

Effect of dietary sophorolipids on growth performance and gastrointestinal functionality of broiler chickens infected with *Eimeria maxima*

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ABSTRACT Two experiments were conducted to evaluate the effects of dietary sophorolipids (SLs) supplementation as antibiotic alternatives on growth performance and gut health of chickens infected with *Eimeria maxima*. In experiment 1, 336 (zero-day-old) male broilers were used. The chickens were weighed and randomly allocated to the following 6 treatment groups with 7 chickens/cage and 8 cages/treatment: control group that received a basal diet (NC), positive control group that received a basal diet and was challenged with *E. maxima* (PC), PC+C18:1 lactonic diacetyled SL (SL1), PC+C18:1 deacetyled SL (SL2), PC+C18:1 monoacetyled SL (SL3), and PC+C18:1 diacetyled SL (SL4). Each SL (200 mg/kg feed) was added to the corresponding treatment group. In experiment 2, 588 (zero-day-old) male broilers were used. The chickens were randomly allocated to the following experimental groups with 10 or 11 chickens/cage and 8 cages/treatment: NC, PC, PC+ monensin at 90 mg/kg feed (MO), PC+SL1 at 200 mg/kg feed (SL1 200), PC+SL1 at 500 mg/kg feed (SL1 500), PC+SL4 at 200 mg/kg feed (SL4 200), and PC+SL4 at 500 mg/kg of feed (SL4 500). The chickens and feed were weighed at 0, 7, 14, 20, and 22 d to determine growth performance. In both experiments, all chickens

except the NC group were orally infected with *E. maxima* (10,000 oocysts/chicken) at d 14. One chicken per cage was euthanized at d 20 to sample jejunal tissue to measure lesion scores, cytokines, and tight junction (TJ) proteins. Excreta samples were collected daily between d 20 and 22 to measure oocyst numbers. Data were analyzed using Mixed Model (PROC MIXED) in SAS. In experiment 1, SLs did not affect the growth of broiler chickens, but SL4 decreased ($P < 0.05$) the lesion score and oocyst number compared to PC chickens. In terms of cytokines and TJ protein gene expression, SLs increased ($P < 0.05$) IL-1 β , IL-6, IL-17F, IL-4, IL-13, occludin, and ZO1 levels compared to PC chickens. In experiment 2, monensin increased ($P < 0.05$) body weight, and decreased ($P < 0.05$) the lesion score and oocyst number compared to the PC group. SL4 500 increased ($P < 0.05$) average daily gain and feed conversion ratio but decreased ($P < 0.05$) lesion score and fecal oocyst number. SL4 decreased ($P < 0.05$) IL-6, IL-17F, TNFSF-15, IL-2, and IL-10 levels but increased ($P < 0.05$) occludin and ZO-1 levels. Overall, dietary SL supplementation, especially SL4, improved growth and gastrointestinal functionality of young broiler chickens, demonstrating significant potential as an antibiotic alternative.

Key words: growth performance, gastrointestinal functionality, antibiotic alternative, sophorolipid, coccidiosi

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INTRODUCTION

Consumer awareness of antimicrobial resistance and food safety and increasing understanding of the close interaction of nutrients, gut microbiota, and the host

immune system in homeostasis of intestinal functionality (Celi et al., 2017) have resulted in the elimination of subtherapeutic use of antibiotic growth promoters (AGP) and anticoccidials in animal agriculture globally. Therefore, commercial poultry companies need innovative methods for raising poultry in the absence of AGP and exploring novel feed additives to replace AGP (Lee et al., 2011; Park et al., 2020). Natural feed additives are needed as alternatives to antibiotics to modulate the microbiome, enhance innate immunity, and reduce financial losses due to infectious diseases

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(Ganguly, 2013; Chan et al., 2015; Gadde et al., 2017b). With an increasing demand for poultry meat, dietary “nutraceutical” supplements from natural sources could reduce financial losses due to enteric diseases such as coccidiosis and necrotic enteritis, which can be pervasive following an AGP ban (Gadde et al., 2017b; Lin et al., 2017). Expanding antibiotic-free poultry production to meet the demand of global poultry meat consumption requires research to develop efficacious vaccines as well as feed additives to reduce the impact of infectious diseases (Kim et al., 2019). Avian coccidiosis, which is caused by several distinct species of *Eimeria*, causes an estimated annual economic loss of more than \$3 billion worldwide and is the primary risk factor for necrotic enteritis (Shirley and Lillehoj, 2012; Kadykalo et al., 2018).

Developing effective antibiotic-free prevention and treatment strategies will require comprehensive understanding of host-pathogen immunobiology. Spholipids (SLs) are one of the most promising glycolipid biosurfactants (Sen et al., 2017). Spholipids that 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 hydrophobic fatty acid tail and a hydrophilic carbohydrate head. The hydrophilic 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). The structure of SLs depends 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 it can be esterified at the 4'' position, giving rise to the lactonic ring structure (Cavalero and Cooper, 2003). SLs have a wide range of antimicrobial activity against

several pathogens. These mechanisms may involve membrane destabilization and increased permeabilization (Bluth et al., 2006) and anti-inflammatory effects through the reduction of cytokine release and initiation of a macrophage response (Napolitano, 2006; Callaghan et al., 2016). However, the research on the antimicrobial activity of SLs has mostly been limited to bacteria and in some cases yeast (Dengle-Pulate et al., 2014; Haque et al., 2016). A detailed understanding of underlying mechanisms by which SLs could serve as antibiotic alternatives in poultry will facilitate research on nutritional strategies to reduce financial losses due to coccidiosis.

We hypothesized that supplementing different types and different doses of SLs would beneficially impact growth, immune response, and integrity of the intestinal barrier of commercial broiler chickens with field coccidiosis. The objective of this study was to evaluate the effects of dietary supplementation on newly hatched chickens infected with *E. maxima*, and the study is the first report about the efficacy of SLs to treat parasites in chickens.

MATERIALS AND METHODS

All experiments were approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee (# 19-018). Figure 1 depicts the schematic outline of the experimental design for these studies.

Chickens and Experimental Design

In experiment 1, a total of 336 newly hatched (zero-day-old, Ross 708) male broiler chickens were purchased from Longenecker's hatchery (Elizabethtown, PA). The

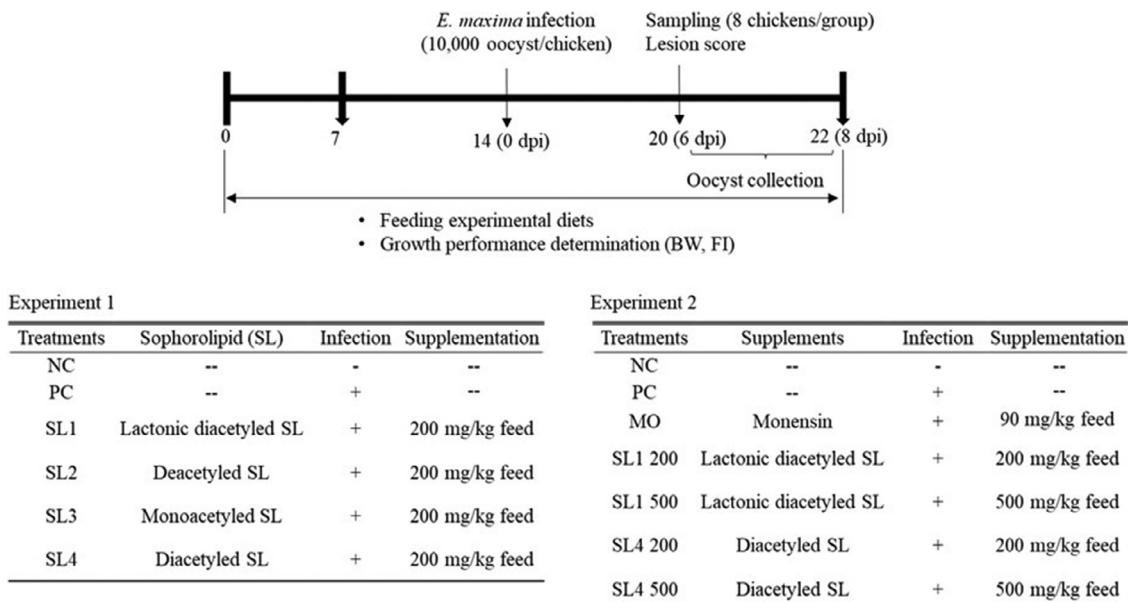


Figure 1. Schematic outline of the experimental design in experiments 1 and 2.

Table 1. Ingredient composition of basal diet in experiments 1 and 2 (as-fed basis, %, unless otherwise indicated).

