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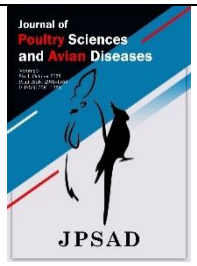

Effect of Glucose Oxidase and a Commercial Bacillus-based Direct Feed Microbial Supplementation on the Productive Performance, Intestinal IgA, Gut Permeability, and Cecal Microbiota of Broiler Chickens

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ABSTRACT

Restrictions on the use of antibiotic growth promoters in broiler chickens have stimulated the search for alternatives, such as probiotics and enzymes. *Bacillus* has benefits for intestinal microbial balance and productive performance; however, less is known about the effects of the enzyme glucose oxidase (GOx) in chickens, and there is scarce information about the combination of both additives on broiler performance and cecal microbiota. Chickens supplemented with either *Bacillus* (10^6 spores/g feed), GOx (100 U/kg feed), the combination of both, or a control group were evaluated. Improvements were observed in performance parameters and gut health, with a reduction in intestinal IgA concentration in the treated groups; however, no difference was noted in gut permeability (serum FITC-d concentration). *Bacillus* and GOx alone increased the cecum microbial Alpha diversity; meanwhile, the Beta diversity from *the Bacillus* group was different from that of the control and *Bacillus*-GOx groups. A reduction in harmful bacteria (Proteobacteria) along with an increase in beneficial bacteria (Firmicutes and Actinobacteria) was observed in the cecal microbiota composition from the treated groups. GOx treatment increased the phylum Actinobacteria. *Bacillus* and GOx can enhance the gut health of chickens by modulating the gut microbiota. However, no synergic effect was seen in the group receiving the additive combination. Further research is needed to more effectively demonstrate the effect.

Keywords: *Bacillus DFM; Glucose oxidase; Broiler chickens; Gut integrity; Microbiota; Intestinal IgA*

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1 Introduction

The demand for animal protein, particularly poultry meat and eggs, is expected to increase significantly as the world's population is projected to reach 10 billion people by 2050, posing a challenge to global food security. Antibiotic growth promoters (AGPs) have been used to accelerate development and improve feed conversion efficiency (1); however, there is growing concern about the emergence of antimicrobial resistance in bacterial populations, as well as the problem of antibiotic contamination in the environment within the framework of "One Health". The absence of AGPs can negatively impact production efficiency. This has created a significant demand for alternatives to them, such as enzymes, organic acids, probiotics, prebiotics, and essential oils (2).

Traditionally, the enzymes have been used to increase nutrient availability, but they have also been used as alternatives to AGPs, such as glucose oxidase -GOx- (3), which is an enzyme that catalyzes the oxidation of β-D-glucose to D-gluconic acid and hydrogen peroxide. In poultry production, GOx acts non-pharmacologically to inhibit the growth of *Clostridium perfringens*, *Salmonella*, and *Escherichia coli* by producing hydrogen peroxide (4), thereby avoiding the problems associated with antibiotic and residual drug resistance (5). GOx also enhances the intestinal barrier and nutrient absorption functions, increases the concentration of secretory IgA, and suppresses inflammation by regulating the expression of inflammatory cytokines. Additionally, it promotes the maturation of crypt-villous structures, improves the function of tight intestinal junctions, and maintains immune homeostasis (6).

Probiotics are viable bacteria that can improve health by modulating the host's immune system, regulating tight junction proteins, aiding mucin production, providing energy through SCFA production, and influencing gut structure, integrity, and function. Probiotics also produce metabolites and antimicrobial compounds, occupy ecological niches within the gut to competitively exclude colonization by other bacteria, including pathogens (7). Spore-forming probiotics, *such as Bacillus* spp. and *Clostridium* spp., are popular in the poultry industry as natural growth promoters due to their ability to encapsulate (8). *Bacillus* has been shown to be effective in promoting intestinal microbial balance and the productive performance of birds (9).

There are scarce references to the combination of GOx and *Bacillus* in chicken feed. Some studies suggest GOx stimulates growth and enhances the probiotic activity of *B. subtilis* by increasing gluconic acid production (10). The combination of GOx and *Bacillus amyloliquefaciens* SC06 exhibited a positive effect on antioxidative capacity and immune function, yielding more beneficial effects than GOx treatment alone (11). GOx and *B. subtilis* induced slightly different functions on improving poultry growth performance (12). Some studies suggest GOx stimulates growth and enhances the probiotic activity of *B. subtilis* by increasing gluconic acid production (10). Therefore, it is hypothesized that combining a DFM containing three species of *Bacillus* and GOx could have a synergistic effect on broiler performance, cecal microbiota, immune response, and gut permeability. The objective of this experiment was to determine the effect of GOx and *Bacillus* supplementation on the productive parameters and intestinal integrity of broiler chickens. The novelty lies in the combination's ability to provide stronger benefits than either additive alone, particularly in improving intestinal microbiota, local humoral immune response, and leaky gut status.

2 Materials and Methods

Four hundred Cobb 500 male broiler chicks from Cobb-Vantress (Siloam Springs, AR) were used, weighed individually, and randomly assigned to one of four groups, n=10 chicks/group and ten replicates per group: Control, *Bacillus*, Glucose oxidase (GOx), and *Bacillus*+Glucose oxidase (*Bacillus*-GOx). The chicks were placed in rearing batteries (4 levels, three cages per level). A gradual reduction in temperature from 32°C to 24°C, with a relative humidity of 55 ± 5%, was used during the first 14 days, and 24°C was maintained from days 15 to 21. Lighting hours were 23, 20, and 18 from days 1 to 4, 5 to 14, and 15 to 21, respectively. All animal handling procedures were conducted under the Institutional Animal Care and Use Committee (IACUC) protocol #15006 at the University of Arkansas, Fayetteville.

