

Scotland's Rural College

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

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1 **INHIBITION OF *CAMPYLOBACTER* BIOFILM AND COLONISATION**

2

3 **TYPLEX[®] Chelate inhibits *Campylobacter Jejuni* Biofilm Formation and Caecal**

4 **Colonization in Broiler Chickens**

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21 **INHIBITION OF *CAMPYLOBACTER* BIOFILM AND COLONISATION**

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23 **TYPLEX® Chelate inhibits *Campylobacter Jejuni* Biofilm Formation and Caecal**

24 **Colonization in Broiler Chickens**

25

26 **ABSTRACT**

27 Reducing *Campylobacter* spp. carriage in poultry is challenging, but essential to

28 control this major cause of human bacterial gastroenteritis worldwide. Although much

29 is known about the mechanisms and route of *Campylobacter* spp. colonization in

30 poultry the literature is scarce on antibiotic-free solutions to combat *Campylobacter*

31 spp. colonization in poultry. *In vitro* and *in vivo* studies were conducted to investigate

32 the role of TYPLEX® Chelate (ferric tyrosine), a novel feed additive, in inhibiting

33 *Campylobacter jejuni* (*C. jejuni*) biofilm formation and reducing *C. jejuni* and

34 *Escherichia coli* (*E. coli*) colonization in broiler chickens at market age. In an *in vitro*

35 study, the inhibitory effect on *C. jejuni* biofilm formation using a plastic bead assay

36 was investigated. The results demonstrated that TYPLEX® Chelate significantly

37 reduces biofilm formation. In an *in vivo* study, 800 broilers (one-day old) were

38 randomly allocated to 4 dietary treatments in a randomised block design, each having

39 10 replicate pens with 20 birds per pen. At day 21, all birds were challenged with *C.*

40 *jejuni* via seeded litter. At day 42, caecal samples were collected and tested for

41 volatile fatty acid (VFA) concentrations, *C. jejuni* and *E. coli* counts. The results

42 showed that TYPLEX® Chelate reduced the carriage of *C. jejuni* and *E. coli* in poultry

43 by 2 and 1 log₁₀ per gram caecal sample, respectively, and increased caecal VFA

44 concentrations. These findings support TYPLEX® Chelate as a novel non-antibiotic

45 feed additive that may help produce poultry with a lower public health risk of
46 *Campylobacteriosis*.

47 **KEY WORDS**

48 Campylobacter, biofilm, volatile fatty acid, feed additive, food safety

49 **INTRODUCTION**

50 *Campylobacter* spp. infections are a major cause of human bacterial gastroenteritis
51 and pose a serious health burden worldwide, accounting for 400-500 million cases of
52 diarrhoea each year (Ruiz-Palacios, 2007). A significant proportion of health care
53 costs are associated with sequelae linked to *Campylobacteriosis* such as Guillain-
54 Barré syndrome, reactive arthritis and irritable bowel syndrome (WHO, 2013).
55 Despite several government programs and awareness campaigns to reduce
56 *Campylobacter* spp. little reduction is reported in the numbers of bacterium in animals
57 and/or animal products in retail outlets (Robyn et al., 2015). It is estimated that
58 foodborne transmission contributes to 58% of the global disease burden (Hald et al.,
59 2015).

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

66 *Campylobacter* infection is dependent on motility mediated by polar flagella A
67 (**FlaA**) and adhesion and biofilm promoting ability of the Major Outer Membrane
68 Protein (**MOMP**) (Ashgar et al., 2007; Muller et al., 2007 and Min et al., 2009;
69 Mahdavi et al., 2014). Considering such idiosyncrasy, it is easy for *Campylobacter* to

70 colonize live animals and survive transitionally in the food chain or in biofilms before
71 reaching the intestinal tract of humans (Buswell et al., 1998; Trachoo and Frank,
72 2002; Miller and Mandrell, 2005; Lehtola et al., 2006; Sanders et al., 2008).

73 Biofilms are accumulations of microorganisms embedded in an extracellular matrix
74 (**ECM**) which adheres to solid biological or non-biotic surfaces (Costerton, 1995;
75 Kalmokoff et al., 2006). The ECM comprises proteins, polysaccharides, nucleic acids
76 and phospholipids (Stoodley et al., 2004). Foodborne bacterial pathogens can either
77 form biofilms on inert surfaces or can reach and integrate into pre-established
78 biofilms.

79 Increase in caecal volatile fatty acid (VFA) concentrations, indicating fermentation by
80 anaerobic bacteria, are found to be negatively correlated with the number of
81 Enterobacteriaceae in broiler chickens (Van Der et al., 2000; Kubena et al., 2001).