Ingredients	Basal diet
Corn	69.01
Soybean meal	23.99
Soybean oil	2.75
Dicalcium phosphate	2.00
Calcium carbonate	1.40
Salt	0.35
Poultry vit mix ¹	0.20
Poultry mineral mix ²	0.15
DL-Methionine	0.10
Choline-chloride, 60%	0.05
Total	100
Calculated values (%)	
CP, %	18.00
Ca, %	1.19
Avail. P, %	0.54
Lys, %	1.00
Met, %	0.42
Cys + Met, %	0.65
ME, Mcal/kg	3.6

Abbreviations: AP, available phosphorus; CP, crude protein.

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.

day after the chicks arrived at the Beltsville ARS facility, they were weighed to adjust to the same body weight (**BW**) per treatment and allocated to 6 dietary treatments in a randomized complete block design. The dietary treatments included a basal corn and soybean diet (**Table 1, NC**), a basal diet for infected chickens (**PC**), PC + C18:1 lactonic diacetyled SL (**SL1**), PC + C18:1 deacetyled SL (**SL2**), PC + C18:1 monoacetyled SL (**SL3**), and PC + C18:1 diacetyled SL (**SL4**). All SLs were obtained from DSM Nutritional Products (Columbia, MD). The dose of SL in each treatment was 200 mg/kg feed. At the beginning of the experiment, each treatment group was composed of eight cages with 7 chickens per cage. The cage size was 0.65 m wide by 0.75 m in length (14 chickens/m²), and all cages were kept in the same room. Cages for NC groups were quarantined in the same room to avoid a cross contamination. The chickens were given ad libitum access to water and feed.

In experiment 2, a total of 588 newly hatched (zero-day-old, Ross 708) male broiler chickens were purchased from Longenecker's hatchery (Elizabethtown, PA). The dietary treatments included NC, PC, PC + monensin at 90 mg/kg feed (**MO**), PC + SL1 at 200 mg/kg feed (**SL1 200**), PC + SL1 at 500 mg/kg feed (**SL1 500**), PC + SL4 at 200 mg/kg feed (**SL4 200**), and PC + SL4 at 500 mg/kg of feed (**SL4 500**). Each treatment group was composed of 8 cages, with four cages containing 10 chickens/cage and another four cages with 11 chickens/cage. Unless mentioned otherwise, all conditions and procedures for the chickens were the same as in experiment 1.

Body Weight and Feed Intake Measurement

The feed was weighed daily and the amount consumed was recorded. The feeders were shaken once per day. The chickens and feed were also weighed at day 0, 7, 14, 20, and 22 to determine growth performance. Dead chickens were removed and weighed daily to calculate mortality and adjust the growth performance data.

Oral Infection With *Eimeria Maxima*

All chickens except for the NC group were infected by oral gavage at 14 d of age with 1.0×10^4 oocysts of *E. maxima* Beltsville strain 41A/chicken, which was maintained at Beltsville ARS as previously described (Lillehoj et al., 2016). A DNA test of *E. maxima* was performed to confirm the purity of strain before oral infection (Haug et al., 2007).

Collection of Intestinal Samples

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

Coccidia Gut Lesion Scoring

Lesion scores were carried out on the jejunum from *Eimeria*-infected chickens following the euthanization per AUP guidelines at d 20, and lesions were scored on a scale from 0 (none) to 4 (high) by four independent observers in a blinded fashion as previously described by Johnson and Reid (1970).

Fecal Oocyst Shedding

For calculating oocyst number, the whole feces on each tray set up under pen were collected between d 20 and 22 (6 and 8 d postinfection [**dpi**]), and the number of sporulated oocysts were determined (Lee et al., 2011) with a McMaster chamber according to the following formula:

$$\text{total oocysts/chicken} = [\text{oocystcount} \times \text{dilution factor} \\ \times (\text{fecal sample volume/counting chamber volume})] / \\ \text{number of chickens per cage.}$$

Isolation of RNA and Reverse Transcription

Total RNA from the jejunal samples was prepared by the protocol as previously described (Park et al., 2022).

Gene Expression Analysis by qRT-PCR

The oligonucleotide primer sequences used for quantitative real-time PCR (qRT-PCR) are listed in Table 2. The differential expression was evaluated for the following cytokines and intestinal TJ proteins in the jejunum: IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17F, IFN- γ , TNFSF-15, JAM-2, occludin, ZO-1, and MUC-2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Amplification and detection were conducted 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 non-specific primer amplification was assessed by including no-template controls. Standard curves were generated with log₁₀ diluted RNA, and the levels of individual transcripts were normalized to those of GAPDH with the Q-gene program (Muller et al., 2002).

Statistical Analysis

Data for each response were analyzed using Mixed Model (PROC MIXED) in SAS (SAS Inst. Inc., Cary NC). The experiment was a randomized complete block design with each cage considered an experimental unit or block factor. The results are given as least squares means and pooled SEM. Probability values less than 0.05 were considered statistically significant. In cases in which growth changed significantly, the means were compared in a pairwise manner (PDIF option). The PDIF option was used to compare significance between groups for other results.

RESULTS

Experiment 1

Growth Performance Initial body weight (BW) between the treatments showed no significant difference ($P > 0.05$), and the dietary SL supplementation did not alter the chickens' BW until d 14 (before *E. maxima* challenge; Table 3). After infection with *E. maxima*, the BW (859 g) of chicken (PC; *E. maxima*-challenged chickens fed a basal diet) at d 22 was decreased ($P < 0.001$) compared to the NC group (552 g; nonchallenged chickens fed a basal diet). The BW of chickens receiving a SL-supplemented diet did not show any significant differences ($P > 0.05$) compared to the PC group (*E. maxima*-challenged chickens fed a basal diet) (Table 3). Dietary SL supplementation, regardless of SL type, did not affect ($P > 0.05$) the average daily gain (ADG) of chickens from d 0 to 14 (Table 3). The ADGs (51.9–14.9 g) of chickens infected with *E. maxima* were significantly decreased ($P < 0.001$) compared to those of chickens fed a basal diet (NC; Table 3). Among *E. maxima*-infected chickens, there was no difference ($P > 0.05$) between the treatment groups (Table 3). During the entire experimental period, neither the SL supplementation nor the *E. maxima* infection affected ($P > 0.05$) average daily feed intake (ADFI) of the chickens (Table 3). The feed conversion ratio (FCR) of chickens from 0 to 14 d did not show any significant differences ($P > 0.05$) among the treatment groups (Table 3). However, chickens infected with *E. maxima* significantly increased ($P < 0.001$) the FCR (1.29–4.57) compared to that of chickens in the NC group (Table 3).

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

Type	Target gene	Primer sequence (5-3)	PCR product size (Kbp)
Reference	GAPDH	F-GGTGGTGCTAAGCGTGTTAT R-ACCTCTGCCATCTCTCCACA	264
Proinflammatory	IL-1 β	F-TGGGCATCAAGGGCTACA R-TCGGGTTGGTTGGTGATG	244
	IL-6	F-CAAGGTGACGGAGGAGGAC R-TGGCGAGGAGGGATTCT	254
	IL-17F	F-TGAAGACTGCCTGAACCA R-AGAGACCGATTCTGATGT	117
Th1	TNFSF-15	F-CCTGAGTATTCCAGCAACGCA R-ATCCACCAGCTTGATGTCACTAAC	292
	IL-2	F-TCTGGGACCACTGTATGCTCT R-ACACCAGTGGGAAACAGTATCA	256
Th2	IFN- γ	F-AGCTGACGGTGGACCTATTATT R-GGCTTTGCGCTGGATT	259
	IL-4	F-ACCCAGGGCATCCAGAAG R-CAGTGCCGGCAAGAAGTT	258
TJ proteins	IL-13	F-CCAGGGCATCCAGAAGC R-CAGTGCCGGCAAGAAGTT	256
	Occludin	F-GAGCCCAGACTACCAAAGCAA R-GCTTGATGTGGAAGAGCTTGTTG	68
	ZO-1	F-CCGAGTCGTTCCAGATCT R-GGAGAAATGTCTGGAATGGTCTGA	63
Mucin	JAM-2	F-AGCCTCAAATGGGATTGGATT R-CATCAACTTGCATTGCTTCA	59
	MUC-2	F-GCCTGCCAGGAAATCAAG R-CGACAAGTTTGCTGGCACAT	59

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

Table 3. Growth performance of chickens fed various sophorolipids (SLs) in experiment 1.

	NC	PC	SL1	SL2	SL3	SL4	SEM	P-value
BW, g								
Initial	37.0	-	37.2	37.5	35.7	35.5	1.2	0.751
D 14	443	433	420	417	417	419	8.9	0.224
D 22 (8 dpi)	859 ^a	552 ^b	563 ^b	554 ^b	550 ^b	566 ^b	15.4	<0.001
ADG, g								
D 0 to 14	29.0	-	27.4	27.1	27.2	27.4	0.6	0.224
D 14 to 22	51.9 ^a	14.9 ^b	17.9 ^b	17.0 ^b	16.6 ^b	18.4 ^b	1.6	<0.001
ADFI, g								
D 0 to 14	37.0	-	37.2	37.5	35.7	34.5	1.1	0.425
D 14 to 22	66.1	61.5	61.7	61.8	60.8	62.6	1.9	0.408
FCR								
D 0 to 14	1.28	-	1.37	1.40	1.31	1.26	0.05	0.375
D 14 to 22	1.29 ^b	4.57 ^a	3.55 ^a	3.81 ^a	3.88 ^a	3.52 ^a	0.39	<0.001

Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; BW, body weight; D, day, dpi, days postinfection; FCR, feed conversion ratio (ADFI/ADG); NC, basal diet; PC, 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 200 mg/kg feed. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*.

