Effects of multicarbohydrase and butyrate glycerides on productive performance, nutrient digestibility, gut morphology, and ileal microbiota in late-phase laying hens fed corn- or wheat-based diets

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ABSTRACT A study was undertaken to determine the effects of supplemental multicarbohydrase (**MC**) and butyrate glycerides (**BG**) on productive performance, nutritional, and physiological responses in laying hens fed corn- or wheat-based diets during a 12-week production period (from 50–62 wk of age). The experiment consisted of a 2 × 2 × 2 factorial arrangement of the treatments with 2 different basal diets (corn- or wheat-based diets), 2 concentrations of MC (0 or 200 mg/kg of diet), and 2 concentrations of BG (0 or 2 g/kg of diet). Each treatment had 6 replicates with 8 hens each. The interactions among diet, MC, and BG were observed for egg production ($P = 0.048$), feed conversion ratio ($P = 0.005$), and ileal *Escherichia coli* count ($P = 0.043$), indicating that the effects of MC and BG on these responses were more marked when wheat-based diet was fed. A diet × MC interaction ($P < 0.05$) was also detected for egg mass, eggshell breaking strength, jejunal viscosity, and digestibility coefficients of fat and ash. Replacing 100% of the corn with wheat in the diets of laying hens negatively affected ($P < 0.05$) yolk color index, eggshell thickness, digesta viscosity, jejunal morphology, and populations of ileal microbiota. By contrast, MC supplementation increased ($P < 0.05$) eggshell thickness, digestibility coefficients of energy and crude protein, and populations of *Lactobacillus* and *Bifidobacterium* spp. in the ileum. Inclusion of BG also resulted in greater ($P < 0.05$) jejunal villus height and villus surface area, and digestibility coefficients of protein and ash, but lower ($P < 0.05$) populations of total bacteria, *Salmonella* and *E. coli* in the ileum. Results indicate that while the complete substitution of corn by wheat has a detrimental effect on productive performance and gut health, the combination of MC and BG may have synergistic effects on improving productive performance and intestinal microbiota in laying hens fed the wheat-based diets during the late laying period.

Key words: laying hen performance, multi-enzyme, butyric acid, digestibility coefficients, intestinal health

2021 Poultry Science 100:101066
https://doi.org/10.1016/j.psj.2021.101066

INTRODUCTION

Wheat is a major component of most animal diets in many countries and also could be considered as a suitable replacement for corn in the diets of broilers and laying hens (Cufadar et al., 2010; Smeets et al., 2018). Although the starch, linoleic acid, and energy contents of wheat are lower than those for corn, the contents of protein, calcium, phosphorus, and some amino acids (such as methionine, lysine, threonine, arginine, and tryptophan) are greater in wheat (National Research Council, 1994). However, the inclusion level of wheat is limited often because of its variable chemical composition and nutritive value, as well as its high nonstarch polysaccharide (**NSP**) content (Annison and Choct, 1991; Choct and Annison, 1992). Exogenous enzyme supplementation of wheat-based diets is a common practice in commercial feeding of poultry to decrease digesta viscosity and extract a greater amount of the available nutrients from the feed consumed, thereby improving feed efficiency and reducing feed costs (Kiarie et al., 2014; Taylor et al., 2018). Several studies have shown the beneficial effects of NSP-degrading enzymes on improving egg production, egg mass, and feed conversion ratio in laying

Received October 8, 2020.
Accepted February 4, 2021.

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hens (Cufadar et al., 2010; Mirzaie et al., 2012; Gonzalez-Ortiz et al., 2017). However, other studies did not observe any beneficial effect of xylanase (Lei et al., 2018) or multienzyme complex containing xylanase, cellulase, β-glucanase, pectinase (Baghban-Kanani et al., 2018) on productive performance or nutrient digestibility of laying hens. Factors that could account for these discrepancies among studies include the type of basal diet used and the enzyme sources and their respective activities. It is also reported that enzyme products have limitations for use in poultry diets because they are not completely stable to the proteolytic and acidic conditions of the intestinal tract (O'Neill et al., 2014) and may not be always cost-effective. Hence, additional dietary adjustments may be needed to further improve NSP utilization in poultry diets.

Recent studies indicated that organic acids could be used to improve the productive performance and gut health in broilers (Panda et al., 2009; Vieira et al., 2018) and laying hens (Sobczak and Kozłowski, 2016; Kazempour and Jahanian, 2017). Among the organic acids, butyric acid seems to play an important role in the improvement of the absorptive surface of intestinal epithelium by stimulating both proliferation and differentiation pathways in epithelial cells that improve nutrient utilization (Abdelqader and Al-Fataftah, 2016; Nari and Ghasemi, 2020). In addition, butyrate has been shown to be the primary energy source for intestinal epithelial cells and plays a vital role in promoting intestinal development and maintaining the integrity of the gut barrier (Liao et al., 2020). In a study on laying hens, Jahanian and Golshadi (2015) reported that butyric acid supplementation of wheat-based diets had positive effects on production performance, ileal nutrient digestibility, and intestinal microbiota. However, to our knowledge, there is no research study to investigate the interactive effects of dietary cereal source, multienzyme supplementation, and butyric acid on productive performance and physiological status of laying hens. It is reported that butyrate could reduce the gastrointestinal pH, which generally leads to better conditions for pepsin activity and more degradation of NSP and phytate (Jahanian and Golshadi, 2015; Nari and Ghasemi, 2020). Therefore, on the basis of these studies, it is hypothesized that supplementing the diet with butyric acid can improve intestinal morphology and microbiota and consequently maximize the effect of NSP-degrading enzymes on nutrient digestibility and productive performance of laying hens, especially when wheat-based diets are fed.

Thus, the objective of this study was to evaluate the effects of multicarbohydrase (**MC**) and butyrate glycerides (**BG**), individually and in combination, on the productive performance, egg quality, nutrient digestibility, jejunal morphology, and ileal microbiota in laying hens fed different basal diets in the late phase of production.