The food-grade glucose oxidase enzyme used in the study is produced by Aspergillus niger (Lab Creative Enzymes, Shirley, NY, USA). The enzyme was added to the feed at a final concentration of 100 U/kg of feed (5). The commercial product used of *Bacillus* (Norum™, Eco-Bio/Euxxis Bioscience LLC, Fayetteville, AR, USA) is a Direct Feed Microbial (DFM) culture composed of spores of *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis*. It was added to the feed at a final concentration of 10^6 spores/g of feed (13). The GOx and DFM were supplied during the whole

experiment (21 days). The feed was adjusted according to the recommendations from Cobb-Vantress (14), Table 1 No AGP nor coccidiostats were used. Chickens had free access to water.

Table 1. Ingredient composition and nutrient content of the maize and soy-based diet used.

Element	Initial diet		
Ingredients	(%)	Calculated análisis	(%)
Corn	51.85	ME (kcal/kg)	3015
Soybean meal	37.66	Ethereal extract	5.88
Dried Distillers Grains	4.00	Crude protein	22.30
Poultry fat	3.24	Lysine	1.18
Calcium carbonate	1.08	Methionine	0.59
Dicalcium phostat	1.01	Treonina	0.77
Sal	0.35	Tryptophan	0.25
DL-methionine	0.29	Total calcium	0.90
L-lisine HCl	0.12	Total phosphorus	0.63
Vitamin premix [a]	0.10	Available phosphorus	0.45
Mineral A premix [b]	0.10	Sodium	0.20
L-Threonine	0.08	Potassium	1.06
Choline chloride	0.06	Chloride	0.27
Baking soda	0.04	Magnesium	0.19
Antioxidant [c]	0.02	Copper	19.20
		Selenium	0.28
		Linoleic acid	1.01

[a] Vitamin premix supplied per kg: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO 64850). [b] Mineral premix supplied per kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10-15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO 64850). [c] Ethoxyquin.

Body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were evaluated at 7, 14, and 21 days of age. Indicators of intestinal integrity were also evaluated, including FITC-d as an indicator of paracellular transport and mucosal barrier dysfunction, as well as total IgA and the composition and diversity of the intestinal microbiota. At the end of the experimental period (21 days), exactly 1 hour before the euthanasia of the chickens by inhaling CO_2, they were weighed to administer by esophageal tube a dose of 8.32 mg of FITC-d/kg of body weight to 20 chickens of each group (2 per replicate). Immediately after death, the chickens were bled through the femoral vein to obtain serum for the determination of FITC-d.

For the quantification of total IgA, a 5 cm segment of the jejunum was taken from Meckel's diverticulum, rinsed with 5 mL of 0.9% saline, and then the rinse was centrifuged at 2,200 × g at four ºC for 10 min. The supernatant was transferred into a 96-well plate and stored at -20°C until further testing.

Considering sequencing cost, statistical power for detecting microbiota differences, and inter-individual variability in the chicken cecum microbial community, the cecal content of six chicks per group was aseptically and individually collected to determine the composition and diversity of the microbiota. The samples were selected from the 20 chickens of each group by generating random numbers in an Excel for Microsoft 365 worksheet, avoiding two from the same replicate and without any pre-selection criteria or stratification by cage or replicate, After taken, the samples were immediately placed in sterile tubes and quickly transferred to liquid nitrogen, further, they were stored at -80°C for subsequent study. The samples were preserved in a nucleic acid stabilization solution (DNA/RNA ShieldTM, Zymo Research, USA).

2.1 Fluorescein dextran-isothiocyanate serum determination (FITC-d)

FITC-d (MW 3-5 KDa; Sigma-Aldrich Co., St. Louis, MO) was used as a marker of paracellular transport and mucosal barrier dysfunction. Serum samples were diluted 1:5 in PBS, and FITC-d concentrations were fluorometrically measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, multimode microplate reader, BioTek Instruments, USA). The fluorescence measurements were then compared with a standard curve with concentrations of serum without FITC-d obtained from the control chickens. FITC-d

concentrations were calculated with the Gen5™ software (BioTek) and expressed as ng of FITC-d/ml of serum (15).

2.2 *Enzyme-linked immunosorbent assay (ELISA) to determine total IgA levels.*

An indirect ELISA was performed to quantify the total IgA in the intestinal lavage of 20 samples per group as previously described (16). The commercial chicken IgA ELISA quantification kit (Catalog E30-103, Bethyl Laboratories Inc., Montgomery, TX, 77356) was used according to the manufacturer's instructions. Samples were thawed at room temperature and diluted (1:100), and 100 µL was added to the respective wells. A standard curve was used to quantify the total IgA in the samples. Absorbance from the plates was measured at 450 nm using an ELISA plate reader (Synergy HT, multimode microplate reader, BioTek Instruments, Inc., Winooski, VT, USA). The total IgA obtained was multiplied by the dilution factor (100) to determine the amount of chicken IgA in the undiluted samples.

2.3 *Analysis of the cecal microbiota*

DNA extraction and PCR: Two hundred milligrams of cecal content from each sample were used for genomic DNA extraction using the QIAamp Rapid Stool DNA Minikit (Catalog 51604, Qiagen Inc., Germantown, MD, USA) following the manufacturer's instructions, with the addition of the microbead blending step (17).

The V3–V4 region of the 16S rRNA gene was amplified from the genomic DNA samples using barcode-tagged universal primers 341F: CCTAYGGGRBGCASCAG and 806R: GGACTACNNGGGTATCTAAT with attached Illumina adapter (18). A library was prepared using the NEBNext Ultra Library Prep Kit (New England Biolabs, Ipswich, MA, USA). The pooled, purified amplicons were sequenced using a 300-cycle MiSeq Illumina with paired-end options at the University of California, Riverside (Riverside, CA, USA) (13).

2.4 *16S rRNA gene sequence analysis*

Paired-end 16S V3-V4 RNA sequencing reads were analyzed in QIIME 2, v. 2020.11 (https://qiime2.org/). The adapter, barcode, and primer sequences were removed before post-analysis using the "Cutadapt" plug-in in QIIME2. Then, the direct and reverse readings of each sample were combined, and quality control was performed. Filtered (low-quality) reads were removed using the Deblur algorithm, version 2022.8.0, to produce ASVs. The ASVs were classified using the Ribosomal Database Project (RDP) 16s rRNA training set (18) and the Bayesian classifier (19). Taxa with an initial confidence of <80% were assigned the name of the last taxonomic level assigned with confidence, followed by "_unclassified. The ASVs that appeared in <5% of the samples were removed for further analysis.

