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Effect of dietary folic acid and energy density on immune response, gut morphology, and oxidative status in blood and breast muscle of broiler chickens

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Abstract

Folic acid (FA) plays an essential role in the functioning of the gut and serves as an antioxidant. Therefore, dietary supplementation with high levels of FA may improve gut health and prevent oxidative stress caused by feeding a high energy density diet to broiler chickens.

Broiler chickens were assigned into eight treatments, consisting of either a normal energy (NE) or high energy (HE) density diet, and four FA levels (2.2, 5, 10, and 15 ppm). Data were analyzed by SAS 16 GLM procedure. Birds fed HE diets had increased ($P<0.05$) plasma concentrations of calcium and albumin but reduced ($P<0.005$) weights of ceca and bursa compared to those fed NE diets. Dietary supplementation with 10ppm FA significantly increased ($P<0.05$) birds' heart weight and bile acid concentration. FA and energy density interactions were significant for jejunal villus height (VH; $P=0.0226$), villus width (VW; $P<0.0001$), and crypt depth (CD; $P=0.0332$). Among the NE group, birds fed 5-15ppm FA had reduced ($P<0.0001$) VW, while in the HE groups, 15ppm FA supplementation resulted in an increased jejunal VH ($P=0.0317$) compared to other treatments. In conclusion, dietary supplementation with increased levels of FA in HE diets could be beneficial for the intestinal health of broiler chickens.

Key Words: Folic acid, energy density, blood biochemistry, jejunal morphology, broiler chickens

INTRODUCTION

Folic acid (FA) is a synthetic form of folate with higher bioavailability than its natural counterpart (Iyer and Tomar 2009). Folate (Vitamin B9) is an essential water-soluble vitamin for a number of metabolic functions, including DNA/RNA synthesis and the provision of methyl groups in numerous reactions (Rossi et al. 2016). It plays a crucial role in the immune system of broiler chickens and is vital for red blood cells and many other cells in the body (Cianciulli et al. 2016). Folate deficiency has long been associated with congenital abnormalities, particularly neural tube defects and neurocristopathies (Melo et al. 2017). The FA requirements of broilers were estimated many years ago to be between 1.3 and 3 mg/kg (Whitehead et al. 1995; Ryu et al. 1995). However, this requirement is likely underestimated in fast-growing and high-breast yield chickens. The effect of FA deficiency on humoral immunity has been widely investigated in animals (Balaghi and Wagner 1992; Balaghi et al. 1993). Dhur et al. (1991) reported that the blastogenic response of T lymphocytes to certain mitogens is decreased, and the thymus is preferentially altered in FA-deficient humans and animals. Nouri et al. (2018) reported that blood glucose and phosphorous levels increased while cholesterol, triglyceride, high-density lipoprotein, and low-density lipoprotein, calcium, and alkaline phosphatase decreased after *in ovo* injection of FA to broiler chicken eggs and subsequently hatched and raised for 42 days. Li et al. (2016) reported that supplemental FA improved the growth performance and folate metabolism of broilers and enhanced their immunoglobulins (IgG and IgM) concentrations. Folic acid also enhanced the relationship between immune function and epigenetic regulation of immune genes (Li et al. 2016). A recent study by Wang et al. (2020) revealed that dietary FA significantly increased villus height, villus height: crypt depth, and villus surface area in the jejunal mucosa of weaned piglets. Munyaka

et al. (2012; 2013) reported that FA modulated immune responses in young laying hens subjected to acute immunological challenge. Folic acid generally has been regarded as a nontoxic vitamin (NRC 1987), and Leeson and Summers (2001) reported that birds are very tolerant to high levels of FA, with up to 5,000 times normal intake being needed to induce toxicity. Meanwhile, few or no studies have investigated the effect of dietary FA and energy density on immune response and antioxidant capacity in broiler chickens.

Studies with different animal species have shown that a high energy density diet can cause oxidative stress, compromise the immune system and gut functionality (Adebowale et al. 2019; Liu et al. 2018; Minaya et al. 2020). Oxidative stress in the blood, cells, or tissues can be dangerous to animals because it can impair normal cellular function, promote tissue damage and lead to lipid peroxidation (Akbarian et al. 2016). Animals experienced oxidative stress when there is an imbalance between excessive reactive oxygen species (ROS) and endogenous antioxidant defense (Schieber and Chandel 2014). The intestinal mucosa has different forms of antioxidant defense, which can be enzymatic, e.g., catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), or non-enzymatic (transient forms) (Circu and Aw 2012). Antioxidant enzymes such as CAT and SOD play important roles in the eradication of ROS. The activities of antioxidant enzymes are bound to change when ROS production level surpasses the capacity of the antioxidant defense system (Lobo et al. 2010). Large production of ROS results in lipid peroxidation, for which malondialdehyde (MDA) is an important indicator. Many literature have discussed extensively the oxidative response of animals when exposed to a high energy density diet. Liang et al. (2020) reported that serum GPX activity and total antioxidant capacity (T-AOC) and liver SOD and CAT significantly decreased in lambs fed a high energy density diet compared to those fed a normal diet. Also, a high energy density diet significantly increased MDA and ROS in lambs (Liang et al.