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

Intestinal Lesion Scores and Fecal Oocyst Shedding

E. maxima-infected chickens had increased ($P < 0.001$) lesion scores (0.3–2.6) in the distal jejunum compared to the NC group chickens (Figure 2A). Dietary SL supplementation, regardless of SL type, significantly decreased (2.6–1.9) the lesion score compared to PC chickens ($P < 0.001$; Figure 2A). The SL4 group had ($P < 0.041$) particularly lower intestinal lesion scores compared to the SL2 (2.1–1.7) and SL3 groups (2.1–1.7; Figure 2A). *E. maxima*-infected chickens (PC) had significantly higher fecal oocyst numbers (0.0 to 1.4×10^5) compared to NC chickens at 6 dpi ($P < 0.001$). However, SL3 ($P = 0.044$, 1.4×10^5 to 7.5×10^4), and SL4 ($P = 0.005$, 1.4×10^5 to 4.4×10^4) supplementation significantly decreased oocyst shedding compared to the PC group (Figure 2B).

Proinflammatory Cytokines *E. maxima* infection of chickens did not change ($P > 0.05$) the jejunal expression levels of IL-1 β , IL-6, and IL-17F compared to the NC group (Figure 3). Dietary SL4 supplementation in the *E. maxima*-infected chickens significantly increased the IL-1 β ($P < 0.001$, 4.6×10^{-4} to 8.5×10^{-3}), IL-6 ($P < 0.001$, 1.5×10^{-4} to 9.1×10^{-4}), and IL-17F ($P < 0.014$, 3.8×10^{-4} to 1.5×10^{-3}) levels in the jejunum compared to all other groups (Figure 3). The SL1 group showed an increased level of IL-6 ($P = 0.049$, 8.8×10^{-5} to 4.0×10^{-4}) and TNFSF-15 ($P < 0.001$, 4.3×10^{-4} to 1.5×10^{-3}) in jejunum compared to that of NC chickens (Figure 3), whereas SL2 or SL3 supplementation did not change the transcription levels of proinflammatory cytokines in jejunum compared to the PC group ($P > 0.05$).

Th1 and Th2 Cytokines *E. maxima* infection significantly increased the jejunal transcription levels of IFN- γ in PC ($P = 0.015$, 4.8×10^{-4} to 6.9×10^{-4}), SL2 ($P = 0.028$, 4.8×10^{-4} to 6.5×10^{-4}), and SL3 ($P = 0.039$, 4.8×10^{-4} to 6.5×10^{-4}) treatment groups compared to the NC group (Figure 4B). The SL1 group had increased IL-2 ($P = 0.048$, 1.7×10^{-4} to 8.7×10^{-4}) and IL-13 ($P < 0.001$, 6.9×10^{-10} to 3.6×10^{-9}) levels compared to NC chickens (Figure 4). The SL4 group

had ($P < 0.013$) significantly higher IL-4 levels (1.2×10^{-7} to 9.1×10^{-7}) in the jejunum compared to the other treatment groups (Figure 4C).

Tight Junction and Mucin Proteins PC chickens did not affect ($P > 0.05$) the transcription levels of TJ proteins and mucin proteins regardless of supplementations (Figure 5). The SL4 group showed increased jejunal occludin ($P < 0.014$, 0.17–0.10) and ZO-1 ($P < 0.001$, 0.19–0.07) levels compared to other chickens (Figure 5).

Experiment 2

Growth Performance The initial BW was not significantly different between the experimental groups ($P > 0.05$; Table 4). However, starting from d 7, the BW (455–414 g) of chickens fed antibiotics (MO group) increased ($P < 0.002$) compared to the chickens of all other groups. The growth-promoting effect of the MO group continued unabated until the final BW measurement, even after the chickens were challenged with *E. maxima*. After infection with *E. maxima*, the BW (809–705 g at 6 dpi and 911–771 g at 8 dpi) decreased ($P < 0.001$) in all groups except for the MO group compared to NC chickens. After *E. maxima* infection, the BW of chickens fed SL1 and SL4 increased compared to PC chickens, although the differences were not statistically significant ($P > 0.05$). The numerical differences in BW of SL-fed chickens showed that SL treatment at 500 ppm is better than 200 ppm.

Before the *E. maxima* challenge, antibiotic supplementation (MO) significantly increased ($P < 0.001$) the ADG (29.5–26.7 g) of chickens compared to those of chickens fed other diets (Table 4). *E. maxima* infection decreased ($P < 0.001$) ADG (65.0 vs. 48.4 g from 0 to 6 dpi, 56.6 vs. 36.8 g from 6 to 8 dpi, and 63.0 vs. 45.8 g in the entire infection period) in all groups (except chickens fed antibiotics) compared to the NC group. However, in the SL1 500 group, ADG (47.4–48.2 g) was ($P = 0.048$) higher than that in the PC group from d 14 to 20

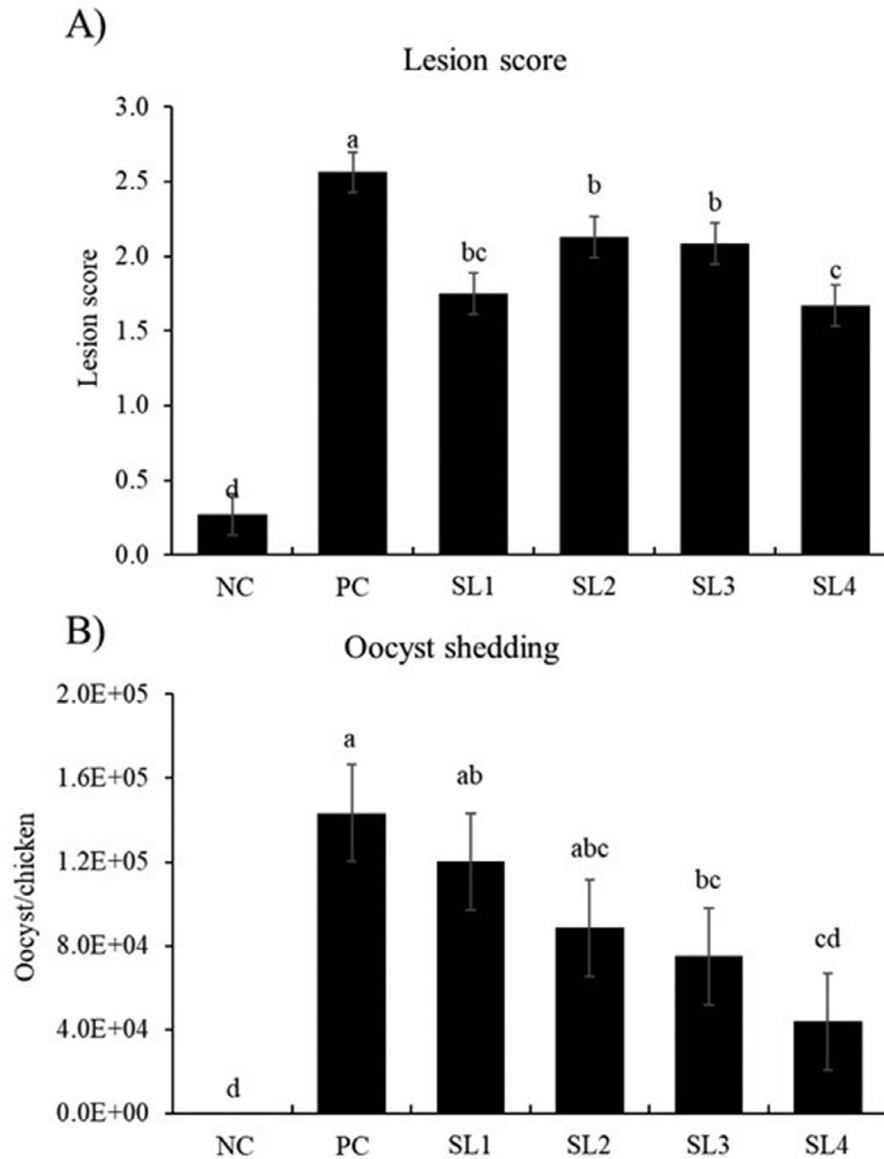


Figure 2. Lesion score and oocyst shedding of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 1. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. ^{a~d} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The lesion score was collected from distal jejunal tissue at d 20 (6 days postinfection: dpi) and fecal sample was collected from 6 to 8 dpi to calculate the oocyst shedding. Abbreviations: NC, basal diet; PC, 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 200 mg/kg feed.

