Effect of dietary supplementation of *Lactobacillus*-fermented *Artemisia princeps* on growth performance, meat lipid peroxidation, and intestinal microflora in Hy-line Brown male chickens

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ABSTRACT The objective of this experiment was to investigate the effect of dietary supplementation of *Lactobacillus*-fermented *Artemisia princeps* (LFA) on growth performance, meat lipid peroxidation, and intestinal microflora in Hy-line Brown male chickens. A total of six hundred twenty-four 1-d-old Hy-Line Brown male chicks were randomly allotted to 3 dietary treatments with 4 replicated pens consisting of 52 chicks. The control diet was formulated to be adequate in energy and nutrients. Two additional diets were prepared by adding 2.5 or 5.0 g/kg of LFA to the control diet. The experimental diets were fed on an ad libitum basis to the birds during 7 wk. Body weight gain and feed intake were recorded at 2 and 7 wk. At the end of the experiment, 2 birds from each treatment were killed by cervical dislocation and the samples for ileal content, breast, and thigh meat were collected for the determination of meat lipid peroxidation and microbial population. Results indicated that increasing inclusion level of LFA in diets improved BW gain (linear and quadratic, $P < 0.05$) and tended to improve feed efficiency (linear and quadratic, $P < 0.10$) of birds during 0 to 7 wk. Feeding the diets containing increasing amounts of LFA to birds reduced (quadratic, $P < 0.05$) thiobarbituric acid-reactive substance (TBARS) values in breast and thigh meat during 15 d of storage. The concentrations of *Lactobacillus* spp. in the ileal content of birds increased (linear and quadratic, $P < 0.05$), but those of *Salmonella* spp. tended to be decreased (quadratic, $P < 0.10$) as inclusion level of LFA in diets increased. These results suggest that dietary LFA may be used as a functional ingredient to improve growth performance, meat lipid stability, and intestinal health of birds.

Key words: *Artemisia princeps*, intestinal microflora, *Lactobacillus* fermentation, Hy-line Brown male chicken, meat lipid peroxidation

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INTRODUCTION

Artemisia species, which belong to the Asteraceae family, are perennial plants that grow in various regions of the world. Historically, this plant has been popular as a traditional herbal medicine for treating bleeding, asthma, circulation diseases, and digestive troubles in many countries (Tan et al., 1998). In recent years, the *Artemisia* species has gained increasing attention as a functional food ingredient for humans and animals because it contains high amounts of bioactive compounds such as polyphenols, terpenoids, steroids, fiber, vitamins, and minerals (Tan et al., 1998; Lee et al., 1999; Yoo et al., 2006). In previous animal experiments, dietary supplementation of *Artemisia* leaves or extracts has shown to improve growth performance of broilers, laying hens, and pigs (Kim et al., 2003; Brisibe et al., 2008); to delay lipid peroxidation in broiler meat during storage (Kim, 2006); and to decrease the concentrations of coliform bacteria and *Escherichia coli* in the ceca of broilers (Khalaji et al., 2011).

Fermentation of plant materials with a microbial inoculum has been widely adopted to develop novel functional ingredients because this process may promote their functional quality such as antioxidant and anti-inflammatory activity (Lee et al., 2008; Wang et al., 2011; Cao et al., 2012). Likewise, fermentation of *Artemisia* leaves resulted in greater anti-inflammatory and anti-allergic properties compared with those shown in fresh *Artemisia* leaves (Lee et al., 2006; Joh et al., 2010). However, there have been limited data pertaining to the effect of dietary supplementation of fermentation products of *Artemisia* species on growth performance, product quality, and intestinal microflora of animals.

In this experiment, therefore, we used *Artemisia princeps* Pampanini, which is known to contain great-

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er amounts of phenolic compounds such as eupatilin and jaceosidin than other *Artemisia* species (Ryu et al., 2005), as a fermentation plant source. *Lactobacillus* was chosen as a microbial inoculum because of its strong fermentation ability and possible probiotic potential. The objective of the current experiment was to determine the effect of dietary supplementation of *Lactobacillus*-fermented *Artemisia princeps* (**LFA**) on growth performance, meat lipid peroxidation level, and intestinal microflora in Hy-line Brown male chickens.