2020). Che et al. (2019) showed a similar result and observed increased plasma concentration of MDA in sows fed with a high energy density diet. Moreover, a high energy density diet significantly increased serum MDA and decreased serum T-AOC of pigeons compared to those fed a lower energy density diet (Hosseinian and Hasanzadeh 2021). Wang et al. (2010) reported that high energy density diets might negatively affect the anti-oxidative status and subsequent performance of cows. Meat quality is associated with oxidative reactions; thus the levels of MDA, T-AOC, and activities of SOD and GPX are indicators of meat quality (Wen et al. 2017). Broiler chickens are more susceptible to oxidative stress due to the genetic selection for large breast muscles and fast growth (Estévez 2015). Previous studies affirmed that oxidative stress could have negative impacts on meat quality and cause myopathies such as wooden breast, color defects, and white striping in broiler breast muscles (Abasht et al. 2016; Salles et al. 2019). The effect of dietary FA and HE density diet on oxidative status in breast muscle of broiler chickens are rarely studied.

While numerous studies have investigated the effects of dietary energy in broiler chickens (Bouvarel et al. 2008; Ale Saheb Fosoul et al. 2016; Papadopoulos et al. 2018), research that examined the ameliorative effects of supplemental FA on immune response, blood biochemical parameters, and intestinal functionality in broiler chickens are uncommon. Thus, whether FA supplementation can mitigate the oxidative stress and immune systems damage caused by feeding a high energy density diet remain poorly understood. The objective of the current study was to determine the effect of increasing levels of FA on blood biochemical parameters, immunoglobulins (IgG and IgM) concentrations, jejunal morphology, immune organ weights, and indicators of oxidative stress (SOD and MDA) in blood and breast muscle of broiler chickens fed normal or high energy density diets.

MATERIALS AND METHODS

Management and Housing

Day-old mixed-sex broiler chickens (Ross 308) were obtained from Atlantic Poultry Incorporated, Port Williams, Nova Scotia. Upon arrival at the Atlantic Poultry Research Center, Dalhousie University Faculty of Agriculture, Truro, NS, chicks were weighed in groups of 25 birds and assigned to floor pens (0.93 m x 2.14 m), at a stocking density of 0.076 m²/bird. Room temperature was monitored daily and was gradually reduced from 30 to 22.6°C from d 0 to 42. The lighting program was set to produce 18 hours of light and 6 hours of darkness throughout the experimental period, and illumination was gradually reduced from 20 lx on d 0 to 5 lx. Broilers were placed in environmentally controlled rooms with the same conditions set for temperature, humidity, light scheduling, and bird density. All chickens were handled and cared for according to the recommendations of the Canadian Council of Animal Care (CCAC 2009) and the experimental methods for this research were approved by Dalhousie University Animal Use and Care Committee (Protocol number 2019-008).

Experimental Diets

Broiler chickens were raised on a phase-feeding program, which consisted of starter (0-14 days), grower (15-24 days), and finisher (25-42 days) and received feed and water *ad libitum*. Diets were fed as crumbled pellets during the starter phase and as pellets during the grower and finisher phases. Birds were randomly sorted into eight dietary treatments, consisting of eight replicate pens per treatment. The experiment was designed as a 2 x 4 factorial design consisting of two levels of dietary energy: 1) Normal (NE) and 2) High energy density (HE) and four levels of supplemental FA (2.2, 5, 10, and 15 ppm) The basal diets were formulated in such a way as to meet typical industry nutrient standards, as suggested by Kuttapan et al. (2012), in the case of Vitamin E

supplementation. The ingredient and chemical compositions of the NE and HE diets used in the study and the procedures for diets' chemical analyses have been previously reported by Adewole and Akinyemi (2021). The HE diets were formulated to contain an additional 100 Kcal/kg ME above the NE diets, and all diets were formulated to have a similar ratio of metabolizable energy (ME) to crude protein and digestible amino acids (Adewole and Akinyemi 2021). The FA inclusion levels were determined based on past studies with laying hens and rats (Munyaka et al. 2013; Zhao et al. 2013). The FA premix was included in the feed formulation at a set amount to achieve 2.2, 5, 10, and 15 ppm in the diets.

