Postprint: Biosafety Evaluation of Pyrroloquinoline Quinone Disodium in Broiler Chickens

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Abstract

This experiment aimed to investigate the effects of dietary supplementation of different levels of pyrroloquinoline quinone disodium (PQQ.Na2) on growth performance, blood physiological and plasma biochemical indices, organ development and histological structure in Arbor Acres (AA) broiler chickens, thereby evaluating the biosafety of PQQ.Na2 in broilers. The experiment adopted a single-factor completely randomized design, in which 240 one-day-old AA broiler chickens were randomly allocated to 4 groups with 6 replicates per group and 10 chickens per replicate (5 males and 5 females). Each group was fed experimental diets containing PQQ.Na2 at supplementation levels of 0, 0.3, 1.5, and 3.0 mg/kg. The experimental period lasted 49 days. The results showed that dietary PQQ.Na2 supplementation level had no significant effects on growth performance, blood physiological and plasma biochemical indices of broilers at 21 and 49 days of age, as well as organ indices at 49 days of age ($P>0.05$), and did not induce histological structural changes in major organs. Therefore, when the highest effective supplementation level of PQQ.Na2 in corn-soybean meal diets for broilers is 0.3 mg/kg, it possesses a 10-fold safety margin and is safe for feeding to broiler chickens.

Full Text

Evaluation of Biological Safety of Pyrroloquinoline Quinone Disodium Salt for Broilers

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Abstract

This experiment was conducted to investigate the effects of dietary pyrroloquinoline quinone disodium salt (PQQ.Na) supplementation at various levels on growth performance, blood physiological parameters, plasma biochemical parameters, organ development, and histological structure in Arbor Acres (AA) broilers, thereby evaluating the biological safety of PQQ.Na for broiler chickens. A total of 240 one-day-old AA broilers were randomly allocated to four groups using a single-factor completely randomized design, with six replicates per group and ten birds per replicate (half male and half female). The experimental groups were fed diets supplemented with PQQ.Na at levels of 0, 0.3, 1.5, and 3.0 mg/kg for a duration of 49 days. The results demonstrated that dietary PQQ.Na supplementation levels had no significant effects on growth performance, blood physiological parameters, or plasma biochemical parameters at 21 and 49 days of age, nor on organ indices at 49 days of age ($P > 0.05$), and did not induce histopathological changes in major organs. Therefore, when the maximum effective supplemental level of PQQ.Na in corn-soybean meal diets for broilers is 0.3 mg/kg, it possesses a 10-fold safety margin and is safe for broiler feeding.

Keywords: pyrroloquinoline quinone disodium salt; biological safety evaluation; broilers

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Pyrroloquinoline quinone (PQQ), discovered in the 1960s, represents another redox enzyme cofactor following nicotinamide [nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP)] and riboflavin [flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)] [1]. PQQ is widely distributed in animals and plants; however, animals cannot synthesize it endogenously and must obtain it primarily through intestinal microbial synthesis and exogenous intake. Following ingestion, PQQ is absorbed in the small intestine and excreted via the kidneys. Exogenous PQQ is essential for maintaining normal physiological functions in animals. Research has revealed that PQQ promotes growth and enhances bacterial tolerance to toxicity and radiation [2-3], provides antioxidant stress protection [4], prevents liver injury [5], and regulates lipid metabolism [6]. Currently classified as a B-vitamin, PQQ is considered an essential nutritional factor for organisms, with pyrroloquinoline quinone disodium salt (PQQ.Na) being its primary form in production and application [7].

Ghirmay [8] observed that dietary supplementation with 0–0.8 mg/kg PQQ.Na in broiler diets improved feed conversion efficiency, slaughter performance at 42 days of age, and plasma antioxidant capacity while reducing plasma lipid peroxide content at 0.2 mg/kg, and enhanced plasma lysozyme activity at 0.1

mg/kg. Sun Limin [9] reported that appropriate dietary PQQ.Na supplementation (0.80 mg/kg) in laying hens increased egg production rate, enhanced antioxidant capacity in both hens and eggs, and showed a trend toward reducing egg cholesterol content. However, current research on PQQ.Na has primarily focused on improving animal growth performance and antioxidant capacity, while safety evaluations have been concentrated on rats and humans [10-11], with no reports on safety assessments for broiler chickens. This study evaluated the effects of dietary PQQ.Na supplementation levels in corn-soybean meal diets on 1–21-day-old and 22–49-day-old broilers by measuring growth performance, blood physiological and plasma biochemical parameters, organ indices, and histological structure changes. The objective was to provide a scientific basis for determining the safety margin of the maximum supplemental level of PQQ.Na in broiler diets to ensure its safe application in broiler production.

