

Effects of a dietary direct-fed microbial and *Ferulago angulata* extract on growth performance, intestinal microflora, and immune function of broiler chickens infected with *Campylobacter jejuni*

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ABSTRACT Colonization of the gastrointestinal tract by potentially pathogenic bacteria and their shedding in animal feces is a fundamental factor for both animal health and human food safety. This study was conducted to evaluate the efficacy of salinomycin (**Sal**), direct-fed microbial (**DFM**), and *Ferulago angulata* hydro-alcoholic extract (**FAE**) against *Campylobacter jejuni* in broiler chickens in a 6-week pilot-scale study. A total of six hundred and seventy two 1-day-old broiler chickens were equally divided into 6 groups (each consisting of 8 replicates of 14 birds): negative control (**NC**; untreated and uninfected); positive control (**PC**; untreated, infected with *C. jejuni*); PC + Sal; PC + DFM; PC + 200 mg/kg of FAE (**FAE200**); or PC + 400 mg/kg of FAE (**FAE400**). All these groups (except NC) were challenged with *C. jejuni* on day 15. The results showed that all experimental treatments improved ($P < 0.05$) average daily gain compared with the PC group, and the

best value was observed in the NC and FAE400 groups throughout the entire experimental period (day 1–42). The overall feed conversion ratio and mortality rate, as well as the population of *C. jejuni* (day 24 and 42) and Coliforms (day 42) in the ileum and cecum, were higher ($P < 0.05$) in broiler chickens fed with the PC diet than for chickens in the other groups, except those in the FAE200 group. Immune responses revealed that among challenged birds, those that were fed diets DFM and FAE400 had significantly higher IgG (day 24 and 42), IgA (day 24), IL-6 (day 24), and gamma interferon (day 24 and 42) concentrations than the PC group. In conclusion, dietary FAE, especially at a high level of inclusion in broiler diet (400 mg/kg), could beneficially influence the immune status, as well as improve growth performance and intestinal microflora under *Campylobacter* challenge, which was comparable to those of Sal and DFM supplements.

Key words: broiler performance, *Campylobacter* challenge, herbal extract, intestinal microflora, immune response

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INTRODUCTION

Over the last decade, the genus *Campylobacter* has become the most identified foodborne pathogen in human gastrointestinal infections. Among the species of *Campylobacter* capable of causing serious diseases in humans, *Campylobacter jejuni* is the most common cause of foodborne diseases associated with farm animals, especially chickens (Saint-Cyr et al., 2017; Šikić

Pogačar et al., 2020). *C. jejuni* colonizes the chicken ceca at high levels following infection, resulting in fecal shedding of the bacterium. This high fecal contamination level, combined with the coprophagic behavior of chickens, leads to the rapid spread of the infection (Ocejo et al., 2017; Smialek et al., 2018). Since the intestinal tract is the primary source of carcass contamination with *Campylobacter*, decreasing its load could minimize the bacterial contamination of poultry products during the slaughter and evisceration processes (Guyard-Nicodème et al., 2016). Although commercial broiler chickens are not currently being vaccinated against campylobacteriosis, new treatments urgently need to be developed that will provide adequate protection against *Campylobacter* colonization while also preventing adverse performance effects. In conventional management, campylobacteriosis is prevented and

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regulated by in-feed (or water) administration of antibiotic supplements, such as salinomycin (**Sal**) and flavo-phospholipol (**Bolder et al., 1999**). However, with increasing pressure to reduce (eliminate) the use of antibiotics and ionophore in poultry production, alternative strategies are required.

Recent evidence revealed that various dietary direct-fed microbial (**DFM**) supplements can influence host gut health and immunity against campylobacteriosis disease (**Smialek et al., 2018; Rahimi et al., 2019, 2020; Mortada et al., 2020**). Some commercial DFM have been also reported to enhance the development of both the intestinal lymphatic system and gastrointestinal epithelium (**Murugesan et al., 2014; Gadde et al., 2017; Chen et al., 2020**). Therefore, it is hypostasized that a healthy population of gut microbes by DFM supplementation may result in better control of invading pathogens within the intestine, which in turn may improve gut health and overall performance in broiler chickens especially under challenging related conditions.

Ferulago angulata, known as Chavir in Iran, is a plant of the *Apiaceae* family and is a native species of the western mountainous regions of Iran. This plant contains flavonoid and phenolic compounds that have a wide range of pharmacological effects, including antioxidant, anti-microbial, anti-inflammatory, and hemostatic activities (**Kiziltas et al., 2017; Lorigooini et al., 2019**). According to previous research, dietary *F. angulata* could enhance growth performance and beneficially affect the intestinal microbial composition and antibody response of broiler chickens (**Rostami et al., 2015**). In this connection, **Bohlouli and Sadeghi (2016)** also reported that the addition of *F. angulata* extract to the diet could effectively improve immunological indices in rainbow trout fingerling. Although the antimicrobial activity of the aerial parts of *F. angulata* against *Salmonella typhi*, *Staphylococcus aureus*, and *Listeria monocytogenes* has been confirmed (**Ghasemi Pirbalouti et al., 2016**), there is still a lack of literature on their use in the control of bacterial diseases such as campylobacteriosis.

Given all the above-mentioned aspects, the objective of this study was to assess and compare the potential effects of Sal, DFM, and *F. angulata* hydroalcoholic extract (**FAE**) on growth performance, intestinal microbiota, and immune response in commercial broiler chickens infected experimentally with *C. jejuni*.