Sample Collection and Analyses

The growth performance aspect of this study has been previously published (Adewole et al., 2021). On d 36, 8 birds/treatment were euthanized by electrical stunning, and exsanguination and plasma samples were collected from one male chicken/pen (i.e., 8 chickens per treatment) in 10 mL heparinized tubes for the determination of blood biochemical parameters. A 1.5 cm tissue section was collected from the middle of the jejunum for histological measurements. On d 42 of age, one male chicken per pen was randomly selected and euthanized by electrical stunning and exsanguination. Five mL of blood was collected into each of a 10 mL heparinized tube and 10 mL serum vacutainer tubes. The weights of the spleen, heart, bursa, liver, and ceca were determined. A 1 cm³ tissue was collected from the cranial part of the right breast muscle and preserved in 2 ml microcentrifuge tubes, and stored at -80°C.

Blood biochemistry analysis. Samples for blood biochemical analysis were centrifuged at 5000 rpm for 10 minutes and shipped on ice to Atlantic Veterinary College, University of Prince Edward Island Pathology Laboratory, where samples were analyzed using cobas® 6000 analyzer series (Roche Diagnostics, IN, USA).

ELISA analysis

Serum immunoglobulins (IgG and IgM) were determined using specific ELISA kits (Bethyl Laboratories, Inc. - catalog number E33-104 (IgG) and E33-102 (IgM); Cedarlane Laboratories). Superoxide dismutase and MDA in plasma and breast muscle tissue samples were determined using Biovision SOD activity kit (Catalog number K335-100; Cedarlane Laboratories) and Biovision MDA assay kit (Catalog number K739-100; Cedarlane Laboratories).

Jejunal morphology measurements

The collected jejunum samples were fixed in formalin, then processed as previously described by Oladokun et al. (2021). To access the villus height (VH), villus width (VW) and crypt depths (CD), measurements were made from the base of the intestinal mucosa to the villus tip, excluding the intestinal crypt for the VH, halfway between the base and the tip for the VW, and from the base upward to the region of transition between the crypt and villi for the CD. The ratio of VH to CD was also determined by measuring the total mucosa thickness. Ten measurements of each component per slide using an image processing and analysis system were carried out (Leica DC480; Leica Microsystems Imaging Solutions Ltd., Concord, ON, Canada).

Statistical analyses

The chicken was the experimental unit for organ weight, blood, and tissue parameters. Data were subjected to analysis of variance using the GLM procedure of SAS with FA level, energy level, and energy level x FA interaction as factors and the following parameters as variables: organ weights, blood biochemistry parameters, IgG, IgM, SOD, and MDA. A normal probability plot of residuals was used to ascertain the normality of all datasets in the same statistical package. Datasets found to be non-normal were thus log base ten transformed. Orthogonal and polynomial contrast

statements were used to determine linear, quadratic, and cubic effects of FA supplementation. P-values less than 0.05 were considered significant. Significant means were separated using Tukey's procedure.

RESULTS

Blood biochemistry parameters

The blood biochemistry parameters of broiler chickens fed varying levels of FA at different dietary energy density levels were presented in Table 1. There were no significant effects of FA and dietary energy on electrolytes and minerals except the significant increase ($P < 0.05$) in calcium and albumin contents in birds fed HE density diet. From the metabolite's profile, we observed a significant increase ($P < 0.05$) in bile acid concentration at 10 ppm FA supplementation and a significant linear increase in aspartate aminotransferase (AST) with FA increase. Creatine kinase (CK) also increased, although not statistically significant, with increasing FA inclusions.

Organ weights, immunoglobulin concentrations, and indicators of oxidative stress

There was no interaction between FA and energy level on serum IgG and serum IgM. Also, no significant effects of FA or energy level were observed for serum IgG and IgM (Table 2). As illustrated in Table 2, there was no effect of FA and energy density on SOD and MDA concentrations in plasma and breast muscle. We examined the effect of FA fed at NE and HE levels on relative organ weights of broiler chickens. There was no interaction between FA and energy level on the weights of the spleen, ceca, and heart, but there was a significant interaction ($P = 0.0016$) between FA and energy density for bursa weight. Compared to the NE group, birds fed a HE diet had significantly reduced ($P < 0.05$) ceca (16.3%) and bursa (10.8%) weights. A

quadratic increase ($P=0.0496$) in bursa weight and a cubic increase ($P=0.0012$) in heart weight were observed with increasing FA levels (Table 3).