(Table 4). An increase in ADG was observed in the SL4 500 group at 8 dpi (from 20 to 22 d) compared to the PC ($P = 0.003$, 33.6 to 46.1 g), SL1 200 ($P = 0.003$, 33.6–46.1 g), SL1 500 ($P = 0.021$, 36.6–46.1 g), or SL4 200 ($P = 0.005$, 34.3–46.1 g) groups (Table 4). During the infection period (14–22 d), ADG (44.0–48.2 g) was ($P = 0.014$) higher in the SL4 500 group compared to that of chickens in the PC group (Table 4). Before the *E. maxima* challenge, feed intake was similar among the treatment groups ($P > 0.05$). During the infection period (14–20 d), ADFI (102–96.7 g) decreased ($P < 0.006$) in all experimental groups, except the MO group, compared to the NC group (Table 4). However, the ADFI in the SL1 200 group was higher compared that observed in the NC ($P = 0.029$, 152–163 g), MO ($P = 0.006$, 148–163 g), and SL4 500 ($P = 0.005$, 146–163 g) groups

from d 20 to 22 (Table 4). During the entire infection period (d 14 to 22), ADFI was ($P < 0.05$) lower in the SL1 and SL4 500 groups compared to the values observed in the NC (vs. SL1 500: 114 to 110 g and vs. SL4 500: 114–108 g), MO (vs. SL1 500: 113–110 g and vs. SL4 500: 113–108 g), and SL1 200 (vs. SL1 500: 113–110 g and vs. SL4 500: 113–108 g) groups (Table 4).

Before the *E. maxima* infection, the FCR (1.07–1.17) was ($P < 0.001$) lower in the MO group compared to the other treatment groups (Table 4). As expected, during the *E. maxima* infection (d 14 to 20), the FCR (1.58–2.41) increased ($P < 0.002$) in all groups, except the MO group, compared to the NC group; moreover, the FCR (2.04–1.95) decreased ($P = 0.025$) in the SL1 500 group compared to the PC group (Table 4). The NC (2.71 to 4.59), MO (2.76 to 4.59), and SL4 500 (3.14

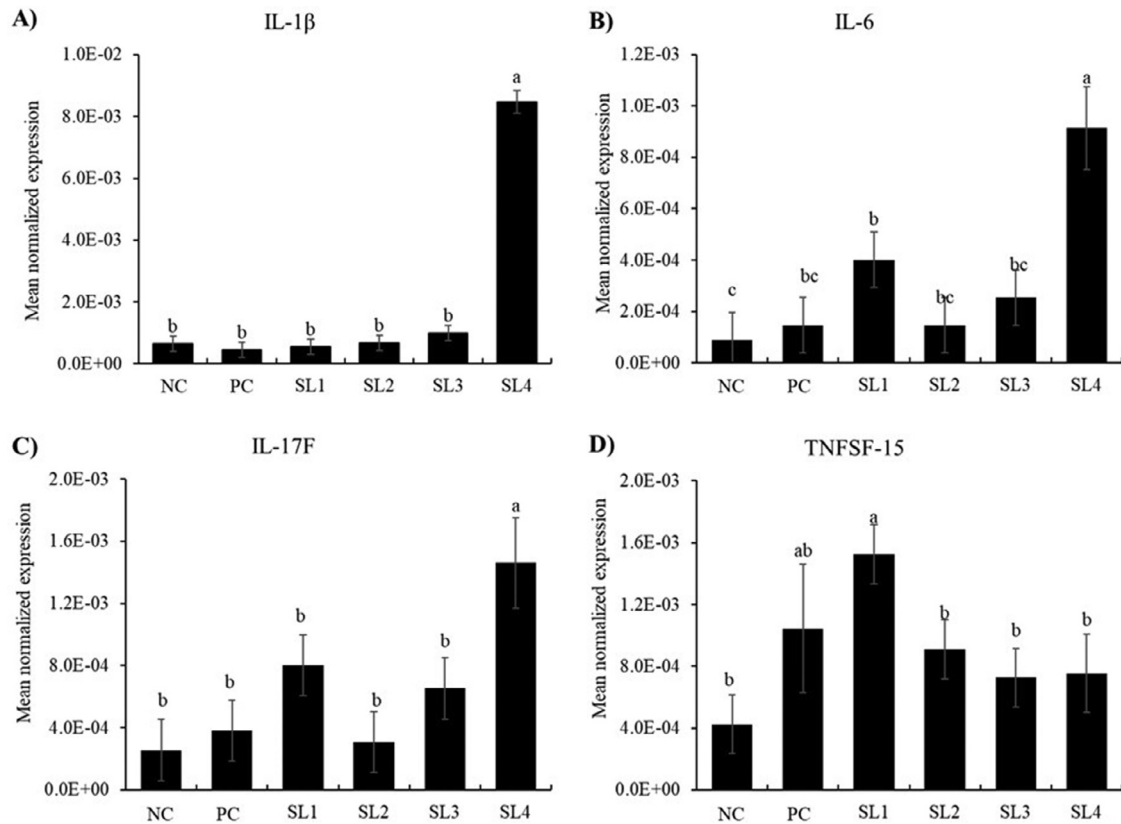


Figure 3. Transcripts of proinflammatory cytokine in jejunum of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 1. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. ^{a-c} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 20 (6 days postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: NC, basal diet; PC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacytyle sophorolipid; SL2, diet supplemented with C18:1 deacytyle sophorolipid; SL3, diet supplemented with C18:1 monoacytyle sophorolipid; SL4, diet supplemented with C18:1 diacytyle sophorolipid; the dose of SL in each treatment was 200 mg/kg feed.

–4.59) groups had ($P < 0.05$) lower FCR values than other groups from d 20 to 22 (Table 4). During the entire infection period (day 14 to 22), the NC (2.42–1.81) and MO (2.42–1.73) groups had ($P < 0.05$) lower FCR than those of other groups. However, the FCR decreased ($P < 0.037$) in the SL1 500 (2.49–2.35) and SL4 500 (2.49–2.26) groups compared to the other groups, except MO group, challenged by *E. maxima* (Table 4).

Intestinal Lesion Scores and Fecal Oocyst Shedding The MO group had ($P < 0.001$) lower lesion scores (2.5–1.4) than the other groups, except NC group (Figure 6A). However, SL supplements also decreased ($P < 0.001$) lesion scores (2.5–1.9) compared to the PC group (Figure 6A). As shown in Figure 6B, oocyst shedding decreased in the MO group compared to the PC group ($P < 0.001$). Similarly, all SL groups showed ($P < 0.001$) a decrease in oocyst numbers (7.1×10^7 to 4.4×10^7) compared to the PC group.

Proinflammatory Cytokines No significant differences in IL-1 β levels at 6 dpi were observed ($P > 0.05$) between the infected groups (Figure 7). IL-6 levels (0.072–0.616) were ($P < 0.001$) higher in the PC group compared to the NC group. However, in the SL4 500 group there was ($P = 0.007$) a decrease in IL-6 levels (0.616–0.334) compared to the PC group (Figure 7). TNFSF-15 levels (0.0024–0.0029) were ($P = 0.023$) higher in the PC

group compared to the NC group; however, all SL groups and the MO group had ($P < 0.001$) lower TNFSF-15 levels (NC vs. SLs: 0.0029 to 0.0008 and PC vs. MO: 0.0029–0.0008) compared to the PC group (Figure 6D). Similarly, IL-17F levels (0.027–0.040) were ($P = 0.015$) higher in the PC group compared to the NC group, whereas the SL4 500 group showed ($P = 0.018$) a decrease in IL-17F levels (0.040–0.020) compared to PC group (Figure 7C).

Th1 and Th2 Cytokines Infection with *E. maxima* increased the IL-2 ($P < 0.001$, 0.0013–0.0037), IL-10 ($P = 0.005$, 2.01–4.79), and IFN- γ ($P = 0.004$, 0.05–0.28) levels in the jejunum of the PC group compared to the NC group at 6 dpi (Figure 8). The SL4 500 supplement resulted ($P = 0.023$) in lower jejunal IL-2 levels (0.0037–0.0014) than those observed in the PC group. SL supplementation decreased ($P < 0.012$) jejunal IL-10 levels (4.79–2.09) regardless of SL type and the dose of SL compared to the PC group. No differences in IL-4 levels were observed ($P > 0.05$) in the MO and SL groups (Figure 8C). IL-10 levels did not differ ($P > 0.05$) between the MO and PC groups (Figure 8D).

