 0.06	0.971
IgA (g/L)	1.15 ± 0.07	1.08 ± 0.05	1.11 ± 0.04	1.16 ± 0.05	0.661
C3 (mg/dL)	31.83 ± 1.73	29.19 ± 2.13	32.36 ± 1.11	33.20 ± 2.08	0.451
C4 (mg/dL)	33.82 ± 2.35	31.65 ± 1.63	30.54 ± 1.79	34.53 ± 4.00	0.684
IL-1β (pg/ml)	36.05 ± 5.18^{a}	26.40 ± 0.88^{b}	37.20 ± 3.21^{ab}	28.01 ± 2.30^{ab}	0.031
TNF-α (pg/ml)	65.12 ± 3.06	68.58 ± 4.96	63.53 ± 4.79	63.53 ± 4.79	0.683
LZM (U/ml)	156.45 ± 10.65^{a}	178.48 ± 6.38^{a}	124.20 ± 7.80^{b}	110.75 ±1 0.53^{b}	<0.001

CT: Control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; TCaP1: Treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; TCaP5: Treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; TCaP10: Treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate; TP: Total protein; ALB: Albumin; GLB: Globulin; A/G: Albumin/globulin; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IgA: Immunoglobulin A; C3: Complement 3; C4: Complement 4; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor α; LZM: Lysozyme.

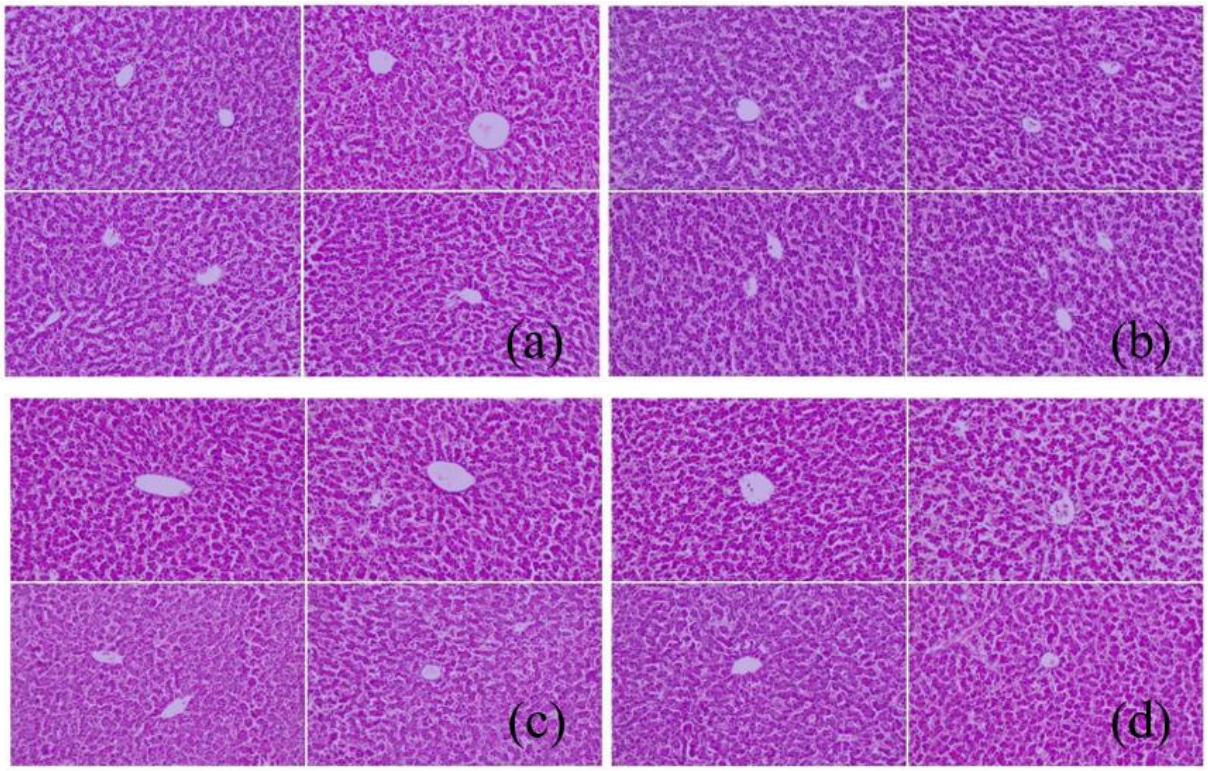

Figure 1. The effects of dietary supplementation of bio-based D-PA on the hepar morphology (×200) broiler chickens during 1-42 days of age. **a:** The control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; **b:** The treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; **c:** The treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; **d:** The treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate.