MATERIALS AND METHODS

All procedures involving bird care and management were performed in accordance with and approved by the Animal Ethics Committee of Ilam University.

Anticoccidial, DFM, and *F. angulata* Extract

Salinomycin (Sacox 60, Intervet/Merck, Millsboro, DE), as an ionophore anticoccidial agent, was included at a rate of 60 mg/kg in all dietary phases for appropriate treatments. Multi-strain DFM (PrimaLac, Star Labs Inc., Clarksdale, MO) containing *Lactobacillus casei*,

Lactobacillus acidophilus, *Bifidobacterium bifidum*, *Streptococcus faecium*, and *Aspergillus oryzae* was added at a dietary level of 1 g/kg. This level of DFM supplementation was selected to warrant survival and establishment in the intestines of treated broilers.

F. angulata plants were collected from Sirvan Mountains (Ilam Province, Iran). After botanical authentication, a voucher specimen was deposited at the Herbarium of Ilam University's Medicinal and Aromatic Plants Research Institute (Herbarium number IURS-1106). We received prior permission from the Forests Range and Watershed Management Organization of Ilam Province, and no endangered or protected species were sampled. The aerial parts of the plant (flower and leaf) were washed with clean water, air-dried, and then ground into a fine powder. Next, the dry powder was macerated at room temperature in ethanol (70:30 ethanol:water, v/v) for 72 h. The ethanol extract of *F. angulata* was evaporated under reduced pressure to remove ethanol and the final product was obtained in a powdered state (32% yield) after lyophilization. Gas chromatography-mass spectrometry (GC 7890, Agilent Tech. Inc., Santa Clara, CA, equipped with a MS 5975C detector and HP-5 ms capillary column) analyses were carried out to detect the components of FAE. The initial column temperature was set at 40°C lasting 3 min, and then programmed at 295°C for 10 min, with a heating rate of 10°C/min. The total analysis time was about 38 min. The main active components of FAE are shown in **Table 1**.

Experimental Design

A total of 672 male Ross 308 broiler chickens were obtained from a local commercial hatchery. Broiler chickens were fed a starter diet from day 1 to 10, a grower diet from day 11 to 24, and a finisher diet from day 25 to 42 (**Table 2**). The non-medicated diets were formulated according to the nutritional recommendation by Ross 308 (2014) and fed in mashed form. The experimental groups were: 1) negative control (**NC**; untreated and uninfected); 2) positive control (**PC**; untreated, infected with *C. jejuni*); 3) PC + 60 mg/kg Sal; 4) PC + 1 g/kg DFM PrimaLac; 5) PC + 200 mg/kg FAE (**FAE200**), and 6) PC + 400 mg/kg FAE (**FAE400**). Each treatment had 8 replicate pens and 14 birds per replicate. On day 1, to avoid contagious bacterial infections, control and challenged birds were housed separately, in identical floor pens (1.4 m × 1.3 m) in the same building, with a solid wall partition isolating them. All tasks were performed with the control chickens first and then with bacteria-challenged chickens.

The room temperature was set at 34°C on the day of arrival, and then reduced by 0.40°C per day until 24°C, where it remained for the rest of the trial. Environmental relative humidity was maintained at 50 to 65% by periodically spraying the walkways with water and adjusting the humidifiers. The lighting program used

Table 1. Major phytocomponents identified by gas chromatography-mass spectrometry in the hydroalcoholic extract of *Ferulago angulata*.

Peak no.	Compounds	RT	Yield (g/kg)
1	Phenol	14.91	9.7
2	Pyrone	17.26	7.2
3	Malic acid	17.83	29.7
4	5-Hydroxymethylfurfural	18.59	46.2
5	Thymol	19.28	24.2
6	Carvacrol	19.65	53.2
7	Benzene, [2-nitro-1-(4-pentenylthio)ethyl]-	20.08	18.3
8	Dodecanoic acid, methyl ester	21.42	11.9
9	Benzocyclooctene, 7,8-dimethyl-	22.16	12.8
10	1,2,3-Benzenetriol (phenol)	22.40	58.4
11	β -D-Glucopyranose, 1,6-anhydro-	23.34	20.8
12	Myristic acid, methyl ester	23.75	17.4
13	Butyraldehyde, semicarbazone	25.07	57.9
14	9-Hexadecenoic acid, methyl ester	25.80	11.6
15	Pentadecanoic acid, 14-methyl-, methyl ester	25.98	55.6
16	Hexadecanoic acid, ethyl ester	26.49	44.2
17	9-Octadecenoic acid, methyl ester	27.86	207.5
18	Methyl stearate	28.02	29.1
19	2H-Pyran-5-carboxamide (heterocyclic compound)	28.34	67.4
20	9,12,15-Octadecatrienoic acid, ethyl ester	28.57	37.2
21	Benzenesulfonothioic acid (thiosulfonic acid)	28.69	66.1
22	1H-1,3-Benzimidazole (heterocyclic compound)	29.20	30.2
23	2-(2-Nitrovinyl)furan	30.38	17.5

Abbreviation: RT, retention time.

was 24L:0D from day 0 to 3 and 23L:1D for the remainder of the experiment.

