

Antimicrobial activity of sophorolipids against *Eimeria maxima* and *Clostridium perfringens*, and their effect on growth performance and gut health in necrotic enteritis

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ABSTRACT The in vitro antimicrobial activity of sophorolipids (SLs) against *Eimeria maxima* and *Clostridium perfringens*, and the in vivo effects of SLs on growth performance and gut health in necrotic enteritis (NE)-afflicted broiler chickens were studied. To test the direct killing effects of SLs on enteric pathogens, 2.5×10^5 freshly prepared sporozoites of each *Eimeria acervulina*, *E. maxima*, and *E. tenella* were placed in each well of a 96-well plate, and the vegetative stage of *Clostridium perfringens* was prepared at 1×10^9 cfu/well. Four different SLs (C18:1 lactonic diacetyled SL [SL1], C18:1 deacetyled SL [SL2], C18:1 monoacetyled SL [SL3], and C18:1 diacetyled SL [SL4]), and 2 anticoccidial chemical controls, decoquinate and monensin, were evaluated at 3 dose levels (125 $\mu\text{g}/\text{mL}$, 250 $\mu\text{g}/\text{mL}$, and 500 $\mu\text{g}/\text{mL}$). Samples were incubated at 41°C for 3 h, and microbial survival ratios were measured by using a cell counter to quantify the number of live microbes stained by fluorescent dye. A total of 336 (0-day-old) male commercial broiler chickens were used to assess the effects of SLs in vivo. Chickens were randomly allocated to 6 treatment groups (7 chickens per cage, 8 cages per treatment) as follows: a control group which received a basal diet (CON), a negative control group

(NC) which received a basal diet and NE challenge, and 4 SL treatment groups with NE (NC+SL1, NC+SL2, NC+SL3, and NC+SL4). The inclusion rates of SLs in each group were 200 mg/kg of feed. NE-induced chickens were orally infected with *E. maxima* (10,000 oocysts/chicken) on d 14, followed by *C. perfringens* (1×10^9 cfu/chicken) on d 19. Disease parameters measured included gut lesion scores, intestinal cytokine production, and level of tight junction protein expression. Data were analyzed using a Mixed Model (PROC MIXED) in SAS. In vitro (Experiment 1), all SLs dose-dependently decreased ($P < 0.001$) the viability of the three species of *Eimeria* sporozoites and *C. perfringens*. In vivo (Experiment 2), dietary SLs increased ($P < 0.001$) body weight and average daily gain of broiler chickens infected with NE. Dietary SL1 and SL4s increased ($P < 0.05$) feed conversion ratio compared to NC. Furthermore, SL1 and SL4 decreased ($P < 0.05$) gut lesion scores in combination with increased expression of IL1 β , IL8, TNFSF15, and IL10 genes ($P < 0.05$) in NE-afflicted chickens. Overall, dietary SLs promoted growth performance, intestinal immune responses, and intestinal barrier integrity of NE-afflicted, young broiler chickens.

Key words: antimicrobial activity, broiler chicken, gut health, necrotic enteritis, sophorolipid

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INTRODUCTION

With an increasing demand for poultry meat and eggs and their antibiotic-free production, the poultry

industry is interested in identifying “nutraceutical” dietary supplements from natural sources that can serve as alternatives to antibiotics. If effective, these may reduce economic losses due to enteric diseases such as coccidiosis and necrotic enteritis (NE) (Lillehoj et al., 2016; Oh et al., 2019), which can become pervasive following antibiotic growth promoter (AGP) bans (Gadde et al., 2017b; Lin et al., 2017). NE is a major enteric disease of poultry caused by infection with *Clostridium perfringens* (Van Immerseel et al., 2004; Cooper and Songer, 2009;

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Timbermont et al., 2011; Lee and Lillehoj, 2022). The development of antibiotic-free NE control-strategies has been slowed due to the difficulty of experimentally reproducing NE in the laboratory using *C. perfringens* infection alone (Collier et al., 2008; Park et al., 2008; Lee et al., 2018). However, experimental NE has been induced by a high-protein diet and intestinal damage after exposure to an infection with *Eimeria* spp. (Williams et al., 2003; Lee et al., 2011; Oh et al., 2018).

Sphorolipids (SLs) which exist in a closed ring lactonic or open acidic structure (Freitas et al., 2018) are produced mainly by yeasts, *Candida bombicola*, *Candida apicola*, and *Rhodotorula bogoriensis* (Chen et al., 2006; Konishi et al., 2007). They are composed of a fatty acid tail (hydrophobic) and a carbohydrate head (hydrophilic) (Sen et al., 2017). The carbohydrate head is composed of a disaccharide sophorose linked by a β -1, 2 bond which is optionally acetylated on the 6' and/or 6'' position (Callaghan, 2017). SL structure is dependent on a terminal or subterminal hydroxylated fatty acid, which is linked β -glycosidically to the sophorose (Callaghan, 2017). The fatty acids' carboxylic end can be free, forming the acidic structure, or can be esterified at the 4'' position giving rise to the lactonic ring structure (Cavalero and Cooper, 2003). Sphorolipids have shown various antimicrobial activities against several bacterial species (Díaz De Rienzo et al., 2016; Silveira et al., 2019). The antimicrobial activities of SLs are mediated by their sugar and lipid portions (surfactant effect) and are associated with changes in or rupture of the bacterial cell membrane. This can result in cytoplasmic leakage and the consequent release of intracellular enzymes such as malate dehydrogenase (Lang et al., 1989; Glover et al., 1999; Kulakovskaya et al., 2014; Zhang et al., 2016). Detailed understanding of the mechanisms by which SLs mediate antimicrobial activity would facilitate the discovery and application of novel nutritional alternatives to replace antibiotics, and reduce economic losses due to enteric diseases such as NE. Due to their antimicrobial effects, we hypothesized that SLs could reduce the viability of *Eimeria* and *C. perfringens* and improve growth performance, immune response, and the integrity of the intestinal barrier in NE-afflicted broiler chickens. The objectives of this study were to evaluate the antimicrobial activities of different SL types against *E. acervulina*, *E. maxima*, *E. tenella*, and *C. perfringens* using in vitro tests, and to evaluate SL in vivo effects on growth performance, intestinal immune response, and gut epithelial integrity following NE challenge infection.

As far as we are aware this study is the first to evaluate the in vitro and in vivo effects of SLs on NE.

MATERIALS AND METHODS

Experiment 1: In Vitro Study

Anticoccidial Assay Against *E. acervulina*, *E. maxima*, and *E. tenella* About 2.5×10^5 freshly prepared sporozoites from sporulated oocysts of each *E. acervulina*, *E. maxima*, and *E. tenella* were placed into each well of a 96-well plate. Decoquinate (Sigma-Aldrich, 1165408, St. Louis, MO) and monensin (Sigma-Aldrich, M5273) were used as positive controls. These were tested alongside 4 novel SLs (C18:1 lactonic diacetyled SL [SL1], C18:1 deacetyled SL [SL2], C18:1 monoacetyled SL [SL3], and C18:1 diacetyled SL [SL4]). All SLs for experiments were produced and provided by DSM Nutritional Products (Columbia, MD). Three different SL doses: low (L, 125 μ g/mL), medium (M, 250 μ g/mL), and high (H, 500 μ g/mL), were combined with freshly prepared live sporozoites and incubated at 41°C for 3 h. Fluorescent dye (AOPI staining solution, CS2-0106) was then added to each mixture at a 1:1 ratio and live sporozoites were counted with a cell counting chamber (Cellometer, Nexcelom Bioscience LLC, Lawrence, MA).