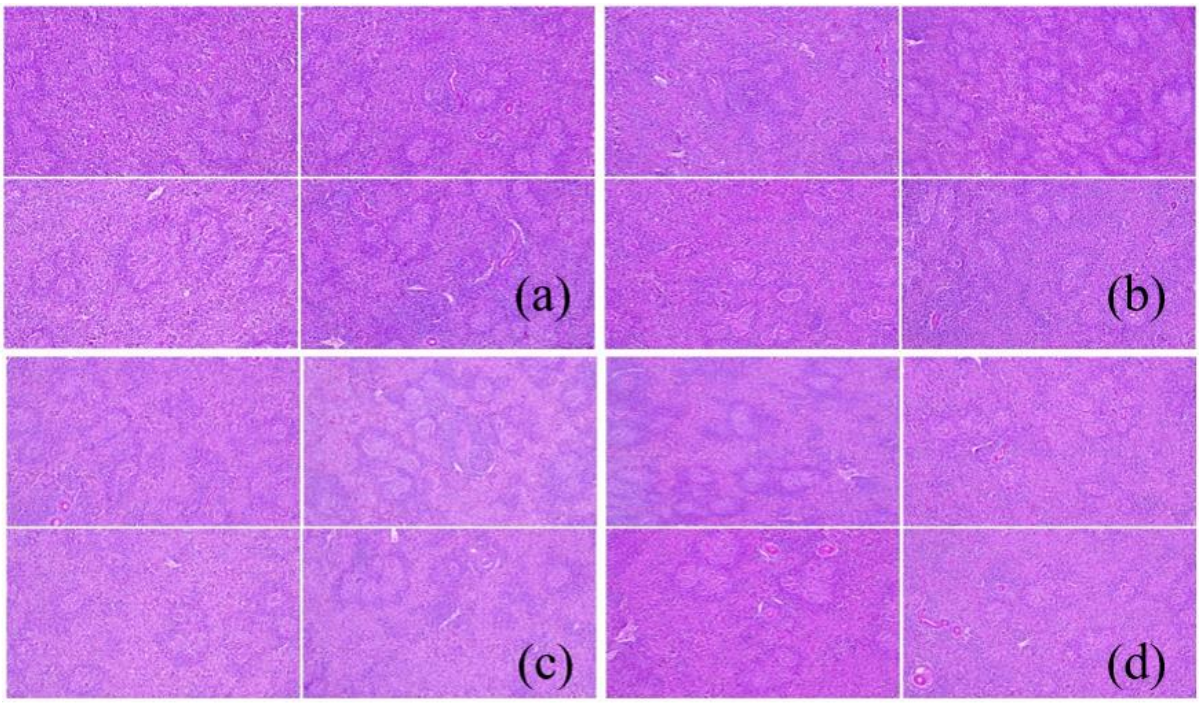

Figure 2. The effects of dietary supplementation of bio-based D-PA on the spleen morphology (×40) of broiler chickens during 1-42 days of age. **a:** The control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; **b:** The treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; **c:** The treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; **d:** The treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate.