2.5 *Statistical analysis*

Performance parameters, total intestinal IgA, and FITC-d were subjected to an analysis of variance (ANOVA) using a fully randomized design, as implemented in SAS 9.3 PROC GLIMMIX with a generalized linear mixed model, with fixed effects for treatments (20). Significant differences between means were determined using Duncan's multiple range test at $p < 0.05$.

For the evaluation of the cecal microbiota, the alpha diversity analysis was calculated based on the Shannon index. Data were normalized using the cumulative sum scale prior to any statistical comparison (21). Significant differences in alpha diversity among groups were calculated using the ANOVA/T test, with a significance level set at $p < 0.05$. The assumptions of homogeneity of variance and normally distributed residuals were examined visually using the conditional Studentized residuals plots. Beta diversity was calculated using the UniFrac weighted distance metric (22), and statistical comparisons between groups were performed with the similarity analysis method (ANOSIM). The *p*-value was calculated with PERMANOVA using 999 permutations. Cluster analysis was performed using principal coordinate analysis (PCoA) to visualise complex multidimensional data and obtain principal coordinates. To determine the differentially abundant phyla and genera among the various groups, MetagenomeSeq was employed, which utilizes an inflated zero Gaussian adjustment model, with a significance level set at $p < 0.05$. LEfSE was used to conduct linear discriminant analysis (LDA), and the multi-group comparison strategy employed was an all-against-all approach.

3 Results

Performance parameters, including total IgA and FITC-d concentrations, were compared using ANOVA and Duncan's multiple range test. The microbiota Alpha diversity was compared using ANOVA/T-test, and Beta diversity was assessed using ANOSIM-PERMANOVA.

3.1 *Performance Parameters*

The productive performance parameters are shown in Table 2. Compared to the control group, only the *Bacillus* group exhibited a higher body weight and weight gain ($p < 0.05$). The GOx and *Bacillus*-GOx groups did not differ from the control or *Bacillus* groups at 14 and 21 days. Despite the increase in body weight and weight gain, the group treated with *Bacillus* didn't show any difference in food intake compared to the other groups ($p>0.05$). The conversion rate was significantly improved ($p < 0.05$, ANOVA + Duncan) in the period from 1 to 14 days in the *Bacillus*-treated group compared to the control group, but not in the other groups.

Table 2. Effect of supplementation with glucose oxidase and Bacillus applied directly to feed on broiler performance parameters.

Variable Days of age	Control	Bacillus	Glucose oxidase	Bacillus + Glucose oxidase
Body weight (g)				
1	41 ± 0.2 [a]	41 ± 0.1 [a]	42 ± 0.2 [a]	41 ± 0.2 [a]
7	119 ± 3 [b]	123 ± 2 [ab]	126 ± 2 [a]	126 ± 2 [a]
14	336 ± 8 [b]	371 ± 6 [a]	360 ± 7 [ab]	359 ± 9 [ab]
21	843 ± 34 [b]	917 ± 11 [a]	909 ± 17 [ab]	902 ± 21 [ab]
Weight gain (g)				
1 – 7	77 ± 3 [b]	81 ± 2 [ab]	84 ± 2 [ab]	85 ± 2 [a]
1 – 14	294 ± 8 [b]	328 ± 7 [a]	317 ± 8 [ab]	318 ± 9 [ab]
1 – 21	801 ± 34 [b]	874 ± 12 [a]	867 ± 17 [ab]	860 ± 20 [ab]
Feed intake (g)				
1 – 7	112 ± 3 [a]	112 ± 1 [a]	115 ± 2 [a]	113 ± 1 [a]
1 – 14	422 ± 4 [a]	429 ± 5 [a]	434 ± 7 [a]	429 ± 4 [a]
1 – 21	1169 ± 9 [a]	1195 ± 24 [a]	1189 ± 11 [a]	1167 ± 12 [a]
Conversion Rate (g)				
1 – 7	0.9 ± 0.01 [a]	0.9 ± 0.01 [a]	0.9 ± 0.02 [a]	0.9 ± 0.01 [a]
1 – 14	1.2 ± 0.03 [a]	1.1 ± 0.02 [b]	1.2 ± 0.03 [ab]	1.2 ± 0.03 [ab]
1 – 21	1.4 ± 0.06 [a]	1.3 ± 0.04 [a]	1.3 ± 0.03 [a]	1.3 ± 0.02 [a]

*Data are expressed as average ± SE.

[abc] Indicates significant differences among treatments within the ranks ($P < 0.05$).

Dosage of administration: Glucose oxidase 100 U/Kg, Norum™ 100 g/ton.

3.2 *Total intestinal IgA and serum FITC-d concentration*

Table 3 shows the results of total intestinal IgA levels and serum FITC-d concentration in broilers. A reduction in the concentration of IgA ($p < 0.05$) was observed in the treated groups (*Bacillus*: 10^4 µg/ml, GOx: 10^1 µg/ml, and combination: 10^1 µg/ml) compared to the positive control group (137 µg/ml). Supplementation with *Bacillus* and GOx, alone or in combination, in the chickens' diet reduced total intestinal IgA concentration at 21 days of age.

Table 3. Total intestinal IgA and serum FITC-d concentration in broilers fed with or without Bacillus and GOx in the diet.

Treatments	Intestinal IgA (µg/mL)	FITC-d (µg/mL)
	Day 21	
Control	137 ± 5 [a]	28 ± 9 [a]
Bacillus	104 ± 8 [b]	39 ± 6 [a]
Glucose oxidase	101 ± 5 [b]	28 ± 7 [a]
Bacillus + Glucose oxidase	118 ± 8 [b]	52 ± 9 [a]

The data expresses the average ± SE of 20 chickens

a-b Different superscripts in the same column indicate a significant difference in $P < 0.05$.

No significant difference was observed in the serum concentration of FITC-d between the control group and the other groups ($p > 0.05$).