C. jejuni Challenge

Birds in the current study were challenged at day 15 because *Campylobacter* spp. is rarely detected in commercial broiler flocks in the first 2 wk and there is a lag

phase before infection can be detected. The reasons for this lag period are not clear, but have been attributed to the protection provided by maternally produced antibodies, in-feed antibiotics, and the development of the intestine as well its microbial community (Cawthraw and Newell, 2010; Skoufos et al., 2019). An infectious and genetically stable strain of *C. jejuni* (ATCC 33291), which was isolated from infected commercial

Table 2. Composition of the basal starter, grower, and finisher diets and their nutrient profile.

Item	Starter (1–10 d)	Grower (11–24 d)	Finisher (25–42 d)
Ingredient, g/kg			
Corn	470.3	596.0	659.9
Wheat	55.8	50.0	50.0
Soybean meal (44% crude protein)	290.2	161.5	102.8
Corn gluten meal (60% crude protein)	100.0	114.8	115.0
Soybean oil	35.0	34.0	30.9
Limestone	14.5	12.3	10.0
Dicalcium phosphate	19.5	18.0	18.3
Sodium chloride	2.0	2.0	2.0
Vitamin premix ¹	2.5	2.5	2.5
Mineral premix ²	2.5	2.5	2.5
DL-Methionine	5.2	5.8	5.7
L-Lysine HCl	2.5	0.6	0.4
Calculated values			
Metabolizable energy (kcal/kg)	2,950	3,000	3,050
Crude protein (g/kg)	220.0	200.0	190.0
Lysine (g/kg)	13.0	12.0	11.0
Methionine (g/kg)	5.6	5.4	5.2
Methionine + cysteine (g/kg)	9.2	9.0	8.8
Calcium (g/kg)	10.4	9.5	9.2
Available phosphorus (g/kg)	5.2	4.4	4.2

¹Provided per kilogram of diet: trans-retinol, 9,000 IU; cholecalciferol, 2,500 IU; α -tocopherol acetate, 45 mg; vitamin K, 5 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B6, 5 mg; vitamin B12, 0.03 mg; nicotinamide, 30 mg; pantothenic acid, 15 mg; folic acid, 1.1 mg; biotin, 0.13 mg; and choline, 450 mg.

²Provided per kilogram of diet: Mn, 100 mg; Fe, 80 mg; Zn, 100 mg; Cu, 10 mg; I, 0.5 mg; Co, 0.2 mg; Se, 0.15 mg.

broilers, was used for the inoculation of birds. This strain was stored at -80°C in peptone broth containing 20% (v/v) glycerol until use. For the challenge experiment, the culture was prepared by the short-term subculture on blood agar and incubation of the plates at 42°C for 48 h and microaerobic (85% N_2 , 10% CO_2 , and 5% O_2) conditions. After incubation, the bacteria were harvested and diluted in PBS, according to a method of Lamb-Rosteski et al. (2008). At 15 d of age, all birds except NC were challenged with 1 mL of inoculum in crop using a 1-mL syringe connected to a stainless steel and sterilized cannula. The inoculum concentration was estimated using an optical density reading at 600 nm. The inoculum was kept on ice until inoculation (maximum 2 h). The bacterial population in the inoculum used to challenge the birds was approximately 10^5 bacteria/mL (De Castro Burbarelli et al., 2017; Oejo et al., 2017). The challenge dose was confirmed by cfu counts of serial 10-fold dilutions on *Campylobacter* blood-free selective agar base. Unchallenged broiler chickens (NC group) were given 1 mL of sterile PBS, producing the same management stress. Biosafety level 2 practices were applied during the trial, and all bacterial culture and inoculum preparation work was performed in a biological safety cabinet.

Growth Performance Parameters

Pen body weight and feed intake were recorded at placement, 10, 24, and 42 d of age for calculation of ADG and ADFI per bird for each replicate pen. Incidences of mortality were recorded daily in order to determine mortality rate. With the body weight of any deceased or culled chickens included, the total ADG and total ADFI for each pen were used for calculating mortality-adjusted feed conversion ratio (FCR) during each feeding period.

Gut Microflora

Prior to inoculation (day 15), and thereafter on day 24 and 42, 1 bird in each pen ($n = 8$ birds from each treatment group) was sacrificed and its ileum and ceca were harvested to determine the populations of *Lactobacillus* spp., *C. jejuni*, and Coliforms in both the ileum and ceca. Subsamples of ileal and cecal digesta (1 g) were instantly collected into glass containers for bacterial enumeration. Briefly, the digesta samples were serially diluted 10-fold in PBS and *Lactobacillus* spp., *C. jejuni*, and Coliforms were enumerated, respectively, on de Man, Rogosa, and Sharpe agar (Merck, 1.05289), blood-free *Campylobacter* agar (CM0739, Oxoid Ltd., Basingstoke, Hampshire, UK) containing selective supplement (SR 155E, Oxoid Ltd.), and MacConkey agar (Merck, 1.05465). Each dilution was plated in duplicate onto appropriate agar plates and the average of 2 bacterial counts was used in the statistical analysis. The MacConkey agar plates were incubated aerobically at 37°C for 24 h, while the de Man, Rogosa, and Sharpe agar plates were incubated anaerobically using a gas pack system (Merck

Anaerocult type A) at 37°C for 72 h. The *Campylobacter* blood-free agar plates were also incubated under microaerobic conditions (85% N_2 , 10% CO_2 , and 5% O_2) at $41.5 \pm 1^{\circ}\text{C}$ for 48 h (Vadalasetty et al., 2018). After incubation, for each bacterial type, the plates were enumerated, and bacterial counts were finally expressed as \log_{10} cfu per gram of ileal digesta. The detection limit was considered as 10^2 bacteria/g of fresh digesta.