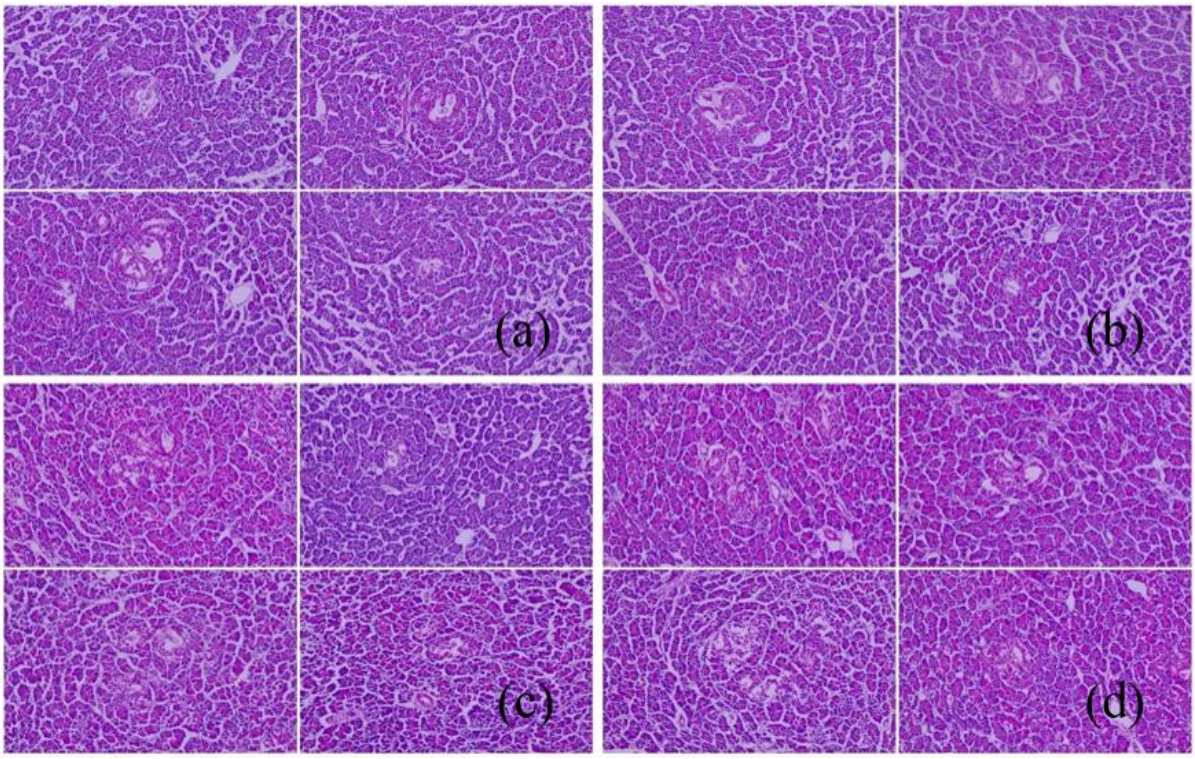

Figure 3. The effects of dietary supplementation of bio-based D-PA on the pancreas morphology(×200) of broiler chickens during 1-42 days of age. **a:** The control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; **b:** The treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; **c:** The treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; **d:** The treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate.

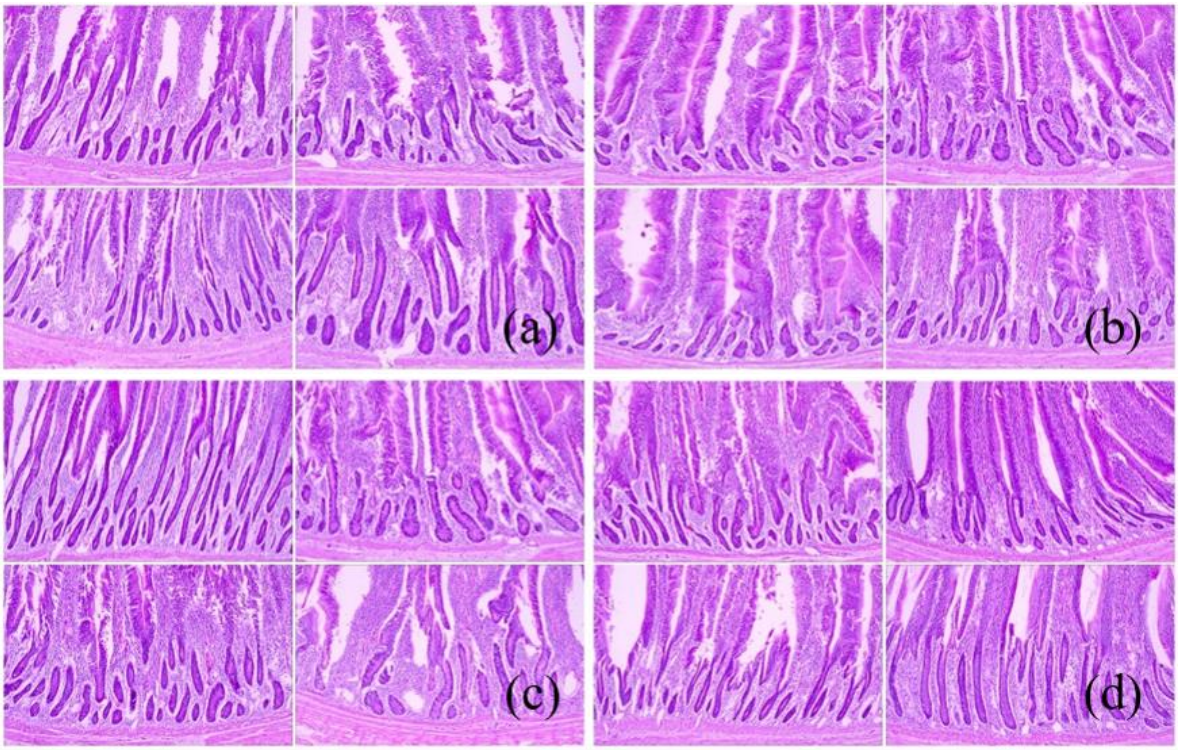

Figure 4. The effects of dietary supplementation of bio-based D-PA on the small intestine morphology (×100) of broiler chickens during 1-42 days of age. **a:** The control group supplemented with 20 mg/kg synthesized calcium D-pantothenate; **b:** The treatment supplemented with 20 mg/kg bio-based calcium D-pantothenate; **c:** The treatment supplemented with 100 mg/kg bio-based calcium D-pantothenate; **d:** The treatment supplemented with 200 mg/kg bio-based calcium D-pantothenate.