3.3 *Cecal microbiota*

3.3.1 *Analysis of microbial diversity*

Figure 1a shows the alpha diversity of the groups as measured by the Shannon index. The mean value in the control group was 2.94 ± 0.09 (Mean ± SEM), which was significantly lower (p < 0.05, ANOVA/T) than in the *Bacillus* and GOx groups (3.72 ± 0.22 and 3.48 ± 0.18, respectively). The *Bacillus*+GOx group did not differ significantly from any of the other groups.

Beta diversity (weighted UniFrac distance) among groups is illustrated in the principal coordinate analysis plot (Figure 1b). Pairwise comparisons show differences between *Bacillus* and control groups (p=0.045, PERMANOVA) and between Bacillus and *Bacillus*-GOx groups (p=0.021, PERMANOVA).

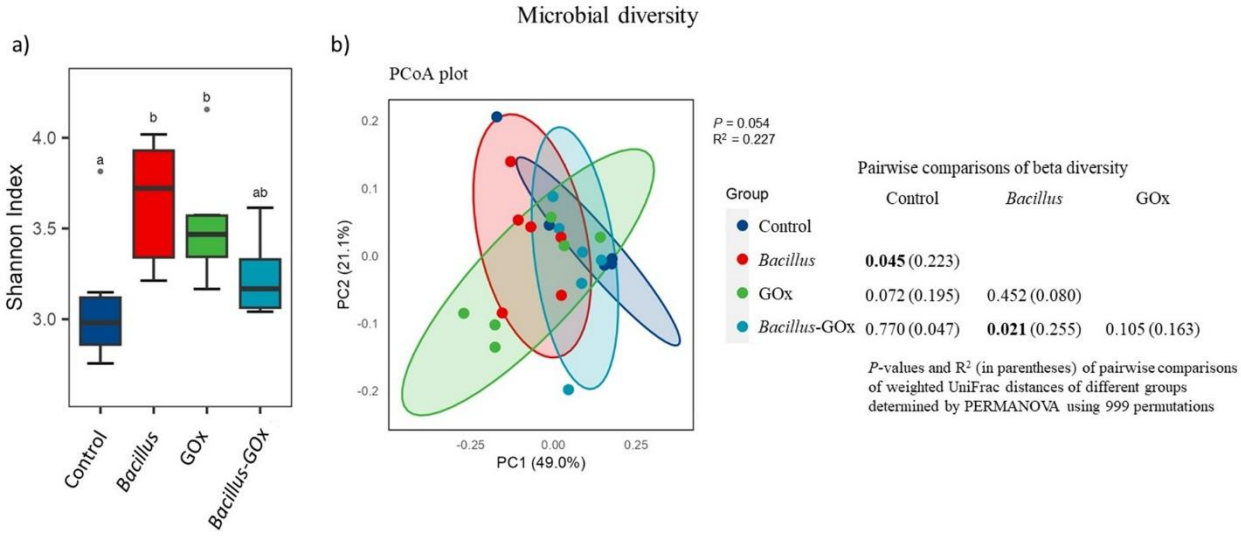

Figure 1. a) Alpha diversity as measured by the Shannon index ([ab] Significant differences were observed among the control group and the Bacillus and GOx groups.) b) PCoA graph showing the microbial community structure among groups.

3.3.2 *Taxonomic assignments*

Firmicutes was the predominant phylum in all groups (control: 88.71%; *Bacillus*: 96.68%, GOx: 92.12%, and *Bacillus* + GOx: 94.53%), followed by Proteobacteria and Actinobacteria, as shown in Figure 2a. Actinobacteria were significantly lower (p<0.05) in the control group compared to the treated groups.

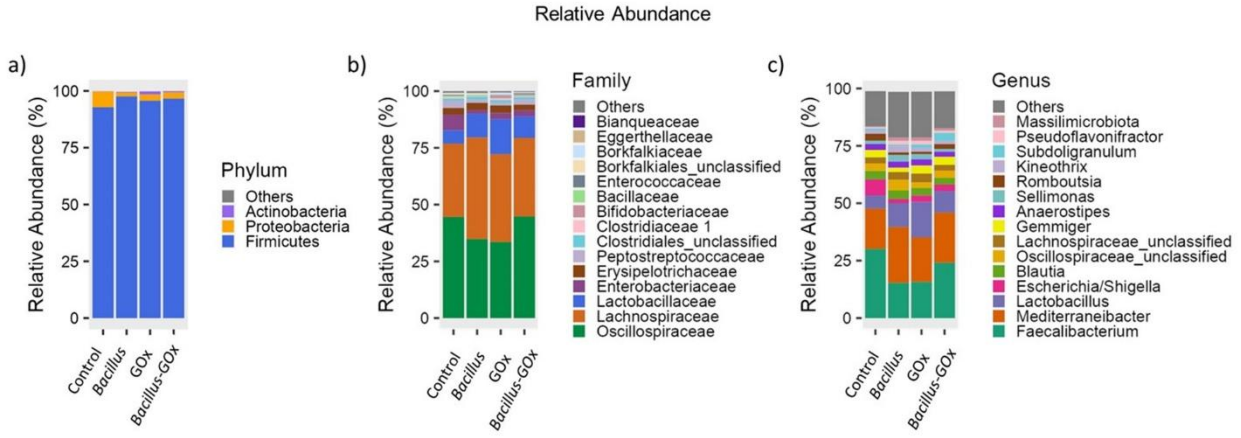

Figure 2. a) Relative abundance of the main phyla in the cecum of broilers on day 21. b) Relative abundance of the main families recovered in the cecum of broilers on day 21. c) Relative abundance of the main genera recovered in the cecum of broilers on day 21. Genres with counts <100 are grouped under "Others".

The bacterial families are presented in Figure 2b, the most abundant in all groups were Oscillospiraceae (predominant in the control group, 46.21% and *Bacillus*+Gox group, 46.09%), Lachnospiraceae (predominant in the *Bacillus* group, 48.23% and GOx, 30.61%) and Lactobacillaceae, which was a significantly increased (p<0.05) in the treated groups compared to the control group (10.11%; *Bacillus*, 14.75%; GOx, 16.89% and *Bacillus*+GOx, 13.02%). There was a significant decrease (p<0.05) in the Enterobacteriaceae family in the *Bacillus* (3.21%), GOx (4.87%), and *Bacillus* + GOx (5.41%) groups compared to the control group (11.23%). Other notable families in the groups are *Erysipelotrichaceae*, *Peptostreptococcaceae*, and *Clostridiales*, among others.