1. Materials and Methods

1.1 Experimental Material

The PQQ.Na product was provided by Shanghai Youbeifu Biotechnology Co., Ltd., with an effective PQQ.Na content exceeding 99%. The PQQ.Na added to experimental diets was in the form of a 0.1% premix product.

1.2 Experimental Design

This experiment employed a single-factor completely randomized design. Three hundred healthy commercial one-day-old Arbor Acres (AA) broilers (half male and half female) were purchased from Beijing Huadu Broiler Company. From these, 240 birds were selected based on body weight and sex and randomly divided into four groups, with six replicates per group and ten birds per replicate (half male and half female).

Based on our laboratory's previous research on the biological efficacy of PQQ.Na in broilers, the appropriate supplemental level of PQQ.Na in corn-soybean meal diets for broilers was determined to be 0.1–0.5 mg/kg. The level of 0.1 mg/kg was considered the minimum effective recommended supplemental level for broiler diets. According to the relevant provisions in the "Guidelines for the Evaluation of Feed and Feed Additive Tolerance in Livestock Target Animals (Trial)" issued by the Ministry of Agriculture, four experimental groups were established: a control group without PQQ.Na supplementation, a group receiving the maximum effective supplemental level (three times the minimum effective recommended level), and two high-level supplementation groups at five and ten times the maximum effective level. Thus, the four dietary PQQ.Na supplemental levels were 0, 0.3, 1.5, and 3.0 mg/kg. The experimental period lasted 49 days and was divided into two phases: 1–21 days of age and 22–49 days of age.

1.3 Experimental Diets

Basal corn-soybean meal diets for the 1–21 days and 22–49 days phases were formulated according to the nutrient recommendations for broilers in the NRC (1994) [12]. The composition and nutrient levels of the basal diets are presented in Table 1. Based on the experimental design, four experimental diets were prepared for each phase by adjusting the amount of cornstarch in the basal diets. All experimental diets were fed to birds in mash form.

1.4 Management Practices

Management practices and routine vaccinations were conducted according to the *AA Broiler Management Manual*. Birds had ad libitum access to feed and water. Health status was observed and recorded daily throughout the experimental period. Any dead birds were immediately necropsied to observe and analyze pathological causes, and feed was weighed accordingly. Body weight and feed remaining were measured by replicate (cage) on days 21 and 49 of the experimental period to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed-to-gain ratio (F/G).

1.5 Sample Collection and Preparation

Prior to diet preparation, samples of corn and soybean meal feed ingredients were collected to analyze crude protein and calcium content, ensuring that the key nutrient contents in the formulated diets matched the formula values as closely as possible. During diet preparation, samples were collected on-site, reduced using the quartering method, ground to pass through a 200-mesh sieve, and stored in self-sealing bags under low-temperature dry conditions for subsequent analysis of dietary crude protein and calcium content.

On days 21 and 49 of the experimental period, all birds were fasted overnight (with water provided). At 08:00 the following day, individual live body weight of each bird was measured after fasting. From each replicate cage, two birds (one male and one female) were selected based on the average body weight of the cage, and 10–15 mL of blood was collected from the wing vein. The first aliquot of anticoagulated blood was used to determine routine blood physiological parameters. The second aliquot of anticoagulated blood was centrifuged at 3,000 r/min for 10 minutes to separate plasma, which was aliquoted and stored at -20 °C for subsequent plasma biochemical analysis. Whole blood or plasma samples from the two birds in each replicate cage were pooled in equal proportions to form one sample for analysis.