MATERIALS AND METHODS

Preparation of Lactobacillus-Fermented Artemisia princeps

The *Artemisia princeps* Pampanini harvested in 2010 was obtained from the Ganghwa Agricultural R&D Center (Incheon, South Korea). The *Artemisia princeps* leaves were first dried and ground finely by Wiley mill (J-NCM, Jisico Co. Ltd., Seoul, Korea). Four strains of *Lactobacillus* spp. including *L. acidophilus* ATCC 496, *L. fermentum* ATCC 1493 (American Type Culture Collection: Virginia, US), *L. plantarum* KCTC 1048 (Korean Collection Type Culture, Daejeon, Korea), and *L. casei* IFO 3533 (Korea Food Research Institute, Daejeon, Korea) were obtained and used to ferment *Artemisia princeps* in this experiment. A 2-mL aliquot of each *Lactobacillus* strain with 10^9 cfu/mL viable counts was cultured in a medium (1 L) containing 10 g of de Man, Rogosa, and Sharpe broth (Difco Laboratories, Francisco Soria Melguizo S.A., Madrid, Spain), 10 g of sucrose, and 980 mL of distilled water, and then was incubated at 36°C for 24 h. For fermentation, 4 kg of dried *Artemisia princeps* was inoculated with 5 L of prepared *Lactobacillus* inoculum in a fermentation vessel, and incubated at 36°C with periodic mixing for 24 h. At the end of the fermentation, fermented *Artemisia princeps* were dried at 60°C to contain approximately 650 g/kg of DM, and subsequently used for the feeding trial. The nutrient compositions of fresh *Artemisia princeps* and LFA were analyzed in duplicate for DM (AOAC, 1990; method 934.01), crude ash (AOAC, 1990; method 942.05), crude fat (AOAC, 1990; method 920.39), CP (AOAC, 1990; method 988.05), and crude fiber (AOAC, 1990; method 978.10) and presented in Table 1.

Birds and Experimental Design

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at Chung-Ang University. A total of six hundred twenty-four 1-d-old Hy-Line Brown male chicks (initial BW = 40 ± 0.25 g) were obtained from a local hatchery and were raised during a 7-wk experimental period. All chicks were housed in a floor pen (width: 2.0 m, length: 2.4 m) in an environmentally controlled room. The experiment was performed as a completely randomized design with 4 replicate pens consisting of 52 chicks. A 2-phase feeding program with a starter diet from 0 to 2 wk and a grower diet from 3 to 7 wk was used in this experiment (Table 2). Within each phase, a control diet was formulated to meet or exceed NRC (1994) requirements of immature Leghorn-type chickens for macro- and micronutrients. Two additional diets were prepared by adding 2.5 or 5.0 g/kg of LFA to the control diet. The LFA was added at the expense of the control diet. The experimental diets were in mash form. The diets and water were available ad libitum. Brooder temperature was adjusted to 32°C, and barn temperature was maintained at 30°C during the first week of the experiment and then gradually decreased to 24°C at the end of the experiment. A 24-h lighting schedule was used during the entire experiment. Body weight gain and feed intake were recorded at 2 and 7 wk of the experiment. Feed efficiency (G:F) was calculated as BW gain divided by feed intake.

Sample Collection and Analysis

At the end of the experiment, 2 birds with a BW close to the pen mean BW (i.e., 8 birds per treatment) were euthanized by cervical dislocation. The gastrointestinal tracts from 8 birds were removed, approximately 10-cm segments from the ileocecal junction were dissected, and ileal content was aseptically collected into an Eppendorf tube. The ileal content was then frozen at −40°C before the analysis.

