TYPLEX® Chelate, a novel feed additive, inhibits *Campylobacter jejuni* biofilm formation and cecal colonization in broiler chickens

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ABSTRACT Reducing *Campylobacter* spp. carriage in poultry is challenging, but essential to control this major cause of human bacterial gastroenteritis worldwide. Although much is known about the mechanisms and route of *Campylobacter* spp. colonization in poultry, the literature is scarce on antibiotic-free solutions to combat *Campylobacter* spp. colonization in poultry. *In vitro* and *in vivo* studies were conducted to investigate the role of TYPLEX® Chelate (ferric tyrosine), a novel feed additive, in inhibiting *Campylobacter jejuni* (*C. jejuni*) biofilm formation and reducing *C. jejuni* and *Escherichia coli* (*E. coli*) colonization in broiler chickens at market age. In an *in vitro* study, the inhibitory effect on *C. jejuni* biofilm formation using a plastic bead assay was investigated. The results demonstrated that TYPLEX® Chelate significantly reduces biofilm formation. In an *in vivo* study, 800 broilers (one d old) were randomly allocated to 4 dietary treatments in a randomized block design, each having 10 replicate pens with 20 birds per pen. At d 21, all birds were challenged with *C. jejuni* via seeded litter. At d 42, cecal samples were collected and tested for volatile fatty acid (VFA) concentrations and *C. jejuni* and *E. coli* counts. The results showed that TYPLEX® Chelate reduced the carriage of *C. jejuni* and *E. coli* in poultry by 2 and 1 $\log_{10}$ per gram cecal sample, respectively, and increased cecal VFA concentrations. These findings support TYPLEX® Chelate as a novel non-antibiotic feed additive that may help produce poultry with a lower public health risk of Campylobacteriosis.

Key words: *Campylobacter*, biofilm, volatile fatty acid, feed additive, food safety

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INTRODUCTION

Campylobacter spp. infections are a major cause of human bacterial gastroenteritis and pose a serious health burden worldwide, accounting for 400 to 500 million cases of diarrhea each year (Ruiz-Palacios, 2007). A significant proportion of health care costs are associated with sequelae linked to Campylobacteriosis, such as Guillain-Barré syndrome, reactive arthritis, and irritable bowel syndrome (WHO, 2013). Despite several government programs and awareness campaigns to reduce *Campylobacter* spp., little reduction is reported in the numbers of the bacterium in animals and/or animal products in retail outlets (Robyn et al., 2015). It is estimated that foodborne transmission contributes to 58% of the global disease burden (Hald et al., 2015).

Chicken, pork, and beef are reported as significant foodborne sources of *Campylobacter* with presence of the pathogen at high concentrations found throughout the food chain (Miller and Mandrell, 2005). The strong adhesion capability of *Campylobacter* strains could partly explain the rapid cross-contamination or re-contamination of food products, and may be the most significant mode of survival for *Campylobacter* in the food chain (Sulaeman et al., 2010).

Campylobacter infection is dependent on motility mediated by polar flagella A (**FlaA**) and adhesion and biofilm-promoting ability of the major outer membrane protein (**MOMP**) (Ashgar et al., 2007; Muller et al., 2007; Min et al., 2009; Mahdavi et al., 2014). Considering such idiosyncrasy, it is easy for *Campylobacter* to colonize live animals and survive transitionally in the food chain or in biofilms before reaching the intestinal tract of humans (Buswell et al., 1998; Trachoo and Frank, 2002; Miller and Mandrell, 2005; Lehtola et al., 2006; Sanders et al., 2008).

Biofilms are accumulations of microorganisms embedded in an extracellular matrix (**ECM**), which adheres to solid biological or non-biotic surfaces (Costerton, 1995; Kalmokoff et al., 2006). The ECM

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comprises proteins, polysaccharides, nucleic acids, and phospholipids (Stoodley et al., 2004). Foodborne bacterial pathogens either can form biofilms on inert surfaces or can reach and integrate into pre-established biofilms.

Increase in cecal volatile fatty acid (**VFA**) concentrations, indicating fermentation by anaerobic bacteria, are found to be negatively correlated with the number of Enterobacteriaceae in broiler chickens (Van Der et al., 2000; Kubena et al., 2001). Several strategies such as reduction of environmental exposure to *Campylobacter* by hygiene and biosecurity measures, water treatment, use of plant-derived feed additives, use of bacteriophage, bacteriocin therapies, vaccination, passive immunization, use of pre- and probiotics, and genetic selection have been investigated to control on-farm *Campylobacter* contamination (Sahin et al., 2003; Chaveerach et al., 2004; Carrillo et al., 2005; Calderon-Gomez et al., 2009; Lin, 2009; Buckley et al., 2010; Hermans et al., 2010; Svetoch and Stern, 2010; Hermans et al., 2011). Despite all these efforts, *Campylobacter* remains a major cause of human gastroenteritis and a priority for the development of new control strategies. In addition, use of antibiotics in both animal and human medicine can influence the development of antibiotic-resistant *Campylobacter* and is increasingly becoming a challenge for food safety and public health (Luangtongkum et al., 2009). The authors postulated that the novel feed additive, TYPLEX® Chelate, a synthetic complex of L-tyrosine and Fe (III), exerts a unique action on enteropathogens by preventing the formation of biofilms at chyme/mucosal and other interfaces. Based on the non-antimicrobial nature of ferric tyrosine, the current studies were designed to demonstrate that TYPLEX® Chelate inhibits *C. jejuni* biofilm formation (*in vitro*), increases cecal VFA concentrations (*in vivo*), and thus has an ability to reduce cecal colonization of *C. jejuni* and *E. coli* in broilers at slaughter.