After blood collection on day 49, all sampled birds were slaughtered. The thymus, bursa of Fabricius, spleen, pancreas, heart, lungs, liver, kidneys, proventriculus, and gizzard were excised. Additionally, the contents of the duodenum, jejunum, and ileum were removed, and these organs were weighed separately to calculate organ indices. The lengths of the duodenum, jejunum, and ileum

were measured to calculate the length indices of each small intestinal segment. Morphological changes in each organ were observed and recorded.

Organ index (%) = (organ weight / live body weight) × 100
Small intestinal segment length index (%) = (length of each segment / total small intestine length) × 100

After weighing the organs, from each group, one male from each of three replicate cages and one female from each of the other three replicate cages were selected. Portions of the liver, kidneys, heart, lungs, and spleen were collected from these birds and fixed in brown bottles containing 4% formalin solution for subsequent sectioning and histological structure observation.

1.6 Laboratory Analyses

1.6.1 Analysis of Feed Ingredients and Diet Samples Samples were digested using wet digestion with concentrated nitric acid and perchloric acid. Calcium content in feed ingredients and diets was determined using an IRIS Intrepid II plasma emission spectrometer (TE, USA). Total phosphorus content in calcium hydrogen phosphate was determined using a colorimetric method [13]. Crude protein content in diets was determined using the Kjeldahl method [14].

1.6.2 Analysis of Whole Blood and Plasma Parameters Blood routine physiological parameters (hemoglobin content, hematocrit, etc.) were determined using a KX-21 automatic blood cell analyzer (SYSMEX, Japan). Plasma biochemical parameters (aspartate aminotransferase, alanine aminotransferase, creatine phosphokinase, alkaline phosphatase activities, and uric acid nitrogen, total protein, albumin, glucose, total bilirubin, creatinine, total cholesterol, and triglyceride contents) were determined using a TBA-40FR automatic biochemical analyzer (Toshiba, Japan).

1.5.3 Histological Examination Histological examination was performed according to methods reported by Deng et al. [15] and Ashraf et al. [16]. The main procedures were as follows: fixed specimens were processed through washing, clearing, paraffin infiltration, and embedding to prepare 5 m sections. After hematoxylin-eosin (HE) staining, histomorphological changes in the liver, kidneys, heart, lungs, and spleen were observed under a microscope, and corresponding tissue images were captured using an image acquisition system.

1.7 Statistical Analysis

All data were analyzed using the General Linear Model (GLM) procedure in SAS 9.0 software [17]. When significant differences were detected, the Least Significant Difference (LSD) method was used to compare differences among groups. Data are presented as means, with 0.05 used as the significance level for all statistical tests in this study.

2. Results

2.1 Effects of Dietary PQQ.Na Supplemental Level on Broiler Growth Performance

As shown in Table 2 , dietary PQQ.Na supplemental level had no significant effects on ADG, ADFI, or F/G of broilers during 1-21 days, 22-49 days, or 1-49 days of age ($P > 0.05$). These results indicate that under the conditions of this experiment, dietary supplementation with different levels of PQQ.Na had no adverse effects on broiler growth performance.

2.2 Effects of Dietary PQQ.Na Supplemental Level on Broiler Blood Physiological Parameters

As shown in Table 3 , dietary PQQ.Na supplemental level had no significant effects on blood hemoglobin content, hematocrit, white blood cell count, or red blood cell count in broilers at 21 or 49 days of age ($P > 0.05$). These results indicate that under the conditions of this experiment, dietary supplementation with different levels of PQQ.Na had no adverse effects on broiler blood physiological parameters.

2.3 Effects of Dietary PQQ.Na Supplemental Level on Broiler Plasma Biochemical Parameters

As shown in Table 4 , dietary PQQ.Na supplemental level had no significant effects on any plasma biochemical parameters in broilers at 21 or 49 days of age ($P > 0.05$). These results indicate that under the conditions of this experiment, dietary supplementation with different levels of PQQ.Na had no adverse effects on broiler plasma biochemical characteristics.

2.4 Effects of Dietary PQQ.Na Supplemental Level on Organ Indices of 49-Day-Old Broilers

As shown in Table 5 , dietary PQQ.Na supplemental level had no significant effects on any organ indices of 49-day-old broilers ($P > 0.05$), and no abnormal morphological changes were observed in any organs. These results indicate that under the conditions of this experiment, dietary supplementation with different levels of PQQ.Na did not affect the development of internal organs in broilers.