Intestinal morphology

The effect of FA fed at different energy levels on jejunal morphology of broiler chickens are presented in Table 4. Results on jejunal morphology showed significant FA and energy density interactions for villus height (VH; $P=0.0226$), width (VW; $P<0.0001$) and crypt depth (CD; $P=0.0332$). Among the NE group, birds fed 5-15 ppm FA had reduced ($P<0.0001$) VW compared to those fed the basal diet. However, among the HE group, 15 ppm FA supplementation resulted in an increased VH ($P=0.0317$) and crypt depth ($P=0.0002$), compared to the basal diet. In addition, a linear increase in VH was observed as FA inclusion level increased. Aside from this, a quadratic reduction in VW was observed as FA inclusion level increased. There was no significant interaction between FA and energy density for VH:CD. However, birds fed 10 ppm FA had the highest VH:CD, while the birds fed with 5ppm FA had the lowest value. Because of the significant interactions, the effects of FA levels on jejunal morphology were presented separately for NE and HE levels in Table 4. It was observed that the effect of FA was more prominent when chickens were fed HE diets compared to when they were fed NE diets.

DISCUSSION

Folic acid is an essential vitamin for many metabolic functions including immune response modulation and antioxidation and can promote gastrointestinal health (Wang et al. 2021a). Previous studies have revealed the importance of FA in piglets (Liu et al. 2011; Wang et al. 2021b), fish (Firouz et al. 2013), rats (Bagnyukova et al. 2008) in terms of blood biochemical characteristics, gut morphology, and indicators of oxidative stress. Whereas the effects of FA on

blood biochemical parameters, immunoglobulins concentrations, gut morphology, immune organ weights, and indicators of oxidative stress in blood and breast muscle of broiler chickens fed NE or HE density diets are rarely studied. The current research proved the importance of FA, on immune and health status of broiler chickens in response to dietary energy density.

Blood biochemical parameters are indicators of health status, changes in the metabolic profile, growth, development, and production level in broiler chickens (Piotrowska et al. 2011). Biochemical parameters such as minerals, proteins, enzymes, metabolites have been reported to be influenced by age, breed, sex (Arzour-Lakehal et al. 2015; Obese et al. 2018), nutrition, season, and geographical location. In the current study, plasma bile acid concentration increased with increasing FA levels and it was significantly higher in 10ppm FA group compared to birds fed basal diet. In an attempt to help facilitate the absorption of dietary lipids and fat-soluble vitamins, the liver converts cholesterol into bile acids, thus reducing plasma cholesterol levels (Boesjes and Brufau 2014; Xu 2016). Bile lipids compositions in poultry are mainly triglycerides and cholesterol esters (Sarikhani et al. 2009). This observation is consistent with the discoveries of Liu et al. (2018) that dietary FA promoted triglycerides hydrolysis. It has been indicated that bile acids can treat lipid metabolism-related diseases, such as hyperlipidemia, dyslipidemia, and non-alcoholic fatty liver disease (Trauner et al. 2010; Fuchs et al. 2016; Salvoza et al. 2016). Ge et al. (2019) demonstrated that bile acids improved chicken meat quality by serving as a dietary emulsifier. In a human study, Prinz et al. (2015) showed total plasma bile acids to be higher in obese compared to normal weight and anorexic subjects and thus are believed to play a role in the mediation of energy expenditure. In our previous report on the effect of FA on white striping (WS) in broiler chickens, it was observed that FA reduced the occurrence of WS in female broiler chickens (Adewole et al. 2021). One of the mechanisms by which FA reduced the incidence of WS could

be traceable to the reduction of blood cholesterol via hepatic conversion to bile acids. On a different metabolic point of view, Liu et al. (2019) reported that FA reduced abdominal fat percentage by decreasing hepatic lipogenesis and suppressing adipocytes proliferation and differentiation. It is possible that the effect of FA on the fillet fat content and therefore, on the degree of WS may be associated with the same cellular and metabolic changes as the ones observed in abdominal fat but this needs further investigation.