Antibacterial Assay Against *C. perfringens* *Clostridium perfringens* was treated with bacitracin methylene disalicylate (BMD, Sigma-Aldrich, 11702) as a positive control and 4 SLs samples in brain-heart infusion broth (BD Difco, 299070, Sparks, MD) at 41°C for 18 h under anaerobic conditions at L (125 μ g/mL), M (250 μ g/mL), and H (500 μ g/mL). After incubation, 100 μ L of the culture solution was spread on differential reinforced Clostridial agar (BD Difco, 264120) plates and incubated at 41°C for 18 h under anaerobic conditions. Colonies of growing bacteria on the plates were counted to determine the survival ratios (%) of *C. perfringens* within each treatment group.

Experiment 2: In Vivo Study

All experiments were approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee (# 20-012).

Chickens and Experimental Design Figure 1 depicts the schematic outline of the experimental design. In experiment 2, a total of 336 newly hatched (0-day-old,

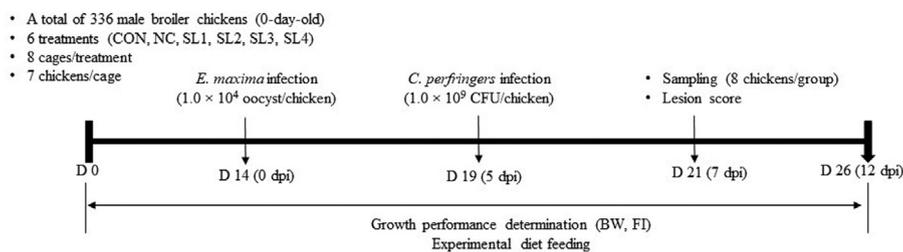


Figure 1. Schematic outline of the experimental design in experiment 2.

Ross 708) male broiler chickens were purchased from Longenecker's hatchery (Elizabethtown, PA). One day after arrival at the Beltsville ARS facility, all chickens were weighed and allocated to 6 dietary treatments in a randomized complete block design. The dietary treatments included a basal diet based on corn and soybean meal (CON), basal diet for infected chickens (NC), CON + SL1, CON + SL2, CON + SL3, and CON + SL4. The dose of SL in each treatment was 200 mg/kg (= 200 ppm) feed. At the beginning of the experiment, each treatment was composed of 8 cages and each cage had 7 chickens. Each cage was 0.65 m in width by 0.75 m in length (14 chickens/m²), and all cages were kept in the same room. Each cage was considered an experimental unit. The chickens were given ad libitum access to water and feed throughout the experiments.

Body Weight and Feed Intake Measurement Feed weights were recorded and the feeders were shaken once per day. The chickens and feed were weighed at 0, 14, 21, and 26 d of age for the computation of growth performance. Dead chickens were removed and weighed daily to calculate mortality and adjust the growth performance data.

Experimental Necrotic Enteritis Model The experimental NE model was based on a previously reported study (Lee et al., 2018). All chickens except those in the CON group were infected with *E. maxima* Beltsville strain 41A (1.0×10^4 oocyst/chicken) by oral gavage on d 14 post-hatch, followed by co-infection with *C. perfringens* strain Del-1 (1.0×10^9 cfu/chicken) by oral gavage on d 19 post-hatch. A DNA test of *E. maxima* was performed to check for the purity of the strain before oral infection (Haug et al., 2007). To facilitate the development of NE, chickens were fed a diet containing 18% CP from 0 to d 19, followed by a diet containing 24% CP from d 19 to the end of the study (Table 1).

Collection of Intestinal Samples Chickens were evenly distributed according to their average body weights to 8 separate cages, and treatment and control groups were randomly assigned for intestinal sample collection at d 21. The chickens were euthanized by cervical dislocation, and their intestines were removed immediately. From each chicken, a small section of the distal jejunum without contents was collected aseptically and stored in RNAlater (Applied Biosystems, Foster City, CA) at -20°C for further analysis.

Gut Lesion Score Intestinal lesion scores were measured from chickens at d 21 following euthanization per guidelines of Animal Use Protocol approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee. Lesions from distal jejunum were scored on a scale from 0 (none) to 4 (high) as described by Park et al. (2021). Lesion scores were evaluated blindly by 4 independent observers.

Isolation of RNA and Reverse Transcription Total RNA from the jejunal samples was prepared by the protocol as previously described (Park et al., 2021).

Gene Expression Analysis by qRT-PCR The oligonucleotide primer sequences used for quantitative real-

Table 1. Ingredient composition of basal diet (as-fed basis, %, unless otherwise indicated).

Ingredients (%)	Low protein (d 0 to 19)	High protein (d 19 to 26)
Corn	69.01	55.78
Soybean meal	23.99	37.03
Soybean oil	2.75	2.97
Dicalcium phosphate	2.00	1.80
Calcium carbonate	1.40	1.51
Salt	0.35	0.38
Poultry vitamin mix ¹	0.20	0.22
Poultry mineral mix ²	0.15	0.15
DL-Methionine	0.10	0.10
Choline-chloride, 60%	0.05	0.06
Total	100	100
Calculated values (%)		
CP, %	18.00	24.00
Ca, %	1.19	1.20
AP, %	0.54	0.51
Lys, %	1.00	1.40
Met, %	0.42	0.49
Cys + Met, %	0.65	0.80
ME, Mcal/kg	3.6	3.5

¹Vitamin mixture provided the following nutrients per kg of diet: vitamin A, 2,000 IU; vitamin D3, 22 IU; vitamin E, 16 mg; vitamin K, 0.1 mg; vitamin B1, 3.4 mg; vitamin B2, 1.8 mg; vitamin B6, 6.4 mg; vitamin B12, 0.013 mg; biotin, 0.17 mg; pantothenic acid, 8.7 mg; folic acid, 0.8 mg; niacin, 23.8 mg.

²Mineral mixture provided the following nutrients per kg of diet: Fe, 400 mg; Zn, 220 mg; Mn, 180 mg; Co, 1.3 mg; Cu, 21 mg; Se, 0.2 mg. CP = crude protein, AP = available phosphorus.

time PCR (qRT-PCR) are listed in Table 2. The various cytokines and intestinal tight junction (TJ) proteins whose differential expression was evaluated in the jejunum included interleukin IL1 β , IL2, IL4, IL6, IL8, IL10, IL13, IL17F, IFN- γ , TNFSF15, JAM2, occludin, ZO1, and MUC2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Amplification and detection were carried out with a Stratagene Mx3000P qPCR system (Agilent Technologies Inc., Santa Clara, CA) and RT² SYBR Green qPCR master mix (Qiagen, Germantown, MD). Each sample was analyzed in triplicate, and nonspecific primer amplification was assessed through the inclusion of no-template controls. Standard curves were generated with log₁₀ diluted RNA, and the levels of individual transcripts were normalized to those of GAPDH using the Q-gene program (Muller et al., 2002).

Statistical Analysis

Data for each response were analyzed using a Mixed Model (PROC MIXED) in SAS (SAS Inst. Inc., Cary, NC). In experiment 2 the design was a randomized complete block design. Each cage was considered an experimental unit. Each cage unit was the block factor. The results are given as least-squares means and pooled SEM. Probability values less than 0.05 were considered to indicate a significant difference. In cases in which the overall effect was significant in terms of growth performance, means were compared in a pairwise manner (PDIFF option). For other results, the PDIFF option was used to test for significant differences between groups.