MATERIALS AND METHODS

Feed Additive

TYPLEX® Chelate (Akeso Biomedical, Inc., Waltham, MA), a complex containing tyrosine and iron (ferric tyrosine), was the novel feed additive used in these studies.

Experimental Diets

Basal iso-nitrogenous and iso-energetic wheat-soyabean meal control diets (T_1) were manufactured as one batch for each feeding phase, i.e., starter (day 0 to 21) and grower (day 21 to 42). Three additional treatments (T_2 to T_4) were generated by addition of TYPLEX® Chelate to T_1 at 0.02, 0.05, and 0.20 g/kg feed, respectively. Diets were manufactured with coccidiostats but contained no veterinary antibiotics. The ingredients, premixes, and the calculated analyses of the starter and grower diets are presented in Table 1. Diets were analyzed for dry matter, nitrogen, ether extract, and iron. Total nitrogen content of diet was determined by the combustion method (AOAC Method 968.06), whereas ether extract was determined in a soxhlet extractor (AOAC Method 922.06). Iron content was determined using inductively coupled plasma–optical emission spectroscopy (AOAC Method 990.08) following digestion, in turn, in concentrated nitric and hydrochoric acid. Colored tracers (Micro-Tracers Inc, San Francisco, CA) were added to TYPLEX™ Chelate at 10% w/w, to enable visual confirmation of TYPLEX™ Chelate content and uniform mixing in feed samples.

Table 1. Composition of basal diets.

Ingredients (% unless otherwise stated)	Starter diets (d 0 to 21)	Grower diets (d 21 to 42)
Barley	10.415	8.315
Wheat	50	55
Soya Ext Hipro	26	23
Full fat Soya Cherwell	5	5
L Lysine HCl	0.4	0.3
DL-methionine	0.4	0.35
L-threonine	0.15	0.15
Soya Oil	4	4.5
Limestone Trucal 52	1.25	1.25
Monocalcium phosphate	1.5	1.25
Salt	0.25	0.25
Sodium bicarbonate	0.15	0.15
Broiler Premix*	0.4	0.4
Robenz 66 G Premix (robenidine coccidiostat)	0.05	0.05
Ronozyme WX (polysaccharidase enzymes	0.02	0.02
Ronozyme P 5000 (CT) (phytase enzymes)	0.015	0.015
Total	100%	100%
Calculated analysis		
Fat (ether extract)	6.39	6.85
Protein	21.84	20.64
Fiber	3.08	3.02
Ash	6.02	5.68
ME-P	12.73	13.04
Total lysine	1.43	1.28
Available lysine	1.33	1.19
Methionine	0.69	0.62
Total methionine and cysteine	1.03	0.95
Threonine	0.91	0.86
Tryptophan	0.25	0.23
Calcium	0.95	0.91
Phosphorus	0.73	0.66
Available phosphorus	0.48	0.42
Sodium chloride	0.30	0.30
Sodium	0.17	0.17
Analyzed nutrient composition		
Dry matter	88.1	88.2
Crude protein†	21.8	20.7
Ether extract	6.66	7.12
Iron (mg/kg)	135	99

*Premix provided per kg: vitamin A, 2,400 IU; vitamin D3, 1,000 IU; vitamin E, 10,000 IU; vitamin K3, 600 mg/kg; vitamin B1, 400 mg/kg; vitamin B2, 1,400 mg/kg; pantothenic acid, 3,000 mg/kg; nicotinic acid, 10,000 mg/kg; vitamin B6, 1,000 mg/kg; vitamin B12, 3,000 μg/kg; folic acid: 200 mg/kg; biotin: 40 mg/kg; copper, 2,000 mg/kg; zinc, 16,000 mg/kg; manganese, 20,000 mg/kg; iodine , 200 mg/kg; selenium, 40 mg/kg; choline choride, 500 g: No added iron in premix.

†Crude protein = Nitrogen x 6.25.