The relative abundance of genera present in the control and treatment groups is shown in Figure 2c. *Faecalibacterium* was the predominant genus in the control group (28.78%) and *Bacillus*+GOx (24.94%), followed by *Mediterraneibacter* (control, 20.13%; *Bacillus*+GOx, 24.12%). However, in the *Bacillus* and GOx groups, the opposite occurs; the *Mediterraneibacter* genus is the predominant one (*Bacillus* group, 24.96%; GOx, 22.38%), followed by *Faecalibacterium* (*Bacillus* group, 19.07%; GOx, 19.23%). *Lactobacillus* occupied the third position, followed by control (8.55%), *Bacillus* (11.89%), GOx (19.81%), and *Bacillus*+GOx (10.04%). The genera *Escherichia/Shigella*, as well as *Romboutsia*, decreased significantly in the treated groups compared to the control group (p < 0.05). In addition, one unclassified genus from the family Oscillospiraceae increased in the *Bacillus* group, *Gemmiger* increased in the GOx group, and *Sellimonas* increased in the *Bacillus*+GOx group. While *Blautia*, unclassified from Lachnospiraceae, and *Anaerostipes* remained similar in all groups.

The results of LEfSE linear discriminant analysis used to evaluate the differences in the relative abundance are shown in Figure 3. The Lachnospiraceae family was more predominant in the *Bacillus* group than the Bifidobacteriaceae in the GOx group. At the genus level, *Sellimonas* (Lachnospiraceae) in the Bacillus group and *Massilimicrobiota* (Erysipelotrichaceae) in the GOx group were increased compared to *Anaeromassilibacillus* (Acutalibacteriaceae) and *Merdimonas* (Lachnospiraceae), respectively. *Lachnospira* was the only genus in the *Bacillus*-GOx group. In the control group, *Mediterraneibacter* (Lachnospiraceae) was found to have a lower relative abundance than *Ruminococcus lactaris* (Lachnospiraceae), *Lactobacillus* (Lactobacillaceae), and *Oscillospiraceae* in *the Bacillus*, GOx, and *Bacillus*-GOx groups, respectively.

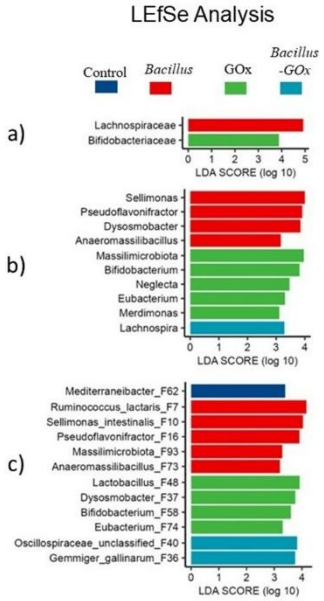

Figure 3. Linear discriminant analysis (LDA) presents the predominant bacteria in each group and the discrimination among them.

4 Discussion

The effect of a commercial product based on three *Bacillus* species, namely *subtilis*, *amyloliquefaciens*, and *licheniformis*, which has a synergistic effect, was investigated (9,13). In this study, the administration of *Bacillus* in broilers improved body weight (+8.7%) and weight gain (+9.1%) on day 21, and feed conversion efficiency (-0.1%) from day 1 to 14, while maintaining feed intake. Another study reported a higher body weight in chickens receiving *Bacillus subtilis,* without a difference in feed intake at three weeks of age (23) and a reduction in feed conversion (24).

In this experiment, GOx alone didn't affect performance parameters, which agrees with other experiments performed in Arbor Acres (AA) chickens at 35 days of age (25) or during days 1-21 (26); nevertheless, 250 U/Kg GOx increased daily body weight gain during days 22–42. In contrast, the use of 50 and 60 U/Kg GOx improved average daily weight gain, while 60 U/Kg improved the ratio of feed to gain in AA chickens during the first three weeks of life (27).

The *Bacillus*-GOx combination in this study had no significant effect on productive performance; similar results were previously reported (28) using GOx and *B. amyloliquefaciens.*

Secretory IgA protects the gut from food and microbial antigens (16). In the present study, total IgA in the control group was higher compared to the treated groups. The lower level of IgA in the *Bacillus* and GOx groups may be related to their anti-inflammatory and immunomodulatory properties (10), as well as the decrease in the Enterobacteriaceae family observed in the treated groups of this experiment. In agreement, an experiment using *Bacillus* (13)resulted in a reduction of intestinal IgA. On the contrary, more IgA was found in broilers receiving GOx (26), and the jejunum of chickens receiving GOx+*Bacillus* (27). This might indicate that IgA production is related to the amount of Enterobacteriaceae present in the gut.

FITC-d serves as a biomarker for intestinal barrier function (15). This study did not show differences among the four groups, which seems normal, since the chickens were not stressed at all. In agreement, no differences were observed between the *Bacillus*-treated and control groups (13). One experiment (28) observed less FITC-d leakage in the gut with GOx +*Bacillus* administration. To our knowledge, this is the first measurement of serum FITC-d after the simultaneous administration of *Bacillus* and GOx.

The cecal microbiota of chickens can significantly influence host health and productivity by regulating nutrient metabolism, immune response, and preventing bacterial invasion (29, 30). Supplementation with *Bacillus* promotes greater microbiota biodiversity in chickens (10, 31), while GOx plays a prebiotic role, mainly by regulating the microbiota (31, 32). The increase in alpha diversity in the groups treated with *Bacillus* and GOx individually is consistent with previous studies and could be indicative of a positive effect of these treatments. The results of the beta diversity analysis showed a significant difference in microbial community structure between the *Bacillus* and the control and the *Bacillus*-GOx groups.