Tight Junction and Mucin Proteins While the expression levels of TJ proteins were similar between the PC and NC groups, SL supplementation, regardless of type

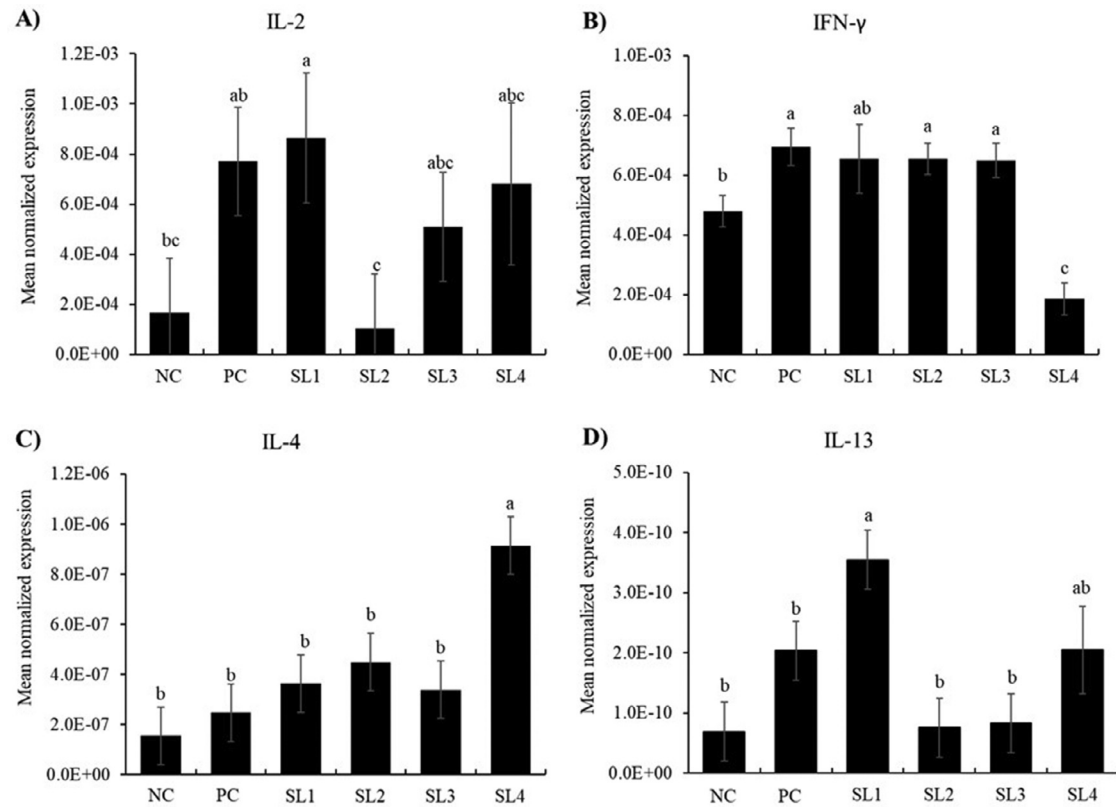


Figure 4. Transcripts of Th1 and Th2 cytokines in jejunum of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 1. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. ^{a-c} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 20 (6 days postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: NC, basal diet; PC, 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 200 mg/kg feed.

and dosage, increased JAM-2 ($P < 0.008$, 0.29–0.75) and occludin ($P < 0.026$, 2.02–4.76) levels compared to the PC group (Figures 9A and 9B).

DISCUSSION

The results of this study are likely the first reported observations on the effect of dietary SL supplements as antibiotic alternatives on growth performance, intestinal immunity, and intestinal barrier integrity of broiler chickens affected by coccidiosis. SLs produced by yeasts such as *Starmerella bombicola*, *Candida bastistaetic*, *C. floricola*, and *C. apicola* (Chen et al., 2006) exist in a form of crude mixtures. Some studies have described varying crude SL mixtures that are antimicrobial, antifungal, anticancer, and spermicide agents through in vitro or in vivo (mainly in mice) tests (Shah et al., 2005; Shao et al., 2012; Ribeiro et al., 2015). SL mixtures have been shown to regulate nitric oxide production and reduce mortality in a rat model of peritoneal sepsis (Bluth et al., 2006). Another study showed that SL mixtures reduce IgE production following nebulizer administration in asthmatic mice (Hagler et al., 2007; Bluth et al., 2008). Such variations among the studies exist due to the compositions of SL species and other biologically active compounds in the mixtures, making

an interpretation of SL studies difficult. Therefore, understanding each property of SLs would allow opportunities to use SLs as antibiotic alternatives on domestic animals that are more susceptible to infectious diseases due to the AGP restriction.

Four types of SLs were tested in experiment 1 to determine their efficacy against *E. maxima* infection. Before *E. maxima* infection, BW, ADG, ADFI, and FCR of chickens fed a SL-supplemented diet did not differ from those of chickens fed a basal diet. At d 22 (6 dpi), BW and ADG of PC chickens were reduced by 35.7 and 71.3%, respectively. The FCR in the PC group also increased 3.5-fold compared to the NC group. In the *E. maxima*-challenged groups, SL supplementation did not affect FCR regardless of the source of SL used. These results showed that the *E. maxima* challenge used in this study was practical to cause coccidiosis. However, the response to the challenge was likely not very severe, as indicated by the differences in ADFI between the experimental groups. These mild conditions frequently occur in commercial chicken farms, and this research may be valuable for practical applications of SLs. Previous studies have reported that SL administration to mice exacerbated the progression of the disease (Chen et al., 2006; Callaghan et al., 2016). However, in the current study, dietary SL supplementation up to 200 ppm revealed no harmful effects on the growth of young broiler chickens.

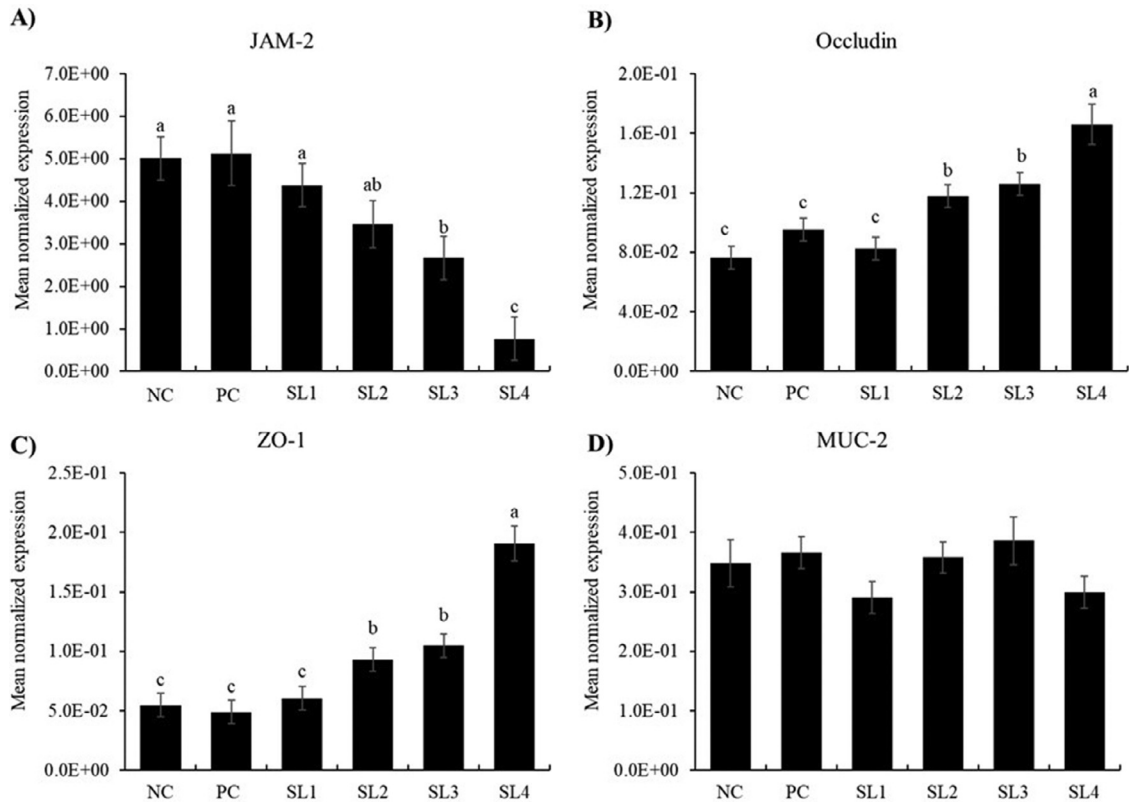


Figure 5. Transcripts of tight junction and mucin protein in jejunum of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 1. All chickens except NC were infected by oral gavage at day 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. ^{a-c} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 20 (6 days postinfection). Transcript levels of the tight junction proteins and mucin protein were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: NC, basal diet; PC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactic diacytyle sophorolipid; SL2, diet supplemented with C18:1 deacytyle sophorolipid; SL3, diet supplemented with C18:1 monoacytyle sophorolipid; SL4, diet supplemented with C18:1 diacytyle sophorolipid; the dose of SL in each treatment was 200 mg/kg feed.

A previous study also demonstrated the dietary safety of up to 200 ppm of SLs, which showed growth- and immune-promoting effects of chickens affected by necrotic enteritis (Park et al., 2022).

