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Additive Effects of Dietary Supplementation with Zeolite and Methyl-Sulfonyl-Methane on Growth Performance and Interleukin Levels of Broiler Chickens

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Silicate minerals are common additives in poultry feed. To assess their effects, we added zeolite (ZEO) and methyl-sulfonyl-methane (MSM) to broiler chicken diets. A total of 960 one-day-old Ross broiler chicks were randomly divided into four dietary groups with six replicates. Each broiler was maintained until it reached 35 days of age. A completely randomized 2 × 2 experimental design was used, with two ZEO (0 and 1.0%) and two MSM (0 and 0.10%) levels. We observed an additive effect (P<0.05) on interleukin-2 (IL-2) concentrations in broiler bursa and serum when both ZEO and MSM were present. Both ZEO or MSM produced significant (P<0.05) increases in body weight, weight gain, and feed intake. Both increased IL-2 and IL-6 levels in the bursa and serum. Neither affected the serum concentrations of albumin, AST, cholesterol, HDL cholesterol, glucose, total protein, or triglycerides. In summary, these results support supplementation with ZEO and MSM in broiler diets, both separately and in combination.

Key words: broiler chickens, interleukin, silicates, methyl sulfonyl methane, performance, zeolite

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Introduction

The poultry industry has long played an important role in providing necessary protein to the world. For the last century, there has been a focus on improving feed efficiency (Safaei *et al*., 2014). Antibiotics have historically been used to increase production (Edens, 2003). However, since 2006, the use of feed additives extracted from natural plants, including aromatic plants and essential oils, has increased in response to European Union legislation restricting antibiotic use of antibiotics in light of resistance. Among the available feed additives, silicate minerals have attracted scientific interest for their potential to improve efficiency. Silicate minerals (zeolite, bentonite, kaolin, sepiolite, perlite, illite, granite, etc.) comprising approximately 90% of soil minerals, are used for their physicochemical properties (Safaei *et al*., 2014).

Zeolite (ZEO), which is primarily composed of SiO_2, improves feed efficiency through its capacity to gain and lose water reversibly; moreover, it has a high cation exchange ability (Shariatmadari, 2008; Katouli *et al*., 2010). Multiple studies have shown that supplementing broiler chicken diets with ZEO improves performance (Al-Nasser *et al*., 2011; Qu *et al*., 2019) and boosts immunity (Zhou *et al*., 2014). Methyl-sulfonyl-methane (MSM) is a natural source of organic sulfur that is growing in popularity as a supplement. It has demonstrated anti-inflammatory and antioxidant effects in murine macrophages activated by lipopolysaccharides, as it decreases the expression of pro-inflammatory mediators (Kim *et al*., 2009). Jang et al. found that a diet supplemented with 0.06% MSM increased daily feed intake in pigs (Jang *et al*., 2006). Likewise, Hui-fang and An-guo (2008) reported that MSM supplementation increased weight gain and feed conversion ratio in ducks raised for meat.

ZEO and MSM supplementation in broiler diets have been combined with other additives for interactive and/or synergistic effects. Combining ZEO with other silicates (bentonite, kaolin, and attapulgite) improves growth and secretory immunoglobulin concentrations in broilers (Katouli *et al*., 2010; Zhou *et al*., 2014). ZEO in combination with dried egg products improves

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total antioxidant capacity and reduces oxidative stress indicators in broiler plasma (Rasheed *et al*., 2020). Interleukins (ILs) are members of the cytokine family that regulate inflammatory and immune responses in both directions; their expression levels are altered in response to pathogens and other antigens (Vaillant and Qurie, 2020). IL-2 and IL-6 stimulate the activation of macrophages and cytotoxic lymphocytes (Bachmann and Oxenius, 2007; Tsukamoto *et al*., 2018).

To our knowledge, no previous work has investigated the combined effects of ZEO and MSM provided as feed additives in broiler diets on performance and ILs concentrations. Therefore, the goal of this study was to determine the interactive effects of ZEO- and MSM-supplemented diets on the performance and concentration of IL-2 and IL-6 in broilers.