The cecum of young chickens is dominated by the phyla Firmicutes, Proteobacteria, and Actinobacteria (33). The relatively higher abundance of Firmicutes in the treated groups from this study agrees with the report of increased Firmicutes and decreased Proteobacteria caused by *Bacillus*-GOx (11). The higher abundance of Firmicutes may be beneficial for intestinal health, as they are known to produce short-chain fatty acids, primarily butyrate. An increased prevalence of Proteobacteria is a marker for dysbiosis and a potential diagnostic criterion for disease (34); this phylum was reduced in the supplemented groups of this experiment.

The phylum Actinobacteria increased in chickens with GOx supplementation compared to the other groups. To our knowledge, this is a significant discovery not previously reported, and it suggests a new potential for modulating the microbiota through GOx supplementation, as Actinobacteria can produce secondary metabolites such as immunomodulators, antibiotics, and anthelmintic enzymes (35). In contrast, a decrease in Actinobacteria was previously reported; however, the dietary addition of GOx improved the rate of nutrient metabolism in broilers (27).

The most prominent bacterial families were Oscillospiraceae in the control and the *Bacillus*+GOx groups, and *Lachnospiraceae* in the *Bacillus* and the GOx groups. This finding does not coincide with any other published reports in chickens. Oscillospiraceae and Lachnospiraceae in the cecum are generally associated with enhanced fermentation of complex carbohydrates, an increase in beneficial metabolites like butyrate, and positive modulation of gut immune and inflammatory responses. However, since these roles could vary with host and environmental contexts, exact implications in chickens or specific conditions may require further study.

Lachnospiraceae has also been associated with high chicken performance (36, 37).

The Lactobacillaceae family increased in the treated groups. This result is consistent with other studies (36, 38), which demonstrated beneficial effects on gut health and immune function in broilers, thereby improving their performance. The Enterobacteriaceae family, which includes pathogenic bacteria such as *Escherichia coli, Salmonella, Shigella,* and *Klebsiell*a, decreased in the treated groups, as previously reported (36, 39). The increase in Enterobacteriaceae has been associated with intestinal inflammation and dysbiosis.

In coincidence with our results, a high abundance of *Faecalibacterium* and *Mediterraneibacter* has been reported (40). *Faecalibacterium* is a commensal genus that produces butyrate in the cecum of young chickens (41) and is thought to have an anti-inflammatory effect, improving the function of the intestinal barrier (42). This genus was higher in the control and *Bacillus*-GOx groups. The genus *Mediterraneibacter* has been less mentioned in scientific literature on avian microbiota. A higher abundance of *Lactobacillus* was seen in the treated groups, especially in the GOx. *Lactobacillus* and *Faecalibacterium* are associated with greater weight gain and improved feed conversion (41); however, these results were observed only in the *Bacillus* group.

Additionally, the LDA-based LEfSE analysis revealed significant differences. Lachnospiraceae and Bifidobacteriaceae were predominant in *the Bacillus* and GOx groups. Similarly, in the *Bacillus* and GOx groups, the cecal bacterial abundance of 4 and 5 genera, respectively, increased compared to the other two groups. For example, the abundance of *Sellimonas* (*Bacillus* group) and *Bifidobacterium* (GOx group) was higher. *Lachnospira* was the only genus that increased in the *Bacillus*-GOx group, and Mediterraneibacter sp. was the only one in the control group.

When commercial chicks are reared in a controlled environment, they tend to develop their gut microbiota from the environment that surrounds them (43). This microbial colonization is crucial for the development and gut health of chicks. Several factors can influence the composition of the gut's microbiota, including diet, environmental management, bedding, air quality, and the presence of other birds and microorganisms in the environment. Exposure to Enterobacteriaceae at the time of hatch can have significant consequences for chicks' gut health in the short term (36, 44). Therefore, the provision of *Bacillus* and GOx in the feed from day 1 can prevent this situation, since the gut microbiota develops rapidly from day 1 to day 3, and most of the organisms found in the mature microbiota are already present around day 7; however, it takes up to 3 weeks before stabilizing (7). For this reason, this experiment considered the first 21 days of life to evaluate the effect of the additives on the cecum microbiota.

The main objective of AGP's replacement in poultry is to maintain or improve the productive performance. This experiment investigated the potential synergy between GOx and a DFM containing three species of *Bacillus*. The Bacillus-DFM alone improved body weight, and a trend of improvement was seen when combining it with GOx. The synergistic effect could be confirmed in trials covering the full rearing period of broiler chickens; therefore, functional gut health evaluations would be justified to explain the pathways for this synergistic effect.

Further research is required into the combined efficacy of GOx and *Bacillus*, including the evaluation of different doses of both additives and a long-term assessment in both controlled and field conditions, as well as consideration of the economic impact on diet formulation.

5 Conclusions

Bacillus-DFM improved the performance of broiler chickens from 1 to 14 days of age.

Bacillus-DFM and GOx, alone or in combination, reduced the amount of total IgA, with none of this affecting gut permeability.

The supplementation of *Bacillus*-DFM and GOx increased the abundance of Firmicutes and reduced the phylum Proteobacteria. Meanwhile, GOx alone increased the phylum Actinobacteria, so it is worth continuing to investigate the potential benefits of this additive.

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Conflict of Interest

The authors declare no competing interests.

Author Contributions

RM-G, JDL, and GT-I conceptualized the study. MC-L, RM-G, and JDL handled the methodology. RM-G, MC-L, JDL, and GT performed the formal analysis. RM-G, BMH, and GT conducted the investigation. RM-G, MC-L, and GTI prepared and wrote the original draft. RM-G, JDL, BMH, and GT contributed to the writing, review, and editing of the manuscript. GT and BMH were responsible for project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

Data Availability Statement

All data analyzed during this study are included in this article. Any other data is available from the corresponding author upon reasonable request.

Ethical Considerations

All animal handling procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) protocol #15006 at the University of Arkansas, Fayetteville.