Table 2. Oligonucleotide primer sequences for real-time quantitative polymerase chain reaction (PCR).

Type	Target gene	Primer sequence (5'-3')	PCR product size (Kbp)
Reference	GAPDH	F-GGTGGTGTAAAGCGTGTAT R-ACCTCTGCCATCTCCACA	264
Proinflammatory	IL1 β	F-TGGGCATCAAGGGCTACA R-TCGGGTTGGTTGGTGATG	244
	IL6	F-CAAGGTGACGGAGGAGGAC R-TGGCGAGGAGGGATTCT	254
	IL8	F-GGCTTGCTAGGGGAAATGA R-AGCTGACTCTGACTAGGAACTGT	200
	IL17F	F-TGAAGACTGCCTGAACCA R-AGAGACCGATTCTGATGT	117
	TNFSF15	F-CCTGAGTATTCCAGCAACGCA R-ATCCACCAGCTTGATGTCACTAAC	292
Th1	IL2	F-TCTGGGACCACTGTATGCTCT R-ACACCAGTGGGAAACAGTATCA	256
	IFN γ	F-AGCTGACGGTGGACCTATTATT R-GGCTTTGCGCTGGATTTC	259
Th2	IL4	F-ACCCAGGGCATCCAGAAG R-CAGTGCCGGCAAGAAGTT	258
	IL10	F-CGGGAGCTGAGGGTGAA R-GTGAAGAAGCGGTGACAGC	272
	IL13	F-CCAGGGCATCCAGAAGC R-CAGTGCCGGCAAGAAGTT	256
TJ proteins	Occludin	F-GAGCCAGACTCAAAGCAA R-GCTTGATGTGGAAGAGCTTGTTG	68
	ZO1	F-CCGCAGTCGTTACAGATCT R-GGAGAATGTCTGGAATGGTCTGA	63
	JAM2	F-AGCCTCAAATGGGATTGGATT R-CATCAACTTGCATTGCTTCA	59
Mucin	MUC2	F-GCCTGCCAGGAAATCAAG R-CGACAAGTTTGCTGGCACAT	59

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; JAM, junctional adhesion molecule; Kbp, kilobases pairs; TJ, tight junction; TNFSF, tumor necrosis factor superfamily; Th, T helper type; ZO, zonula occludens.

RESULTS

Experiment 1

Anticoccidial Activity Against *Eimeria acervulina*, *maxima*, and *tenella*

Vehicle control (CON) was

measured as 2.5×10^5 sporozoites/mL of each *Eimeria* spp (Figures 2A–2C). In *E. acervulina* sporozoites (Figure 2A), decoquinatate and monensin as coccidiostats linearly decreased ($P < 0.001$) the survival ratio of *E. acervulina* sporozoites in proportion to these doses

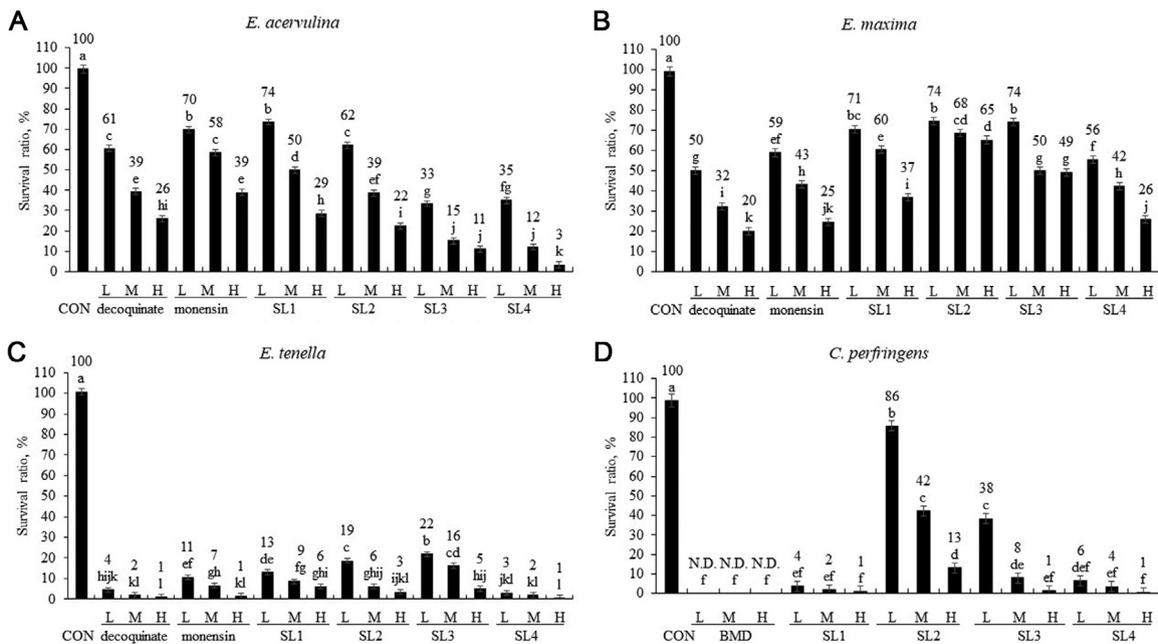


Figure 2. Anticoccidial and antibacterial efficacies of sophorolipid against *Eimeria* sporozoites and *C. perfringens* in experiment 1. BMD, bacitracin methylene disalicylate; CON, vehicle control (2.5×10^5 sporozoites/mL and 1.0×10^9 CFU/mL of *Clostridium perfringens*); H, high dose (500 μ g/mL); L, low does (125 μ g/mL); M, medium dose (250 μ g/mL); SL1, C18:1 lactonic diacetyled sophorolipid, SL2, C18:1 deacetyled sophorolipid; SL3, C18:1 monoacetyled sophorolipid; SL4, C18:1 diacetyled sophorolipid.

compared to CON. Survival ratio in decoquinate compared to CON was decreased ($P < 0.001$) by 61, 39, and 26% at L (125 $\mu\text{g}/\text{mL}$), M (250 $\mu\text{g}/\text{mL}$), and H (500 $\mu\text{g}/\text{mL}$), respectively. In the case of monensin, the equivalent survival ratios compared to CON were ($P < 0.001$) 70, 58, and 39% (Figure 2A). All SLs dose-dependently decreased ($P < 0.001$) the survival ratio of *E. acervulina* sporozoites compared to CON. L, M, and H of SL1 decreased ($P < 0.001$) the number of *E. acervulina* sporozoites by 70, 58, and 29%, respectively, compared to CON. SL2 treatment also decreased ($P < 0.001$) the *E. acervulina* sporozoites by 62% (L), 39% (M), and 22% (H) compared to CON. In the case of SL3, L, M, and H of SL3 decreased ($P < 0.001$) the survival ratio of *E. acervulina* sporozoites by 33, 15, and 11%, respectively, compared to CON. In *E. maxima* sporozoites (Figure 2B), decoquinate and monensin treatments dose-dependently decreased ($P < 0.001$) the survival ratio of *E. maxima* sporozoites compared to CON. All SLs linearly decreased ($P < 0.001$) *E. maxima* sporozoite in proportion to their doses compared to CON. L, M, and H of SL1 significantly decreased ($P < 0.001$) the survival ratio of *E. maxima* sporozoites by 71, 60, and 37%, respectively, compared to CON. In the case of SL2, the survival ratio of *E. maxima* sporozoite were dose-dependently decreased ($P < 0.001$) from 74 to 65% compared to CON. L, M, and H SL3 linearly decreased ($P < 0.001$) the survival ratio of *E. maxima* sporozoite by 74, 50, and 49%, respectively, compared to CON as well as SL4 was 56, 42, and 26%. In the case of *E. tenella* sporozoites (Figure 2C) decoquinate and monensin treatments dose-dependently decreased ($P < 0.001$) the survival