Materials and Methods

Sampling, birds, and management

This study was conducted at the Poultry Experimental Station of the Department of Animal Sciences, Jeonbuk National University, Republic of Korea. All protocols were approved by the Jeonbuk National University Institutional Animal Care and Use Committee (JBNU 2022-040).

Clinoptilolite (ZEO) collected from a natural mine was supplied by Davistone Co., Ltd. (Busan, Republic of Korea). Its primary components are SiO_2 (73.2%) and Al_2O_3 (14.8%) (Table 1). MSM was supplied by Natural Engineering Inc. (Anyang, Republic of Korea). Feed additives were powdered before use. A total of 960 one-day-old Ross broiler chicks were allotted to an experimental windowless house with wire-floored cages (235 × 180 cm; 9.5 birds/m^2). The ambient temperature was maintained at 33.5°C for the first 3 days, whereupon it was gradually decreased by 3 °C per week until 23 °C was reached, at which point it was kept constant until the end of the experiment. A completely randomized experimental design, involving a 2 × 2 factorial arrangement consisting of two ZEO levels (0 and 1.0%) and two MSM levels (0 and 0.10%) was created, with six replicates of 40 birds per experimental unit (i.e., 240 birds per factor). All birds had feed and water provided *ad libitum*. Diets were based on corn and soybean meal and formulated to meet the nutrient requirements of broilers, as recommended by the guidelines set forth in the Korean Feeding Standard for Poultry (2017). The proximate compositions of the formulated basal diets, which were divided into three stages: pre-starter (0–7 d), starter (8–21 d), and grower (22–35 d), are shown in Table 2.

Table 1. **Composition of zeolite (ZEO)**

Ingredient	%
SiO_2	73.2
Al_2O_3	14.8
Na_2O	4.57
K_2O	4.26
CaO	1.37
Fe_2O_3	0.610
MgO	0.160
Ignition loss	0.880
Mineral chemical composition (%)	
Calcium	0.600
Potassium	0.190
Sodium	0.071
Magnesium	0.020
Iron	0.280
Zinc	0.001
Phosphorus	0.010

Sample processing and Analyses

Body weight and feed intake were measured weekly. Using these data, the weight gain and feed intake were calculated at the end of each growth stage. The feed conversion ratio was calculated as feed intake per weight gain.

At the end of the experiment, six birds were randomly se-

Table 2. **Basal diet ingredients and chemical composition**

Composition (g/kg)	Pre-starter	Starter	Grower
Corn	432	491	556
Soybean meal	378	340	291
Wheat bran	74.5	60.7	39.7
Wheat	54.4	48.0	47.5
Soybean oil	14.1	16.4	25.5
Mono-di calcium phosphorus	16.2	14.1	12.1
Limestone	12.4	11.3	10.3
Corn gluten meal	6.90	6.90	6.90
Salt	2.90	2.90	2.90
Vitamin premix[1]	1.00	1.00	1.00
Mineral premix[2]	1.00	1.00	1.00
Lysine-HCl	2.10	2.10	2.10
L-arginine	1.00	1.00	1.00
DL-Methionine	2.80	2.50	2.10
Threonine	1.00	1.00	1.00
Valine	0.500	0.500	0.500
Total	100	100	100
Chemical composition			
Metabolic energy (kcal/kg)	3,050	3,100	3,200
Crude protein (%)	23.0	21.5	19.5
Total calcium (%)	0.970	0.880	0.790
Available phosphorus (%)	0.450	0.400	0.350
Total methionine (%)	0.630	0.570	0.510
Total lysine (%)	1.46	1.36	1.22
Total threonine (%)	0.950	0.890	0.820
Total arginine (%)	1.60	1.48	1.32
Total valine (%)	1.10	1.03	0.93

[1]Contains per kg: vitamin A, 12,000 IU; vitamin D3, 5,000 IU; vitamin K3, 3 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; biotin, 0.2 mg; folic acid, 0.2 mg; niacin, 70 mg; pantothenic acid, 20 mg. [2] Contains per kg; Cu, 20 mg; Co, 0.5 mg; Fe, 50 mg; I, 1,300 mg; Mn, 120 mg; Se, 0.3 mg; Zn, 100 mg.