Intestinal lesion scores and BW gain are commonly used as clinical measurements to evaluate the severity of coccidiosis (Zhu et al., 2000; Park et al., 2020). In experiment 1, chickens (PC) infected with *E. maxima* exhibited high lesion scores, indicating severe, extensive destruction of the gut epithelium in the area of Meckel's diverticulum. Infected chickens fed SL diets, regardless of SL source, showed lower lesion scores at 6 dpi compared to chickens in the PC group. *E. maxima* infection increased fecal oocyst output; however, SL3 and SL4 supplementation resulted in a notable decrease in fecal oocyst output. For SL1 and SL2, oocyst output decreased compared to the PC group, but this difference was not statistically significant. These results suggest that SLs may directly reduce the number of *E. maxima*.

In terms of the antimicrobial activity of SLs, their sugar and lipid portions (surfactant effect) are associated with changes or ruptures in the cellular membrane (Kulakovskaya et al., 2014). SLs induce the release of cellular cytoplasmic content, including intracellular enzymes such as malate dehydrogenase (Lang et al., 1989; Glover et al., 1999; Kulakovskaya et al., 2014; Zhang et al., 2016). The antimicrobial activity of SLs

can be influenced depending on the concentration, treatment time, composition of fatty acids, the predominance of acidic and lactic forms, and the sugar group of the molecule (Morya et al., 2013; Lydon et al., 2017). In general, lactic SLs are more efficient at reducing surface tension and are better antimicrobial agents (Shah et al., 2007; Paulino et al., 2016), whereas acidic SLs have better foaming properties (Lang et al., 2000). Acetyl groups can also lower the hydrophilicity of SLs and enhance their antiviral and cytokine stimulating effects (Shah et al., 2005).

Eimeria infection activates chickens' innate and acquired immune response, which involves the secretion of various chemokines and cytokines (Lillehoj, 1998). Cytokines, small immune-regulatory peptides, aid in cell-to-cell communication during immune responses and have been identified as essential biomarkers of gastrointestinal functionality (Celi et al., 2019). Therefore, understanding the cytokine pathways could make immune manipulation feasible in order to promote the growth of domestic animals. In experiment 1 of this study, *Eimeria*-challenged chickens fed SL4 chickens showed increased expression of jejunal IL-1 β , IL-6, and IL-17F at 6 dpi compared to PC chickens. The SL1 group had a higher TNFSF-15 levels compared to the other SL groups. IL-1 β is an important pro-inflammatory cytokine that is produced mainly by activated

Table 4. Growth performance of chicken fed a diet supplemented with sophorolipids (SLs) in experiment 2.

	NC	PC	MO	SL1		SL4		SEM	P-value
				200	500	200	500		
BW (g)									
Before infection									
Initial	40.3	-	40.0	40.3	40.0	40.3	40.2	0.3	0.893
D 7	140 ^b	-	150 ^a	138 ^b	141 ^b	140 ^b	139 ^b	1.8	0.001
D 14	417 ^b	-	455 ^a	417 ^b	418 ^b	409 ^b	410 ^b	3.9	<.001
After infection									
D 20 (6 dpi)	809 ^b	695 ^c	866 ^a	707 ^c	716 ^c	700 ^c	706 ^c	9.6	<.001
D 22 (8 dpi)	911 ^b	756 ^c	963 ^a	767 ^c	782 ^c	762 ^c	789 ^c	12.9	<.001
ADG (g/d)									
Before infection									
D 0 to 7	14.3 ^b	-	15.7 ^a	13.8 ^b	14.2 ^b	13.8 ^b	14.0 ^b	0.3	0.001
D 7 to 14	39.6 ^b	-	43.7 ^a	39.8 ^b	39.7 ^b	38.5 ^b	38.7 ^b	0.5	<.001
D 0 to 14	26.9 ^b	-	29.5 ^a	26.8 ^b	27.2 ^b	26.2 ^b	26.5 ^b	0.3	<.001
After infection									
D 14 to 20	65.0 ^b	47.4 ^d	68.6 ^a	48.5 ^{cd}	49.8 ^c	47.9 ^{cd}	48.2 ^{cd}	1.0	<.001
D 20 to 22	56.6 ^a	33.6 ^c	54.0 ^b	33.6 ^c	36.6 ^c	34.3 ^c	46.1 ^b	3.0	<.001
D 14 to 22	63.0 ^a	44.0 ^c	65.3 ^a	45.3 ^{bc}	46.8 ^{bc}	44.9 ^c	48.2 ^b	1.2	<.001
ADFI (g/d)									
Before infection									
D 0 to 7	18.7	-	19.2	18.7	19.3	18.7	19.2	0.3	0.235
D 7 to 14	42.9	-	44.2	42.7	44.2	44.0	43.8	0.6	0.198
D 0 to 14	30.6	-	31.5	31.0	31.7	31.5	31.5	0.4	0.201
After infection									
D 14 to 20	102 ^a	96.1 ^b	102 ^a	97.1 ^b	96.8 ^b	96.4 ^b	97.2 ^b	1.4	0.001
D 20 to 22	152 ^b	150 ^{ab}	148 ^b	163 ^a	155 ^{ab}	159 ^{ab}	146 ^b	4.0	0.030
D 14 to 22	114 ^a	109 ^b	113 ^a	113 ^a	110 ^b	111 ^{ab}	108 ^b	2.0	0.029
FCR									
Before infection									
D 0 to 7	1.31 ^a	-	1.22 ^b	1.35 ^a	1.33 ^a	1.33 ^a	1.35 ^a	0.02	<.001
D 7 to 14	1.08 ^a	-	1.01 ^b	1.07 ^a	1.11 ^a	1.14 ^a	1.14 ^a	0.02	<.001
D 0 to 14	1.14 ^a	-	1.07 ^b	1.14 ^a	1.17 ^a	1.19 ^a	1.19 ^a	0.01	<.001
After infection									
D 14 to 20	1.58 ^c	2.04 ^a	1.49 ^d	2.01 ^{ab}	1.95 ^b	2.01 ^{ab}	2.01 ^{ab}	0.04	<.001
D 20 to 22	2.71 ^b	4.75 ^a	2.76 ^b	4.66 ^a	4.23 ^a	4.73 ^a	3.14 ^b	0.34	<.001
D 14 to 22	1.81 ^c	2.48 ^a	1.73 ^c	2.51 ^a	2.35 ^b	2.48 ^a	2.26 ^b	0.05	<.001

Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; BW, body weight; D, day; dpi, days postinfection; FCR, feed conversion ratio (ADFI/ADG); NC, basal diet; PC, basal diet for infected chickens; MO, 90 mg monensin/kg feed, SL1, diet supplemented with C18:1 lactonic diacylated sophorolipid, SL4, diet supplemented with C18:1 diacylated sophorolipid, 200 = 200 mg/kg feed, 500 = 500 mg/kg feed. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*.

^{a-c}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.

macrophages. It plays a vital role in the innate immune responses by recruiting inflammatory cells (Hong et al., 2006). IL-6, produced by T cells, monocytes, and macrophages, functions as proinflammatory and anti-inflammatory cytokines and promotes Th17 cell differentiation (Waititu et al., 2014). IL-6 expression has also been reported to aid in populations of heterophils that are more capable of responding to and eliminating pathogens (Swaggerty et al., 2004; Hong et al., 2006). Therefore, released cytokines have very tight networks for transferring signals on immune sensing.

The current study also investigated the changes in IL-2, IL-4, IL-13, and IFN- γ levels related to the activation of Th1 and Th2 cells. IL-4 also drives the differentiation of macrophages into M2 while inhibiting classical activation to the M1 phenotype (Ramani et al., 2015). Chickens infected with *E. maxima* and receiving a basal diet (PC group) showed increased INF- γ levels compared to chickens in the NC group (basal diet without *Eimeria* challenge). In *E. maxima*-infected chickens, SL1 upregulated IL-13 levels compared to the chickens in the PC group. SL2 downregulated IL-2 levels compared to those of chickens in

the PC group. SL4 downregulated IFN- γ levels and upregulated IL-4 levels compared to the PC group. In general, IL-4 is known to decrease IFN- γ (Ramani et al., 2015). This response was confirmed in the SL4 treatment group (Experiment 1).