Immunological Measurements

On day 24 and 42, 2 birds per pen were randomly selected and blood samples were collected from the wing vein. The blood samples were kept at room temperature (approximately 25°C) for 2 h and then centrifuged at $580 \times g$ for 10 min. Serum immunoglobulin (IgA, IgG, and IgM) levels were measured on flat-bottomed 96-well plates by ELISA using chicken-specific IgA, IgG, and IgM ELISA quantitation kits (Bethyl Laboratories Inc., Montgomery, TX). The concentrations of IL-6 and gamma interferon ($\text{IFN-}\gamma$) in serum samples were also measured using the commercially available chicken cytokine ELISA kits (R&D Systems, Wiesbaden, Germany). All measurements were performed at least in triplicate and according to the manufacturer's instructions.

Statistical Analysis

All the data were statistically analyzed as a completely randomized design using GLM procedures of SAS 9.4 (SAS Institute Inc., Cary, NC) with a pen as an experimental unit. All data were tested for outliers and normality of the residuals by employing the UNIVARIATE procedure of SAS. Mortality was converted to the square root of $n + 1$ prior to analysis (Si et al., 2001). Mean separation was conducted by the Tukey's multiple comparison test with differences deemed significant at $P < 0.05$.

RESULTS

Chemical Composition of FAE

The main phytocomponents present in FAE recognized by gas chromatography-mass spectrometry analysis are shown in Table 1.

Among the active compounds in FAE, 9-octadecenoic acid methyl ester (207.5 g/kg); 2H-pyran-5-carboxamide (67.4 g/kg); benzenesulfonothioic acid (66.1 g/kg); 1,2,3-benzenetriol (58.4 g/kg); butyraldehyde, semicarbazone (57.9 g/kg); pentadecanoic acid, 14-methyl-, methyl ester (55.6 g/kg); and carvacrol (53.2 g/kg) were the major components.

Growth Parameters

The effects of the experimental treatments on broiler performance during different growing periods are presented in Table 3. Dietary treatments did not

significantly affect the ADG, ADFI, and FCR during day 1 to 10. From day 11 to 24, the ADG in all experimental groups, except FAE200 group, was greater ($P = 0.019$) than that in the PC group. During the finisher phase (day 25–42) and the entire (day 1–42) experimental period, all experimental groups showed improved ADG ($P < 0.05$) compared with the PC group, with the highest values recorded in the NC and FAE400 groups. The PC birds also consumed less feed ($P < 0.05$) compared with the NC and FAE400 groups during the grower, finisher, and overall experimental periods.

From day 11 to 24, broiler chickens in the Sal and DFM groups exhibited a lower FCR ($P = 0.026$) than birds in the PC group. During the finisher phase, the FCR in the NC and FAE400 was better ($P = 0.015$) than that in the PC group. Moreover, the overall FCR and mortality rate were greater ($P < 0.05$) in broiler chickens in the PC group than for chickens in the other groups, except those in the FEA200 group. By comparison, the overall mortality was also lower ($P < 0.001$) for birds in the NC group than the DFM and FAE200 groups.

Intestinal Microbiota

The effects of dietary treatments on the populations of *Lactobacillus* spp., *C. jejuni*, and Coliforms in the ileum and cecum on day 15 (prior to inoculation), 24, and 42 are presented in Table 4. On day 15, although there were no treatment effects ($P > 0.05$) on *Lactobacilli* counts in the ileum, the cecal *Lactobacilli* count was highest ($P = 0.039$) in broiler chickens that were fed the DFM diet and differed from all other experimental treatments, except for broiler chickens fed the FAE200 diet. On day 24 and 42, broiler chickens in the DFM group also had higher ($P < 0.05$) ileal and cecal *Lactobacilli* counts than broiler chickens in the PC diet.

However, at the same ages, the cecal *Lactobacilli* counts in the DFM group were similar ($P > 0.05$) to those in the NC group.

No *C. jejuni* was found in the ileal and cecal samples in the NC treatment on day 15 and 24 d of age. Among the challenged groups, the *C. jejuni* count in the ileum was lower ($P < 0.05$) in all treatment groups compared with that in the PC group, and the lowest bacterial counts were observed in the Sal and FAE400 groups at 24 and 42 d of age. In the cecum, all experimental groups, except FAE 200 group, exhibited lower *C. jejuni* counts ($P < 0.001$) compared to the PC group at 24 d of age. Moreover, on day 42, cecal *C. jejuni* counts were significantly lower ($P = 0.002$) in all treatment groups than in the PC group. By comparison, birds in all challenged groups exhibited higher ($P < 0.05$) ileal and cecal *C. jejuni* counts than birds in the NC group on day 42.

Although there were no significant differences among the experimental groups in the ileal Coliform counts at 15 and 24 d of age, all supplemented groups had lower ileal Coliform counts ($P = 0.028$) compared to the PC group on day 42. On day 24 and 42, all experimental groups, except the FAE200 group, also exhibited lower cecal Coliform counts ($P < 0.05$) compared with the PC group, and the Sal and FAE400 treatments had the lowest Coliform counts.