lected from each group and euthanized by cervical dislocation. The spleen and bursa were collected and stored at −20°C. Total RNA (approximately 50 mg) was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were measured using a NanoDrop ND-2000 (Thermo Scientific, Waltham, MA, USA). Complementary DNA was synthesized using 1 µg of total RNA and the PrimeScript® RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan), according to the manufacturer's instructions. The IL-2 primers (Table 3) were evaluated using routine PCR before use in real-time PCR (TaKaRa PCR Thermal Cycler Dices, Takara). To quantify the expression of chicken IL-2 mRNA, real-time quantitative PCR was conducted using laStratagene MX 3000P (Agilent Technologies, Santa Clara, CA, USA) with denaturation step at 95°C for 30 s, then 40 cycles of amplification at 95 °C for 5 s and a primer-specific annealing/extension temperature for 30 s. Relative mRNA expression was calculated by comparing the number of thermal cycles required to generate threshold amounts of product (PCR-ct). PCR-ct was calculated for chicken IL-2 and normalized to the expression RNA polymerase-II (RP-II) (Eltahan *et al*., 2017); we confirmed that RP-II expression was not altered under our experimental conditions. IL-2 mRNA expression was calculated as $2^{-\Delta\Delta PCR\text{-}ct}$, as previously described (Schmittgen and Livak, 2008).

Blood samples were collected between 8:00 and 9:00 AM by puncturing the wing veins of six hens from each group at the end of the experiment. Samples were collected using sterile syringes and then centrifuged. The resulting serum was stored at -20°C. Serum IL-2 and IL-6 concentrations were analyzed using chicken ELISA kits (Elabscience Biotechnology Co., Ltd., E-EL-Ch0120 and E-EL-Ch0228, respectively, Houston, TX, USA), according to the manufacturer's instructions. Absorbances were read at 450 nm using a microplate spectrophotometer (BioTek ELX 800, Winooski, VT, USA). Serum concentrations of albumin, AST aminotransferase (AST), cholesterol, glucose, protein, and triglyceride were measured via colorimetry using an Automatic Biochemical Analyzer (Thermo Scientific).

Statistical analysis

The experiment was conducted using a completely randomized design with a factorial structure. Data were statistically analyzed in a 2 × 2 factorial design using the General Linear Models (GLM) procedure in SAS software (version 9.2, SAS Inst. Inc., Cary, NC, USA). Differences, including the interactions, were considered significant using Duncan's multiple range test if their *P*-values were <0.05.

Results and Discussion

Growth performance

Performance data are presented in Table 4. No interactions were observed between dietary ZEO and MSM for any of the evaluated parameters. Increased body weight, weight gain, and feed intake (all $P<0.05$) were observed in broilers fed either dietary ZEO or MSM than in those fed the basal diet. These results are consistent with those from an earlier study (Al-Nasser *et al*., 2011), in which supplementation with 1.0% ZEO increased weight gain and feed intake. Qu *et al*. (2019) demonstrated that 1% ZEO increased weight gain and feed intake of broilers aged 1 to 21 days and increased the duodenal villus height-to-crypt depth ratio (VH:CD) at 42 days. Furthermore, others have shown that dietary ZEO is involved in many biochemical processes through ion exchange, adsorption, and catalysis in the gut (Papaioannou *et al*., 2005), and has been shown to improve poultry gut microflora (Khambualai *et al*., 2009). Our results may also be attributable to increased feed retention time, as this would allow enzymatic action over a longer period, improving digestibility (Tatar *et al*., 2008).

For MSM, Jiao *et al*. (2017) reported that supplementation to broiler diets of 0.1% MSM increased weight gain and feed intake. However, no difference in CP digestibility was observed. Hui-fang and An-guo (2008) found that MSM increased daily gain during the second and third weeks of supplementation in ducks. Yan *et al*. (2020) showed that Pekin ducks fed a diet supplemented with 0.3% MSM gained more weight than those without MSM. MSM supplementation has been shown to increase the expression of growth-related proteins, including IGF-1, STAT5b, and Jak2, in osteoblastic cells and MSCs (Joung *et al*., 2012). MSM also improves nutrient digestibility (Cho *et al*., 2005), which may account for its effects on weight. In summary, our findings suggest that supplementation with either dietary ZEO or MSM produces a similar benefit in broiler performance.