82 Several strategies such as reduction of environmental exposure to *Campylobacter* by
83 hygiene and biosecurity measures, water treatment, use of plant-derived feed
84 additives, use of bacteriophage, bacteriocin therapies, vaccination, passive
85 immunization, use of pre- and probiotics and genetic selection have been investigated
86 to control on-farm *Campylobacter* contamination (Sahin et al., 2003; Chaveerach et
87 al., 2004; Carrillo et al., 2005; Calderon-Gomez et al., 2009; Lin, 2009; Buckley et
88 al., 2010; Svetoch and Stern, 2010; Hermans et al., 2010; Hermans et al., 2011).

89 Despite all these efforts *Campylobacter* remains a major cause of human
90 gastroenteritis and a priority for the development of new control strategies. In
91 addition, use of antibiotics in both animal and human medicine can influence the
92 development of antibiotic-resistant *Campylobacter* and is increasingly becoming a
93 challenge for food safety and public health (Luangtongkum et al., 2009). The authors
94 postulated that the novel feed additive, TYPLEX[®] Chelate, a synthetic complex of L-

95 tyrosine and Fe (III), exerts a unique action on enteropathogens by preventing the
96 formation of biofilms at chyme/mucosal and other interfaces. Based on the non-
97 antimicrobial nature of ferric tyrosine the current studies were designed to
98 demonstrate that TYPLEX[®] Chelate inhibits *C. jejuni* biofilm formation (*in vitro*),
99 increases caecal VFA concentrations (*in vivo*) and thus has an ability to reduce caecal
100 colonization of *C. jejuni* and *E. coli* in broilers at slaughter.

101 MATERIALS AND METHODS

102 *Feed additive*

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

105 *Experimental diets*

106 Basal iso-nitrogenous and iso-energetic wheat-soyabean meal control diets (T₁) were
107 manufactured as one batch for each feeding phase *i.e.* starter (day 0-21) and grower
108 (day 21-42). Three additional treatments (T₂ to T₄) were generated by addition of
109 TYPLEX[®] Chelate to T₁ at 0.02, 0.05 and 0.20 g/kg feed, respectively. Diets were
110 manufactured with coccidiostats but contained no veterinary antibiotics. The
111 ingredients, premixes and the calculated analyses of the starter and grower diets are
112 presented in Table 1. Diets were analysed for dry matter, nitrogen, ether extract and
113 iron. Total nitrogen content of diet was determined by the combustion method
114 (AOAC Method 968.06) whereas ether extract was determined in a soxhlet extractor
115 (AOAC Method 922.06). Iron content was determined using Inductively Coupled
116 Plasma – Optical Emission Spectroscopy (AOAC Method 990.08) following
117 digestion, in turn, in concentrated Nitric and Hydrochloric acid. Coloured tracers
118 (Micro-Tracers Inc, San Francisco) were added to TYPLEXTM chelate at 10% w/w, to

119 enable visual confirmation of TYPLEX™ chelate content and uniform mixing in feed
120 samples.

121

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

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

128 ***Extraction of TYPLEX® chelate from the experimental diets.*** 10 g aliquots of
129 feed from all experimental diets (T₁ to T₄) for each phase were homogenised to very
130 fine particles (around 100 µm) and mixed with 50 ml (1:5) of buffer (20 mM
131 KCl/HCl; pH 3.4). The feed/buffer mixtures were autoclaved (high-pressure saturated
132 steam at 121°C for around 15–20 minutes, 3 cycles). The high heat and pressure of
133 the autoclave resulted in extraction of the TYPLEX® Chelate from the feeds while
134 retaining its physical properties. The resulting suspensions were then filtered using
135 filter paper (25 µm filter). The pH of all filtered samples was measured and found to
136 be between 6.2 to 6.5.

137 ***Bead Assay.*** *C. jejuni* strain NCTC11168 was grown overnight in 3 ml of sterile brain
138 heart infusion (**BHI**) broth under microaerophilic conditions (85% nitrogen, 10%
139 carbon dioxide and 5% oxygen) at 42°C on modified *Campylobacter*-selective
140 charcoal cefoperazone desoxycholate (**CCDA**). The optical density at a wavelength of
141 600 nm (**OD₆₀₀**) of these cultures were measured, then they were used to inoculate a
142 feed extract + BHI suspension (1:4 dilution) to achieve a final OD₆₀₀ of 0.02.