In vitro assessment of C. jejuni biofilm inhibition in a simulated gut environment using a plastic bead assay

A plastic bead assay (O'Toole and Kolter, 1998; Stepanovic et al., 2000) with some modifications for suitability with *C. jejuni* was carried out *in vitro* to show that the TYPLEX® Chelate extracted from the experimental diets has an inhibitory effect on *C. jejuni* biofilm formation.

Extraction of TYPLEX® Chelate from the experimental diets. 10 g aliquots of feed from all experimental diets (T_1 to T_4) for each phase were homogenized to very fine particles (around 100 μm) and mixed with 50 mL (1:5) of buffer (20 mM KCl/HCl; pH 3.4). The feed/buffer mixtures were autoclaved (high-pressure saturated steam at 121°C for around 15 to 20 min, 3 cycles). The high heat and pressure of the autoclave resulted in extraction of the TYPLEX® Chelate from the feeds while retaining its physical properties. The resulting suspensions were then filtered using filter paper (25 μm filter). The pH of all filtered samples was measured and found to be between 6.2 and 6.5.

Bead assay. *C. jejuni* strain NCTC11168 was grown overnight in 3 mL of sterile brain heart infusion (**BHI**) broth under microaerophilic conditions (85% nitrogen, 10% carbon dioxide, and 5% oxygen) at 42°C on modified *Campylobacter*-selective charcoal cefoperazone desoxycholate (**CCDA**). The optical density at a wavelength of 600 nm ($\mathbf{OD_{600}}$) of these cultures was measured, then the cultures were used to inoculate a feed extract + BHI suspension (1:4 dilution) to achieve a final OD_{600} of 0.02.

Two sterile plastic beads per dietary treatment were placed into 2.5 mL of BHI, with 0.5 mL of the extracted feeds and inoculated with *C. jejuni*. The beads were then incubated at 42°C for 48 hours. Following incubation, the beads were gently washed twice (5x dipping each) in phosphate buffered saline (**PBS**), placed into 1 mL of PBS, and vigorously vortexed for 30 s prior to centrifuging at 3,000 rpm. The PBS containing bacterial cells released from the biofilm was serially diluted, and 3 × 5 μl aliquots of each dilution were spotted onto CCDA agar plates for quantification (ThermoFisher plates, 3 plates/bead, 2 beads/treatment, 6 samples/treatment). The plates were subsequently incubated microaerophilically at 42°C for 48 h, and colonies were counted and expressed as cfu/mL. The same experiment was carried out in triplicate.

Seeder litter challenge to colonize chicken with C. jejuni in vivo

To confirm *in vitro* findings, an *in vivo* study was designed to investigate the efficacy of TYPLEX® Chelate under farm conditions. A total of 800 male broiler chickens (Ross 308) was allocated randomly to pens in 4 dietary treatments (T_1 to T_4), with pens distributed using a randomized complete block design. Each treatment had 10 replicate floor pens with 20 chicks per pen. Birds were reared on fresh wood shavings in clean pens having European Union maximum stocking density at 42 d of 38 kg/m^2. At 21 d of age, all birds were challenged with *C. jejuni* seeded litter. Diets were fed ad libitum for 42 d in mash form; body weight and feed intake of each replicate pen were recorded to calculate the global zootechnical data (d 0 to 39). At the end of the study (d 42), all birds were humanely killed through cervical dislocation, and cecal samples were collected from 10 birds/pen and used for *C. jejuni* and *E. coli* enumeration, and 2 birds/pen for cecal VFA analysis.

The *in vivo* study (AU AE 37–2016) was carried out under the Animals Scientific Procedures Act (1986) and approved by the ethical review committee of Scotland's Rural College (**SRUC**).

Seeded litter challenge procedure. Approximately 20 kg of used poultry litter was taken from a recently completed broiler study. The litter (not tested for any pathogen) was placed in an oven at 80°C until a constant weight was obtained, then divided into 400 g batches in 40 trays (each tray was approximately 38 × 28 × 8 cm). Each dried reused litter tray was reconstituted with 1,000 mL of deionized water. Trays were then seeded with a mixture of Mueller-Hinton (**MH**) broth, *Campylobacter* suspension (4.5×10^5 cfu *C. jejuni*/mL), and dried hen droppings (20 mL: 10 mL: 10 g). The hen droppings were dried in the same way as the litter. *Campylobacter* isolates were obtained from cecal samples taken from 3 different commercial poultry farms and stored at -80°C in bead cryopreservation vials (Technical Service Consultants, Lancashire, UK). *C. jejuni* (individual strains were not identified) were resuscitated on blood agar (**BA**) No. 2 with horse blood plates (Oxoid PB0114). These cultures were used to prepare lawn plates on further BA plates, incubated for 40 to 48 h at 41.5°C, in boxes with a microaerophilic atmosphere generation system (CampyGen, Oxoid, Hampshire, UK). The lawn plates were harvested by adding 5 mL MH broth, gently detaching the culture with a sterile spreader, and decanting to a container. The suspension was then adjusted with further MH broth to OD_{600} of 0.19 to 0.21 (approximately 1.5×10^5 cfu.ml^{-1}). The bacterial suspension, broth, and droppings were mixed and spread evenly on the top of the litter tray. The tray was then placed in a pen near the feeder.