Immune Responses

The effects of dietary treatments on serum concentrations of IgG, IgA, and IgM in broiler chickens are shown in Table 5. The birds infected with *C. jejuni* (PC group) had greater ($P < 0.05$) serum concentrations of IgA (day 24) and IgG (day 24 and 42) than the uninfected birds (NC group). Among the infected birds, the serum IgA and IgG levels in the Sal, DFM, and FAE400 groups were higher ($P < 0.05$) compared with the PC group

Table 3. Growth performance observed in broiler chickens infected with *Campylobacter jejuni* at 15 d of age and provided with diets supplemented with Sal, DFM, and *Ferulago angulata* extract at levels of 200 and 400 m/kg (FAE200 and FAE400, respectively).

Item	NC ¹	PC ²	Sal	DFM	FAE200	FAE400	SEM	P-value
ADG (g)								
Day 1–10	22.7	22.5	22.9	23.4	22.5	23.2	0.73	0.451
Day 11–24	54.3 ^a	46.8 ^b	53.7 ^a	52.4 ^a	49.1 ^{a,b}	52.2 ^a	2.06	0.019
Day 25–42	85.7 ^a	74.1 ^c	82.8 ^{a,b}	84.0 ^{a,b}	79.7 ^b	85.4 ^a	1.65	<0.001
Day 1–42	60.2 ^a	52.5 ^c	58.8 ^{a,b}	59.0 ^{a,b}	55.9 ^b	59.5 ^a	1.14	0.023
ADFI (g)								
Day 1–10	26.8	26.7	26.7	27.6	26.8	27.1	0.54	0.373
Day 11–24	80.4 ^a	73.2 ^b	77.4 ^{a,b}	76.1 ^{a,b}	74.4 ^b	79.8 ^a	2.28	0.014
Day 25–42	170.0 ^a	158.5 ^b	168.9 ^a	168.6 ^a	163.8 ^{a,b}	168.2 ^a	3.36	0.008
Day 1–42	106.0 ^a	98.7 ^b	104.5 ^{a,b}	104.2 ^{a,b}	101.4 ^{a,b}	105.1 ^a	2.49	0.037
Feed conversion ratio								
Day 1–10	1.18	1.19	1.17	1.18	1.19	1.17	0.025	0.625
Day 11–24	1.48 ^{a,b}	1.56 ^a	1.44 ^b	1.45 ^b	1.52 ^{a,b}	1.53 ^{a,b}	0.031	0.026
Day 25–42	1.98 ^b	2.14 ^a	2.04 ^{a,b}	2.01 ^{a,b}	2.06 ^{a,b}	1.97 ^b	0.061	0.015
Day 1–42	1.76 ^b	1.87 ^a	1.78 ^b	1.76 ^b	1.81 ^{a,b}	1.77 ^b	0.035	0.022
Mortality (Day 1–42, %)	3.12 ^c	13.54 ^a	5.21 ^{b,c}	8.33 ^b	9.38 ^{a,b}	5.21 ^{b,c}	1.35	<0.001

^{a–c}Means within a row not sharing the same superscript are different at $P < 0.05$. Values are means of 8 replicates (pens) per treatment.

Abbreviations: DFM, direct-fed microbial; Sal, salinomycin.

¹Negative control (not treated and uninfected).

²Positive control (not treated, but infected).

Table 4. Microbial profile in ileum and ceca (log cfu/g fresh digesta) on day 15, 24, and 42, observed in broiler chickens infected with *Campylobacter jejuni* at 15 d of age and provided with diets supplemented with Sal, DFM, and *Ferulago angulata* extract at levels of 200 and 400 m/kg (FAE200 and FAE400, respectively).

Item	NC ¹	PC ²	Sal	DFM	FAE200	FAE400	SEM	P-value
<i>Lactobacillus</i> spp.								
Ileum								
Day 15	7.65	7.90	7.75	8.38	8.15	7.96	0.35	0.234
Day 24	7.44 ^{a,b}	6.97 ^b	6.87 ^b	7.88 ^a	7.36 ^{a,b}	7.26 ^{a,b}	0.26	0.017
Day 42	7.25 ^a	6.05 ^b	5.88 ^b	7.18 ^a	6.58 ^{a,b}	6.63 ^{a,b}	0.31	0.009
Ceca								
Day 15	8.83 ^b	8.85 ^b	8.70 ^b	9.41 ^a	9.04 ^{a,b}	8.76 ^b	0.18	0.039
Day 24	8.62 ^a	7.47 ^b	7.52 ^b	8.90 ^a	8.31 ^{a,b}	7.96 ^{a,b}	0.37	0.023
Day 42	8.43 ^a	7.35 ^{b,c}	7.05 ^c	8.56 ^a	7.69 ^b	7.71 ^b	0.20	0.005
<i>C. jejuni</i>								
Ileum								
Day 15	ND ³	ND	ND	ND	ND	ND	-	-
Day 24	ND ^d	8.55 ^a	5.93 ^c	6.41 ^{b,c}	7.26 ^b	6.04 ^c	0.42	<0.001
Day 42	4.75 ^d	8.84 ^a	6.32 ^c	6.85 ^{b,c}	7.33 ^b	6.22 ^c	0.29	<0.001
Ceca								
Day 15	ND	ND	ND	ND	ND	ND	-	-
Day 24	ND ^c	8.47 ^a	7.15 ^b	7.24 ^b	7.58 ^{a,b}	6.90 ^b	0.47	<0.001
Day 42	5.84 ^c	9.05 ^a	7.22 ^b	7.53 ^b	7.77 ^b	7.31 ^b	0.51	0.002
Coliforms								
Ileum								
Day 15	6.59	6.67	6.31	6.44	6.30	6.09	0.30	0.232
Day 24	6.87	7.16	6.15	6.39	6.64	6.23	0.47	0.089
Day 42	6.94 ^{a,b}	7.12 ^a	5.79 ^c	6.26 ^{b,c}	6.30 ^{b,c}	5.98 ^c	0.29	0.028
Ceca								
Day 15	7.25	7.31	7.09	6.98	7.14	6.88	0.19	0.177
Day 24	7.15 ^{b,c}	8.13 ^a	6.92 ^c	7.26 ^{b,c}	7.85 ^{a,b}	6.84 ^c	0.31	0.008
Day 42	7.39 ^{b,c}	8.35 ^a	6.75 ^c	7.33 ^{b,c}	7.93 ^{a,b}	6.83 ^c	0.42	0.037