143 Two sterile plastic beads per dietary treatment were placed into 2.5 ml of BHI, with
144 0.5 ml of the extracted feeds and inoculated with *C. jejuni*. The beads were then
145 incubated at 42°C for 48 hr. Following incubation, the beads were gently washed
146 twice (5x dipping each) in phosphate buffered saline (**PBS**), placed into 1 ml of PBS
147 and vigorously vortexed for 30 s prior to centrifuging at 3000 rpm. The PBS
148 containing bacterial cells released from the biofilm was serially diluted and 3 x 5 µl
149 aliquots of each dilution were spotted onto **CCDA** agar plates for quantification
150 (Thermo-Fisher plates, 3 plates/bead, two beads/treatment, 6 samples/treatment). The
151 plates were subsequently incubated microaerophilically at 42°C for 48 hrs and
152 colonies were counted and expressed as cfu/ml. The same experiment was carried out
153 in triplicate.

154 *Seeder litter challenge to colonise chicken with C. jejuni in vivo*

155 To confirm *in vitro* findings, an *in vivo* study was designed to investigate the efficacy
156 of TYPLEX[®] Chelate under farm conditions. A total of 800 male broiler chickens
157 (Ross 308) were allocated randomly to pens in 4 dietary treatments (T₁ to T₄), with
158 pens distributed using a randomized complete block design. Each treatment had 10
159 replicate floor pens with 20 chicks per pen. Birds were reared on fresh wood shavings
160 in clean pens having European Union maximum stocking density at 42 d of 38 kg/m².
161 At 21 days of age all birds were challenged with *C. jejuni* seeded litter. Diets were fed
162 *ad libitum* for 42 days in mash form; body weight and feed intake of each replicate
163 pen were recorded to calculate the global zootechnical data (days 0-39). At the end of
164 the study (day 42), all birds were humanely killed through cervical dislocation and
165 caecal samples were collected from 10 birds/pen and used for *C. jejuni* and *E. coli*
166 enumeration, and 2 birds/pen for caecal VFA analysis.

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

170 ***Seeded litter challenge procedure.*** Approximately 20 kg of used poultry litter
171 was taken from a recently completed broiler study. The litter (not tested for any
172 pathogen) was placed in an oven at 80°C until a constant weight was obtained, then
173 divided into 400 g batches in forty trays (each tray was approximately 38 × 28 × 8
174 cm). Each dried reused litter tray was reconstituted with 1000 ml of deionised water.
175 Trays were then seeded with a mixture of Mueller-Hinton (MH) broth,
176 *Campylobacter* suspension (4.5×10^5 cfu *C. jejuni*/ml) and dried hen droppings (20
177 ml: 10 ml: 10g). The hen droppings were dried in the same way as the litter.
178 *Campylobacter* isolates were obtained from caecal samples taken from three different
179 commercial poultry farms and stored at -80°C in bead cryopreservation vials
180 (Technical Service Consultants, UK). *C. jejuni* (individual strains were not identified)
181 were resuscitated on Blood Agar No. 2 with Horse Blood (BA) plates (Oxoid, UK).
182 These cultures were used to prepare lawn plates on further BA plates, incubated for
183 40 – 48 hrs at 41.5°C, in boxes with a microaerophilic atmosphere generation system
184 (CampyGen, Oxoid, UK). The lawn plates were harvested by adding 5 ml MH broth,
185 gently detaching the culture with a sterile spreader and decanting to a container. The
186 suspension was then adjusted with further MH broth to OD₆₀₀ of 0.19 – 0.21
187 (approximately 1.5×10^5 cfu.ml⁻¹). The bacterial suspension, broth and droppings
188 were mixed and spread evenly on the top of the litter tray. The tray was then placed in
189 a pen near the feeder.

190 ***Isolation and enumeration of C. jejuni from caeca.*** A sterile scalpel was used
191 to cut off the blind end of both caecal sacks from each chicken. For each sample, 0.5g

192 of content from each caecal sac (in total 1g) was weighed out into sterile Universal
193 bottles. At each sampling, a total of 2g caecal content was diluted with 4 ml of sterile
194 Maximum Recovery Diluent (**MRD**), which was added to each Universal container
195 and mixed thoroughly. This constituted the 1:2 dilutions (w/v). A further 8 serial
196 dilutions were made in MRD. Then, 5-10 μ L of each dilution was spotted on CCDA,
197 MRS, Brilliance or chromogenic plates (Oxoid PO0119) and left to dry. Plates were
198 incubated microaerophilically at $41.5^{\circ}\text{C} \pm 2.0^{\circ}\text{C}$ for 24-48 hrs. Following incubation,
199 plates were assessed for the presence or absence of thermotolerant *Campylobacter*
200 species. In addition, plates of an appropriate dilution level were selected and colonies
201 enumerated. As a confirmatory measurement, two colonies from each presumptively
202 positive plate were selected and sub-cultured onto paired blood agar plates (Oxoid
203 PB0114). These plates were incubated at 37°C for 48 hrs, one plate aerobically and
204 one plate microaerophilically. The presence of *C. jejuni* was indicated by lack of
205 growth aerobically and colonies with *Campylobacter* morphology that grew
206 microaerophilically. In addition, Gram stains were performed on all presumptively
207 *Campylobacter* positive samples. Oxidase strips (Oxoid MB0266) were used to
208 further confirm that the samples were oxidase positive.