Intestinal microflora was measured by the quantitative PCR method as described by Kim et al. (2011a,b). In short, total genomic DNA was isolated from 250 mg of ileal content by using an UltraClean Fecal DNA Kit (MO BIO Laboratory, Carlsbad, CA), according to the manufacturer's instructions. Sample genomic DNA was used as a template for PCR amplification using SYBER Green PCR technology in an ABI 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA). The 16S rRNA primers for *Clostridium perfringens*, *Escherichia coli*, *Lactobacillus* spp., and *Salmonella* spp. were used for the amplification. Standard curves were constructed using the PCR product of the 16S rRNA gene from the 4 target genomic DNA preparation at 1, 10, 100, and 1,000 pg/μL. Absolute quantification

Table 1. Analyzed composition of fresh *Artemisia princeps* and *Lactobacillus*-fermented *Artemisia princeps* (LFA)[1]

Composition	*Artemisia princeps* leaves	LFA
DM (g/kg)	922	644
Crude ash (g/kg)	100	74
Crude fat (g/kg)	24	28
CP (g/kg)	156	187
Crude fiber (g/kg)	276	177

[1]Nutrient composition was analyzed in duplicate for DM (AOAC, 1990; method 934.01), crude ash (AOAC, 1990; method 942.05), crude fat (AOAC, 1990; method 920.39), CP (AOAC, 1990; method 988.05), and crude fiber (AOAC, 1990; method 978.10).

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Table 2. Composition and nutrient content of experimental diets (as-fed basis)

Item	Starter diet (0 to 2 wk)	Grower diet (3 to 7 wk)
Ingredient (g/kg)		
Ground corn	481.9	439.0
Wheat shorts	87.1	184.2
Soybean meal	333.0	271.0
Rapeseed meal	18.0	30.0
Animal fat	50.0	45.0
L-Lys-HCl	2.5	2.6
L-Thr	1.0	2.5
Dicalcium phosphate	15.0	13.0
Salt	2.5	2.6
Ground limestone	6.0	6.1
Nonstarch polysaccharide enzyme	0.5	0.5
Sodium bicarbonate	1.0	2.0
Phytase	0.5	0.5
Vitamin-mineral premix[1]	1.0	1.0
Total	1,000.0	1,000.0
Nutrient content[2]		
ME (MJ/kg)	12.56	13.02
CP (g/kg)	215.1	198.2
Lys (g/kg)	12.5	11.3
Met + Cys (g/kg)	9.0	8.5
Ca (g/kg)	9.0	9.0
Total P (g/kg)	8.5	8.5

[1]Provided per kilogram of the complete diet: vitamin A (from vitamin A acetate), 12,500 IU; vitamin D_3, 2,500 IU; vitamin E (from DL-α-tocopheryl acetate), 20 IU; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_6, 3 mg; vitamin B_{12}, 18 μg; calcium pantothenate, 8 mg; folic acid, 1 mg; biotin, 50 μg; niacin, 24 mg; Zn (as ZnO), 60 mg; Mn (as $MnSO_2{\cdot}H_2O$), 50 mg; Fe (as $FeSO_4{\cdot}7H_2O$), 50 mg; Cu (as $CuSO_4{\cdot}5H_2O$), 6 mg; Co (as $CoCO_3$), 250 μg; I [as $Ca(IO_3)_2{\cdot}H_2O$], 1 mg; Se (as Na_2SeO_3), 150 μg.

[2]The values for CP were analyzed and other values were calculated from NRC (1994).

was performed based on the standard curves that were established by amplification of the known amounts of target DNA.

The breast and thigh meat samples (without skin) from 4 birds were also collected and analyzed for meat lipid peroxidation. The extent of lipid peroxidation in breast and thigh meat samples was determined by measuring thiobarbituric acid-reactive substance (**TBARS**) values at 0, 4, 8, and 15 d of the storage at 4°C. The meat samples were placed individually in plastic wrap in a refrigerator during the storage. The TBARS values were measured by thiobarbituric acid colorimetric methods as described by Ahn et al. (1998) with a minor modification. Briefly, 3 g of meat sample was weighed into a 50-mL test tube and homogenized with 15 mL of distilled water by using a homogenizer (model 985370, Biospec Products Inc., Bartlesville, OK) for 20 s at high speed. Thereafter, 50 μL of butylated hydroxyanisole (7.2%) and 5 mL of thiobarbituric acid-trichloroacetic acid solution (20 m*M* thiobarbituric acid in 15% trichloroacetic acid) were added to the test tube. The tubes were heated in 90°C hot water for 15 min, cooled, and centrifuged at 2,000 × *g* for 15 min at 4°C. Absorbance of the supernatant was measured at 532 nm with a spectrophotometer (UNIKON 933, Kontron Co. Ltd., Milan, Italy). The TBARS values were calculated from the standard curve constructed with 1,1,3,3-tetraethoxypropane and were expressed as milligrams of malondialdehyde per kilogram of meat samples.