^{a-d}Means within a row not sharing the same superscript are different at $P < 0.05$. Values are means of 8 replicates (pens) per treatment.

Abbreviations: DFM, direct-fed microbial; Sal, salinomycin.

¹Negative control (not treated and uninfected).

²Positive control (not treated, but infected).

³ND = non-detectable ($<10^2$ bacteria/g). The non-detected count was considered zero for the purpose of statistical analysis.

on day 24. The serum IgG level in the DFM and FAE400 groups was also higher ($P = 0.001$) compared with the PC group on day 42, whereas the IgA and IgM levels were not different ($P > 0.05$).

The serum concentrations of IL-6 and IFN- γ on day 24 to 42 are presented in [Figure 1](#). The serum concentration of IL-6 between the different experimental groups was not different ($P > 0.05$) on day 42. In contrast, birds infected with *C. jejuni* (PC group) had higher

($P < 0.05$) serum concentrations of IL-6 (day 24) and IFN- γ (day 24 and 42) than the NC birds. Among the challenged groups, serum IL-6 and IFN- γ concentrations in broiler chickens in the DFM and FAE400 groups were higher than the corresponding values in the PC group on day 24. Birds that received any of the test additives, except 200 mg/kg of FAE, also had higher ($P < 0.05$) serum IFN- γ concentration compared to that in the PC group on day 42 ([Figure 1B](#)).

Table 5. Serum concentrations of IgA, IgG, and IgM on day 24 and 42, observed in broiler chickens infected with *Campylobacter jejuni* at 15 d of age and provided with diets supplemented with Sal, DFM, and *Ferulago angulata* extract at levels of 200 and 400 m/kg (FAE200 and FAE400, respectively).

Item	NC ¹	PC ²	Sal	DFM	FAE200	FAE400	SEM	P-value
Day 24								
IgA (mg/mL)	0.46 ^c	0.63 ^b	0.80 ^a	0.79 ^a	0.72 ^{a,b}	0.82 ^a	0.06	0.012
IgG (mg/mL)	0.92 ^c	1.52 ^b	2.09 ^a	2.26 ^a	1.83 ^{a,b}	2.23 ^a	0.19	0.008
IgM (mg/mL)	0.36	0.45	0.56	0.59	0.48	0.61	0.13	0.178
Day 42								
IgA (mg/mL)	0.73	0.80	0.82	0.87	0.78	0.93	0.08	0.092
IgG (mg/mL)	1.31 ^c	2.08 ^b	2.68 ^{a,b}	3.02 ^a	2.56 ^{a,b}	2.93 ^a	0.26	0.001
IgM (mg/mL)	0.54	0.67	0.78	0.90	0.86	1.02	0.22	0.087

^{a-c} Means within a row not sharing the same superscript are different at $P < 0.05$. Values are means of 8 replicates (pens) per treatment.

Abbreviations: DFM, direct-fed microbial; Sal, salinomycin.

¹Negative control (not treated and uninfected).

²Positive control (not treated, but infected).

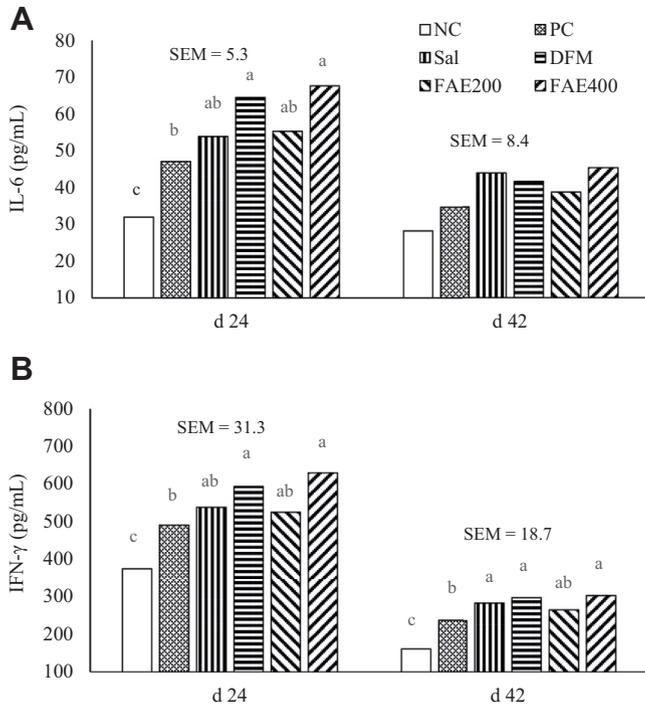


Figure 1. Serum concentrations of (A) IL-6 and (B) IFN- γ in broiler chickens after challenge with *Campylobacter jejuni* at 15 d of age. Means with different letters differ significantly ($P < 0.05$). Means represent 8 replicates (pens) per treatment. Abbreviations: DFM, direct-fed microbial supplemented diet, infected (1 g/kg of diet); FAE200, *Ferulago angulata* extract supplemented feed, infected (200 mg/kg of diet); FAE400, *F. angulata* extract supplemented feed, infected (400 mg/kg of diet); IFN, interferon; NC, negative control (non-treated, uninfected); PC, positive control (non-treated, infected); SAL, salinomycin supplemented diet, infected (60 mg/kg of diet).