MATERIALS AND METHODS

Multicarbohydrase and Butyric Acid Sources

The MC enzyme used in this study (MultiBehzyme) was produced by the Institute of Animal Nutrition, AKAM Faravardehaye Bahman Arad Co. (Karaj, Iran), for the trial and applied at a dose rate of 200 mg/kg of feed. The preparation (in powder form) contained endo-1,4-β-xylanase originating from *Bacillus subtilis* (EC 3.2.1.8), β-glucanase originating from *Bacillus amyloliquefaciens* (EC 3.2.1.6), pectinase originating from *Bacillus licheniformis* (EC 3.2.1.15), and α-amylase originating from *Bacillus amyloliquefaciens* (EC 3.2.1.1). The enzyme was added in diets to provide a guaranteed minimum of 2000 U xylanase, 800 U β-glucanase, 600 U pectinase, and 500 U amylase per kg of feed. One xylanase, glucanase, or pectinase unit is defined as the amount of enzyme which releases 1 μmol of total reducing sugar equivalents (as xylose, glucose, or galacturonic acid) from wheat arabinoxylan, barley glucan, or pectin, respectively, per min at pH 5.0 and 50°C. One amylase unit is defined as the amount of enzyme required to release 1 μmol glucosidic linkage per min at pH 7.0 and 37°C.

The BG supplementation used in this trial was provided in the butyric acid glycerides form as mono-, di-, and tri-acyl glycerol (Baby C4; Silo Industria Zootecnica, Florence, Italy) and was mixed with the feed at 2 g/kg.

Diets and Experimental Design

The experimental protocols used in the present study were approved by the Arak University Institutional Animal Care and Use Committee. A total of 384 Bovans White laying hens (average weight = 1,640 ± 16 g, 48 wk old) were obtained from a 2000 hen flock based on similar weights and production rates. After 2 wk of adaptation, the hens were weighed individually and randomly assigned to 8 treatment diets in a completely randomized design with a 2 × 2 × 2 factorial arrangement of treatments. The factors were 2 different basal diets (corn- or wheat-based diets), 2 concentrations of MC (0 or 200 mg/kg of diet), and 2 concentrations of BG (0 or 2 g/kg of diet). The experimental period lasted 12 wk (from 50–62 wk of age). A total of 48 cages with dimensions of 90 × 60 × 40 cm, with 2 divisions of 45 × 60 × 40 cm, provided with linear feeders and nipple drinkers, were used. Each cage had 8 hens at an average stocking density of 675 cm^2/hen. All birds were housed in an environmentally controlled house with the minimal temperature maintained at 20°C. The house had controlled ventilation and lighting (16 h of light per day). All hens were provided with

feed and water ad libitum throughout the experiment. All diets were fed in mash form and were formulated to meet the nutrient recommendation of laying hens as per the Bovans Nutrition Management guide (2017) during the period of 50 to 62 wk of age. The chemical composition of the corn and wheat used in this trial is shown in Table 1. The ingredient composition and nutrient contents of the experimental diets are presented in Table 2.

Chemical Analysis

Representative samples of main feed ingredients (corn, wheat, and soybean meal), as well as experimental diets, were collected for measuring the contents of dry matter (DM; method 930.15), crude fat by extract in ether (method 920.39), crude protein (6.25 × N; method 984.13), crude fiber (method 978.10), crude ash (method 942.05), calcium (method. 968.08), total phosphorus (method 946.06), and nitrogen-free extract according to AOAC (2006). Gross energy determination was performed in an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL) standardized with benzoic acid. The contents of neutral detergent fiber and acid detergent fiber were determined using a fiber analyzer (A200, Ankom, Technology Corp., Fairport, NY) following the procedures of Van Soest et al. (1991). The total starch and d-xylose contents of the experimental diets were measured using commercial kits (Megazyme International). The arabinoxylan content was calculated using the following equation based on Mirzaie et al. (2012): arabinoxylan (%) = xylose (%) × 100/62. Total amino acid contents of feed ingredients and experimental basal diets were analyzed (Evonik Industries, Evonik Degussa GmbH, Hanau-Wolfgang, Germany). Coefficients for amino acid digestibility and apparent metabolizable energy corrected to zero nitrogen balance (**AMEn**) values for feedstuffs were in accordance with the values reported by AminoDat (2015).

The recovery percentage of enzymes in feed was determined by the enzyme producer. Xylanase activity in the MC-supplemented diets was assayed by measuring the reducing sugar released from xylan with a reaction mixture, consisting of 250 μL of 1% xylan, 150 μL of McIlvine buffer (pH 5.0), and 100 μL of crude cell extract (Miller, 1959). The β-glucanase and pectinase activities were assayed using the same method, except that xylan was substituted by β-glucan (for β-glucanase assay) or galacturonic acid (for pectinase assay) as a substrate at the reaction temperature of 50°C. The α-amylase activity was determined according to the method of Okolo et al. (1995), with reaction mixture consisting of 1.25 mL of 1% soluble starch, 0.25 mL 0.1 M acetate buffer, 0.25 mL of distilled water, and 0.25 mL of crude enzyme extract.

Table 1. Determined chemical composition (g/kg, on dry matter basis) of corn and wheat samples used in the experimental diets.

Item[1]	Corn	Wheat
Dry matter	908.3	917.8
Crude protein	83.0	130.2
Ether extract	45.0	23.0
Crude fiber	23.0	30.0
Total ash	12.0	17.0
Calcium	0.3	0.5
Total phosphorous	2.3	2.7
Starch	671.0	590.0
d-Xylose	19.0	32.0
Arabinoxylan[2]	30.6	51.6
Neutral detergent fiber	106.0	149.0
Acid detergent fiber	31.0	41.0
Total lysine	2.5	3.8
Total methionine	1.7	1.8
Total cysteine	1.8	2.8
Total sulfur amino acids	3.5	4.6
Total threonine	2.8	3.7
Total valine	3.7	5.5
Total tryptophan	0.6	1.5
Total arginine	3.9	6.3
Gross energy, MJ/kg	18.6	18.1

[1]Mean of 3 samples.
[2]Calculated as d-xylose (g/kg) × 100/62.

Productive Performance

Feed intake was measured on a cage basis every week, and daily feed intake per bird was determined on a replication total feed consumption basis for the 12-week feeding period and for the number of days during the period. The number and weight of eggs laid were recorded daily from 50 to 62 wk of age. The egg production percentage was expressed as an average hen-day production. Egg mass was calculated by multiplying the hen-day egg production by the average egg weight. Feed conversion ratio (**FCR**) was also calculated as the ratio of feed intake per unit of the egg mass.