Statistical Analysis

All data were analyzed by ANOVA according to completely randomized design (Steel et al., 1997) using the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Outlier data were identified according to Steel et al. (1997), using the UNIVARIATE procedure of SAS, but no outliers were detected. The pen was an experimental unit for growth performance data, whereas the individual bird was an experimental unit for meat TBARS and microbial population data. Dietary treatment was a fixed effect in all statistical models. The LSMEANS procedure was used to calculate mean values. The orthogonal polynomial contrast test was performed to determine linear and quadratic effects of increasing inclusion level of LFA in diets on each measurement. Significance and tendency for statistical tests were set at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

RESULTS AND DISCUSSION

Growth Performance

During the initial 2 wk of the experiment, BW gain and feed intake were not influenced by inclusion of LFA in diets, but there was a quadratic relationship ($P < 0.05$) between inclusion level of LFA and feed efficiency (Table 3). During 3 to 7 wk of the experiment, BW gain increased (linear and quadratic, $P < 0.05$) with inclusion level of LFA and a tendency for improved feed efficiency (linear and quadratic, $P < 0.10$) was observed as

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Table 3. Growth performance of Hy-line Brown male chickens fed the diet containing *Lactobacillus*-fermented *Artemisia princeps* (LFA)[1]

Item	LFA (g/kg)			SEM	*P*-value	
	0.0	2.5	5.0		Linear	Quadratic
0 to 2 wk						
BW gain (g/bird)	133	138	130	3.3	0.553	0.113
Feed intake (g/bird)	238	225	233	6.9	0.567	0.227
Feed efficiency[2]	0.56	0.62	0.56	0.019	0.994	0.034
3 to 7 wk						
BW gain (g/bird)	715	773	752	10.6	0.038	0.014
Feed intake (g/bird)	2,101	2,072	2,079	47.4	0.745	0.770
Feed efficiency[2]	0.34	0.37	0.36	0.008	0.096	0.061
0 to 7 wk						
BW gain (g/bird)	848	911	882	10.3	0.047	0.005
Feed intake (g/bird)	2,339	2,297	2,311	48.5	0.690	0.644
Feed efficiency[2]	0.36	0.40	0.38	0.008	0.089	0.020

[1]Data are least squares means of 4 observations per treatment.

[2]Feed efficiency = G:F.

inclusion level of LFA in diets increased. There was no effect of inclusion level of LFA in diets on feed intake of birds. For overall experiment, increasing inclusion level of LFA in diets increased BW gain (linear and quadratic, $P < 0.05$), and tended to improve feed efficiency (linear and quadratic, $P < 0.10$).