Isolation and enumeration of C. jejuni from ceca. A sterile scalpel was used to cut off the blind end of both cecal sacks from each chicken. For each sample, 0.5 g of content from each cecal sac (in total 1 g) was weighed out into sterile universal bottles. At each sampling, a total of 2 g cecal content was diluted with 4 mL of sterile maximum recovery diluent (**MRD**), which was added to each universal container and mixed thoroughly. This constituted the 1:2 dilutions (w/v). A further 8 serial dilutions were made in MRD. Then, 5 to 10 μL of each dilution were spotted on CCDA,

De Man, Rogosa and Sharpe (MRS), Brilliance, or chromogenic plates (Oxoid PO0119) and left to dry. Plates were incubated microaerophilically at 41.5°C ± 2.0°C for 24 to 48 hours. Following incubation, plates were assessed for the presence or absence of thermotolerant *Campylobacter* species. In addition, plates of an appropriate dilution level were selected and colonies enumerated. As a confirmatory measurement, 2 colonies from each presumptively positive plate were selected and sub-cultured onto paired blood agar plates (Oxoid PB0114). These plates were incubated at 37°C for 48 h, one plate aerobically and one plate microaerophilically. The presence of *C. jejuni* was indicated by lack of growth aerobically and colonies with *Campylobacter* morphology that grew microaerophilically. In addition, Gram stains were performed on all presumptively *Campylobacter* positive samples. Oxidase strips (Oxoid MB0266) were used to further confirm that the samples were oxidase positive.

In the case of pre-inoculation testing, at d 16, cloacal swabs (one bird/pen) and overshoes (one overshoe/pen) were tested for presence and absence of *C. jejuni* using the same procedure as reported for *C. jejuni* enumeration.

Cecal volatile fatty acid analysis The cecal digesta were gently flushed out in 30 mL universal sample containers and immediately stored at -80°C until analyzed. Samples were ground in the presence of solid CO_2 to ensure homogeneity. They were then shaken with a known volume of water to extract the fatty acids. The extracts were spiked with 4-methylvaleric acid as an internal standard and then passed to gas chromatography (HP 5890 Series II GC; Agilent J & W 30 m × 0.535 mm × 1.00 micon HP-FFAP column; FID detector) to determine the individual component composition by comparison with a series of standard solutions that also were spiked with an internal standard. Acetic, n-butyric, propanoic, n-valeric, iso-butyric, and iso-valeric acid were detected, and results were expressed as mg/kg.

Statistics

For the *in vitro* tests, a single 5 μl aliquot extracted from a bead in a serially diluted manner, grown on a CDDA plate, was the statistical unit of measurement. A randomized complete block design with 10 blocks and 4 treatments was used for *in vivo* studies. The individual bird sampled was the experimental unit for microbiological (*C. jejuni* and *E. coli*) and VFA analysis, whereas the pen was the experimental unit for growth performance data. The microbiological data were $\log_{10}$ transformed prior to analysis. Data obtained were subjected to analysis of variance (**ANOVA**) using a GenStat 16 statistical software package (IACR, Rothamstead, Hertfordshire, UK). Significance among treatments was determined using orthogonal polynomial contrasts. Where correlations are presented (identified using Genstat), all r^2 are significant to at least $P < 0.05$ unless otherwise stated. All statements of significance are based on the probability level of $P \leq 0.05$.

Table 2. Calculated and analyzed value of TYPLEX® Chelate in experimental diets.

Treatment	Calculated value	Analyzed value of TYPLEX® Chelate in starter diets (g/kg)	Analyzed value of TYPLEX® Chelate in grower diets (g/kg)
T_1	0	0	0
T_2	0.02	0.02	0.02
T_3	0.05	0.06	0.04
T_4	0.20	0.19	0.26

Table 3. Inhibitory effect of TYPLEX® Chelate on *C. jejuni* (NCTC11168) biofilm formation using plastic beads.

Treatment	Starter diet Log_{10} cfu/mL	Grower diet Log_{10} cfu/Ll
T_1	5.608	5.573
T_2	5.082	5.194
T_3	5.285	4.129
T_4	5.123	4.583
SEM	0.040	0.076
P-value's for contrast		
T_1 versus T_2	<0.001	<0.001
T_1 versus T_3	<0.001	<0.001
T_1 versus T_4	<0.001	<0.001
Linear	<0.001	

N°. replicates/treatment = 18; cfu = colony forming units.
SEM = standard error of the mean.
T_1 = Control (HCl/KCl) ; T_2 = 0.02 g/kg TYPLEX® Chelate ; T_3 = 0.05 g/kg TYPLEX® Chelate; T_4 = 0.20 g/kg TYPLEX® Chelate.