Egg Quality Parameters

To measure egg quality parameters, a total of 24 eggs from each treatment group (4 eggs per replication) were randomly sampled and weighed at the end of the study when the hens were 62 wk of age. The measurements were made during the first 24 h after eggs were laid. The eggshell breaking strength (kg/cm^2) was measured by an eggshell strength tester (OSK 13473; Ogawa Seiki Co., Ltd., Tokyo, Japan). After that, all eggs were weighed and broken open to separate albumen and yolk. The chalazae and any adhering albumen were carefully removed from the yolk, and then, the yolk weight was measured with a digital scale (0.01 g precision). The egg yolk scores were evaluated visually using the Roche color fan scale (Roche Ltd., Basel, Switzerland; where 15 = dark orange and 1 = light pale). Albumen length was measured with a digital caliper (0.01 mm precision), and the Haugh unit was calculated with the formula given by Haugh (1937). The eggshells were weighed after air-drying at room temperature for 48 h. Eggshell thickness was a mean value of measurements at 3 points on the egg (air cell, equator, and sharp end) measured by using a caliper (0.01 mm precision).

Nutrient Digestibility

The acid insoluble ash (**AIA**) marker was used to measure ileal nutrient digestibility in accordance with the method reported by McCarthy et al. (1974). All diets were supplemented with Celite (5 g/kg), as a source of AIA, for 4 d from day 80 to day 84 of main experimental period. At the end of the study, 2 laying birds from each cage design treatment were randomly selected and killed by cervical dislocation. The ileal contents, from vitelline diverticulum to the ileocecal junction, were collected, pooled within a replicate cage, and immediately stored at −20°C until further analysis could be performed. The samples were subsequently freeze-dried and ground with a hammer mill (model 5543 GEN; Isfahan Dasht, Isfahan, Iran) to pass through a 0.5-mm screen before chemical analysis. Chemical analysis of the ileal digesta followed the same methods as those used for the samples from feedstuffs and diets. The amount of AIA in the diet and ileal digesta was measured according to McCarthy et al. (1974). The apparent ileal digestibility (**AID**) of nutrients in the diets was calculated by the following equation:

$$\mathrm{AID}(\%) = [1 - (\mathrm{AIA}_{\mathrm{diet}} / \mathrm{AIA}_{\mathrm{digesta}}) \times (\mathrm{Nutr}_{\mathrm{digesta}} / \mathrm{Nutr}_{\mathrm{diet}})] \times 100,$$

where $\mathrm{AIA}_{\mathrm{diet}}$ and $\mathrm{Nutr}_{\mathrm{diet}}$ are the concentrations of AIA and nutrient in the diet (%) and $\mathrm{AIA}_{\mathrm{digesta}}$ and $\mathrm{Nutr}_{\mathrm{digesta}}$ represent the concentrations of the same AIA and nutrient in the ileal digesta (%).

Jejunal Digesta Viscosity and Histomorphology

At 62 wk of age, the jejunum samples (tissue and digesta samples) from those hens sampled for nutrient digestibility determinations were collected for digesta viscosity and villus micrometry analysis (n = 12 hens from each treatment group). The viscosity of the jejunal digesta was read immediately after the collection. The digesta samples from birds within a cage were pooled and mixed to achieve a homogenous mixture, which was then centrifuged at 3,600 × *g* for 10 min (Jia et al., 2008). A 0.5 mL aliquot of the supernatant was analyzed for viscosity with a digital viscometer (model DV-III; Brookfield Engineering Laboratories Inc., Middleboro, MA).

For morphological assessment, approximately 2 cm segments of the jejunum at the midpoint location (between the point of entry of the bile ducts and vitelline diverticulum) were collected, flushed with distilled water to remove the intestinal contents, and fixed in 10%

Table 2. Composition of corn- and wheat-based basal diets, and their calculated and determined analysis (as-fed basis), fed to laying hens (weeks 50–62).

Item, (g/kg, unless stated otherwise)	Basal diet		Analyzed nutritive value[5], g/kg	Basal diet	
	Corn	Wheat		Corn	Wheat
Corn	575.4	-	Crude protein	178.8	179.1
Wheat	-	643.3	Ether extract	45.8	50.7
Soybean meal	287.1	202.3	Crude fiber	29.3	31.0
Soybean oil	14.2	29.0	Total ash	113.6	118.3
Dicalcium phosphate	12.9	13.2	Total phosphorous	5.8	5.7
Oyster shell	95.5	95.5	Calcium	38.2	38.9
Salt (NaCl)	1.7	1.3	Starch	387.8	380.1
Sodium bicarbonate	1.2	2.0	d-Xylose	15.6	26.4
Vitamin and mineral premix[1]	5.0	5.0	Arabinoxylan[6]	25.2	42.5
DL-Methionine	1.3	1.3	Neutral detergent fiber	97.2	121.4
L-Lysine HCl	0.3	1.4	Acid detergent fiber	40.8	42.6
L-Threonine	0.4	0.7	Total lysine	8.7	8.5
Kaolin[2]	5.0	5.0	Total methionine	3.8	3.6
Calculated nutritive value			Total cysteine	3.1	3.2
AMEn, MJ/kg	11.3	11.3	TSAA	6.9	6.8
Crude protein	17.6	17.6	Total threonine	6.5	6.3
Calcium	40.0	40.0	Total valine	8.0	7.7
Nonphytate phosphorous	3.4	3.4	Total tryptophan	1.9	2.1
Digestible TSAA[3]	6.3	6.3	Total arginine	10.6	10.2
Digestible lysine	7.5	7.5			
Digestible threonine	5.6	5.6			
Sodium	1.6	1.6			
DEB[4], mEq/kg	250	250			

[1]Supplied per kg of diet: all-trans-retinyl acetate, 8,800 IU; cholecalciferol, 2,500 IU; α-tocopherol acetate, 6.6 mg; menadione sodium bisulfite, 2.5 mg; thiamine mononitrate, 1.5 mg; riboflavin, 4.4 mg; nicotinic acid, 20 mg; calcium D-pantothenate, 8 mg; pyridoxine, 2.5 mg; folic acid, 1.1 mg; cyanocobalamine, 0.08 mg; biotin, 0.15 mg; choline chloride, 400 mg, Mn (from manganese sulfate), 60 mg; Fe (from ferrous sulfate), 30 mg; Zn (from zinc sulfate), 66 mg; Cu (from copper sulfate), 6 mg; I (from potassium iodate), 0.8 mg; Se (from sodium selenite), 0.2 mg.

[2]Dietary supplements (multi-carbohydrase and butyric acid) were added to aliquots of the basal diet at the expense of an inert filler (kaolin).

[3]Total sulfur amino acids.