Dietary supplementation of *Artemisia* species has been reported to improve BW gain and feed efficiency in broilers and productive performance in laying hens (Brisibe et al., 2008). It is suggested that positive effects of dietary *Artemisia* species on birds' performance may result from its high concentrations of phytochemicals such as flavonoids, terpenoids, steroids, vitamins, and minerals (Tan et al., 1998; Lee et al., 1999; Yoo et al., 2006), which have potentials of antioxidant (Bilia et al., 2006), immune system modulator (Chew, 1995; Khalaji et al., 2011), stress alleviation (Bagchi et al., 1999), and antimicrobial activity (Khalaji et al., 2011). To our knowledge, however, there have been no data pertaining to the effect of dietary fermentation products of *Artemisia* species on growth performance of birds, and therefore, it is difficult to compare previous data with those determined in this experiment. It is suggested, however, that fermentation processes of plant materials are able to elevate the efficacy of their antioxidant and anti-inflammatory properties to a level greater than that in the raw materials (Lee et al., 2008; Wang et al., 2011; Cao et al., 2012). Similar improvements in anti-inflammatory and anti-allergic activity were also observed for fermented *Artemisia* products compared with fresh nonfermented *Artemisia* leaves (Lee et al., 2006; Joh et al., 2010). Therefore, one reason for improved BW gain and feed efficiency by inclusion of LFA in diets may be associated with elevated antioxidant and anti-inflammatory properties of LFA. This may also be the reason why inclusion of relatively small amounts (2.5 or 5.0 g/kg) of LFA in diets showed significant positive effects on growth performance of birds in this experiment. Furthermore, the LFA used in this experiment may have contained a certain amount of viable or inactive *Lactobacillus* spp., although they were not measured in this experiment. *Lactobacillus* spp. have been widely appreciated as potential probiotic bacteria and dietary supplementation of *Lactobacillus* spp. have been reported to improve growth performance of birds (Panda et al., 2006; Nakphaichit et al., 2011). There is also the implication that dead or inactive probiotic bacteria may improve performance and the health status of animals, possibly via similar mechanisms operating in animals fed viable probiotic bacteria (Wagner et al., 2000; Huang et al., 2004). As a consequence, improved BW gain and feed efficiency by feeding diets containing LFA to birds is also likely due to the presence of *Lactobacillus* spp. in LFA.

Lipid Peroxidation in Breast and Thigh Meat

As expected, the TBARS values measured in breast and thigh meat increased from 0 to 15 d of the storage, regardless of dietary treatments (Table 4). Increasing inclusion level of LFA in diets decreased (quadratic, $P < 0.05$) the TBARS values in breast meat at 8 and 15 d of the storage. A similar result of decreased (quadratic, $P < 0.05$) TBARS values in thigh meat at 8 d of the storage was observed. The average TBARS values during 15 d of the storage decreased (quadratic, $P < 0.05$) in both breast and thigh meat with inclusion level of LFA in diets.

Decreased TBARS values in breast and thigh meat by feeding the diet containing LFA is likely caused by high concentrations of antioxidant compounds such as terpenoids and flavonoids in the LFA. A similar result was also observed by Cao et al. (2012) who reported that increasing inclusion of *Aspergillus niger*-fermented Ginkgo leaves, which are also high in flavonoids and terpenoids, in diets decreased lipid peroxidation levels in broiler breast meat. In this experiment, it was expected that increasing inclusion of LFA in diets linearly decreased meat TBARS values; however, a quadratic response was observed in this experiment. The reason

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Table 4. Thiobarbituric acid-reactive substance (TBARS) values in breast and thigh meat of Hy-line Brown male chickens fed the diet containing *Lactobacillus*-fermented *Artemisia princeps* (LFA)[1]

	LFA (g/kg)				*P*-value	
Item	0.0	2.5	5.0	SEM	Linear	Quadratic
Breast meat[2] (mg of MDA/kg)						
0 d	0.070	0.064	0.067	0.004	0.588	0.446
4 d	0.121	0.095	0.112	0.012	0.611	0.188
8 d	0.150	0.125	0.136	0.006	0.106	0.025
15 d	0.205	0.183	0.196	0.006	0.284	0.026
Average[3]	0.137	0.117	0.128	0.004	0.198	0.021
Thigh meat[2] (mg of MDA/kg)						
0 d	0.078	0.077	0.078	0.003	0.979	0.745
4 d	0.133	0.126	0.126	0.006	0.375	0.627
8 d	0.172	0.139	0.157	0.006	0.136	0.010
15 d	0.221	0.205	0.216	0.009	0.752	0.251
Average[3]	0.151	0.137	0.144	0.003	0.190	0.024

[1]Data are least squares means of 4 observations per treatment.

[2]The TBARS values are quantified as malondialdehyde (MDA) equivalents per gram of meat sample at 0, 4, 8, and 15 d of the storage at 4°C.