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References

1. Li C, Cai H, Li S, Liu G, Deng X, Bryden WL, et al. Comparing the potential of Bacillus amyloliquefaciens CGMCC18230 with antimicrobial growth promoters for growth performance, bone development, expression of phosphorus transporters, and excreta microbiome in broiler chickens. Poultry Science. 2022;101(11):102126.[PMID: 36099660] [PMCID: PMC9474562] [DOI]
2. Aslam B, Khurshid M, Arshad MI, Muzammil S, Rasool M, Yasmeen N, et al. Antibiotic Resistance: One Health One World Outlook. Front Cell Infect Microbiol. 2021;11:771510.[PMID: 34900756] [PMCID: PMC8656695] [DOI]
3. Wu S, Chen X, Li T, Ren H, Zheng L, Yang X. Changes in the gut microbiota mediate the differential regulatory effects of two glucose oxidases produced by Aspergillus niger and Penicillium amagasakiense on the meat quality and growth performance of broilers. Journal of Animal Science and Biotechnology. 2020;11(1):73.[PMID: 32647570] [PMCID: PMC7336442] [DOI]
4. Muniyappan M, Chen N, Liu Y, Kim IH. The Effect of Dietary Glucose Oxidase Supplementation on Production Performance, Egg Quality and Nutrient Digestibility in Laying Hens. Brazilian Journal of Poultry Science. 2022;24[DOI]
5. Qu W, Liu J. Effects of glucose oxidase supplementation on the growth performance, antioxidative and inflammatory status, gut function, and microbiota composition of broilers fed moldy corn. Frontiers in Physiology. 2021;12:646393.[PMID: 34220529] [PMCID: PMC8244819] [DOI]
6. Bauer JA, Zámocká M, Majtán J, Bauerová-Hlinková V. Glucose oxidase, an enzyme "Ferrari": Its structure, function, production and properties in the light of various industrial and biotechnological applications. Biomolecules. 2022;12(3):472.[PMID: 35327664] [PMCID: PMC8946809] [DOI]
7. Khan S, Moore RJ, Stanley D, Chousalkar KK. The gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety. Applied and Environmental Microbiology. 2020;86(13):e00600-20.[PMID: 32332137] [PMCID: PMC7301851] [DOI]
8. Popov IV, Algburi A, Prazdnova EV, Mazanko MS, Elisashvili V, Bren AB, et al. A review of the effects and production of spore-forming probiotics for poultry. Animals. 2021;11(7):1941.[PMID: 34209794] [PMCID: PMC8300232] [DOI]
9. Hernandez-Patlan D, Solis-Cruz B, Latorre JD, Merino-Guzman R, Morales Rodríguez M, Ausland C, et al. Whole-genome sequence and interaction analysis in the production of six enzymes from the three Bacillus strains present in a commercial direct-fed microbial (NorumTM) using a Bliss Independence test. Frontiers in Veterinary Science. 2022;9:784387.[PMID: 35274019] [PMCID: PMC8902298] [DOI]
10. Wang W, Xie R, Cao Q, Ye H, Zhang C, Dong Z, et al. Effects of glucose oxidase on growth performance, clinical symptoms, serum parameters, and intestinal health in piglets challenged by enterotoxigenic Escherichia coli. Frontiers in Microbiology. 2022;13:994151.[PMID: 36267185] [PMCID: PMC9578003] [DOI]
11. Wang Y, Wang B, Zhan X, Wang Y, Li W. Effects of glucose oxidase and its combination with B. amyloliquefaciens SC06 on intestinal microbiota, immune response and antioxidative capacity in broilers. Animal. 2022;16(3):100473.[PMID: 35218993] [DOI]
12. Gao S, Zhang L, Zhu D, Huang J, Yang J, Jiang J, et al. Effects of glucose oxidase and Bacillus subtilis on growth performance and serum biochemical indices of broilers exposed to aflatoxin B1 and endotoxin. Animal Feed Science and Technology. 2022;286:115186[DOI]
13. Adhikari B, Hernandez-Patlan D, Solis-Cruz B, Kwon YM, Arreguin MA, Latorre JD, et al. Evaluation of the antimicrobial and anti-inflammatory properties of Bacillus-DFM (NorumTM) in broiler chickens infected with Salmonella Enteritidis. Frontiers in Veterinary Science. 2019;6:282.[PMID: 31508436] [PMCID: PMC6718558] [DOI]
14. Cobb V. Cobb500 Broiler Performance & Nutrition Supplement. Siloam Springs: Cobb-Vantress Inc.; 2022.
15. Baxter MF, Merino-Guzman R, Latorre JD, Mahaffey BD, Yang Y, Teague KD, et al. Optimizing fluorescein isothiocyanate dextran measurement as a biomarker in a 24-h feed restriction model to induce gut permeability in broiler chickens. Frontiers in Veterinary Science. 2017;4:56.[PMID: 28470003] [PMCID: PMC5396023] [DOI]
16. Merino-Guzmán R, Latorre JD, Delgado R, Hernandez-Velasco X, Wolfenden AD, Teague KD, et al. Comparison of total immunoglobulin A levels in different samples in Leghorn and broiler chickens. Asian Pacific Journal of Tropical Biomedicine. 2017;7(2):116-20[DOI]
17. Kogut MH, Lee A, Santin E. Microbiome and pathogen interaction with the immune system. Poultry Science. 2020;99(4):1906-13.[PMID: 32241470] [PMCID: PMC7587753] [DOI]
18. Maguey-González JA, Liu J, Zhang G, Latorre JD, Hernández-Ramírez JO, de Jesús Nava-Ramírez M, et al.