[4]DEB (dietary electrolyte balance) = (Na+, mEq/kg + K+, mEq/kg) – CL−, mEq/kg.

[5]Mean of 3 samples per diet.

[6]Calculated as d-xylose (g/kg) × 100/62.

buffered formalin (Majdolhosseini et al., 2019). After dehydration and infiltration with solidified paraffin wax, a 5-μm cross-section was made using a microtome (Typ 1400; Leitz, Wetzlar, Germany), placed on a glass slide, and stained with hematoxylin-eosin using standard histological techniques. The tissue slides were then analyzed with a light microscope (Olympus, CX31, Shinjuku, Tokyo, Japan) and the villus height (**VH**), villus width (**VW**), and crypt depth (**CD**) were measured for each intestinal segment using an image-analysis software (QWinPlus v. 3.1.0; Leica Cambridge Ltd., Cambridge, UK). Jejunal morphometric variables were measured from 2 sections per bird and a minimum of 10 villi and 10 crypts per section. For the VW, the width of the selected villus was taken in 3 different locations (top, middle and bottom) section of the villus and averaged to make the VW of the single villus. Data from the VH and CD was used to obtain the VH/CD ratio. The villus surface area (**VSA**) was calculated using the equation of Majdolhosseini et al. (2019): $2\pi \times (VW/2) \times VH$.

Gut Microbiota

On week 62, the subsamples of ileal digesta (1 g) from 2 birds within a cage (n = 12 hens from each treatment group) were instantly collected into glass containers for bacterial enumeration in the ileum. Briefly, the ileal samples were serially diluted in sterile saline solution (0.85% NaCl). Bacteria were enumerated on plate count agar (total bacteria), de Man Rogosa Sharpe agar (*Lactobacillus* spp.), Bifidus Selective Medium agar (*Bifidobacterium* spp.), eosin methylene blue agar (*Escherichia coli*), and *Salmonella-Shigella* agar (*Salmonella* spp.). Each dilution was plated in duplicate onto appropriate agar plates and the average of 2 bacterial counts was used in the statistical analysis. Plates were then incubated at 39°C, for 24–48 h aerobically (plate count, eosin methylene blue, and *Salmonella-Shigella* agars) or 48–72 h anaerobically (Man Rogosa Sharpe and Bifidus Selective Medium agars), and colonies were counted (Jahanian and Golshadi, 2015; Rostami et al., 2015). Bacterial counts were finally expressed as log10 cfu per gram of ileal digesta.

Statistical Analysis

The data were subjected to a 3-way analysis of variance for a 2 × 2 × 2 factorial arrangement of treatments using the General Linear Models procedures of SAS 9.3 package (SAS Institute Inc., 2010). The model included the main effects of basal diet, MC level, and BG level, and their interactions. The experimental unit differed in accordance with the measured parameters. For performance traits and egg quality parameters, the experimental unit was cage, whereas individual laying hen data were used for nutrient digestibility, digesta viscosity, histomorphology, and gut microbiota. Normality and homogeneity of variances were evaluated by Shapiro-Wilk and Levene tests, respectively. Mean separation was conducted by the Tukey's post hoc analysis with differences deemed significant at $P < 0.05$. The results are presented as the means with standard errors of the means.

RESULTS

Feedstuff and Diet Analysis

Table 1 presents the chemical analysis of the corn and wheat used in the present study. There were apparent differences in the analyzed nutrients content, gross energy, and amino acid profile between the corn and wheat. When wheat was compared with corn, the crude fiber, NDF, ADF, d-xylose, and arabinoxylan levels in the wheat (30, 149, 41, 32, and 51.6 g/kg DM, respectively) was higher than their concentrations in the corn (23, 106, 31, 19, and 30.6 g/kg DM, respectively). By contrast, the determined values of starch and gross energy of wheat were lower than those values determined for corn.

Analyzed enzyme activity recovery in the MC-supplemented corn-based diets for xylanase, β-glucanase, pectinase, and amylase were 114, 95, 87, and 122%, respectively. The respective values were 105, 110, 106, and 89% for MC-supplemented wheat-based diets.

Productive Performance

The productive performance of laying hens fed the experimental diets is presented in Table 3. During the experimental period (50–62 wk of age), the interaction among diet, MC, and BG was observed for egg production ($P = 0.048$) and FCR ($P = 0.005$), being better in hens fed on wheat-based diets supplemented with MC and BG than in hens fed on wheat-based diets without MC and BG. The 2-way diet × MC interactions were also detected for egg production ($P = 0.013$) and egg mass ($P = 0.007$), indicating that the effect of MC on these responses were more marked in hens fed on wheat-based diets during the period 50 to 62 wk of age. An interaction between the diet and BG was also observed on FCR ($P = 0.038$), which improved with BG addition in the wheat-based diets. In terms of evaluating the main effect, although productive parameters were not influenced by the type of basal diet, dietary supplementation of MC increased both egg production ($P < 0.001$) and egg mass ($P < 0.001$); consequently improved FCR ($P = 0.001$) compared with nonsupplemented laying hens. Similarly, the inclusion of BG resulted in higher egg weight ($P = 0.019$) and egg mass ($P = 0.005$), but lower FCR ($P < 0.001$).

Egg Quality

With regard to egg quality parameters (Table 4), albumin height, Haugh units, yolk weight, and eggshell weight were not affected ($P > 0.05$) by diet, MC, BG, or their interactions. By contrast, the yolk color index and shell thickness were higher ($P = 0.038$ and

Table 3. Effects of dietary treatments on productive performance of laying hens from 50 to 62 wk of age.