ratio of *E. tenella* sporozoites compared to CON. L, M, and H of SL1 dose-dependently decreased ($P < 0.001$) the survival ratio of *E. tenella* sporozoites by 13, 9, and 6%, respectively, compared to CON. SL2 treatments not only decreased survival ratio of the *E. tenella* sporozoites linearly by 19% (L), 6% (M), and 3% (H), respectively, compared to CON, but L, M, and H of SL3 also decreased ($P < 0.001$) the survival ratio of *E. tenella* sporozoites by 22, 16, and 5%, respectively, compared to CON. SL4 also decreased them by 3% (L), 2% (M), and 1% (H) compared to CON.

Antibacterial Activity Against *Clostridium Perfringens* Vehicle control (CON) was measured as 1.0×10^9 cfu/mL of *C. perfringens* (Figure 2D). *C. perfringens* was not detected in any BMD treatments regardless of the dose applied. In the case of SL1 treatment, L, M, and H of SL1 dose-dependently decreased ($P < 0.001$) the survival ratio of *C. perfringens* by 4, 2, and 1%, respectively, compared to CON. SL2 treatments were ($P < 0.001$) 86% (L), 42% (M), and 13% (H). SL3 treatments not only decreased the survival ratio of *C. perfringens* dose-dependently by 38% (L), 8% (M), and 1% (H) compared to CON, but SL4 treatments also decreased them by 6, 4, and 1% compared to CON.

Experiment 2

Growth Performance Initial body weight (BW) did not differ ($P > 0.05$) statistically among treatments (Table 3). Before NE-induction, no treatments changed chicken BWs. After NE-induction chicken BWs

Table 3. Growth performance of necrotic enteritis-induced chicken fed a diet supplemented with sophorolipids (SLs) in experiment 2.

	CON	NC	SL1	SL2	SL3	SL4	SEM	P-value
BW, g								
Initial	39.5	39.1	39.5	39.3	39.0	39.4	0.3	0.893
D 14 (0 dpi)	329	332	332	328	332	327	7.5	0.993
D 21 (7 dpi)	581 ^a	485 ^b	503 ^b	482 ^b	491 ^b	488 ^b	9.9	<0.001
D 26 (12 dpi)	1018 ^a	741 ^d	825 ^b	781 ^c	790 ^{bc}	806 ^{bc}	14.4	<0.001
ADG, g								
D 0 to 14	20.8	20.8	21.1	20.5	20.8	20.5	0.55	0.957
D 14 to 21	35.9 ^a	21.8 ^c	24.4 ^b	21.8 ^c	22.8 ^{bc}	23.0 ^{bc}	0.97	<.001
D 21 to 26	72.8 ^a	42.6 ^c	53.7 ^b	49.9 ^b	49.8 ^b	52.9 ^b	2.07	<.001
Infection (d 14 to 26)	54.5 ^a	32.1 ^d	39.2 ^b	35.9 ^c	36.2 ^{bc}	38.0 ^{bc}	1.17	<.001
Overall (d 0 to 26)	43.1 ^a	28.6 ^d	33.1 ^b	30.8 ^c	31.0 ^c	32.1 ^{bc}	0.71	<.001
ADFI, g								
D 0 to 14	32.3	33.5	32.9	33.8	33.4	34.0	0.64	0.604
D 14 to 21	65.3	61.6	60.5	59.8	60.6	60.0	1.88	0.320
D 21 to 26	118 ^a	90.3 ^b	91.4 ^b	89.5 ^b	89.5 ^b	90.9 ^b	2.40	<.001
Infection (d 14 to 26)	92.0 ^a	76.0 ^b	76.0 ^b	74.8 ^b	75.1 ^b	75.4 ^b	1.69	<.001
Overall (d 0 to 26)	71.9 ^a	61.6 ^b	61.6 ^b	61.1 ^b	61.1 ^b	61.3 ^b	1.22	<.001
FCR, g								
D 0 to 14	1.55	1.60	1.57	1.64	1.64	1.64	0.04	0.274
D 14 to 21	1.81 ^d	2.81 ^a	2.50 ^c	2.79 ^{abc}	2.8 ^{ab}	2.67 ^{abc}	0.11	<.001
D 21 to 26	1.63 ^b	2.08 ^a	1.70 ^b	1.83 ^{ab}	1.85 ^{ab}	1.74 ^b	0.09	0.022
Infection (d 14 to 26)	1.72 ^c	2.45 ^a	2.10 ^b	2.31 ^{ab}	2.33 ^{ab}	2.21 ^{ab}	0.09	<.001
Overall (d 0 to 26)	1.66 ^c	2.17 ^a	1.93 ^b	2.09 ^a	2.10 ^a	2.02 ^{ab}	0.06	<.001

Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; BW, body weight; CON, basal diet; D, day, dpi, days postinfection; FCR, feed conversion ratio (ADFI/ADG); NC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacetyled sophorolipid; SL2, diet supplemented with C18:1 deacetyled sophorolipid; SL3, diet supplemented with C18:1 monoacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid.

The dose of SL in each treatment was 0.2 g/kg feed. All chickens except CON were infected by oral gavage at d 14 and 19 with 1.0×10^4 oocysts/chicken of *E. maxima* and 1.0×10^9 CFU/chicken of *C. perfringens*, respectively.

^{a-d}Means in the same row with different superscripts differ ($P < 0.05$) and the difference was reevaluated by PDIFF option in SAS when P -value between treatments was less than 0.05.

regardless of SLs supplementation were decreased ($P < 0.001$) compared to CON at both of d 21 (581–490 g) and 26 (1,018–789 g). Among NE-induced chicken groups, there was no detectable change in BW at d 21 regardless of SL type, however, all SLs increased ($P < 0.001$) BW (741–801 g) of chickens compared to the NC at d 26. Among SL groups, BW (825–781 g) of chickens fed SL1 was significantly increased ($P = 0.024$) compared to that of SL2. In a similar manner to chicken BW, the average daily gain (ADG) of chickens did not differ among treatments at d 14. NE-induction significantly decreased ($P < 0.001$) the ADG (54.5–36.3 g) of chickens compared to CON in the entire infection duration. SL1-fed chickens showed increased ADG compared to the NC ($P = 0.049$, 21.8–24.4 g) and SL2 ($P = 0.048$, 21.8–24.4 g) groups at d 14 to 21. All SL types increased ($P = 0.016$) the ADG (42.6–51.6 g) of chickens compared to that of NC at d 21 to 26. In average daily feed intake (ADFI), all of treatments did not change ($P > 0.05$) ADFI of chickens by d 21. NE-induced chickens after d 21, regardless of treatment type, decreased ($P < 0.001$) ADFI (92.0–75.5 g) compared to that of CON. In the case of feed conversion ratio (FCR), there was no difference ($P > 0.05$) between treatments before infection (d 14). NE-induced chickens (NC) significantly increased ($P < 0.002$) FCR (1.72–2.28) compared to that of CON during the entire infection period. SL1-fed chickens showed significantly decreased FCR compared to that of NC ($P = 0.049$, 2.81–2.50) and SL3 ($P = 0.048$, 2.79–2.50) at d 14 to 21. SL1- ($P = 0.007$, 2.08–1.70) and SL4- ($P = 0.044$, 2.08–1.74) fed chickens showed decreased FCR compared to NC at d 21 to 26.