[3]Average TBARS values measured at 0, 4, 8, and 15 d of the storage.

for this observation is not clear, but it may be related to the differences in total fat concentrations and fatty acid composition of meat when birds were fed the diets containing different amounts of LFA. Cao et al. (2012) reported that body fat content of birds decreased with inclusion of fermented Ginko leaves and the birds fed the diet containing 10 g/kg of fermented Ginkgo leaves had greater concentrations of polyunsaturated fatty acids in the breast meat than birds fed the diet containing 4 g/kg of fermented Ginkgo leaves.

Microbial Population

In the ileal content of birds, increasing inclusion level of LFA in diets increased (linear and quadratic, $P < 0.05$) the concentrations of *Lactobacillus* spp. (Table 5). A tendency (quadratic, $P < 0.10$) for decreased concentrations of *Salmonella* spp. was observed as inclusion level of LFA in diets increased. The concentrations of *Clostridium perfringens* and *Escherichia coli* in the ileum content were not affected by inclusion of LFA in diets.

Previous in vitro experiments reported that *Artemisia* extracts or oils contained antimicrobial compounds against various microbes such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* spp., and *Bacillus* spp. (Zheng et al., 1996; Lopes-Lutz et al., 2008; Yun et al., 2008), and some protozoan parasites (Brisibe et al., 2008). This result was confirmed by an in vivo experiment of Khalaji et al. (2011) who reported that inclusion of 10 g/kg of *Artemisia* leaves in diets significantly decreased the concentrations of coliform bacteria and *Escherichia coli* in the ceca of broilers. In the current experiment, however, we failed to detect any significant effect on *Escherichia coli* concentrations in the ileal content of birds by feeding the diet containing LFA. Interestingly, the concentrations of *Lactobacillus* spp. in the ileal content of birds increased with inclusion level of LFA in diets. The possible reason for this observation is that because the LFA used in this experiment was fermented with *Lactobacillus* spp., it may contain viable *Lactobacillus* spp., which is able to aid in the colonization of *Lactobacillus* spp. in the gastrointestinal tract of birds.

The concentrations of *Salmonella* spp. in the ileum of birds decreased with inclusion level of LFA in diets. *Salmonella* spp. are a common cause of foodborne microbial diseases for humans, and their contamination of poultry meat and eggs is one of the greatest clinical concerns in poultry production (Gaggìa et al., 2010). We speculated that decreased concentrations of *Salmonella* spp. in the ileal content of birds may result from increased concentrations of *Lactobacillus* spp. by feeding diets containing LFA. It was reported that feed-

Table 5. Microbial population in the ileal content of Hy-line Brown male chickens fed the diet containing *Lactobacillus*-fermented *Artemisia princeps* (LFA)[1]

	LFA (g/kg)				*P*-value	
Population ($\log_{10}$ cfu/g)	0.0	2.5	5.0	SEM	Linear	Quadratic
Lactobacillus spp.	5.43	7.68	7.63	0.340	0.001	0.022
Clostridium perfringens	1.54	0.99	1.17	0.429	0.551	0.504
Escherichia coli	3.58	3.19	3.49	0.211	0.770	0.211
Salmonella spp.	2.74	1.29	1.94	0.453	0.247	0.093

[1]Data are least squares means of 4 observations per treatment. Data for each observation were average values obtained from 2 birds per replicated pen.

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ing *Lactobacillus*-based probiotic cultures decreased the colonization of *Salmonella* spp. in the ceca of broilers (Higgins et al., 2007, 2008). However, it is difficult to explain why growth-inhibitory effects of dietary LFA were limited in *Salmonella* spp. and did not extend to *Escherichia coli* and *Clostridium perfringens* in this experiment because dietary *Lactobacillus* spp. have been known to depress the growth of various pathogenic bacteria including *Escherichia coli* and *Clostridium perfringens* in the gastrointestinal tracts of animals (Murry et al., 2006; Gaggìa et al., 2010; Nakphaichit et al., 2011).

Conclusion

The results of the current experiment indicate that dietary supplementation of LFA improves growth performance and meat lipid stability of birds. Dietary LFA has probiotic potentials by increasing *Lactobacillus* spp. concentrations and decreasing *Salmonella* spp. concentrations in the gastrointestinal tract of birds. As a consequence, dietary LFA is considered a valuable functional ingredient to improve performance, meat quality, and intestinal health of birds.

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