Basal diet	MC[1] level	BG[2] level	Egg weight g	Egg production %	Egg mass g/hen/d	Feed intake g/hen/d	FCR[3]
Corn	−	−	63.4	84.0^{c}	53.2	110.6	2.08^{b,c}
Corn	−	+	64.5	83.4^{c}	53.8	112.5	2.09^{b,c}
Corn	+	−	63.9	84.4^{b,c}	53.9	113.3	2.10^{a,b}
Corn	+	+	64.3	85.2^{a,b,c}	54.8	109.4	2.00^{b,c,d}
Wheat	−	−	63.0	79.8^{d}	50.2	111.1	2.21^{a}
Wheat	−	+	63.9	85.0^{a,b,c}	54.3	108.1	1.99^{c,d}
Wheat	+	−	63.9	87.2^{a,b}	55.7	111.8	2.01^{b,c,d}
Wheat	+	+	64.6	87.7^{a}	56.6	110.0	1.94^{d}
SEM			0.45	1.08	0.76	1.61	0.033
Main effect means							
Basal diet							
Corn			64.0	84.24	53.9	111.4	2.07
Wheat			63.8	84.92	54.2	110.3	2.04
SEM			0.22	0.54	0.38	0.80	0.017
MC level							
−			63.7	83.0^{b}	52.9^{b}	110.6	2.09^{a}
+			64.2	86.1^{a}	55.3^{a}	111.1	2.01^{b}
SEM			0.22	0.54	0.38	0.80	0.017
BG level							
−			63.5^{b}	83.8	53.3^{b}	111.7	2.10^{a}
+			64.3^{a}	85.3	54.9^{a}	109.9	2.01^{b}
SEM			0.22	0.54	0.38	0.80	0.017
Significance							
Basal diet			0.569	0.376	0.591	0.306	0.232
MC level			0.126	<0.001	<0.001	0.620	0.001
BG level			0.019	0.053	0.005	0.138	<0.001
Diet × MC			0.313	0.013	0.007	0.515	0.057
Diet × BG			0.908	0.082	0.097	0.550	0.038
MC × BG			0.506	0.268	0.196	0.331	0.697
Diet × MC × BG			0.699	0.048	0.107	0.128	0.005

[a-d]Means within each column with no common superscript differ ($P < 0.05$).
[1]Multicarbohydrase.
[2]Butyrate glycerides.
[3]Feed conversion ratio.

$P = 0.032$, respectively) for hens fed corn-based diets than for hens fed wheat-based diets. There were main effects of both MC ($P = 0.024$) and BG ($P = 0.035$) on eggshell thickness, so that the dietary inclusion of either of the 2 supplements improved shell thickness of the eggs. A diet × MC interaction was also observed for eggshell breaking strength ($P = 0.019$), suggesting that the response to MC supplementation was more pronounced in the wheat-based diet groups than those of the corn-based diet groups.

Jejunal Digesta Viscosity and Ileal Nutrient Digestibility

Data on jejunal digesta viscosity and AID of nutrients are shown in Table 5. As presented, no interactions were observed ($P > 0.05$) between the diet, MC, and BG for digesta viscosity and nutrient digestibility. By contrast, the AID of crude fat ($P = 0.047$) and crude ash ($P = 0.037$), as well as jejunal digesta viscosity ($P = 0.002$), were influenced by basal diet × MC interaction, so that supplemental MC was more effective in improving these parameters in hens fed wheat-containing diets than in those fed on corn-containing diets. Jejunal digesta viscosity ($P < 0.001$) and the AID of crude ash ($P = 0.031$) were affected by the type of basal diet, so that an increase in the digesta viscosity and a decrease in the ash digestibility was observed in hens fed on wheat-based diets. In the MC-supplemented group, the digesta viscosity was decreased ($P < 0.001$) and the AID of energy, crude protein, and crude fat was increased ($P = 0.034$, $P = 0.018$, and $P = 0.005$, respectively), compared with the nonsupplemented group. Inclusion of BG also increased the AID of crude protein ($P = 0.045$) and crude ash ($P = 0.009$), but did not affect ($P > 0.05$) the AID of DM, energy, and crude fat, or the viscosity of jejunal digesta.

Gut Morphology

Morphological characteristics in the jejunum of laying hens fed the experimental diets are presented in Table 6. The main effect of MC supplementation, two-way

Table 4. Effects of dietary treatments on egg quality parameters of laying hens at 62 wk of age.

			Albumin height		Yolk weight		Shell weight	Shell thickness	EBS[3]
Basal diet	MC[1] level	BG[2] level	mm	Haugh unit	g	Yolk color	g	mm	kg/cm^2
Corn	−	−	5.68	68.92	17.95	7.25	6.37	0.336	3.36
Corn	−	+	6.01	73.16	18.50	7.38	6.25	0.358	3.44
Corn	+	−	5.80	70.72	19.28	8.00	6.33	0.355	3.35
Corn	+	+	5.85	69.06	19.09	7.25	6.50	0.352	3.31
Wheat	−	−	5.46	68.91	18.61	6.13	5.83	0.314	3.06
Wheat	−	+	5.81	73.85	18.67	6.63	6.19	0.337	3.31
Wheat	+	−	6.05	70.99	18.67	6.38	6.24	0.342	3.54
Wheat	+	+	6.19	71.98	18.77	6.88	6.14	0.354	3.60
SEM			0.361	2.06	0.440	0.645	0.224	0.0088	0.129
Main effect means									
Basal diet									
Corn			5.83	70.47	18.70	7.47[a]	6.36	0.350[a]	3.37
Wheat			5.88	71.43	18.68	6.50[b]	6.09	0.337[b]	3.38
SEM			0.181	1.03	0.219	0.322	0.112	0.0044	0.064
MC level									
−			5.74	71.21	18.43	6.84	6.16	0.336[b]	3.30
+			5.97	70.68	18.95	7.13	6.30	0.351[a]	3.45
SEM			0.181	1.03	0.219	0.322	0.112	0.0044	0.064
BG level									
−			5.75	69.88	18.63	6.94	6.19	0.337[b]	3.33
+			5.97	72.01	18.76	7.03	6.27	0.350[a]	3.42
SEM			0.181	1.03	0.219	0.322	0.112	0.0044	0.064
Significance									
Basal diet			0.865	0.510	0.939	0.038	0.099	0.032	0.898
MC level			0.369	0.718	0.100	0.540	0.363	0.024	0.089
BG level			0.395	0.150	0.678	0.838	0.623	0.035	0.336
Diet × MC			0.332	0.668	0.163	0.946	0.819	0.191	0.019
Diet × BG			0.922	0.569	0.867	0.377	0.738	0.503	0.463
MC × BG			0.527	0.097	0.579	0.633	0.792	0.138	0.403
Diet × MC × BG			0.942	0.741	0.531	0.633	0.239	0.611	0.855

[a,b]Means within each column with no common superscript differ ($P < 0.05$).
[1]Multicarbohydrase.
[2]Butyrate glycerides.
[3]Eggshell breaking strength.

interaction effects of diet × MC, diet × BG, MC × BG, and the three-way interaction of diet × MC × BG were not significant for all morphological parameters. Regarding the main effects of the basal diet, jejunal VH, and VH/CD ratio were lower ($P = 0.006$ and $P < 0.001$, respectively), whereas CD was greater ($P = 0.017$) in hens fed on wheat-based diets than in those fed corn-based diets. Supplemental BG also increased VH ($P = 0.015$) and VSA ($P = 0.042$) in the jejunum.