Lesion Scores of Distal Jejunum NE-induced chickens regardless of supplementation type had significantly increased ($P < 0.001$) jejunal lesion score (0.20 to 2.22) compared to that of CON at d 21 (7 d postinfection: dpi; Figure 3). Among SLs, SL1 (vs. NC: $P = 0.043$,

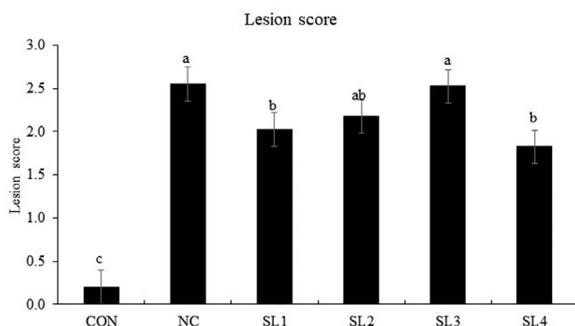


Figure 3. Lesion score of necrotic enteritis-induced chickens fed diet supplemented with various sophorolipids in experiment 2. The dose of SL in each treatment was 0.2 g/kg feed. All chickens except CON were infected by oral gavage at d 14 and 19 with 1.0×10^4 oocysts/chicken of *E. maxima* and 1.0×10^9 CFU/chicken of *C. perfringens*, respectively. ^{a-c} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The lesion score was collected from distal jejunal tissue at d 21 (7 d postinfection: dpi). Abbreviations: CON, basal diet; NC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacetyled sophorolipid; SL2, diet supplemented with C18:1 deacetyled sophorolipid; SL3, diet supplemented with C18:1 monoacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid.

2.55 to 2.03 and vs. SL4: $P = 0.041$, 2.53 to 2.03) and SL4 (vs. NC: $P = 0.012$, 2.55 to 1.83 and vs. SL3: $P = 0.015$, 2.53 to 1.83) supplementation decreased jejunal lesion scores of chickens compared to those of NC and SL3.

Proinflammatory Cytokines NE-induced chickens (NC) without supplementations numerically increased ($P = 0.098$) IL1 β expression level in the distal jejunum compared to that of CON (Figure 4A). SL1 ($P = 0.002$, 3.5×10^{-3} to 1.3×10^{-3}), SL2 ($P = 0.003$, 3.5×10^{-3} to 1.3×10^{-3}), SL3 ($P = 0.011$, 3.5×10^{-3} to 1.7×10^{-3}), and SL4 ($P = 0.026$, 3.5×10^{-3} to 1.9×10^{-3}) supplementations significantly decreased IL1 β levels compared to that of NC (Figure 4A). Co-infection with *E. maxima* and *C. perfringens* and SLs supplementation did not change ($P > 0.05$) the IL6 level of the distal jejunum at d 21 compared to that of CON (Figure 4B). All SL types decreased (SL1: $P = 0.009$, 6.9×10^{-2} to 1.0×10^{-2} SL2: $P = 0.018$, 6.9×10^{-2} to 1.3×10^{-2} SL3: $P = 0.025$, 6.9×10^{-2} to 1.3×10^{-2} and SL4: $P = 0.028$, 6.9×10^{-2} to 5.0×10^{-3}) IL8 level compared to that of NC (Figure 4C). SL1- ($P = 0.014$, 3.9×10^{-3} to 2.2×10^{-3}), SL2- ($P = 0.018$, 3.9×10^{-3} to 2.2×10^{-3}), and SL4- ($P = 0.010$, 3.9×10^{-3} to 2.3×10^{-3}) fed chickens had a decreased TNFSF15 level in the jejunum compared to that of NC (Figure 4d).

Th1 Cytokines NE-induced chickens (NC) had significantly increased IL2 ($P = 0.003$, 1.1×10^{-3} to 1.6×10^{-3}), IFN- γ ($P < 0.001$, 2.8×10^{-4} to 6.6×10^{-4}), and IL10 ($P = 0.023$, 4.8×10^{-5} to 9.6×10^{-5}) levels in the distal jejunum compared to those of CON (Figure 5). SL1- ($P = 0.002$, 1.6×10^{-3} to 1.1×10^{-3}) and SL2- ($P = 0.002$, 1.6×10^{-3} to 1.1×10^{-3}) fed chickens had a significantly decreased IL2 level compared to that of NC (Figure 5A). All SL types did not alter ($P > 0.05$) IFN- γ level compared to that of NC (Figure 5B), whereas all SL types decreased ($P < 0.001$) IL10 (9.6×10^{-5} to 1.5×10^{-5}) level (Figure 5C).

Tight Junction and Mucin Proteins JAM2 levels were not affected by NE-induction or SL supplementations (Figure 6A), whereas occludin (1.2×10^{-1} to 6.9×10^{-2}) ZO1 (1.3×10^{-1} to 5.8×10^{-2}), and MUC2 (2.8×10^{-1} to 2.0×10^{-1}) levels decreased ($P < 0.001$) with NE-induction compared to CON. SL3- ($P = 0.010$, 6.9×10^{-2} to 9.0×10^{-2}) and SL4- ($P < 0.001$, 6.9×10^{-2} to 1.3×10^{-1}) fed chickens showed a significantly increased occluding level compared to that of the NC (Figure 6B). In the case of SL4 supplementation, the ZO1 level (5.8×10^{-2} to 1.3×10^{-1}) also increased ($P < 0.001$) compared to that of NC (Figure 6C).

DISCUSSION

Sophorolipids produced by yeasts such as *Starmerella bombicola*, *Candida bastistaetic*, *C. floricola*, and *C. apicola* (Chen et al., 2006) exist as crude mixtures. The effects of crude SL mixtures as antibacterial, antifungal, anticancer, and spermicidal agents are described in vitro