DISCUSSION

In addition to farm biosecurity steps, the public health importance of *C. jejuni* infection and the emergence of multi-antibiotic-resistant species of *Campylobacter* require new nutritional and management strategies to minimize the carriage of *C. jejuni* in live birds. To our knowledge, this is the first report evaluating the protective effect of DFM and FAE as feed additives against the *C. jejuni* challenge compared to Sal. The most obvious symptom of *C. jejuni* colonization is growth retardation that is described by weight loss (Awad et al., 2014), and there is also a highly significant correlation between *Campylobacter* positivity and poorer FCR in broiler chickens (Sparks, 2016; Khattak et al., 2018). On the other hand, the damage to the intestinal tissue decreases nutrient absorption and thus reduces feed intake and body weight, and eventually results in poor growth performance (Sweeney et al., 2017; Skoufos et al., 2019).

Results of the current study showed that the overall ADG and FCR of broilers treated with DFM were significantly better than the challenged chickens that did not receive any additives but were similar to the chickens that were infected and had received Sal. In line with the results of the current experiment, Rahimi et al. (2019) reported that adding DFM to the diet of turkey poulters challenged with *C. jejuni* could minimize its

negative effects on growth performance. Direct-fed microbial strains are able to compete for binding sites and occupy the most common receptors on the mucus of the gut wall. This could decrease intestinal colonization by *Campylobacter* and reduce their shedding and, subsequently, may effectively control the spread and prevalence of these bacteria in poultry (Saint-Cyr et al., 2017; Smialek et al., 2018). Therefore, the reason for improving the ADG and FCR in DFM treatment may be related to its effect on the microbial population of the gastrointestinal tract, in which case better bird growth will be provided by balancing the microbial population and protective effects against the pathological consequences of *Campylobacter*.

In the current experiment, there were also significant positive effects of a high level of FAE on ADG and FCR, which were similar to those observed in the Sal and DFM groups. In addition, among the challenged groups, the Sal and FAE400 groups had the lowest mortality rate, which was comparable to that in the NC group. It is well-known that phenolic compounds such as thymol and carvacrol cause degradation of cell membrane lipids in the presence of molecular oxygen, leading to changes in the membrane permeability to ions, which is accompanied by cell death as a result of inhibition of cellular enzyme discharge (Nazzaro et al., 2013; Kachur and Suntres, 2019). Natural bioactive compounds such as flavonoids, tannins, and saponins have also displayed high antioxidant and anti-inflammatory activities, which can protect the intestinal epithelium from oxidative and inflammatory damages (Scheurer et al., 2013; Tungmunnithum et al., 2018). The better ADG and FCR values in the *Campylobacter*-challenged broilers fed the high-FAE diet supported the above-mentioned results. The decrease in the mortality rate of the FAE400 group in the present study could be also explained by the higher serum IgA and IgG concentrations in the respective groups than those in the PC group.

Changes in nutrient composition of the diet, together with the microbiota to which the post-hatch chick is exposed, are key factors that determine the intestinal microbiome (Sweeney et al., 2017; Rubio, 2019). In this study, all dietary supplementation was used from the day-of-hatch, and birds were fed the additive until the end of the trial. Supplementing the broiler diet with Sal, DFM, and FAE400 in this study positively altered the intestinal microbiota by decreasing the proliferation of pathogenic bacteria in the ileum and ceca as compared with the PC birds on day 24 and 42 (9 and 27 d post-infection). Birds that received DFM supplementation also exhibited the highest ileal and cecal *Lactobacilli* counts at 42 d of age, indicating the ability of DFM to increase the growth of potentially beneficial bacteria. Corroborating our findings, Saint-Cyr et al. (2017) found that broiler chickens inoculated with a DFM strain *Lactobacillus salivarius* SMXD51 exhibited a reduction in *Campylobacter* colonization of the chicken ceca at 14 and 35 d of age. Although the exact mechanism of action is not fully understood, the capacity of DFM bacteria to adhere and compete for adhesion sites in the intestinal epithelium and the capacity to

produce organic acids and effective antibacterial compounds might result in the impairment of pathogenic bacteria colonization in the gut (Willis and Reid, 2008; Arsi et al., 2015; Chen et al., 2020).