Ileal Microbial Population

The effect of dietary treatments on bacterial populations of the ileum is given in Table 7. The three-way interaction effect of diet × MC × BG ($P = 0.043$), and two-way interaction effects of diet × MC ($P = 0.006$) and diet × BG ($P = 0.001$) were significant for ileal *E. coli* count. However, no other interaction effects were found for the other bacterial species studied. The populations of *lactobacillus* spp. and *Bifidobacterium* spp. in ileal contents were not affected by the type of basal diet; however, ileal enumerations of total bacteria ($P < 0.001$), *E. coli* ($P < 0.001$), and *Salmonella* spp. ($P = 0.006$) were greater in hens fed on wheat-based diets than in those fed corn-based diets. By contrast, the addition of MC increased the populations of *Lactobacillus* spp. ($P = 0.036$) and *Bifidobacterium* spp. ($P = 0.046$), but decreased *E. coli* count ($P = 0.003$) in ileal contents. Dietary supplementation with BG also decreased the populations of total bacteria ($P = 0.015$), *E. coli* ($P < 0.001$), and *Salmonella* spp. ($P < 0.001$).

DISCUSSION

The beneficial impact of NSP-degrading enzymes in wheat-based diets was expected because the wheat-containing diets have higher NSP content than corn-containing diets. This was confirmed by a significant interaction between basal diet and MC supplementation for egg production, egg mass, and FCR (Table 3). Our present study and several previous reports (Cufadar et al., 2010; Mirzaie et al., 2012; Gonzalez-Ortiz et al.,

Table 5. Effects of dietary treatments on jejunal viscosity and apparent ileal digestibility coefficients (AIDC) of nutrients in laying hens at 62 wk of age.

			AIDC of nutrients					
			Viscosity	Dry matter	Energy	Crude protein	Crude fat	Crude ash
Basal diet	MC[1] level	BG[2] level	cP[3]			%		
Corn	−	−	3.86	71.75	71.37	66.48	78.67	52.47
Corn	−	+	3.74	72.27	72.12	69.87	79.47	54.95
Corn	+	−	3.69	73.13	72.93	70.15	79.28	52.33
Corn	+	+	3.67	72.70	75.28	70.32	80.12	54.62
Wheat	−	−	5.92	70.80	69.52	65.90	76.35	47.13
Wheat	−	+	5.43	71.27	71.47	68.07	77.82	51.90
Wheat	+	−	4.40	72.33	72.35	68.42	80.70	52.97
Wheat	+	+	4.32	72.55	73.13	69.41	80.23	53.82
SEM			0.246	1.029	1.484	1.138	0.947	1.350
Main effect means								
Basal diet								
Corn			3.74[b]	72.46	72.93	69.20	79.38	53.59[a]
Wheat			5.01[a]	71.74	71.62	67.95	78.77	51.45[b]
SEM			0.123	0.515	0.742	0.569	0.473	0.675
MC level								
−			4.74[a]	71.52	71.12[b]	67.58[b]	78.08[b]	51.61
+			4.02[b]	72.68	73.43[a]	69.76[a]	80.08[a]	53.43
SEM			0.123	0.515	0.742	0.569	0.473	0.675
BG level								
−			4.47	72.00	71.54	67.74[b]	78.75	51.23[b]
+			4.29	72.20	73.00	69.42[a]	79.41	53.82[a]
SEM			0.123	0.515	0.742	0.569	0.473	0.675
Significance								
Basal diet			<0.001	0.326	0.220	0.127	0.369	0.031
MC level			<0.001	0.119	0.034	0.018	0.005	0.064
BG level			0.219	0.794	0.172	0.045	0.331	0.009
Diet × MC			0.002	0.733	0.956	0.937	0.047	0.037
Diet × BG			0.206	0.834	0.931	0.906	0.814	0.825
MC × BG			0.335	0.683	0.918	0.179	0.482	0.287
Diet × MC × BG			0.213	0.811	0.514	0.523	0.467	0.336

[a,b]Means within each column with no common superscript differ ($P < 0.05$).
[1]Multicarbohydrase.
[2]Butyrate glycerides.
[3]cP = centipoise (1 cP = 1/100 dyne s/cm^2).

2017) showed positive productive performance responses to NSP-degrading enzymes in wheat-based diets. Dietary inclusion of NSP-degrading enzymes, especially xylanase, could degrade fiber structures of wheat-based diets, resulting in a reduction in intestinal viscosity and subsequently increased the nutrient digestibility of birds (Moss et al., 2020). The enzyme can also decrease the possible antinutritional effects caused by NSP in wheat-based diets by increasing the access to the cell contents for endogenous digestive enzymes (Smeets et al., 2018).

A novel finding of the present study is that interactions were observed among diet, MC, and BG for egg production and FCR, indicating that the simultaneous inclusion of MC plus BG in the wheat-based diets significantly improved egg production and FCR, whereas no significant improvements were detected in the corn-based diets. Although the reason for these improvements in egg production and FCR with MC addition with 2 g/kg BG in the wheat-based diet is unknown, it seems that BG could intensify the effect of MC and led to improved egg-laying performance. The low pH environment in the gastrointestinal tract caused by BG intake (Panda et al., 2009) may positively affect the activity of NSP-degrading enzymes and improve nutrition utilization of laying hens in the present study. Consistent with the present findings, Jahanian and Golshadi (2015) reported that supplementation of butyric acid into the wheat diet may further enable NSP hydrolysis by inhibiting the formation of protein- and starch-NSP complexes, resulting in improved nutrient utilization and better laying performance. Butyric acid is also reported to improve the integrity of the epithelial cell line and the absorptive surface of the intestine by stimulation of cell proliferation and differentiation in the intestinal epithelium, leading to better nutrient utilization (Aristimunha et al., 2020; Nari et al., 2020). Although a cost benefit analysis was not performed in the present study, the data from this study suggest that the combination of MC and BG in the wheat-based diets may improve economic concerns in

Table 6. Effects of dietary treatments on jejunum morphology of laying hens at 62 wk of age.