2.5 Effects of Dietary PQQ.Na Supplemental Level on Histological Structure of Major Organs in Broilers

As shown in Figure 1 [Figure 1: see original paper], no histological structural changes were observed in the hearts of 49-day-old broilers across all groups. All groups exhibited smooth epicardium; myocardial cells appeared as short columns with tight, orderly arrangement and uniform staining; intercalated discs and transverse striations showed no inflammatory cell infiltration; and nuclei were round or oval-shaped, centrally located within muscle cells.

As shown in Figure 2 [Figure 2: see original paper], no histological structural changes were observed in the livers of 49-day-old broilers across all groups. All groups exhibited regularly structured hepatic lobules with polygonal prism morphology; hepatocytes were radially arranged around central veins with clear boundaries and uniform reticular structure; and hepatic cords and sinusoids were intact and clearly defined.

As shown in Figure 3 [Figure 3: see original paper], no histological structural changes were observed in the spleens of 49-day-old broilers across all groups. All groups exhibited clear and complete splenic structure with no changes in the number or diameter of splenic nodules; no differences were observed in central artery diameter or periarterial lymphatic sheath thickness.

As shown in Figure 4 [Figure 4: see original paper], no histological structural changes were observed in the lungs of 49-day-old broilers across all groups. Lungs in all groups appeared bright red and were covered by serous membrane; tertiary bronchial epithelial cells showed no inflammatory exudate, with surface microvilli of uniform thickness and distribution; lymphocytes within atrial septa formed lymphoid nodules; respiratory capillaries were uniformly sized with no foreign matter in the lumen and smooth epithelial cell surfaces; and capillaries surrounding respiratory capillaries were abundant.

As shown in Figure 5 [Figure 5: see original paper], no histological structural changes were observed in the kidneys of 49-day-old broilers across all groups. All groups exhibited glomeruli in the cortical region that were spherical with moderate volume and normal structure, surrounded by clear Bowman's capsules with distinct capsular spaces; renal tubular epithelial cells showed normal structure with homogeneous red cytoplasmic staining; and collecting duct epithelial cells in medullary bodies exhibited relatively homogeneous cytoplasm.

These results demonstrate that under the conditions of this experiment, dietary supplementation with PQQ.Na at various levels did not induce histological structural changes in the microscopic structure or morphology of major organs in broilers.

3. Discussion

3.1 Effects of Dietary PQQ.Na Supplemental Level on Broiler Growth Performance

Research has shown that PQQ functions similarly to vitamins [18], and its growth-promoting effects may be related to enhanced expression of RSA genes involved in growth and developmental signal transduction [19], making it an essential nutritional factor for animal growth, development, and reproduction. Zhang et al. [7] investigated three dietary treatments for broilers: oxidized duck oil, fresh duck oil, and oxidized duck oil supplemented with 0.4 mg/kg PQQ.Na , finding that PQQ.Na supplementation tended to improve growth performance in the oxidized duck oil group, though not significantly. Wang et al. [20] re-

ported that dietary supplementation with 0–0.4 mg/kg PQQ.Na had no significant effects on broiler growth performance during early, late, or overall periods. Samuel et al. [21] obtained similar results when supplementing broiler diets with 0–0.8 mg/kg PQQ.Na . The present study found that dietary PQQ.Na supplemental levels had no significant effects on broiler growth performance, consistent with previous research, indicating that dietary PQQ.Na supplementation had no adverse effects on broiler growth under our experimental conditions.

3.2 Effects of Dietary PQQ.Na Supplemental Level on Broiler Blood Physiological Parameters

Blood hemoglobin content and hematocrit in animals can serve as indicators of overall health status. The present study found that dietary PQQ.Na supplemental levels had no significant effects on hemoglobin content, hematocrit, or other blood parameters in broilers. Wang et al. [22] administered PQQ at high, medium, and low doses (3.8, 7.5, and 15.0 mg/kg, equivalent to 300 times the human recommended dose) to mice via gavage for 90 days and observed no significant differences compared to the blank control group, consistent with our findings. These results indicate that dietary PQQ.Na supplementation had no adverse effects on broiler blood physiological parameters under our experimental conditions.