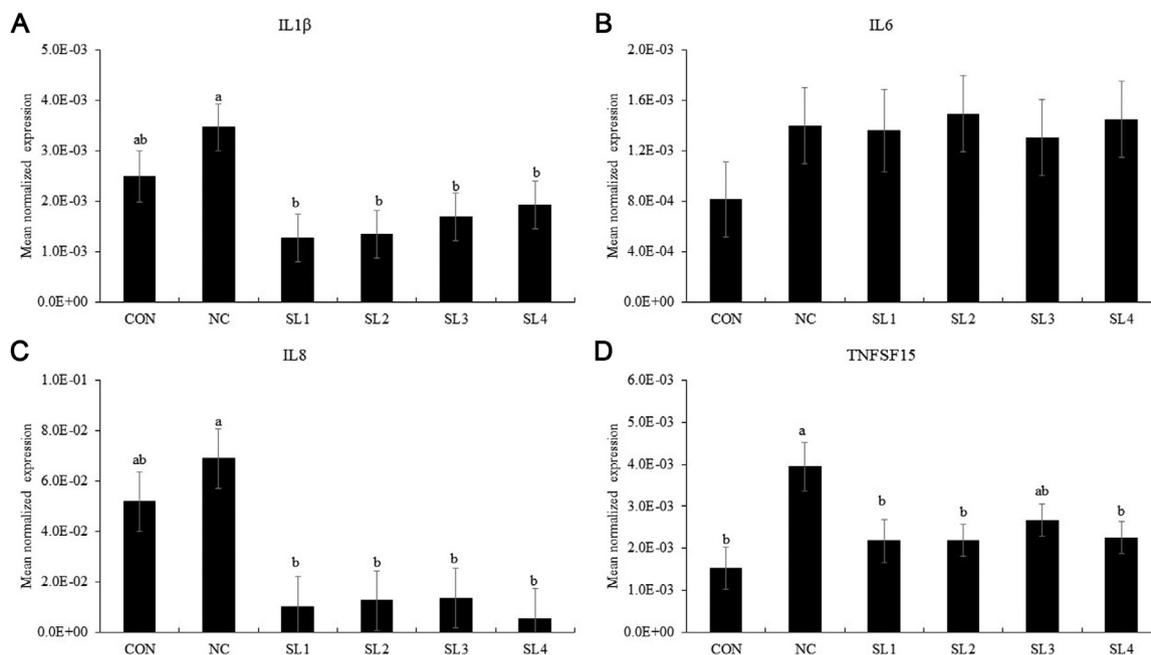


Figure 4. Transcripts of proinflammatory cytokine in jejunum of necrotic enteritis-induced chickens fed diet supplemented with various sophorolipids in experiment 2. The dose of SL in each treatment was 0.2 g/kg feed. All chickens except CON were infected by oral gavage at d 14 and 19 with 1.0×10^4 oocysts/chicken of *E. maxima* and 1.0×10^9 CFU/chicken of *C. perfringens*, respectively. ^{a-c} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 21 (7 d postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: CON, basal diet; NC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacetyled sophorolipid; SL2, diet supplemented with C18:1 deacetyled sophorolipid; SL3, diet supplemented with C18:1 monoacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid.

or in vivo, primarily in mice (Shah et al., 2005; Sleiman et al., 2009; Shao et al., 2012; Ribeiro et al., 2015; Díaz De Rienzo et al., 2016; Sen et al., 2017; Silveira et al., 2019). In the case of SL antibacterial

activity, gram-positive bacteria were more susceptible to SL action than gram-negative bacteria (Díaz De Rienzo et al., 2016; Silveira et al., 2019). It was speculated that the different layer and charge properties of

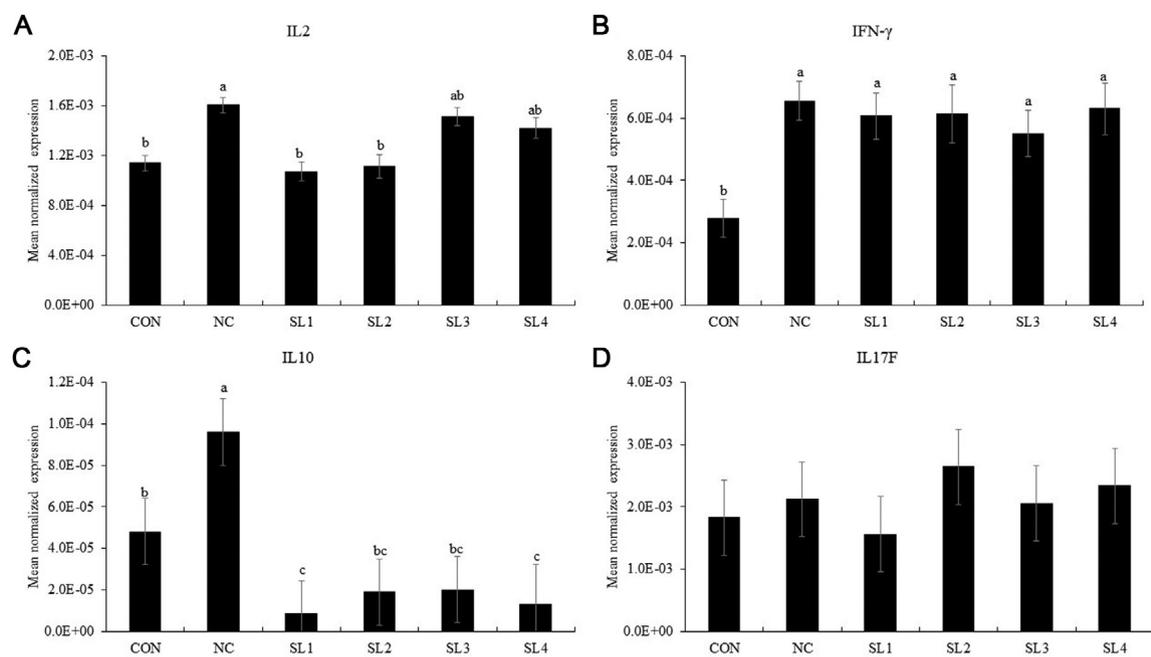


Figure 5. Transcripts of Th1 cytokines in jejunum of necrotic enteritis-induced chickens fed diet supplemented with various sophorolipids in experiment 2. The dose of SL in each treatment was 0.2 g/kg feed. All chickens except CON were infected by oral gavage at d 14 and 19 with 1.0×10^4 oocysts/chicken of *E. maxima* and 1.0×10^9 CFU/chicken of *C. perfringens*, respectively. ^{a-c} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 21 (7 d postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: CON, basal diet; NC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacetyled sophorolipid; SL2, diet supplemented with C18:1 deacetyled sophorolipid; SL3, diet supplemented with C18:1 monoacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid.