RESULTS

Nutrient composition and analyzed TYPLEX® Chelate of the experimental diets were within the expected range and are presented in Tables 1 and 2, respectively.

Inhibitory Impact of Extracted TYPLEX® Chelate from Feeds on C. Jejuni Biofilm Formation

In vitro data showed that the addition of TYPLEX® Chelate significantly reduced the ability of *C. jejuni* to adhere to plastic beads and form a biofilm (Table 3). For the starter diet, the addition of TYPLEX® Chelate extract at all concentrations reduced linearly the number of *C. jejuni* cells compared to the control group (0.5, 0.3, and 0.5 $\log_{10}$ cfu/g reduction**,** respectively, $P < 0.001$). Similar results were observed for the grower diet; the addition of TYPLEX® Chelate extract at 0.02 g/kg, 0.05 g/kg, and 0.2 g/kg reduced linearly the number of *C. jejuni* cells compared to the control group (0.4, 1.4, and 1.0 $\log_{10}$ cfu/g reductions, respectively, $P < 0.001$).

Table 4. Effect of dietary treatments on growth performance of broilers.[1]

Item	Starter phase (d 0 to 21)			Grower phase (d 21 to 39)			Overall performance (d 0 to 39)		
	AWG[2] (kg/bird)	AFI[3] (kg/bird)	FCR[4] (kg/kg)	AWG (kg/bird)	AFI (kg/bird)	FCR (kg/kg)	AWG (kg/bird)	AFI (kg/bird)	FCR (kg/kg)
Treatment									
T_1	0.773	1.077	1.400	0.381	2.948	1.611	2.618	4.025	1.544
T_2	0.821	1.076	1.314	0.428	2.915	1.579	2.695	3.991	1.495
T_3	0.776	1.024	1.327	0.461	2.865	1.533	2.660	3.890	1.471
T_4	0.829	1.085	1.316	0.479	2.943	1.543	2.748	4.028	1.472
SEM	0.014	0.016	0.027	0.020	0.036	0.023	0.029	0.047	0.016
P-values for contrast[5]									
T_1 vs. T_2	0.002	0.979	0.004	0.027	0.361	0.182	0.02	0.484	0.007
T_1 vs. T_3	0.842	0.003	0.012	<0.001	0.031	0.002	0.181	0.009	<0.001
T_1 vs. T_4	<0.001	0.584	0.005	<0.001	0.889	0.007	<0.001	0.94	<0.001
Linear	0.011	0.614	0.010	0.579	0.579	0.002	0.001	0.559	<0.001

[1]All means are average of 10 pens per treatment.
[2]AWG = Average weight gain.
[3]AFI = Average feed intake.
[4]FCR = Feed conversion ratio.
T_1 = Control; T_2 = 0.02 g/kg TYPLEX® Chelate; T_3 = 0.05 g/kg TYPLEX® Chelate; T_4 = 0.20 g/kg TYPLEX® Chelate.
[5]Significance level ($P \leq 0.05$).

Campylobacter Infection and Bird Performance

The seeded litter challenge model was successful in horizontal transfer of *C. jejuni* in birds within pens. Pre-inoculation cloacal swabs and samples cultured from overshoes were negative, whereas all cecal samples from all pens cultured positive for *C. jejuni*. The growth performance of birds during the starter (d 0 to 21), grower (d 21 to 39), and overall growth period (d 0 to 39) is presented in Table 4. During the pre-challenge (starter phase), average weight gain (**AWG**) of all birds fed the TYPLEX® Chelate supplemented diet was consistently higher compared to the T_1 control group ($P < 0.05$). However, the differences were significant only when birds fed T_2 and T_4 (0.02 and 0.20 g/kg TYPLEX® Chelate) were compared with T_1 ($P < 0.05$). This improvement in AWG also resulted into 3.6% lower ($P < 0.05$) mortality adjusted feed conversion ratio (**FCR**) values compared with T_1. During the *Campylobacter* challenged period (grower phase), all birds fed TYPLEX® Chelate (T_2, T_3, and T_4) had 19.6% higher ($P < 0.05$) AWG compared to T_1 (control group). This increase in AWG also was translated into 5.7% improvement in FCR of all birds fed TYPLEX® Chelate (T_2, T_3, and T_4) supplemented diets compared to the control group (T_1). The average feed intake (**AFI**) over the entire trial period was lower ($P < 0.05$) for T_3 (0.05 g/kg TYPLEX® Chelate) compared to T_1 control. This shift in AFI resulted in significantly lower ($P < 0.05$) FCR for birds in the T_3 group compared to control. The overall growth performance depicted similar trends and showed 2.9 and 4.9% linear improvement ($P < 0.05$) in AWG in birds fed T_2 and T_3 (0.02 and 0.20 g/kg TYPLEX® Chelate), respectively, and 4.1% lower ($P < 0.05$) FCR for birds fed all TYPLEX® Chelate diets (T_2, T_3, and T_4) compared to control (T_1). The overall AWG and FCR values were positively correlated ($r^2 = 0.693$, 0.822, respectively; $P < 0.05$), whereas AFI showed no correlation ($r^2 = 0.03$; $P > 0.05$) with the inclusion level of TYPLEX® Chelate in the diet. The birds remained healthy throughout the experimental period, and the percentage mortality for treatments 1 to 4 was 6.0, 6.5, 3.5, and 6.5%, respectively. Mortality was not associated with treatment ($P > 0.05$).