The plasma concentrations of calcium (Ca) and albumin were higher in chickens fed HE diets than in those fed NE diets in the current study. The increased plasma albumin concentration can be explained by the rapid production and utilization of albumin for the synthesis of tissue proteins in the chickens consuming HE diets. Our result is in agreement with a previous study by Wang et al. (2020), who found that increasing dietary energy was associated with a linear increase in serum albumin in lambs. Albumin has been recognized as one of the essential proteins, majorly known for activities associated with protein synthesis, substrate transport, toxin neutralization, and colloidal osmotic pressure (Levitt and Levitt 2016) during the growth and development process. The study conducted by Donnen et al. (1996) revealed that serum albumin concentration increased in children fed HE density gruels, which is in agreement with our findings. Albumin has been reported to help maintain a balance in metabolic changes during periods of rapid growth in chickens (Tothova et al. 2019). Consumption of HE density diets by chickens predisposes them to increased metabolic demand associated with accretion of muscle tissues within a short time (Stojevic et al. 2000). It has also been documented that nutritional changes, diet composition, and metabolic rate can impact the blood composition of protein fractions (Tothova et al. 2019). The increased plasma Ca concentration in chickens fed HE diets observed in this present study can be explained by the higher amount of analyzed Ca in the HE diets (1.01%) than in the NE diets

(0.86%) during the finisher phase. It has been previously demonstrated by Shafey et al. (2007) that increased dietary Ca resulted in increased plasma total Ca, Ca consumption, and excretion in broiler chickens. The most profuse minerals in the body are Ca and P, with 99% of Ca and 80% of P stored in the skeleton, where they perform a crucial role in bone development and mineralization (Veum 2010). The remaining Ca is located in the extracellular fluid, plasma, and within cells, where they perform fundamental roles in metabolism, blood coagulation, enzyme stimulation, neuromuscular function, muscle contraction, cell adhesion, and intracellular signaling (Veum 2010). There was a significant linear increase in AST with FA increase. Creatine kinase (CK) also increased, although not statistically significant, with increasing FA inclusions. A high AST is indicative of liver damage (Hoffman and Solter 2008). Similarly, Bona et al. (2018) reported that the increased in AST levels above 230U/L is indicative of hepatic damage in birds. It is not clear why AST linearly increased with FA, and this needs to be investigated. The combined increases of AST and CK could simply be attributed to higher muscle turnover rates linked to higher growth rates (Senanayake et al. 2015) rather than due to hepatocellular and/or muscle damage.

Intake of HE density diet has been recognized as one of the important causes of oxidative stress in animals such as pigeons (Hosseinian and Hasanzadeh, 2021), piglets (Adebowale et al., 2015), rats (Jiang and Jiang, 2018), cows (Wang et al., 2010), and sheep (Liang et al., 2020). This current study revealed that energy level and FA have no effect on plasma and breast muscle MDA and SOD levels in broiler chickens. Our evidence supports a previous study by Cetin et al. (2019), who observed that plasma MDA was not influenced by increasing dietary energy levels. The recent study by Hosseinian and Hasanzadeh, (2021) established that oxidative stress induced by dietary energy level depends on diet composition and formulation. The discrepancy among studies might be due to the diet composition, animal species, and sample collection. The different responses of

SOD activities in plasma, liver, and heart reported by Lin et al. (2006) suggest tissue specificity. No previous studies involved the effect of HE diet on breast muscle MDA and SOD, so we could not compare our results in this aspect.

Interaction between energy level and FA supplementation was observed for relative bursa weight, with birds fed HE diet having lower bursa weight compared to those fed NE diets in the current study. The inclusion of 15 ppm FA counteracted the negative effect of HE diets by increasing the relative weight of the bursa. The bursa is regarded as an immune organ, and changes in its weight may be an indication of immune system complications. Adebowale et al. (2019) demonstrated an increased tendency for a reduced immune status in piglets fed a HE density diet and suggested that HE density diets could induce immune impediments and provoke oxidative stress. A quadratic increase in bursa weight observed with increasing FA level could be an indication that FA supplementation was able to repair the immune damage caused by inflammation due to the HE density diets. Folic acid is a useful free radical scavenger and, if present in the body at a sufficient amount, can protect bio-constituents from free radical damage and oxidative stress (Joshi et al. 2001). Lipopolysaccharide-induced inflammation was also attenuated by FA supporting FA anti-inflammatory proprieties (Feng et al. 2011; Munyaka et al. 2013). On the other hand, the heart was significantly enlarged in broilers fed 10ppm FA compared to birds fed 15ppm FA. This enlargement of the heart at 10ppm FA instead of at 15ppm FA suggests that the first group of birds could have suffered from cardiomyopathy and that a dose of 15ppm alleviated this myopathy. It is interesting to note that birds on the HE diets had reduced ceca weight compared to those fed the NE diets. The ceca is known as the site where significant microbial fermentation of undigested feed components occurs (Svihus 2014). To balance the energy content of the NE diets, soybean meal and corn were replaced with vegetable oil, thereby reducing the amount of fiber that

can be fermented in the ceca. It is well known that soybean is a major source of indigestible oligosaccharides (Chen et al. 2013; Zhong et al. 2019), and a reduction in its content could reduce microbiota fermentation in the lower gut of chickens and hence a reduction in ceca weight. In our previous study (Adewole and Akinyemi 2021), it was demonstrated that dietary energy density significantly altered the population of few ceca microbes in broiler chickens.