In accordance with previous studies about cytokine release after SL administration, incubating multiple concentrations of SLs (3–100 $\mu\text{g}/\text{mL}$) with VK-2 cells for 6 h increased IL-1 and IL-8, and the lactonic 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 (Bluth et al., 2006; Mueller et al., 2006). These studies demonstrated that SL might mediate immune response inflammation. In experiment 1, contrary to the expectation that SLs would suppress of proinflammatory cytokines, SL1 and SL4 upregulated these cytokines. Releasing cytokines diverts nutrients in the feed being utilized for chicken growth to produce and utilize immune proteins, which

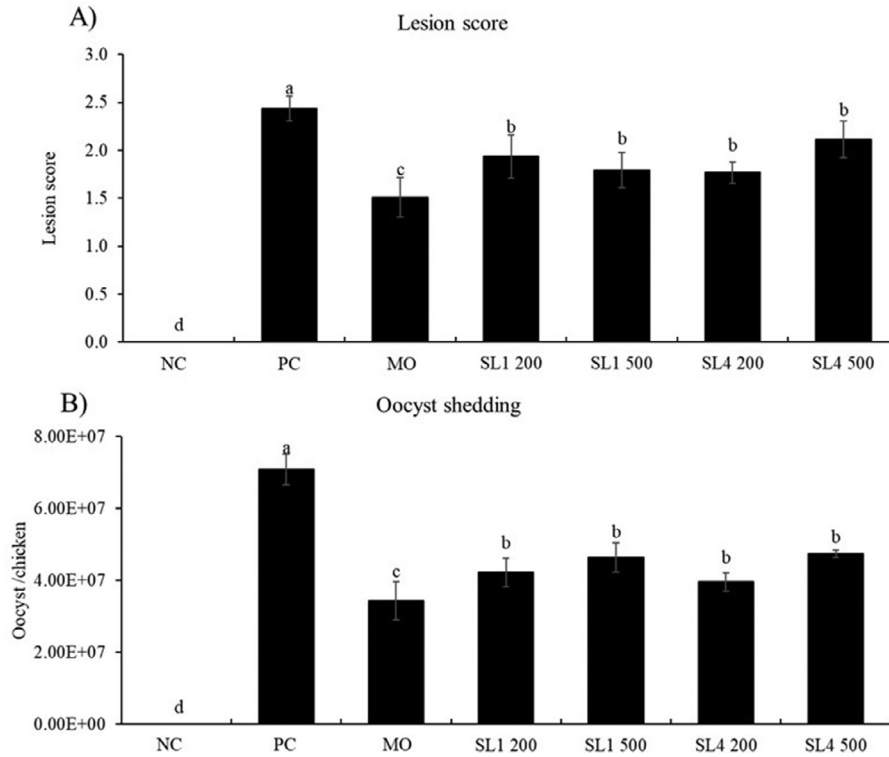


Figure 6. Lesion score and oocyst shedding of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 2. ^{a~d} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The lesion score was collected from distal jejunal tissue at d 20 (6 days postinfection: dpi) and fecal sample was collected from 6 to 8 dpi to calculate the oocyst shedding. Abbreviations: MO, 90 mg monensin/kg feed; NC, basal diet; PC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid; 200, 200 mg/kg feed; 500, 500 mg/kg feed. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*.

leads to a reduction in growth. The growth of the chickens in the current study did not show any discrepancy between *E. maxima*-infected groups. Based on the results, chickens in the SL1 and SL4 groups may have

exhibited a greater tolerogenic response compared to others. Also, for cytokines related to Th1 and Th2, the SL1 and SL4 chickens showed greater expression of these cytokines in the *E. maxima*-infected groups. Increasing

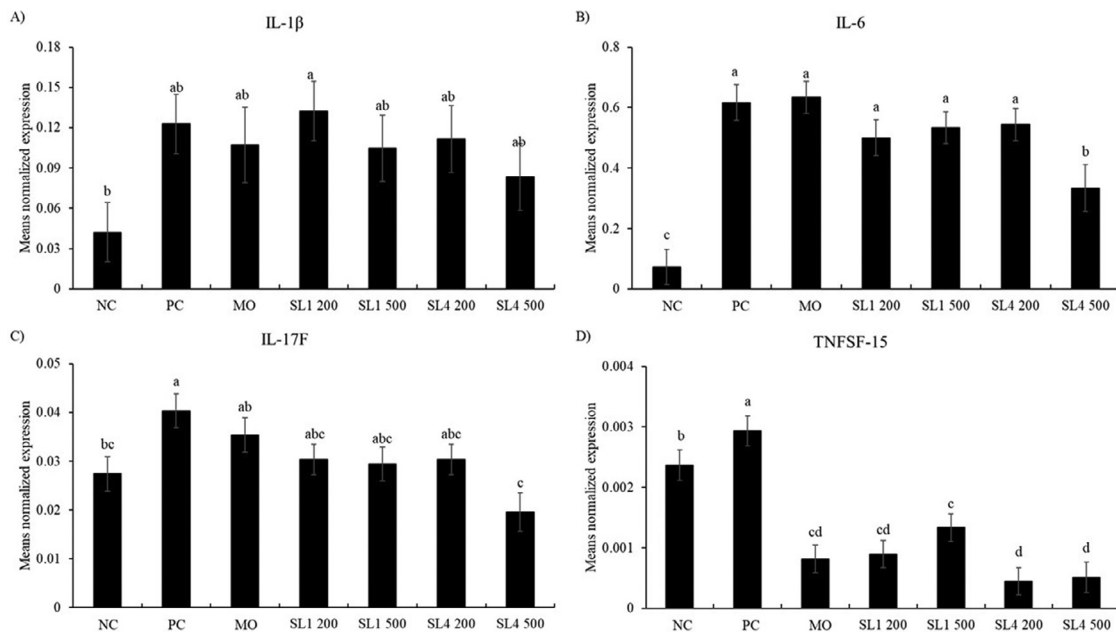


Figure 7. Transcripts of proinflammatory cytokine in jejunum of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 2. ^{a~d} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 20 (6 days postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: MO, 90 mg monensin/kg feed; NC, basal diet; PC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactonic diacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid; 200, 200 mg/kg feed; 500, 500 mg/kg feed. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*.

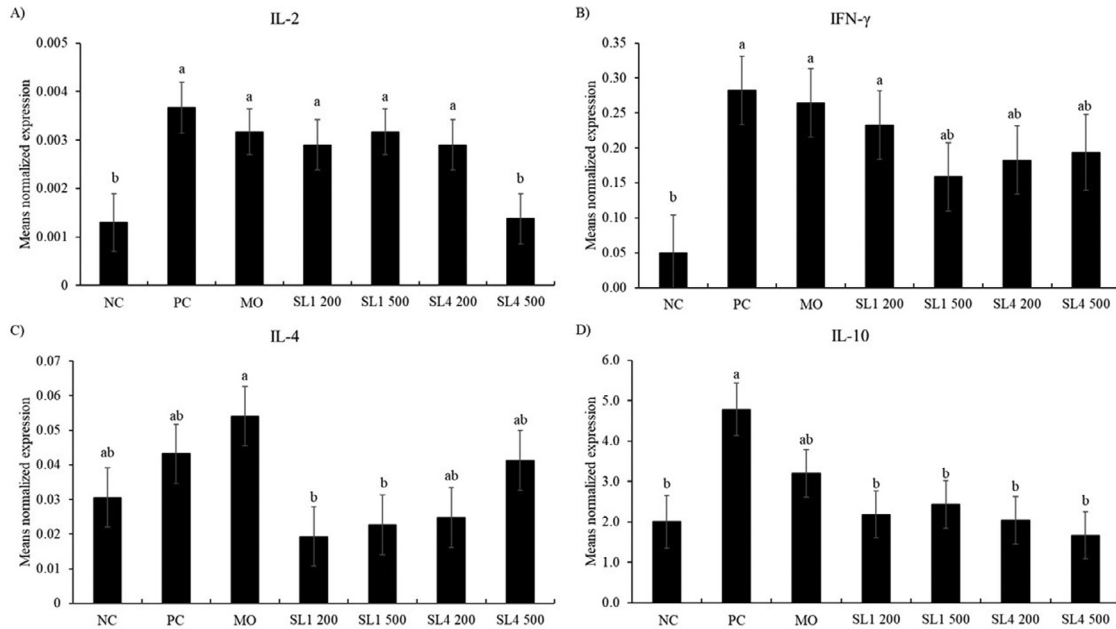


Figure 8. Transcripts of Th1 and Th2 cytokines in jejunum of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 2. ^{a~b} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 20 (6 days postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: MO, 90 mg monensin/kg feed; NC, basal diet; PC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactic diacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid; 200, 200 mg/kg feed; 500, 500 mg/kg feed. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*.

cytokines strengthens a sensing capacity against pathogens, promoting the signal transference related to proinflammatory.