Interleukins and serum biochemical parameters

The effects of ZEO and MSM on broiler interleukin (IL) levels are shown in Table 5. We documented interaction effects ($P<0.05$) in groups fed ZEO and MSM on IL-2 mRNA in the bursa and serum IL-2. These results are consistent with those of Uzunoglu and Yalcin (2019), who found that the silicate sepiolite, in combination with betaine, improved broiler IgG levels synergistic effects. Kececi *et al*. (1998) reported that broilers fed ZEO in addition to polyvinylpolypyrrolidone (PVPP) had more

Table 3. **PCR primers**

Gene	Accession no.		Sequences 5′−3′	Product size (bp)
IL-2	NM_204153.1	Forward	ACTCTGCAGTGTTACCTGGG	140
		Reverse	CCGGTGTGATTTAGACCCGT	
RP-II	NM_001006448.1	Forward	CGACGGTTTGATTGCACCTG	161
		Reverse	CAATGCCAGTCTCGCTAGTTC	

Primers were designed with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/) for interleukin-2 (IL-2) while according to Eltahan et al., 2017 RNA polymerase II has been designed to serve as an internal control gene which expressed constant (RP-II).

Table 4. **Additive effects of supplementation with ZEO and MSM on the performance of broilers aged 35 d**

ZEO (%)	MSM (%)	Body weight (g)	Weight gain (g)	Feed intake (g)	Feed conversion ratio
0	0	1,922	1,878	3,170	1.69
1.0	0	2,014	1,970	3,275	1.66
0	0.10	2,034	1,990	3,307	1.66
1.0	0.10	2,089	2,045	3,379	1.65
Main effect					
ZEO (%)					
0		1,968 [b]	1,924 [b]	3,222 [b]	1.68
1.0		2,061 [a]	2,018 [a]	3,343 [a]	1.66
MSM (%)					
0		1,978 [b]	1,934 [b]	3,238 [b]	1.68
0.10		2,051 [a]	2,007 [a]	3,327 [a]	1.66
Probability > F					
Source of variation					
ZEO		0.003	0.003	0.006	0.383
MSM		0.012	0.012	0.030	0.396
ZEO × MSM		0.466	0.466	0.655	0.716

[a,b] Means in each column with no common superscript differ significantly at $p<0.05$. ZEO: zeolite, MSM: methyl sulfonyl methane.

Table 5. **Additive effects of supplementation with ZEO and MSM on interleukin (IL) levels in broilers**

ZEO (%)	MSM (%)	IL-2 mRNA in spleen	IL-2 mRNA in bursa	IL-2 in blood (pg/ml)	IL-6 in blood (pg/ml)
0	0	0.643	0.948	184	161
1.0	0	0.910	1.463	214	190
0	0.10	0.926	1.634	203	186
1.0	0.10	1.061	1.677	237	211
Main effect					
ZEO (%)					
0		0.785	1.291 [b]	199	176 [b]
1.0		0.986	1.660 [a]	220	198 [a]
MSM (%)					
0		0.777	1.288 [b]	193 [b]	174 [b]
0.10		0.994	1.573 [a]	226 [a]	200 [a]
Probability > F					
Source of variation					
ZEO		0.140	<0.001	0.137	0.039
MSM		0.192	0.008	0.029	0.018
ZEO × MSM		0.634	0.030	0.009	0.856

[a,b] Means in each column with no common superscript differ significantly at $p<0.05$. ZEO: zeolite, MSM: methyl sulfonyl methane.

thrombocytes at 21 days than broilers fed diets supplemented with either ZEO or PVPP. ZEO in combination with other silicate minerals (bentonite, kaolin, or attapulgite) is used to supplement broiler diets to improve growth and secretory immunoglobulin concentrations (Katouli *et al*., 2010; Zhou *et al*., 2014). Additionally, combining MSM with dried egg product improved total antioxidant capacity and reduced oxidative stress indicators in broiler plasma synergistically (Rasheed *et al*., 2020). In our experiment, the increased levels of IL-2 in the bursa and blood induced by combining ZEO and MSM might be explained by a positive chemical interaction between the two additives.