The intestinal health of poultry is associated with overall health, welfare, and performance (Carrasco et al. 2019). Intestinal health indicators such as VH, CD, and VH:CD have been found to be related with the absorptive capacity of the small intestine (Gungor and Erener 2020). Our results showed significant interactions between FA and energy density for VH, VW, and CD. Among the NE group, birds fed 5-15ppm FA had reduced ($P<.0001$) VW compared to those fed the basal diet, while among the HE group, 15ppm FA supplementation resulted in an increased VH ($P=0.0317$) and CD ($P=0.0002$), compared to the basal diet. Wang et al. (2021b) revealed the dietary supplementation of FA (3, 9, and 18 ppm) also increased the VH, VH:CD of piglets, though the dietary energy content was not mentioned in the study. Contrary to the findings of Wang et al. (2021b), Liu et al. (2011) reported that FA supplementation did not affect the intestinal morphology, including the VH and CD. A previous finding observed that increasing maternal FA supplementation significantly enhanced the ratio of small intestinal weight to live body weight and the thickness of the intestinal muscle layer in the offspring (Li et al. 2020). Variation in results might be as a result of different FA inclusion levels and species used. Aside FA, effects of other kinds of vitamins have also been reported on the intestinal morphology of animals. Yildiz et al. (2017) observed that Vitamin E supplementation can contribute to regulating small intestinal functions in broilers by decreasing mass cell growth. Another study revealed that vitamin C enhanced intestinal health by improving villus morphology (Gan et al. 2020). Most nutrients are

absorbed in the villi, and VH is an indicator of the absorptive ability of intestinal mucosa (Attia et al. 2020). New epithelial cells are produced in the crypts, which is key to the maintenance of tissue homeostasis (Williams et al. 2015). Li et al. (2018) reported that the small intestine epithelium integrity and functions depend on the fast renewal of epithelial cells carried out by the crypts. Measurement of crypt depth also indicates the likely maturity and functional capacity of enterocytes on the villi (Hampson 1986). The increased VH observed in chickens fed HE with 15 ppm FA inclusion level in the present study suggests an increase in surface area, which implies better digestive and greater absorptive ability of the small intestine (Shang et al. 2020) while increasing CD relates to the absorption of more nutrients and is associated with better feed efficiency and growth performance of chickens (Lisnahan and Nahak 2020). The positive effect of FA on intestinal morphology of broiler chickens fed HE diets seen in this current study is similar to our observation with bacitracin methylene (BMD) in our previous study (Adewole and Akinyemi 2021), which demonstrated that the negative effect of HE density diets on intestinal structure was ameliorated by the administration of BMD. Supplemental folic acid might serve as a potential alternative feed additive in place of BMD in enhancing intestinal health for better production efficiency in poultry industry but more research is needed to affirm this.

In conclusion, the results of the current study suggest that 15 ppm FA supplementation in broiler chicken diets could repair immune damage and can be beneficial to the intestinal health and integrity of birds fed HE density diets.

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Table 1. Effect of folic acid fed at different energy levels on blood biochemistry parameters in broiler chickens