Basal diet	MC[1] level	BG[2] level	Villus height µm	Villus width µm	Crypt depth µm	VH/CD[3]	VSA[4] mm^2
Corn	−	−	1,176.0	121.8	130.1	9.09	0.448
Corn	−	+	1,258.0	115.6	127.0	10.17	0.457
Corn	+	−	1,207.4	120.7	125.6	9.92	0.457
Corn	+	+	1,262.9	125.9	128.1	10.13	0.505
Wheat	−	−	1,024.6	118.8	145.6	7.12	0.385
Wheat	−	+	1,166.2	123.4	141.4	8.36	0.449
Wheat	+	−	1,076.6	119.9	140.5	7.74	0.403
Wheat	+	+	1,189.5	125.1	141.8	8.58	0.472
SEM			54.68	6.32	8.29	0.715	0.032
Main effect means							
Basal diet							
Corn			1,226.1[a]	121.0	127.7[b]	9.83[a]	0.466
Wheat			1,114.2[b]	121.8	142.3[a]	7.95[b]	0.427
SEM			27.33	3.16	4.15	0.357	0.016
MC level							
−			1,156.2	119.9	136.0	8.68	0.435
+			1,184.1	122.9	134.0	9.09	0.459
SEM			27.33	3.16	4.15	0.357	0.016
BG level							
−			1,121.1[b]	120.3	135.4	8.47	0.423[b]
+			1,219.1[a]	122.5	134.6	9.31	0.471[a]
SEM			27.33	3.16	4.15	0.357	0.016
Significance							
Basal diet			0.006	0.860	0.017	<0.001	0.089
MC level			0.475	0.506	0.730	0.421	0.286
BG level			0.015	0.622	0.883	0.104	0.042
Diet × MC			0.802	0.723	0.959	0.977	0.858
Diet × BG			0.454	0.550	0.920	0.699	0.406
MC × BG			0.723	0.506	0.637	0.534	0.635
Diet × MC × BG			0.989	0.555	0.998	0.820	0.701

[a,b]Means within each column with no common superscript differ ($P < 0.05$).
[1]Multicarbohydrase.
[2]Butyrate glycerides.
[3]villus height/crypt depth.
[4]VSA, villus surface area = $2\pi \times$ (villus width/2) $\times$ villus height.

the egg-laying industry by improving egg production and feed conversion ratio.

As presented in Table 4, the type of diet did not influence any of the egg quality parameters studied, except for egg yolk index and eggshell thickness, which were increased when a corn-based diet was fed. In agreement with our results, previous studies have shown that feeding corn-containing diets increased yolk color compared with wheat-containing diets (Pérez-Bonilla et al., 2011; Ghasemi et al., 2014). The increasing effect of corn-based diets on Roche color fan values of the yolks is linked to the high content of carotenoid pigments (especially carotenes and xanthophyll) within the yellow corn varieties that impart a yellow or orange color to egg yolk (Perry et al., 2009). In addition, it seems that the absorption of dietary minerals is greater from corn-based diets than that of wheat-based diets because of the lower NSP content of former diets and better absorption of nutrients including minerals within the gastrointestinal tract (resulted from a lower digesta viscosity). As a high efficiency of calcium absorption is one of the key factors in achieving optimal eggshell quality in laying hens especially in the last third of the laying cycle (Akbari Moghaddam Kakhki et al., 2019), this could be a possible explanation for the improved eggshell thickness in the aged hens fed the corn-based diet in the present study.

In the present study, a two-way interaction between diet and MC was observed for eggshell breaking strength. Because optimizing eggshell mineralization is an important factor affecting the eggshell strength, the effectiveness of MC in improving the eggshell strength may be correlated with the utilization of minerals, especially calcium. It is possible that the production of xylose, arabinose, and arabinoxylo-oligomers from arabinoxylan hydrolysis contributes to beneficial modifications in the pH and microbiota of the intestine and thus increases calcium solubility and absorption (Li et al., 2017; Park and Carey, 2019). In agreement with our results, previous studies (Sobczak and Kozłowski, 2016; Kazempour and Jahanian, 2017) reported that dietary supplementation with butyric acid could increase

Table 7. Effects of dietary treatments on ileal microbiota composition (log cfu/g fresh digesta) of laying hens at 62 wk of age.

Basal diet	MC[1] level	BG[2] level	Total bacteria	*Lactobacillus* spp.	*Bifidobacterium* spp.	*Escherichia coli*	*Salmonella* spp.
Corn	−	−	6.36	5.04	4.29	4.88^{d}	4.11
Corn	−	+	6.21	5.09	4.76	4.92^{d}	3.66
Corn	+	−	6.23	5.09	4.80	5.01^{c,d}	4.07
Corn	+	+	6.17	5.31	4.84	4.74^{d}	3.38
Wheat	−	−	7.21	4.57	4.22	6.85^{a}	4.61
Wheat	−	+	6.57	4.94	4.48	5.48^{b,c}	4.10
Wheat	+	−	7.08	5.17	4.74	5.70^{b}	4.34
Wheat	+	+	6.35	5.16	4.71	5.09^{c,d}	3.73
SEM			0.219	0.177	0.230	0.180	0.191
Main effect means							
Basal diet							
Corn			6.24^{b}	5.13	4.67	4.89^{b}	3.81^{b}
Wheat			6.80^{a}	4.96	4.54	5.78^{a}	4.20^{a}
SEM			0.109	0.088	0.115	0.089	0.096
MC level							
−			6.59	4.91^{b}	4.44^{b}	5.53^{a}	4.12
+			6.46	5.18^{a}	4.77^{a}	5.14^{b}	3.88
SEM			0.109	0.088	0.115	0.089	0.096
BG level							
−			6.72^{a}	4.97	4.51	5.61^{a}	4.28^{a}
+			6.32^{b}	5.12	4.70	5.06^{b}	3.72^{b}
SEM			0.109	0.088	0.115	0.089	0.096
Significance							
Basal diet			<0.001	0.175	0.413	<0.001	0.006
MC level			0.401	0.036	0.046	0.003	0.086
BG level			0.015	0.212	0.266	<0.001	<0.001
Diet × MC			0.754	0.292	0.821	0.006	0.540
Diet × BG			0.068	0.858	0.668	0.001	0.963
MC × BG			0.994	0.667	0.268	0.356	0.544
Diet × MC × BG			0.767	0.280	0.825	0.043	0.786

[a-d]Means within each column with no common superscript differ ($P < 0.05$).
[1]Multicarbohydrase.
[2]Butyrate glycerides.

eggshell thickness in laying hens fed corn-soybean meal-based diets. This improvement could be explained by a greater increase in mineral and protein absorption after dietary supplementation with BG in the present study.