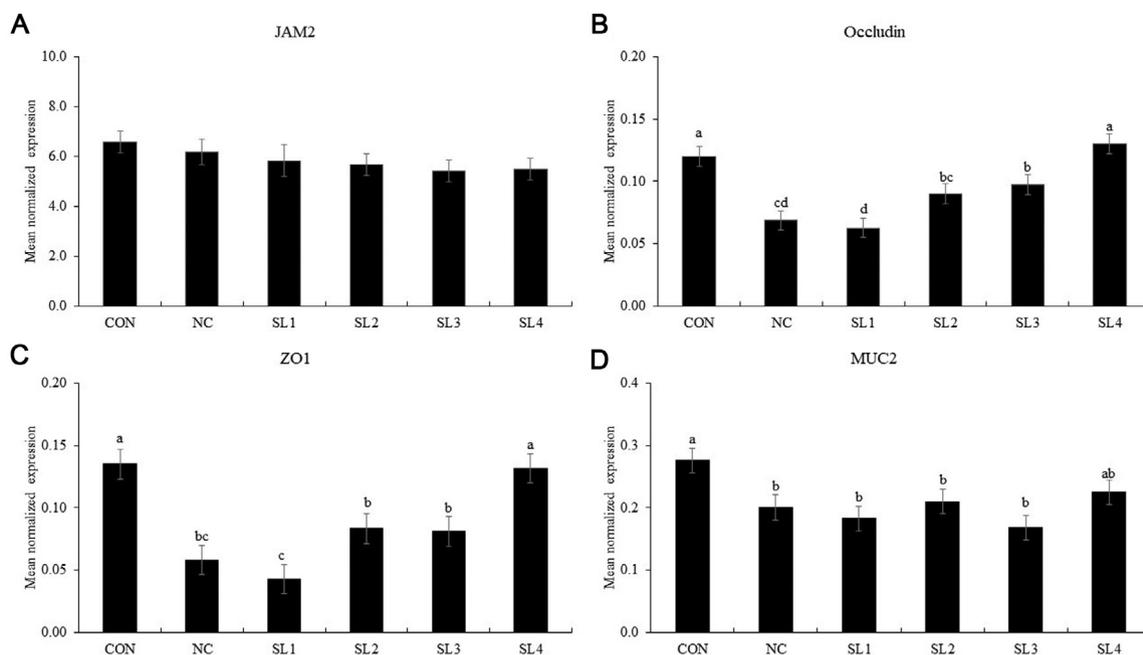


Figure 6. Transcripts of tight junction and mucin protein in jejunum of necrotic enteritis-induced chickens fed diet supplemented with various sophorolipids in experiment 2. The dose of SL in each treatment was 0.2 g/kg feed. All chickens except CON were infected by oral gavage at d 14 and 19 with 1.0×10^4 oocysts/chicken of *E. maxima* and 1.0×10^9 CFU/chicken of *C. perfringens*, respectively. ^{a-d} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 21 (7 d postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: CON, basal diet; NC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacetyled sophorolipid; SL2, diet supplemented with C18:1 deacetyled sophorolipid; SL3, diet supplemented with C18:1 monoacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid.

the cell membrane make it more difficult for SL to interact with the cell envelope of gram-negative than gram-positive bacteria (Dengle-Pulate et al., 2014). Although the antimicrobial mechanism of action of SLs was not explained exactly in these studies, it is speculated that leakage of cytoplasmic contents in targeted microbes, and the consequent release of intracellular enzymes can be induced by changes to or rupture of the cellular membrane due to the actions of SL (Kulakovskaya et al., 2014; Zhang et al., 2016; Freitas et al., 2018). The in vitro results in the current study are likely the first report of the anticoccidial efficacy of SLs. Among the three types of sporozoite studied (*E. acervulina*, *E. maxima*, and *E. tenella*), *E. tenella* was the most sensitive to the anticoccidial effects of decoquinate, monensin, and all types of SL supplementation. Decoquinate and monensin dose-dependently (125–500 $\mu\text{g}/\text{mL}$) reduced *E. tenella* sporozoite numbers by more than 95 and 90%, respectively, compared to vehicle control. Among SLs, SL4 showed similar anticoccidial ability to decoquinate. SL1 and SL2 also showed strong anticoccidial ability against the *E. tenella* sporozoite, which was reduced by more than 88% compared to the control. Although SL3 among these treatments showed the weakest anticoccidial activity against *E. tenella* sporozoite, it still reduced numbers by more than 78%. The next most sensitive sporozoite strain to the anticoccidial activity of SLs was *E. acervulina*. Among treatments against *E. acervulina* sporozoite, SL3 and SL4 in low dose (125 $\mu\text{g}/\text{mL}$) showed an anticoccidial effect of more than 75% compared to the control and more than 90% at the highest dose (500 $\mu\text{g}/\text{mL}$). These remarkable anticoccidial

efficacies surpassed those of decoquinate and monensin. SL1 and SL2 also showed anticoccidial abilities quite similar to those of decoquinate and monensin. The anticoccidial activity of decoquinate and monensin against the *E. maxima* sporozoites was similar to that of *E. acervulina*. The survival ratio of the *E. maxima* sporozoite in SL treatments was slightly elevated compared to that of *E. acervulina*. Despite this, the anticoccidial efficacies of SL1 and SL4 in high doses (500 $\mu\text{g}/\text{mL}$) were greater than 63% compared to CON.

In terms of antibacterial activity, *C. perfringens* was eliminated by BMD treatment. SL1 and SL4, regardless of their doses, showed strong antibacterial ability, eliminating more than 95% of *C. perfringens* compared to CON. SL2 and SL3 also showed antibacterial ability against *C. perfringens*, however, not at the levels of SL1 and SL4.

Sophorolipids have been documented to have a wide range of antimicrobial activity against several pathogenic bacteria, such as *Bacillus licheniformis* (Solaiman et al., 2016), *B. subtilis* (Díaz De Rienzo et al., 2015), *C. perfringens* (Silveira et al., 2019), *Escherichia coli* O157:H7 (Zhang et al., 2017), *Pseudomonas aeruginosa* (Hoa et al., 2017), and *Staphylococcus aureus* (Díaz De Rienzo et al., 2016). Different kinds of SLs have different properties and reveal a wide range of physiological functions depending on the microorganisms producing them (Díaz De Rienzo et al., 2016). Important factors affecting SL properties include the solubilization of hydrophobic compounds, heavy metal binding, virulence factors, cell signaling (quorum sensing), and biofilm formation (Franzetti et al., 2011;

Díaz De Rienzo et al., 2015, 2016). The remarkable antimicrobial ability of the SLs we tested in vitro suggested that they can serve as antibiotic alternatives for chickens susceptible to enteric diseases such as coccidiosis and NE. Therefore, these SLs were further investigated in experiment 2, which quantified the in vivo effect of SLs on growth performance and gut health of NE-induced broiler chicken. The results of this experiment are probably the first observations of dietary SL supplementation on the growth performance, intestinal immunity, and intestinal barrier integrity of NE-induced broiler chickens infected with *E. maxima* and *C. perfringens*.

In experiment 2, 200 mg SL/kg feed, regardless of SL type, revealed no harmful effect on the growth performance of broiler chickens. NE-induced chickens lost approximately 19% of BW compared to CON fed a basal diet at 7 dpi. Up to this point, SLs did not influence the BW of NE-induced chickens. However, all SL supplementations improved the BW of chickens compared to NC chickens (SL1:11.3%, SL2:5.3%, SL3:6.6%, and SL4:8.8%) at 12 dpi. This implies that SL supplementation may help the recovery of chickens after infection. The BW changes also affected the ADG of chickens. NE-induced chickens (NC) had a 40% lower ADG compared to CON, whereas SL1 supplementation improved the ADG of NC by 12% from d 14 to 21. After that period, SLs showed their growth-promoting efficacy in comparison to NE-infected chickens (NC) from d 21 to 26. SL supplementations improved ADG of chickens between 17 and 26% compared to that of NE-induced chicken (NC) in this period. In the case of ADFI, there were no changes detected between treatments even though NE induction reduced the ADG of chickens compared to CON, and simultaneously, SL supplementations improved the ADG of NE-induced chickens. The ADFI of chickens in all treatments was sharply reduced between 7 dpi and 12 dpi compared to CON, and there was no detectable difference among treatments. Our results for ADFI implied that the increased ADG of chickens due to SL supplementations did not come from their feed intake. The FCR was calculated by ADFI/ADG, thus changes to ADFI or ADG normally affect FCR. The FCR also followed the trend of ADG and ADFI, namely, NE-induction impaired the FCR of chickens during the entire period, whereas SL1 and SL4 improved FCR by 20% compared to the NC. Interestingly, our results demonstrated that the FCR was improved by feed supplements like SL1 and SL4 because the ADFI in that time was not changed among NE-infected groups.