We observed significant ($P<0.05$) increases in bursa IL-2 and blood IL-6 levels in groups supplemented with either ZEO or MSM (Table 5). These results are consistent with those of Lim *et al*. (2017), who reported high IL-2 and IL-6 concentrations in broilers fed diets supplemented with 0.10% silicate minerals. Moghadam *et al*. (2008) showed that the addition of the silicate sodium bentonite to an aflatoxin-containing diet significantly improved antibody production against inflammatory bowel disease (IBD) in broilers. Yan *et al*. (2020) confirmed that 0.3% MSM

Table 6. **Additive effects of supplementation with ZEO and MSM on broiler blood profiles**

ZEO (%)	MSM (%)	ALB (g/dl)	AST (g/dl)	CHOL (mg/dl)	HDL (mg/dl)	GLU (mg/dl)	TP (g/dl)	TG (mg/dl)
0	0	1.53	315	151	107	277	3.59	47.3
1.0	0	1.56	229	158	114	264	3.54	42.1
0	0.10	1.55	284	159	117	280	3.59	40.6
1.0	0.10	1.45	258	159	118	295	3.32	41.9
Main effect								
ZEO (%)								
0		1.54	272	154	111	271	3.56	44.7
1.0		1.50	271	159	117	287	3.45	41.3
MSM (%)								
0		1.54	299	155	112	279	3.59	43.9
0.10		1.51	243	158	116	279	3.43	42.0
Probability > F								
Source of variation								
ZEO		0.257	0.988	0.530	0.257	0.088	0.281	0.455
MSM		0.399	0.107	0.644	0.456	0.937	0.116	0.676
ZEO × MSM		0.146	0.381	0.646	0.596	0.145	0.286	0.487

[a]ZEO: zeolite, MSM: methyl sulfonyl methane, ALB: albumin, AST: aspartate amino transferase, CHOL: cholesterol, HDL: High density lipoprotein cholesterol, GLU: glucose, TP: total protien, TG: triglyceride.

supplementation increased serum concentrations of IL-2 and IL-6. Others have found that 0.2% MSM significantly increased white blood cell and lymphocyte counts (Jiao *et al*., 2017).

These changes are important because ILs play an important role in intercellular communication between leukocytes (Akdis *et al*., 2016). IL-2 and IL-6 give rise to an immune response that leads to the multiplication of activated T and B lymphocytes, as well as antibody production (Yan *et al*., 2020). Accordingly, our results suggest that dietary supplementation with ZEO or MSM enhances IL-2 and IL-6 levels, which should improve general health status.

None of the blood parameters examined were influenced by dietary supplementation with ZEO and MSM or by the interaction between them (Table 6). These results are consistent with those of Eleroğlu *et al*. (2011), who reported that 1% ZEO supplementation did not exert any significant effect on glucose, cholesterol, protein, uric acid, Ca, or P. However, Safaeikatouli *et al*. (2011) reported that 3% ZEO had no significant effect on serum HDL cholesterol, triglycerides, or urea, but increased serum albumin and protein levels in broilers. Lotf *et al*. (2004) reported that glucose, protein, and AST concentrations were affected by increasing ZEO from 2 to 6% in broilers. Such varied results may be due to differences in the levels of ZEO provided in the tested diets.

With respect to MSM, our findings were similar to those of a previous study (Yan *et al*., 2020), which found no significant effect of MSM supplementation ranging from 0.05 to 0.10% on AST concentrations in broilers. Lim *et al*. (2018) reported that 0.1 to 0.4 MSM had no effect on serum profiles (glucose, protein, triglyceride, cholesterol, and albumin) of laying hens. Our study showed that supplementation with ZEO, MSM, or both had no negative effects on serum biochemical parameters. Therefore, these results suggest that both ZEO and MSM are appropriate for use as feed additives, either separately or in combination, and cause no negative effects.

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Author Contributions

Chun Ik Lim conducted experiments, analyzed data, and wrote the paper. Kyeong Seon Ryu designed experiments and revised the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

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