Table 5. Cecal microbial counts at 42 d of age.[1]

Treatment	*Campylobacter* spp.[1] log10 cfu/g	*E. coli*[1] log10 cfu/g
T_1	5.86	7.83
T_2	5.03	7.24
T_3	3.81	7.05
T_4	3.74	6.64
SEM	0.296	0.191
P-values for contrast[2]		
T_1 vs. T_2	0.005	0.002
T_1 vs. T_3	<0.001	<0.001
T_1 vs. T_4	<0.001	<0.001
Linear	<0.001	<0.001

[1]All means are average of 20 (2 x culture plates/treatment). *Campylobacter* spp. cultured on CCDA medium, *E. coli* cultured on chromogenic agar; CFU = colony forming unit; SEM = standard error of the mean; T_1 = Control; T_2 = 0.02 g/kg TYPLEX® Chelate; T_3 = 0.05 g/kg TYPLEX® Chelate; T_4 = 0.20 g/kg TYPLEX® Chelate.
[2]Significant level ($P \leq 0.05$).

Effect of TYPLEX® Chelate on cecal C. jejuni and E. coli colonization. Microbiological analyses of cecal samples showed a 0.8, 2.1, and 2.1 log10 cfu/g linear reduction ($P < 0.05$) in *C. jejuni* colonization in TYPLEX® Chelate supplemented T_2, T_3, and T_4 treatment groups, respectively, compared to T_1 control (Table 5). Similarly, 0.6, 0.8, and 1.2 $\log_{10}$ cfu/g linear reduction ($P < 0.05$) in *E. coli* counts was recorded in birds that received 0.02, 0.05, and 0.20 g/kg TYPLEX® Chelate (T_2, T_3, and T_4) compared with T_1 control

Table 6. Effect of experimental diets on the concentrations of volatile fatty acid (VFA; mg/kg) in the cecal content of broilers at 42 d of age.

Item	Acetic acid	n-Butyric acid	Propionic acid	n-Valeric acid	Iso-valeric acid	Iso-butyric acid	Total VFA†
Treatment							
T_1	4785	1222	1048	179	214	126	7573
T_2	6009	2139	1232	228	212	135	9954
T_3	5979	2032	1199	255	310	175	9567
T_4	5497	1728	1155	237	256	144	9018
SEM	387.9	188.9	95.2	30.3	37	18.09	622.4
P-values for contrast[1]							
T_1 vs. T_2	0.003	<0.001	0.059	0.115	0.965	0.635	<0.001
T_1 vs. T_3	0.003	<0.001	0.118	0.015	0.012	0.009	0.002
T_1 vs. T_4	0.072	0.010	0.266	0.062	0.252	0.331	0.024
Linear	0.091	0.022	0.342	0.040	0.058	0.108	0.050

Means represents 2 birds per pen and 10 pens/treatment.
[1]Significance level ($P \leq 0.05$).
SEM = Standard error of differences of means.
†Total VFA = sum of all individual volatile fatty acids (VFA).

(Table 5). The *Campylobacter* spp. and *E. coli* counts were positively correlated with the inclusion level of TYPLEX® Chelate (r^2 = 0.931, 0.952, respectively; $P < 0.05$).

Effect of dietary TYPLEX® Chelate on cecal VFA concentrations. Individual and total VFA concentrations in the cecal digesta are presented in Table 6. Total VFA concentrations increased by 31 and 26% ($P < 0.05$) in birds fed 0.02 and 0.05 g TYPLEX® Chelate/kg feed, respectively, compared to the control group. This increase was due to a general increase in most acids. Thus, feeding birds diets containing 0.02 and 0.05 g TYPLEX® Chelate/kg feed increased ($P < 0.05$) the concentration of acetic acid by 26 and 24%, respectively, compared to the non-supplemented T_1 control. No additional increase in acetic acid concentration was observed when the inclusion rate of TYPLEX® Chelate was further increased from 0.05 to 0.20 g/kg feed. Propionic acid concentrations also were consistently higher in birds fed TYPLEX® Chelate diets, but the differences were not significant ($P > 0.05$) when compared with the T_1 control. Differences in the concentration of n-butyric acid were more profound, and the concentration linearly increased ($P < 0.05$) by 75, 66, and 41% in birds T_2, T_3, and T_4 fed diets containing 0.02 and 0.05 and 0.20 g TYPLEX® Chelate/kg feed, respectively, compared with T_1 control. Also, n-valeric, iso-butyric, and iso-valeric acids were produced in very small quantities, but followed the same trend, with significantly greater concentrations in birds fed T_3 diets containing 0.05 g TYPLEX® Chelate/kg feed compared to T_1 control.