In addition to SLs' growth-promoting effects, NE-induction of chickens increased distal jejunum lesion had higher scores compared to that of CON. However, among infected groups, SL1 and SL4 showed protective effects against NE-induction through reduction of the lesion score. Based on the antimicrobial properties of SLs against *E. maxima* and *C. perfringens* in experiment 1 (in vitro), as expected, our results of the lesion score imply that antimicrobial activity of SL1 and SL4 directly reduce the number of *E. maxima* and *C.*

perfringens in the chicken intestine. Many studies indicate that a growth-promoting effect in infected chickens is associated with directly reducing the number of pathogens using antimicrobial feed supplements (Lee et al., 2018; Oh et al., 2019; Park et al., 2020). Our results showing growth-promoting effects and lesion score reductions associated with antimicrobial SL supplementation support this pattern.

Cytokines, small immune-regulatory peptides that aid in cell-to-cell communication during immune responses, have been identified as important biomarkers of gastrointestinal functionality (Celi et al., 2019). Therefore, cytokines can play an important role in the regulation of the immune system, and an understanding of cytokine pathways may increase the feasibility of achieving growth promoting effects in domestic animals through immune response manipulations. Necrotic enteritis activates chickens' innate and acquired immune response, which involves the secretion of various chemokines and cytokines (Fasina and Lillehoj, 2019). In the current study, NE-induction without SLs elevated proinflammatory cytokine levels, although only the increase in TNFSF15 was statistically significantly. Most SLs suppressed the release of these pro-inflammatory cytokines. The local responses initiated by the production of proinflammatory cytokines increase vascular permeability, induce expression of adhesion permeability, and induce local production of chemokines (Kogut, 2000). IL1 β is an important proinflammatory cytokine that is produced mainly by activated macrophages and plays an important role in the innate immune responses through recruitment of inflammatory cells (Hong et al., 2006a). Interleukin-8 stimulate the recruitment of inflammatory cells like polymorphonuclear leukocyte and thus known to play a major role in the initiation and progression of any inflammation (Chand et al., 2020). Tumor necrosis factor- α is one of the most pleiotropic cytokines in mammals, but has yet to be identified in avian species (Takitomo et al., 2005; Hong et al., 2006b; Park et al., 2007). TNF superfamily 15 was induced in vivo following LPS injection and showed cytotoxic activity against the L929 cell line and cultured chicken fibroblast cells, suggesting that it may function as a substitute for TNF- α (Hong et al., 2006b). In addition to the changes in the expression of various proinflammatory cytokines, we also investigated the alterations in IL2, IL10, IL17F, and IFN- γ levels that relate to activation of Th1 cells. Similarly to the results for proinflammatory cytokines, NE-induction upregulated cytokines related to Th1 whereas SLs, especially SL1, suppressed the release of IL2 and IL10 compared to NC. In avian species, IL-2 generally indicates lymphocyte proliferation, activation of NK cells, and clearance of intracellular pathogens (Susta et al., 2015). *Eimeria* stimulates IL-10 expression in the small intestine and caecum of infected chickens, suppressing their immune response, and facilitating disease progression (Lessard et al., 2020). In previous studies of cytokine release after SL administration, incubating multiple concentrations of SLs (3–100 μ g/mL) with VK-2 cells for 6 h increased IL1 and IL8 levels,

and lactic SL induced more cytokine production compared to acidic SL (Shah et al., 2005). In another study, LPS stimulated rat alveolar macrophage cells (NR8383) were cultured in the presence or absence of SLs for 12, 24, 36, and 48 h. TNF- α was significantly decreased in the LPS + SL group compared to the LPS group at 12 to 24 h but trended upward at 36 to 48 h. Proinflammatory cytokines IL1 β followed the same pattern (Mueller et al., 2006). These studies showed that SLs may mediate immune responses associated with inflammation in vitro. There have been no cases of analysis about cytokine release on SLs from in vivo studies until lately. In terms of the relationship between growth and immune responses in chickens, Klasing (2007) reported that a cytokine storm induces metabolic changes, including increased protein degradation in skeletal muscle, thereby diverting nutrients from the muscle and other tissues so that they are made available for the increased demands of leukocytes and the production of protective proteins. Consequently, these responses decrease growth performance and directly influenced the success of poultry production. In practice, under equal feed intakes, a vigorous acute-phase immune response in chickens has been estimated to account for 10% of nutrient use (Klasing, 2007). Jiang et al. (2010) have reported that LPS-challenged chickens (1 mg LPS per kg of body weight at 14, 16, 18, and 20 d of age) show a 22% decrease in body weight gain during the challenge; 59% of the loss is accounted for by decreased feed intake, and the remaining 41% is attributed to immune response-related factors (Broom and Kogut, 2018). Similarly, the current study demonstrated that SL supplementations suppressed cytokine production associated with the immune response to NE-induction, and promoted growth by preventing the diversion of feed nutrients for the production of immune factors.

Intestinal epithelial integrity can be damaged by many factors related to disease and stress. The damage reduces nutrient absorption, induces pathogenic invasion, and increases inflammatory disease, which ultimately leads to reduced growth (Yegani and Korver, 2008). The intestinal epithelium is composed of a single layer of columnar epithelial cells that are tightly bound by intercellular junctional complexes (Song et al., 2014). Tight junctions include four integral transmembrane proteins (occludin, claudin, JAM, and tricellulin) that interact with cytosolic scaffold proteins, which in turn bind the actin cytoskeleton (Ulluwishewa et al., 2011). According to Al-Sadi et al. (2011), occludin plays important roles in the assembly and maintenance of TJs and the regulation of intestinal permeability. In TJ proteins in the current study, NE-induced chickens showed lower occludin and ZO1 levels compared to that of CON. The result implies that NE-induced chickens' experienced epithelial rupture in the jejunum. SL4 supplementation enhanced occlusion levels compared to that of NC. Gadde et al. (2017a) have suggested that increased TJ protein expression in chickens improves intestinal barrier function and provides optimal gut health. In contrast, Callaghan (2017) reported that

acidic SL dose-dependently downregulated ZO-1 tight junction protein in incubated HT29 cancer cells, however lactic SL treatments failed to have any effect on HT29 tight junctions until higher doses. In another study, bio-surfactant decreased the permeability of TJs in human intestinal epithelial Caco-2 Cells (Mine and Zhang, 2003). It is too early to evaluate the direct efficacy of SLs on TJ proteins because of the paucity of information available in this area. However, it could be argued that the observed effect of SL supplements on TJ proteins might occur indirectly through the efficacy of SLs in modulating the intestinal immune response, which has a cascade reaction with TJ proteins.

In conclusion, all SLs showed antimicrobial activity against *E. acervulina*, *E. maxima*, *E. tenella*, and *C. perfringens* in vitro. The antimicrobial activity of SLs, especially SL1 and SL4, improved in vivo growth performance, reduced lesion development in the distal jejunum, suppressed pro-inflammatory cytokine release, and enhanced tight junction protein expression in the jejunum of NE-induced chickens co-infected with *E. maxima* and *C. perfringens*. Overall, dietary sophorolipid supplementation promoted growth, intestinal immune responses, and intestinal barrier integrity of young broiler chickens during a NE challenge, and therefore shows potential as an antibiotic alternative.

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Author contributions: SO, PC, and HL designed the research. SO, and HL conducted the research. IP, and HN conducted formal analysis. IP, and HL analyzed data. IP, and HL wrote an original draft. IP, PC, and HL edited the original draft. All authors read and approved the final manuscript.

DISCLOSURES

PC was employed by DSM Animal Nutrition and Health. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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