3.3 Effects of Dietary PQQ.Na Supplemental Level on Broiler Plasma Biochemical Parameters

Plasma total protein and albumin contents can reflect protein synthesis and metabolism status in the body. Plasma alanine aminotransferase and aspartate aminotransferase are important indicators of liver function; when liver damage occurs, plasma activities of these enzymes increase. Plasma creatine kinase activity reflects the level of myocardial cell damage. Alkaline phosphatase, primarily present in bone and liver, dephosphorylates substrates to produce phosphate, which then combines with calcium for bone deposition; alkaline phosphatase activity increases when liver or osteoblast damage occurs [23]. The present study found that dietary PQQ.Na supplemental levels had no significant effects on plasma biochemical parameters including alanine aminotransferase, aspartate aminotransferase, creatine kinase, alkaline phosphatase activities, and total protein, creatinine, total bilirubin, and glucose contents. These results are similar to those reported by Sun [9] and Wang et al. [20], indicating that dietary PQQ.Na supplementation had no adverse effects on broiler plasma biochemical parameters.

3.4 Effects of Dietary PQQ.Na Supplemental Level on Organ Indices of 49-Day-Old Broilers

Immune organ indices and digestive organ indices are important reference indicators for evaluating animal immune and digestive functions. The present study found that dietary PQQ.Na supplemental levels had no significant effects on

immune organ indices or digestive organ indices in broilers. Samuel et al. [21] also reported that dietary PQQ.Na levels did not affect relevant immune organ indices (thymus, spleen, and bursa of Fabricius) or lysozyme activity in broilers. These results indicate that dietary PQQ.Na supplementation does not affect the development of major internal organs in broilers.

3.5 Effects of Dietary PQQ.Na Supplemental Level on Histological Structure of Major Organs in Broilers

Organ histological structure is an important indicator of animal metabolism. In this experiment, dietary supplementation with different levels of PQQ.Na did not induce histological structural changes in the microscopic structure or morphology of major organs such as the heart in broilers. Wang et al. [22] administered three levels of PQQ (3.8, 7.5, and 15.0 mg/kg) to mice via gavage, with the control group receiving a normal diet at a gavage volume of 10 mL/kg body weight, and observed only minor changes in mouse organs without significant histopathological lesions. Zhao [24] reported that treating 30-week-old Hy-Line Brown laying hen liver cells with 50–400 nmol/L PQQ.Na did not significantly affect cell survival rates compared to normal controls, indicating that 50–400 nmol/L PQQ.Na did not produce toxic effects on primary cultured chicken liver cells in vitro. These findings are generally consistent with our results, indicating that dietary PQQ.Na supplementation does not cause structural changes in important tissues of broilers.

The present study demonstrated that dietary supplementation with 0, 0.3, 1.5, and 3.0 mg/kg PQQ.Na did not adversely affect broiler growth performance, plasma biochemical parameters, blood physiological parameters, or the development of immune organs and major organs. Therefore, we recommend that the maximum effective supplemental level of PQQ.Na in broiler diets be 0.3 mg/kg, which provides a 10-fold safety margin and is safe for broiler feeding. Some studies have shown that PQQ, as a growth factor, can activate transcription proteins and transcription factors important for cell growth, proliferation, and differentiation, thereby promoting animal growth [25]. However, this experiment found that dietary PQQ.Na supplementation did not affect broiler growth performance, possibly due to differences in animal breeds and environmental conditions, as well as inherent tolerance in animals. Therefore, at the supplementation levels used in this experiment, PQQ.Na did not produce effects on various broiler parameters.

4. Conclusion

Dietary supplementation with 0–3.0 mg/kg PQQ.Na had no adverse effects on broiler growth performance, blood routine parameters, plasma biochemical parameters, or major organ development, and no histopathological changes were observed in major internal organs. No toxic reactions occurred in any birds. Therefore, we recommend that the maximum effective supplemental level of

PQQ.Na in broiler diets be 0.3 mg/kg, which provides a 10-fold safety margin and is safe for broiler feeding.

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