DISCUSSION

The public health significance of *C. jejuni* infection and emergence of multi-antibiotic resistant species of *Campylobacter* demands development of new control strategies in addition to farm biosecurity measures to lower carriage of *C. jejuni* in live animals. To our knowledge, this is the first study to report that TYPLEX® Chelate inhibits *C. jejuni* biofilm formation. The *in vitro* assay showed that TYPLEX® Chelate (extracted from experimental diets) in a simulated gut environment reduced the ability of *C. jejuni* to adhere to plastic beads and form a biofilm.

It is known that MOMP binds to multiple host cell membranes by promoting biofilm formation and autoaggregation. It is the biofilm-forming ability of *C. jejuni* that enables the organism to survive in the environment and enter the food chain (Joshua et al., 2006). Based on our *in vitro* results, it is likely that TYPLEX® Chelate inhibits FlaA and MOMP-mediated adhesion of *C. jejuni* as evidenced by the reduction in biofilm formation. The ability of *C. jejuni* to attach to surfaces and grow in biofilms, where they are protected from antibiotics, biocides, and other chemical or physical challenges, is a key factor in persistence of infection in humans (Costerton et al., 1999; Stewart, 2002; Stoodley et al., 2004). As the TYPLEX® Chelate inhibits biofilm formation, it can therefore be helpful in farming and food processing to reduce cross-contamination of food products. This strategy offers an antibiotic-free method that will not encourage the emergence of resistance in pathogenic bacteria within the host organism. The inhibitory effect of TYPLEX® Chelate on biofilm formation showed the same pattern in starter and grower diets.

The birds in the current study were challenged at d 21 because *Campylobacter* spp. is rarely detected in commercial flocks of less than 3 wk of age, regardless of production methods (Kazwala et al., 1990), species (Allen et al., 2011; Umar et al., 2016), or biosecurity measures (Allain et al., 2014). The lag phase in colonization of poultry, even in the presence of positive birds, suggests that a biological mechanism of colonization resistance may be present in young birds due to maternal antibodies (Cawthraw and Newell, 2010). The relatively dry litter in the poultry house also may limit the ability of *Campylobacter* to survive in the small volume of excreta produced by birds in the first few wk of life (Sparks, 2016). However, when the flock is infected,

the majority of birds become colonized within 4 to 7 d after infection of the first bird (Sahin et al., 2015), and the overall prevalence rises as high as 100% at slaughter age (Barrios et al., 2006), without any apparent clinical manifestations in the chicken host (Kaino et al., 1988).

The incidence of human Campylobacteriosis often is associated with consumption of poultry products contaminated with *C. jejuni* and this, in turn, is linked to the number of *C. jejuni* present in the ceca of the bird (Wagenaar et al, 2006; Neal-McKinney et al., 2014). As few as 500 cells of C. *jejuni* can cause infection (Black et al., 1988). There is no evidence for a "safe" level of *Campylobacter* contamination, as the minimum infectious dose will always be strain-specific. In general, it is considered that the risk of Campylobacteriosis increases as the number of *Campylobacter* on the bird increases (EFSA, 2009). Therefore, it is possible to reduce the incidence of human infection by lowering the number of *C. jejuni* in birds bound for the food supply. In this study, use of TYPLEX® Chelate in broiler diets at 0.05 and 0.20 g/kg feed reduced the counts of cecal *C. jejuni* by up to 2 $\log_{10}$ cfu/g sample the counts of cecal *C. jejuni* in broilers aged 42 days. According to food safety risk analysis studies, this level of reduction in colonization may be able to reduce the public health risk associated with human Campylobacteriosis (Rosenquist et al., 2003; EFSA, 2011).