According to the results of the current study, although broiler chickens fed with the FAE400 diet had lower ileal and cecal Coliforms and *C. jejuni* counts than chickens fed with the PC diet on day 24 and 42, their *C. jejuni* counts were still higher than those of chickens fed with the NC diet. However, broiler chickens receiving the FAE400 diet handled the challenge during the infection period better than birds in the PC group with regard to the population of intestinal pathogenic bacteria at 24 and 42 d of age, which was comparable to the birds fed Sal, an in-feed anticoccidial. These results indicate that the high dietary FEA level (400 mg/kg) may have contributed to a lower prevalence and load of *Campylobacter* and Coliforms at market age. The antimicrobial activities of *F. angulata* have also been demonstrated by other investigators (Rostami et al., 2015; Ghasemi Pirbalouti et al., 2016; Mumivand et al., 2019). Previous reports of in vitro studies also showed that essential oils from different herbal plants, such as thyme, orange, rosemary, and clove oils were effective against both *Salmonella* and *Campylobacter* spp. (Thaniserry et al., 2014; Thaniserry and Smith, 2014), but no studies have yet examined the effect of FAE. Under in vivo studies, no consistent results were observed regarding the efficiency of herbal products on gut microbiota in broiler chickens even though active herbal components are commonly recognized as antimicrobial agents. For example, a decrease in the ileal counts of *Escherichia coli* and *Clostridium* spp. along with an increase in the count of *Lactobacilli* was observed by supplementation of broiler diet with elecampane rhizome extract (Abolfathi et al., 2019). El-Ashram and Abdelhafez (2020) also reported that the counts of Coliform, *Lactobacilli*, and *Enterococcus* were decreased, not affected, and increased, respectively, as a result of phytogetic supplementation. In contrast, no changes were observed in the cecal counts of *Lactobacillus*, *Bifidobacterium*, Coliform, and *Clostridium* spp. in broiler chickens after 6 wk feeding with a phytogetic blend (Oso et al., 2019). Therefore, it is hypothesized that the in vivo antimicrobial property of active components from herbs can be influenced by basal diet, environmental conditions, and hygiene practices. The antimicrobial properties of FEA against pathogenic bacteria, especially *C. jejuni*, in this study are supposed to promote a healthy gut, which in turn could be correlated with improved post-hatch performance.

As presented in Table 5 and Figure 1, serum IgA (day 24), IgG (day 24 and 42), IL-6 (day 24), and IFN- γ (day 24 and 42) concentrations in broiler chickens infected with *Campylobacter* were significantly greater than those of the uninfected birds. Similarly, antibody- and cell-mediated immunity are reported to be activated by infection with *Clostridium perfringens* (Du et al., 2016; Tian et al., 2016), *E. coli* (Huang et al., 2019), and *Salmonella enteritidis* (Shanmugasundaram et al., 2020). Antibodies have been suggested to minimize the

invasion of epithelial cells by pathogenic bacteria when bacteria come into direct contact with local antibodies before entering the host (Lee et al., 2012). Serum immunoglobulins are important indicators to evaluate the humoral immunity of animals. Increased immunoglobulin concentrations can activate complement components to enhance chemotaxis or phagocytosis of immune cells in birds and thus protect them against infections (Long et al., 2020). In addition, the innate immune system plays an important role in host defense against infection. Both IL-6 and IFN- γ are important indicator cytokines that initiate cell-mediated immune responses and have broad biological activities in immune regulation, hematopoiesis, and inflammation (Park et al., 2008; Kishimoto, 2010). According to the results of this experiment, all dietary supplements, except 200 mg/kg FAE, have the potential to positively affect immune function in broiler chickens challenged with *Campylobacter* by increasing IgA and IgG levels. In addition, the DFM and FAE400 groups exhibited higher serum IL-6 and IFN- γ concentrations than the PC group. Similar results have been demonstrated in *Campylobacter*-challenged broilers in which administration of multispecies DFM (PoultryStar ME), composed of *Lactobacillus reuteri*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, and *Enterococcus faecium*, could improve the transforming growth factor- β 4 mRNA content at 14 d post-infection (Mortada et al., 2020). Recent evidence indicates that DFM may enhance host defenses against infection because of the bacteria's effect on host immunity and gut integrity under enteric pathogen challenge (De Oliveira et al., 2019; Šikić Pogačar et al., 2020).

Our results also indicated that supplementation of 400 mg/kg FAE in the broiler diet could result in enhancement of resistance to *C. jejuni* infection, probably by enhancing both cellular and humoral immune responses. Limited research has been performed on the impacts of *F. angulata* and their extracts on poultry health-related parameters during bacterial challenge. A previous report indicates that the use of 8 g/kg *F. angulata* powder in broiler diet may promote humoral immunity by increasing total anti-sheep red blood cell hemagglutinin titers (Rostami et al., 2015). In another study in hemp ducks, a mixed extract of 3 local herbs (*Cortex Fraxini*, *Pulsatilla chinensis*, and *Eucommia ulmoides*) increased the serum concentrations of IgG, IgA, and IL-2 (Bai et al., 2019). Gut microflora composition is reported to be directly responsible for improvement in intestinal health via antagonization of pathogenic bacteria, and modulation of immunity (Moraes et al., 2019). Therefore, positive changes in the intestinal microflora caused by FAE400 treatment may be directly related to the best broiler immune function, but how this relationship may influence the resistance to *Campylobacter* is not clear.

CONCLUSIONS

In conclusion, the high FAE dose helped the *Campylobacter*-challenged broiler chickens to perform as well as

the chickens receiving DFM and Sal, and better than chickens in the PC group. Supplementing broiler diet with Sal, DFM, and a high dose (400 mg/kg) of FAE altered the intestinal microbiota by reducing the proliferation of pathogenic bacteria at 24 and 42 d of age. Moreover, feeding broiler chickens with DFM and FAE400 diets could positively influence immune response, which may be one of the mechanisms of their beneficial effects on intestinal health. However, further studies are required to clarify whether the restorative effect of supplements is caused by specific modes of action against *C. jejuni* infection or by immunomodulatory activities.

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DISCLOSURES

None of the authors have any conflict of interest to declare.

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