Assessment of the impact of humic acids on intestinal microbiota, gut integrity, ileum morphometry, and cellular immunity of turkey poults fed an aflatoxin B1-contaminated diet. Toxins. 2024;16(3):122.[PMID: 38535788] [PMCID: PMC10975313] [DOI]
19. Qin B, Xia Y, Wang S, Du X. A novel Bayesian classification for uncertain data. Knowledge-Based Systems. 2011;24(8):1151-8[DOI]
20. Littell RC, Stroup WW, Freund RJ. SAS for Linear Models. Nashville, TN: John Wiley & Sons; 2002.
21. Paulson JN, Stine OC, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. Nature Methods. 2013;10(12):1200-2.[PMID: 24076764] [PMCID: PMC4010126] [DOI]
22. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology. 2014;15(12):550.[PMID: 25516281] [PMCID: PMC4302049] [DOI]
23. Rivera-Pérez W, Barquero-Calvo E, Chaves AJ. Effect of the use of probiotic Bacillus subtilis (QST 713) as a growth promoter in broilers: An alternative to bacitracin methylene disalicylate. Poultry Science. 2021;100(9):101372.[PMID: 32316269] [PMCID: PMC7222747] [DOI]
24. Saleh AA, Paray BA, Dawood MAO. Olive cake meal and Bacillus licheniformis impacted the growth performance, muscle fatty acid content, and health status of broiler chickens. Animals. 2020;10(4):695[DOI]
25. Zhao Y, Li P, Chen N, Liu Y, Liu D, Guo Y. Effects of housing systems and glucose oxidase on growth performance and intestinal health of Beijing You Chickens. Poultry Science. 2021;100(4):100943.[PMID: 33652241] [PMCID: PMC7921002] [DOI]
26. Meng Y, Huo H, Zhang Y, Bai S, Wang R, Zhang K, et al. Effects of dietary glucose oxidase supplementation on the performance, apparent ileal amino acids digestibility, and ileal microbiota of broiler chickens. Animals. 2021;11(10):2909.[PMID: 34679930] [PMCID: PMC8532941] [DOI]
27. Wu S, Li T, Niu H, Zhu Y, Liu Y, Duan Y, et al. Effects of glucose oxidase on growth performance, gut function, and cecal microbiota of broiler chickens. Poultry Science. 2019;98(2):828-41.[PMID: 30169708] [DOI]
28. Wang Y, Wang Y, Xu H, Mei X, Gong L, Wang B, et al. Direct-fed glucose oxidase and its combination with B. amyloliquefaciens SC06 on growth performance, meat quality, intestinal barrier, antioxidative status, and immunity of yellow-feathered broilers. Poultry Science. 2018;97(10):3540-9.[PMID: 30085304] [DOI]
29. Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract: Influence on health, productivity and disease. Applied Microbiology and Biotechnology. 2014;98(10):4301-10.[PMID: 24643736] [DOI]
30. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, et al. The chicken gastrointestinal microbiome. FEMS Microbiology Letters. 2014;360(2):100-12[PMCID: 25263745] [DOI]
31. Madden JAJ, Hunter JO. A review of the role of the gut microflora in irritable bowel syndrome and the effects of probiotics. British Journal of Nutrition. 2002;88(S1):S67-S72.[PMID: 12215182] [DOI]
32. Zhao Y, Fu J, Li P, Chen N, Liu Y, Liu D, et al. Effects of dietary glucose oxidase on growth performance and intestinal health of AA broilers challenged by Clostridium perfringens. Poultry Science. 2022;101(1):101553.[PMID: 34852314] [PMCID: PMC8639461] [DOI]
33. Ranjitkar S, Lawley B, Tannock G, Engberg RM. Bacterial succession in the broiler gastrointestinal tract. Applied and Environmental Microbiology. 2016;82(8):2399-410.[PMID: 26873323] [PMCID: PMC4959490] [DOI]
34. Shin NR, Whon TW, Bae JW. Proteobacteria: Microbial signature of dysbiosis in gut microbiota. Trends in Biotechnology. 2015;33(9):496-503.[PMID: 26210164] [DOI]
35. Al-Shaibani MM, Radin Mohamed RMS, Sidik NM, Enshasy HAE, Al-Gheethi A, Noman E, et al. Biodiversity of secondary metabolites compounds isolated from phylum Actinobacteria and its therapeutic applications. Molecules. 2021;26(15):4504.[PMID: 34361657] [PMCID: PMC8347454] [DOI]
36. Huang S, Chen J, Cui Z, Ma K, Wu D, Luo J, et al. Lachnospiraceae-derived butyrate mediates protection of high fermentable fiber against placental inflammation in gestational diabetes mellitus. Science Advances. 2023;9(44):eadi7337.[PMID: 37922350] [PMCID: PMC10624355] [DOI]
37. Barekatain R, Chrystal PV, Nowland T, Moss AF, Howarth GS, Van TTH, et al. Negative consequences of reduced protein diets supplemented with synthetic amino acids for performance, intestinal barrier function, and caecal microbiota composition of broiler chickens. Animal Nutrition. 2023;13:216-28.[PMID: 37388459] [PMCID: PMC10300400] [DOI]
38. Azad MAK, Sarker M, Li T, Yin J. Probiotic species in the modulation of gut microbiota: An overview. BioMed Research International. 2018;2018:9478630.[PMID: 29854813] [PMCID: PMC5964481] [DOI]
39. Bilal M, Achard C, Barbe F, Chevaux E, Ronholm J, Zhao X. Bacillus pumilus and Bacillus subtilis promote early maturation of cecal microbiota in broiler chickens. Microorganisms. 2021;9(9):1899.[PMID: 34576794] [PMCID: PMC8465073] [DOI]
40. Shi L, Li Y, Liu Y, Jia H. Alterations of gut microbiota and cytokines in elevated serum diamine oxidase disorder. Medicine. 2022;101(50):e31966.[PMID: 36550793] [PMCID: PMC9771309] [DOI]
41. Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME. Microbiota, gut health and chicken productivity: What is the connection? Microorganisms. 2019;7(10):374.[PMID: 31547108] [PMCID: PMC6843312] [DOI]
42. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proceedings of the National Academy of Sciences. 2008;105(43):16731-6.[PMID: 18936492] [PMCID: PMC2575488] [DOI]
43. Richards P, Fothergill J, Bernardeau M, Wigley P. Development of the caecal microbiota in three broiler breeds. Frontiers in Veterinary Science. 2019;6:201.[PMID: 31294039] [PMCID: PMC6603203] [DOI]
44. Chasser K, McGovern K, Duff A, Graham B, Briggs W, Rodrigues D, et al. Evaluation of day of hatch exposure to various Enterobacteriaceae on inducing gastrointestinal inflammation in chicks through two weeks of age. Poultry Science. 2021;100(7):101193.[PMID: 34087698] [PMCID: PMC8182431] [DOI]