DISCUSSION

The chemical synthesis method is the predominant manufacturing route of commercial D-PA product; however, the common chemical synthesis method uses highly toxic raw materials and releases cyanide-containing wastewater. The deteriorating environment and energy deficiency encourage scientists to develop eco-friendly microbial fermentation methods (Postaru et al., 2015; Acevedo-Rocha et al., 2019). The studies available on the toxicity of chemically synthesized D-PA have shown that the redundant pantothenic acid was secreted through urine in about three hours, thus the chemically synthesized D-PA was safe for humans and animals (Spies et al., 1940; Shigeta et al. 1966), however, the safety of D-PA obtained from the microbial fermentation method has not been reported yet.

In the current study, dietary supplementation of 5-fold and 10-fold doses of D-PA obtained from the microbial fermentation method increased the ADG and F/G of broilers during 22-42 days of age and F/G during 1-42 days of age. Similar findings had been reported previously, where dietary deficiency of pantothenic acid led to poor growth performance, and supplementation with D-PA improved growth in broiler chickens, pullets, ducks, and fish (Lepkovsky et al., 1945; Beer et al., 1963; Southern and Baker; 1981; Qian et al., 2015; Tang et al., 2021). However, the optimal requirements of D-PA differed due to the animal species, feed content of pantothenic acid, and the health status of animals. There was no difference in growth performance between the CT group and the TCaP1 group in the current study, indicating that the bio-based D-PA and synthetic D-PA have the same effect of promoting growth.

The blood physiological and biochemical indicators can reflect the nutritional metabolism and health status of poultry, although they vary due to the health status of broilers (Siddon and Tormey, 2019). The former studies indicated that tissue pantothenic acid decreased when diet pantothenic acid deficiency occurred in fish and ducks, thus leading to other functional changes in organs (Qian et al., 2015; Tang et al., 2020a). Tang et al. (2021) reported that pantothenic acid deficiency in duck diets resulted in abnormal glucose metabolism and elevated uric acid content. The effects of dietary supplementation of D-PA on the blood physiological and serum biochemistry parameters, particularly using D-PA obtained from microbial fermentation, were rarely reported. In the present study, no dose-dependent regularity was observed for WBC, GRA, GRA%, LYM%, MID, MCV, RDW-SD, and MPV in different treatments. The serum antioxidant parameters and hormone parameters were not affected and histological observations of the liver, spleen, pancreas, and small intestines showed that the organs of broiler chickens were in the healthy status. Thus, the differences in blood physiological and biochemical changes may be caused by experimental errors and further studies are need to verify the reason of the blood physiological and biochemical changes. Organ index and histomorphology are important parameters that reflect the impact of different feed treatments on animal development and organ function status (Selim et al., 2021). Pantothenic acid deficiency could cause the metabolic disorders of carbohydrates, lipids, and proteins, thus may affect the organ development and function (Wang et al., 2016; Tang et al., 2021). In the present investigation, the tissue structures of the organs of broiler chickens from the test groups were clear, and no abnormal changes, such as inflammatory cell infiltration and fibrous tissue hyperplasia were observed, indicating that the 10-fold dose supplementation level of the novel bio-based D-PA was safe to broiler chickens. These results in the present study were in consensus with the former reports that pantothenic acid is a water-soluble vitamin that is difficult to store in its original form in the body and almost innocuous to animals (Spies et al., 1940; Shigeta et al. 1966; Wang et al., 2016). Moreover, dietary supplementation of D-PA can improve the growth performance and feather quality while decrease the dermatosis and mortality of poultry (Hegsted et al., 1949; Beer et al., 1963).

CONCLUSION

Under the condition of this experiment, the addition of a novel bio-based D-PA product produced by genetically engineered bacteria (*E. coli* K12) to the diet was safe at the supplementation level of 200 mg/kg without adverse effects on broiler chickens. The application of novel bio-based D-PA products could reduce environmental pollution and promote the growth performance of broilers. In general, the supplementation of the 10-fold recommended dose of D-PA in the diet showed no adverse effect on the growth performance and health status of broilers. Further studies are needed to verify the safety of the novel bio-based D-PA product on other animals.

DECLARATIONS

Authors' contributions

G.L. and X.X. designed the experiment, G.L. and C.L. conducted the experiment, H.Z. completed data analysis; L. Z. supplied resources, M.L. wrote the original draft manuscript, G.L. corrected the manuscript, L.Z. supervised the project. All authors have checked the collected, and analyzed data and agreed on the submission of this article.

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Availability of data and materials

The data that support the findings of this study are available on reasonable request from the corresponding author.

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Conflicts of interests

The authors declare no conflicts of interest.

Ethical considerations

The authors have avoided plagiarism, misconduct, data fabrication/falsification, and double submission/publication and have given consent to publish this article.

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