Many factors related to disease and stress can damage intestinal epithelial integrity. This can lead to decreased nutrient absorption, increased pathogenic invasion and inflammatory disease, and a decrease in growth

(Yegani and Korver, 2008). The intestinal epithelium, which serves as a physical barrier against invading pathogens and intraluminal toxins (Ulluwishewa et al., 2011; Song et al., 2014), is composed of a single layer of columnar epithelial cells that are tightly bound by intercellular junctional complexes. These junctional complexes maintain the integrity of the epithelial barrier by

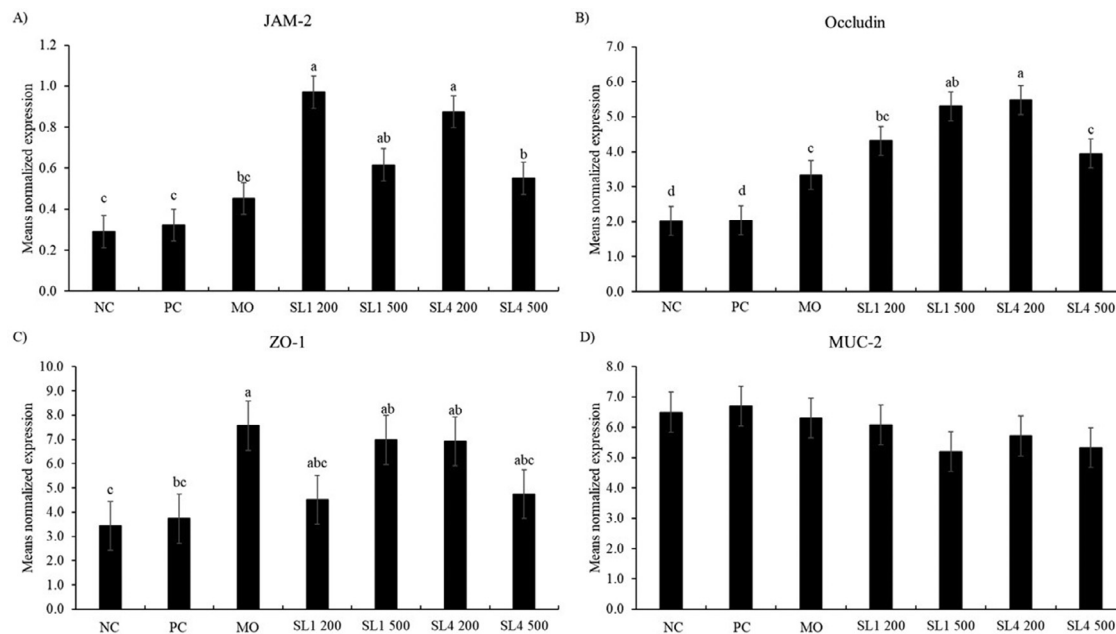


Figure 9. Transcripts of tight junction and mucin protein in jejunum of chickens fed diet supplemented with various sophorolipid during infection with *E. maxima* in experiment 2. ^{a~d} Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean \pm SEM ($n = 8$). The data were collected at d 20 (6 days postinfection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: MO, 90 mg monensin/kg feed; NC, basal diet; PC, basal diet for infected chickens; SL1, diet supplemented with C18:1 lactic diacetyled sophorolipid; SL4, diet supplemented with C18:1 diacetyled sophorolipid; 200, 200 mg/kg feed; 500, 500 mg/kg feed. All chickens except NC were infected by oral gavage at d 14 with 1.0×10^4 oocysts/chicken of *E. maxima*.

regulating paracellular permeability and are composed of TJs, gap junctions, adherent junctions, and desmosomes (Gadde et al., 2017a). TJs 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; Lee et al., 2015). JAM-2 and occludin play important roles in the assembly and maintenance of TJs and the regulation of intestinal permeability, as shown by increased paracellular permeability to macromolecules in knockout mice (Al-Sadi et al., 2011; Lee et al., 2015). In experiment 1, *E. maxima* infection did not change TJ proteins levels; however, SL4 chickens showed the greatest occludin and ZO-1 levels compared to the other groups. Gadde et al. (2017a) have suggested that increased TJ protein expression in chickens improves intestinal barrier function and provides optimal gut health. Callaghan (2017) reported on the efficacy of SLs on TJ proteins by incubating cancer cells (HT29) with SLs. In this study, acidic SL dose-dependently disrupted HT29 via downregulation of ZO-1. However, lactonic SL treatments increased the ZO-1. Due to the lack of information available in this area, more research is required to evaluate the direct effect of SLs on TJ proteins.

In experiment 1, SL4 supplements showed the highest efficacy in improving intestinal immune responses and barrier integrity, followed by SL1. SL4 is also structurally similar to SL1. However, since a 200-ppm dose of SLs did not improve the growth of young broiler chickens, we decided to test higher doses in a new experiment. Thus, SL1 and SL4 were tested at 200 ppm and 500 ppm in experiment 2.

In experiment 2, chickens in the MO group (monensin) showed a rapid BW change, in which BW increased by 7% at 7 d compared to the CON group. This effect was sustained throughout the study even after *E. maxima*-infection. The BW was 9, 7, and 6% higher in the MO group compared to the NC group at d 14, 20, and 22, respectively. The slight percentage reduction observed between d 14 and 22 in the MO group was likely due to the *E. maxima*-infection. As expected, the *Eimeria* challenge reduced the BW in the PC group by 14 and 17% at d 20 and 22, respectively, compared to the NC group. The BW of SLs-fed chickens did not differ from the chickens in the NC group before *E. maxima*-infection and the PC group after the infection. However, the BW results for the SL groups demonstrated the safety of the SLs at 500 ppm for chickens and confirmed the safety of 200 ppm of SL1 and SL4 as previously observed in experiment 1. In experiment 2, the ADG of MO group showed a similar positive pattern as observed for the BW. Despite the clear results of the effect of monensin on BW and ADG in this study, not all studies have shown consistent responses from monensin supplementation. For example, Li et al. (2004) reported that *Eimeria* oocysts consisting of *E. tenella*, *E. maxima*, and *E. acervulina*, were resistant to monensin (100 mg/kg of feed), sensitive to both salinomycin (60 mg/kg) and laso-lacid (90 mg/kg), and partially sensitive to maduramicin

(5 mg/kg) and semduramicin (25 mg/kg). Valotteau et al. (2017) showed that 40 mg monensin/kg feed-fed chickens increased their daily weight gain by 27% at 6 dpi (23 d of age) compared to untreated control chickens infected with *Eimeria* spp. Despite these inconsistent results on using monensin, the reason why monensin was selected in the current study was that monensin showed (not published) the better results in our previous study on various antibiotics (decoquinat, diclazuril, monensin, and salinomycin) against *Eimeria* spp which are managed in our Lab.

In current study, the ADG was reduced by 30% at 8 dpi in *E. maxima*-infected chickens compared to unchallenged chickens (NC group). In contrast with experiment 1, *E. maxima* infection inhibited ADFI by 4.4% at 8 dpi compared to the NC group. Changes in ADFI observed in experiment 2 suggest that the impact of the *Eimeria* challenge might have been stronger than in experiment 1. Nevertheless, SL1 200 improved ADFI by almost 4% at 8 dpi. Before the chickens were challenged with *E. maxima*, the MO group showed lower FCR values than the other groups. This difference was maintained even after the *E. maxima* challenge. In experiment 2, the *E. maxima* challenge increased FCR by 40% in the PC group at 8 dpi compared to the NC group. Notably, the FCR was reduced by 5.2 and 9.0% at 8 dpi in the SL1 500 and SL4 500 groups compared to the PC group. The observed improvement in the FCR in the SL1 500 and SL4 500 groups could potentially contribute to feed cost savings. The growth results from both experiments indicate that the 200 ppm of SLs did not result in significant differences in growth. Moreover, the growth result observed in experiment 2 suggests more than 500 ppm of SLs should be tested to pinpoint the optimal effects of SLs through linear and quadratic responses.

The SLs regardless of the inclusion amount or SL types showed lower lesion scores and oocyst shedding compared to the PC group, but their abilities on the reduction of lesion score and oocyst shedding were not as much as the MO group. In experiment 2, SLs, especially SL4 500, showed suppression of immune responses activated due to the oral administration of *E. maxima*. All of the proinflammatory cytokines except IL-1 β were upregulated in *E. maxima*-infected chickens (PC group). Monensin and SL supplementation downregulated TNFSF-15 expression, suggesting that the inflammatory responses against *E. maxima*-invasion were suppressed. In addition, SL4 500 also downregulated IL-6 and IL-17F levels. Similarly, in the case of cytokines related to Th1 and Th2, *E. maxima* infection upregulated most cytokines measured in this study (except IL-4), whereas antibiotics did not influence these cytokines. IL-2 and IL-10 levels were downregulated in the SL4 500 group compared to the PC group. These findings indicate that 500 ppm of SL4 may improve cellular immune responses by suppressing the expression of IL-2 and IL-10.

In conclusion, dietary monensin supplementation resulted in maintained growth performance in young broiler chickens challenged with *E. maxima*. During the

E. maxima infection, dietary 500 mg C18:1 diacetyled sophorolipid/kg feed (SL4) reduced the number of *E. maxima* in the jejunum of chicken, suppressed cytokine release during proinflammatory responses, and enhanced TJ protein expression in the jejunum. Overall, dietary SL supplementation maintains growth performance, positively modulates intestinal immune responses, and maintains the intestinal barrier integrity of young broiler chickens during a coccidiosis challenge. These results demonstrate the potential for using SLs as an alternative to antibiotics for poultry.

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Author contributions: IP, SO, PC and HL designed the research. IP, SO, and HL conducted the research. IP, and DG 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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