	Folic Acid level (ppm) ¹					Orthogonal Polynomial Contrasts P-value			Energy level			Energy*FA ³
	2.2	5	10	15	SEM ²	Linear	Quadratic	Cubic	Normal	High	SEM ²	
Electrolytes and minerals, mmolL ⁻¹												
Sodium	145	149	148	145	2.12	0.3630	0.7802	0.8158	145	148	1.50	0.3306
Potassium	4.99	5.01	4.73	4.89	0.21	0.8179	0.3652	0.6261	4.90	4.91	0.15	0.9259
Chloride	106	109	108	106	1.79	0.3408	0.7412	0.2137	107	108	1.27	0.6928
Calcium	2.66	2.69	2.72	2.52	0.07	0.7326	0.4819	0.0642	2.56b	2.73a	0.05	0.8990
Phosphorus	1.40	1.49	1.47	1.47	0.06	0.3209	0.6942	0.7516	1.45	1.46	0.04	0.7324
Magnesium	0.80	0.80	0.82	0.79	0.02	0.6955	0.9795	0.4414	0.81	0.79	0.02	0.2484
Metabolites, mmolL ⁻¹												
Urea	0.24	0.26	0.30	0.25	0.02	0.7649	0.1844	0.0399	0.25	0.28	0.01	0.4454
Creatinine	0.63	1.13	1.13	0.81	0.04	0.4520	0.7901	0.3631	0.75	1.09	0.25	0.2834
Glucose	14.3	15.2	14.7	14.6	0.22	0.0244	0.6040	0.2148	14.6	14.8	0.16	0.4866
Cholesterol	2.98	2.97	3.09	2.99	0.10	0.7933	0.5386	0.5677	2.94	3.08	0.07	0.5363
Iron	14.8	14.9	15.9	15.3	0.55	0.8588	0.1946	0.4321	15.6	14.8	0.39	0.7148
Bile acids	14.2b	15.5ab	18.3a	15.7ab	1.09	0.7890	0.0532	0.0650	16.6	15.2	0.77	0.0863
Uric acid	289	306	335	344	19.4	0.4872	0.0365	0.9054	313	324	13.73	0.2484
Total Bilirubin	0.50	0.31	0.44	0.38	0.13	0.2683	1.0000	1.0000	0.47	0.34	0.09	0.2986
Enzymes, UL ⁻¹												
Amylase	397	578	466	587	113	0.1945	0.7309	0.7213	472	542	79.92	0.2763
Alkaline phosphatase	3996	4446	3667	3912	460	0.4423	0.3525	0.8907	3797	4212	325.50	0.0883
Creatine kinase	14865	21154	17850	24324	3250	0.0868	0.3477	0.3701	18679	20418	2298	0.8601
Aspartate Aminotransferase	263	329	295	312	20.8	0.0250	0.7239	0.8628	285	315	14.69	0.7496
Gamma-Glutamyl Transferase	11.9	13.4	11.4	11.4	0.85	0.2611	0.1370	0.7065	11.4	12.7	0.60	0.6036
Lipase	18.3	25.3	18.6	21.2	2.77	0.8588	0.1946	0.4321	20.2	21.5	1.96	0.1224
Proteins, gL ⁻¹												
Total protein	25.3	25.5	26.1	25.8	0.79	0.9162	0.4811	0.6997	25.0	26.3	0.56	0.9658
Albumin	11.3	11.6	11.7	11.3	0.29	0.5677	0.8311	0.2551	11.2b	11.8a	0.21	0.5185
Globulin	14.0	13.9	14.4	14.4	0.55	0.8800	0.3696	0.9599	13.8	14.5	0.39	0.9957

¹Basal, Basal diet; 5ppmFA, diet containing 5 ppm folic acid; 10ppmFA, diet containing 10 ppm folic acid; 15ppmFA, diet containing 15 ppm folic. ²Standard error of the mean. ³Energy x folic acid interaction p-value. In a row, means assigned different lowercase letters are significantly different, $P < 0.05$ (Tukey's procedure). $n = 8$.

Table 2. Effect of folic acid fed at different energy levels on serum immunoglobulin concentrations and indicators of oxidative stress in plasma and breast muscle of broiler chickens

	Folic Acid level (ppm) ¹					Orthogonal Polynomial Contrasts P-value			Energy level			Energy*FA ³
	2.2	5	10	15	SEM ²	Linear	Quadratic	Cubic	Normal	High	SEM ²	
Serum IgG (ng/ml)	46.0	47.7	45.2	47.5	1.775	0.4631	0.8572	0.3719	46.1	47.1	1.257	0.8692
Serum IgM (ng/ml)	21.6	22.4	22.0	22.3	0.578	0.8299	0.6541	0.3415	22.2	22.0	0.409	0.9140
Plasma MDA ⁴ (nmol/mL)	77.7	98.1	93.1	84.2	8.598	0.8326	0.0946	0.5213	80.5	96.1	6.083	0.3720
Breast muscle MDA ⁴ (nmol/mg)	0.15	0.10	0.13	0.16	0.02	0.4283	0.0644	0.9631	0.14	0.14	0.02	0.6019
Plasma SOD ⁵ Activity (%)	84.0	79.1	87.5	74.3	3.42	0.3180	0.2364	0.0174	83.9	78.5	2.42	0.3334
Breast muscle SOD ⁵ Activity (%)	92.5	91.4	84.3	86.8	4.24	0.1657	0.6756	0.6008	87.0	90.5	3.00	0.1498

¹Basal, Basal diet; 5ppmFA, diet containing 5 ppm folic acid; 10ppmFA, diet containing 10 ppm folic acid; 15ppmFA, diet containing 15 ppm folic. ²Standard error of the mean. ³Energy x folic acid interaction p-value. ⁴Malondialdehyde. ⁵Superoxide dismutase. In a row, means assigned different lowercase letters are significantly different, P < 0.05 (Tukey's procedure). n = 8.