As presented in Table 5, the interactions between the basal diet and MC supplementation on digesta viscosity and AID of crude fat and crude ash were significant, a finding that suggests that MC was more effective in diets based on wheat than in diets based on corn. Similar to the above studies, our results showed that jejunal digesta viscosity was increased with the substitution of corn by wheat in the diet, while was decreased by NSP-degrading enzymes, results that are in line with most research conducted with wheat-containing diets for laying hens (Mirzaie et al., 2012), broilers (Kiarie et al., 2014; Ayres et al., 2018), and turkeys (Ingelmann et al., 2018). Our results also confirm the results of a recent study (Lu et al., 2020), indicating that the presence of arabinoxylan reduces the nutrient digestibility and that the addition of a carbohydrase mixture (rich in xylanase and arabinofuranosidase) to the broiler diets improves the overall digestibility of nutrients. The improvements in nutrient digestibility by enzyme supplementation in the wheat-based diet in the present study could be mainly related to the degradation of arabinoxylans and xylans, the major components of NSP in wheat grain, which increase the digesta viscosity and lead to the possible adverse effects on the digestion and absorption of nutrients in the gastrointestinal tract (Annison and Choct, 1991; Choct and Annison, 1992). These findings might be directly associated with improvements in productive performance and eggshell strength of laying hens fed wheat-SBM-based diets supplemented with multienzyme preparation.

In the present study, dietary supplementation with BG resulted in improvements in digestibility of dietary protein and ash. One possible mechanism by which BG supplementation can improve the protein and ash digestibility is through reduction of gastrointestinal pH, which generally leads to better conditions for pepsin activity and more degradation of NSP and phytate (Jahanian and Golshadi, 2015; Vieira et al., 2018; Nari and Ghasemi, 2020). As noted, supplemental BG increased jejunal VH and VSA in this study. It seems that improving the gut morphology is another possible mechanism, by which BG could improve ileal nutrient digestibility.

Measurement of intestinal mucosal morphology can help in evaluating the absorptive surface area for nutrient uptake (Geyra et al., 2001). In this study, feeding wheat-based diets significantly decreased VH and VH/CD ratio, but increased CD in the jejunum as compared with the corn-based diets. It seems that an increase in the levels of dietary NSP (wheat inclusion to diet) could negatively alter the morphological development of the intestine by increasing the digesta viscosity (Sharifi et al., 2013). However, dietary multienzyme did not affect the morphological parameters in the jejunum, suggesting that the positive effect on egg-laying performance caused by multienzyme was not associated with an increase in the jejunal absorptive surface area. As seen in Table 6, the inclusion of BG in laying hen diets exerted a positive impact on the intestinal morphology, as reflected by increased VH and VSA of the jejunum. There are limited data available in the literature evaluating the influence of dietary BG supplementation on intestinal morphology of laying hens. However, results obtained in this study are consistent with other researchers, who reported improved intestinal morphology with butyrate glycerides in broiler chickens (Nari et al., 2020) and Japanese quail (Elnesr et al., 2019). The beneficial effects of butyric acid on intestinal morphology may be due to the ability to facilitate cell differentiation in the intestinal epithelium, as butyrate is known to be a strong promoter of cell differentiation (Kang et al., 2011). In addition, the improvements in jejunal morphology can be attributed to the protective effect of butyric acid against intestinal inflammation as proposed by Zou et al. (2019), who reported that dietary supplementation of BG could decrease the growth of intestinal pathogenic bacteria, leading to a lower inflammatory response and improvement in the histological structure of the intestinal mucosa.

The gut microbiota plays a central role in host nutrition and health maintenance through enhancing supply, digestion, and absorption of nutrients, protecting against pathogen colonization, and modulating metabolic and immune functions (Marchesi et al., 2016). As presented in Table 7, *E. coli* counts were affected by basal diet × MC × BG interaction, so that simultaneous dietary supplementation with MC and BG had more pronounced suppressive effects on ileal *E. coli* count of hens fed wheat-based diets compared with those on corn-based diets. Because changes in the intestinal microbiota can influence nutrient absorptive capacity, this may partially explain the improvements in egg production and FCR that were observed in hens fed on wheat-based diets supplemented with MC and BG. Although the antibacterial mechanism of BG is not fully understood, it could be attributed to a decrease in the acid buffering capacity and pH in the intestinal tract of birds, thereby inhibiting the growth of pathogenic bacteria (Panda et al., 2009; Nari et al., 2020).

Interestingly, our results showed that the numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. in the ileal digesta were increased by dietary supplementation of multienzyme. In a study on broiler chickens, Liu and Kim (2017) observed that dietary xylanase addition increased *Lactobacillus* numbers, while decreased *E. coli* populations in the ileum and cecum. By contrast, Rebolé et al. (2010) showed that supplementation of an enzyme blend (xylanase, β-glucanase, and glucanase) in broiler chickens fed wheat- or barley-based diets did not affect the ileum and cecum composition of bacterial phyla. The possible reason for these results in our study may be that the MC enzyme preparation used in the present study hydrolyzes NSP compounds, such as arabinoxylans and β-glucans, to release oligosaccharides, which can act as prebiotics and encourage the growth of lactic acid bacteria in the intestine.

CONCLUSIONS

In conclusion, the results of the present study indicated that replacing 100% of the corn with wheat in laying hen diets negatively affected egg quality and gut morphology during a 12-week laying period. By contrast, improvements in productive performance and gut microbiota were observed when adding both multicarbohydrase and butyrate glycerides to wheat-based diets, but not when added to corn-based diets. However, the ability of multicarbohydrase, butyric acid, or their combination to ameliorate the negative impact of wheat-based diets in laying hen diets is still limited and, therefore, more studies are needed in this area.

ACKNOWLEDGMENTS

This work was funded by a grant (grant no. 97-12356) from Arak University (Arak, Iran). Authors of this article are grateful to the management board of the AKAM Faravardehaye Bahman Arad Co. (Karaj, Iran), especially Dr. Bahman Goudarzi as the managing director of the company, for providing all the materials that made possible the implementation of this experiment. The authors also thank Dr. Farhad Khaligh for his assistance with regard to bird management and sample collection.

DISCLOSURES

The authors declare that the research was performed in the absence of any financial or commercial relationships that could be construed as a potential conflict of interest.

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