One of the mechanisms known to reduce *Enterobacteriaceae* (Gram-negative bacteria) in the intestinal microflora is the bacteriostatic effect of VFA in the ceca (Van Der et al., 2000). The current study showed that feeding broilers diets supplemented with 0.02 and 0.05 g TYPLEX® Chelate/kg feed resulted in increased ($P < 0.05$) cecal concentrations of total VFA, mainly due to higher production of acetic and n-butyric acid. High concentrations of VFA are indicative of fermentations by obligate anaerobic bacteria, reported to be an important source of energy for enterocytes and vital for intestinal health (Sunkara et al., 2012). It has been reported that increased concentrations of VFA lower the intestinal pH, which is associated with suppression of pathogens (Kubena et al., 2001, Rehman et al., 2007). VFA not only affect host functions but also serve as a carbon source for the endogenous bacteria, and at high concentrations can exhibit toxic effects on bacteria. Chickens are omnivores, and the diversity of microbial communities in their intestinal tracts is related to their lifestyle. It has been proposed that the different nutritional requirements for maintaining homeostasis of the microbiota communities in the gut would be due to different fermentable substrates available in the terminal ilea (Fang et al., 2012). Among the bacterial fermentation end products in the chicken cecum, butyrate is of particular importance because of its nutritional properties for epithelial cells and pathogen inhibitory effects in the gut (Sun and O'Riordan, 2013). In the current study, the reduction in the proportion of acetic, butyric, and valeric acids in the challenged unsupplemented control group (T_1) could be due to *C. jejuni*. It has been reported that *Campylobacter* colonization reduces butyrate, iso-butyrate, valerate, and iso-valerate in the cecum (Awad et al., 2016).

Furthermore, translocation of *E. coli* to the liver, spleen, and cecum increases in birds infected with *C. jejuni* (Awad et al., 2016). Results from some of the epidemiological studies also reported an increase of *E. coli* in chicken carcasses that were infected with *Campylobacter* (Duffy et al., 2014). The results from this study suggest that *Campylobacter* infection may have an influence on the development of other microbial populations, such as *E. coli*, illustrated by the data on TYPLEX® Chelate, used at higher doses. At 0.20 g/kg feed, TYPLEX® Chelate not only caused a 2.1 $\log_{10}$ cfu/g reduction in cecal *C. jejuni* colonization, but also resulted in 1.2 $\log_{10}$ cfu/g reduction in *E. coli* populations. In a previous study, we found that inclusion of TYPLEX® Chelate in broiler diets without coccidiostats caused a significant reduction in coccidial oocyst counts and elevated the microbial dominance at 52 to 54% guanine + cytosine in comparison with non-supplemented diets, indicative of high-performing healthy birds (Khattak et al., 2017). These results support the notion that TYPLEX® Chelate may reduce the activities of pathogenic bacteria but favor beneficial bacteria and thus enhance growth performance. In addition, the assessment of minimum inhibitory concentrations (**MIC**) of TYPLEX® Chelate to *C. jejuni*, *E. coli*, and *Salmonella enterica* showed that TYPLEX® Chelate did not inhibit the growth of these pathogens (personal communications with Dr. Juha Apajalahti; Alimetrics Group Ltd). The MIC values were higher than 400 mg/L for *C. jejuni* and higher than 200 mg/L for *E. coli* and *Salmonella*, indicating that none of the enteric pathogens tested was inhibited by TYPLEX® Chelate at concentrations relevant for animal feed applications. It is possible that the TYPLEX® Chelate does not kill these pathogens but reduces their ability to adhere to the gut mucosa and thus lowers their chances to compete with beneficial bacteria.

The addition of TYPLEX® Chelate to the diet consistently improved broiler AWG and FCR compared to non-supplemented control birds. It has been reported that *C. jejuni* can cause a significant decrease in weight gain of poultry (Awad et al., 2014a) and that there is a highly significant association between *Campylobacter* positivity and poorer FCR (Sparks, 2016). *Campylobacter* infection is found to significantly downregulate the gene expression of the sodium/glucose cotransporter (SGLT-1), peptide transporter (PepT-1), glucose transporter (GLUT-2), cationic amino acid transporter (CAT-2), excitatory amino acid transporter (EAAT-3), and the $_L$-type amino acid transporter (y^+LAT-2) in different parts of the gut (Awad et al., 2014b). Such decreased nutrient absorption not only explains the negative effect of *Campylobacter* colonization on body weight, but this also could be crucial for the persistence of *Campylobacter* itself. To colonize and invade, *C. jejuni*

bacteria require adequate nutrients, mainly amino acids and Ca^{2+} (Fang et al., 2012; Awad et al., 2015). Therefore, reduction in the intestinal uptake of nutrients by an avian host may increase carbon and nitrogen sources that are essential for bacterial growth (Guccione et al., 2008). The present study suggests that TYPLEX® Chelate also may promote growth performance by enhancing intestinal nutrient absorption and reducing colonization of *C. jejuni*. However, in addition to on-farm environmental conditions and host immune response (Lin, 2009), any negative effects on broiler body weights due to *Campylobacter* also could be strain-specific, as different isolates of *C. jejuni* can have different colonization potential (Hermans et al., 2011).

In conclusion, the data from the current studies suggest that TYPLEX® Chelate reduces gut colonization of *C. jejuni* by inhibiting biofilm formation, increases the bacteriostatic effect of VFA, and improves intestinal absorptive function. By reducing their adhesion power, it is likely that the spread of *C. jejuni* through other biological and non-biological routes would be reduced. In addition, disabling biofilm resistance may enhance the ability of existing antibiotics to fight infections that are refractory to current treatments and eventually help to reduce human cases of Campylobacteriosis.

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