Table 3. Effect of folic acid fed at different energy levels on relative organ weights (g/kg BW) of broiler chickens

	Folic Acid level (ppm) ¹					Orthogonal Polynomial Contrasts P-value			Energy level			Energy*FA ³
	2.2	5	10	15	SEM ²	Linear	Quadratic	Cubic	Normal	High	SEM ²	
Spleen	0.79	0.73	0.81	0.80	0.044	0.3318	0.2924	0.9295	0.78	0.79	0.03	0.6879
Ceca	5.16	4.98	4.78	5.34	0.380	0.9862	0.9818	0.2752	5.51 ^a	4.61 ^b	0.27	0.4519
Bursa	1.56	1.48	1.55	1.84	0.084	0.9119	0.0496	0.0202	1.67 ^a	1.49 ^b	0.06	0.0016
Heart	4.80 ^{ab}	5.06 ^a	5.01 ^a	4.42 ^b	0.128	0.7537	0.1102	0.0012	4.94	4.68	0.09	0.1418

¹Basal, Basal diet; 5ppmFA, diet containing 5 ppm folic acid; 10ppmFA, diet containing 10 ppm folic acid; 15ppmFA, diet containing 15 ppm folic. ²Standard error of the mean. ³Energy x folic acid interaction p-value. In a row, means assigned different lowercase letters are significantly different, P < 0.05 (Tukey's procedure). n = 8.

Table 4. Effect of folic acid fed at different energy levels on jejunal morphology in broiler chickens

	Folic Acid level (ppm) ¹					Orthogonal Polynomial Contrasts P-value			Energy level		Energy*FA ³	
	2.2	5	10	15	SEM ¹	Linear	Quadratic	Cubic	Normal	High		SEM ²
Overall												
Villus height (mm)	1.55	1.57	1.63	1.63	0.032	0.0365	0.8085	0.1801	1.55 ^b	1.65 ^a	0.023	0.0226
Villus width (mm)	0.15 ^a	0.12 ^b	0.11 ^b	0.12 ^b	0.003	<.0001	<.0001	<.0001	0.13 ^a	0.12 ^b	0.002	<.0001
Crypt depth (mm)	0.17 ^b	0.18 ^a	0.17 ^{ab}	0.19 ^a	0.003	0.2833	0.7688	0.0011	0.17	0.18	0.003	0.0332
VH:CD ⁴	0.97 ^a	0.93 ^b	0.98 ^{ab}	0.95 ^{ab}	0.009	0.0391	0.7575	0.1218	0.95	0.97	0.013	0.8496
Normal energy density												
Villus height(mm)	1.51	1.52	1.63	1.51	0.047	0.0869	0.1936	0.6666	-	-	-	-
Villus width (mm)	0.18 ^a	0.12 ^b	0.11 ^b	0.12 ^b	0.006	<.0001	<.0001	<.0001	-	-	-	-
Crypt depth (mm)	0.17	0.18	0.17	0.17	0.005	0.6531	0.6276	0.6274	-	-	-	-
VH:CD ⁴	0.95	0.93	0.97	0.94	0.002	0.1496	0.6876	0.3535	-	-	-	-
High energy density												
Villus height (mm)	1.58 ^b	1.62 ^{ab}	1.63 ^{ab}	1.76 ^a	0.044	0.2163	0.2874	0.0128	-	-	-	-
Villus width (mm)	0.13 ^a	0.12 ^{ab}	0.11 ^b	0.12 ^{ab}	0.003	0.0569	0.0081	0.0853	-	-	-	-
Crypt depth (mm)	0.16 ^b	0.19 ^a	0.17 ^b	0.20 ^a	0.006	0.2967	0.9505	<.0001	-	-	-	-
VH:CD ⁴	0.99	0.94	0.98	0.96	0.018	0.1406	0.3934	0.2037	-	-	-	-

¹Basal, Basal diet; 5ppmFA, diet containing 5 ppm folic acid; 10ppmFA, diet containing 10 ppm folic acid; 15ppmFA, diet containing 15 ppm folic. ²Standard error of the mean. ³Energy x folic acid interaction p-value. ⁴Villus height to crypt depth. In a row, means assigned different lowercase letters are significantly different, P < 0.05 (Tukey's procedure). n = 8.

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