209 In case of pre-inoculation testing, at day 16 cloacal swab (1 bird/pen) and overshoes
210 (1 overshoe/pen) were tested for presence and absence of *C. jejuni* using the same
211 procedure as reported for *C. jejuni* enumeration.

212 ***Caecal volatile fatty acid analysis.*** The caecal digesta were gently flushed out
213 in 30 ml universal sample containers and immediately stored at -80°C until analysed.
214 Samples were ground in the presence of solid CO_2 to ensure homogeneity. They were
215 then shaken with a known volume of water to extract the fatty acids. The extracts
216 were spiked with 4-methylvaleric acid as an internal standard and then passed to the

217 Gas Chromatography (HP 5890 Series II GC; Agilent J & W 30m × 0.535mm × 1.00
218 micron HP-FFAP column; FID detector) to determine the individual component
219 composition by comparison with a series of standard solutions which were also spiked
220 with an internal standard. Acetic, *n*-butyric, propanoic, *n*-valeric, *iso*-butyric and *iso*-
221 valeric acid were detected and results were expressed as mg/kg.

222 ***Statistics***

223 For the *in vitro* tests, a single 5 µl aliquot extracted from a bead in a serially-diluted
224 manner, grown on a CDDA plate was the statistical unit of measurement. A
225 randomized complete block design with ten blocks and four treatments was used for
226 *in vivo* studies. The individual bird sampled was the experimental unit for
227 microbiological (*C. jejuni* and *E. coli*) and VFA analysis whereas the pen was the
228 experimental unit for growth performance data. The microbiological data were log₁₀
229 transformed prior to analysis. Data obtained were subjected to Analysis of Variance
230 (ANOVA) using a GenStat 16 statistical software package (IACR, Rothamstead,
231 Hertfordshire, UK). Significance between treatments was determined using
232 orthogonal polynomial contrasts. Where correlations are presented (identified using
233 Genstat), all r^2 are significant to at least $P < 0.05$ unless otherwise stated. All
234 statements of significance are based on the probability level of $P \leq 0.05$.

235

RESULTS

236 Nutrient composition and analysed TYPLEX[®] Chelate of the experimental diets were
237 within the expected range and are presented in Tables 1 and 2 respectively.

238 ***Inhibitory impact of extracted TYPLEX[®] Chelate from feeds on C. jejuni biofilm*** 239 ***formation***

240 *In vitro* data showed that the addition of TYPLEX[®] Chelate significantly reduced the
241 ability of *C. jejuni* to adhere to plastic beads and form a biofilm (Table 3). For the

242 starter diet, the addition of TYPLEX[®] Chelate extract at all concentrations reduced
243 linearly the number of *C. jejuni* cells compared to the control group (0.5, 0.3 and 0.5
244 log₁₀ cfu/g reduction, respectively, P < 0.001). Similar results were observed for the
245 grower diet; the addition of TYPLEX[®] Chelate extract at 0.02 g/kg, 0.05 g/kg and 0.2
246 g/kg reduced linearly the number of *C. jejuni* cells compared to the control group (0.4,
247 1.4 and 1.0 log₁₀ cfu/g reductions, respectively, P < 0.001).

248 ***Campylobacter infection and bird performance***

249 The seeded litter challenge model was successful in horizontal transfer of *C. jejuni* in
250 birds within pens. Pre-inoculation cloacal swabs and samples cultured from overshoes
251 were negative, whereas all caecal samples from all pens cultured positive for *C.*
252 *jejuni*. The growth performance of birds during the starter (days 0-21), grower (days
253 21 to 39) and overall growth period (days 0 to 39) is presented in Table 4. During the
254 pre-challenge (starter phase), average weight gain (AWG) of all birds fed the
255 TYPLEX[®] Chelate supplemented diet were consistently higher compared to the
256 T1control group (P < 0.05). However, the differences were only significant when
257 birds fed T2 and T4 (0.02 and 0.20g/kg TYPLEX[®] Chelate) were compared with T1
258 (P < 0.05). This improvement in AWG also resulted into 3.6% lower (P < 0.05)
259 mortality adjusted feed conversion ratio (FCR) values compared with T1. During the
260 *Campylobacter* challenged period (grower phase) all birds fed TYPLEX[®] Chelate
261 (T2, T3 and T4) had 19.6% higher (P < 0.05) AWG compared to T1 (control group).
262 This increase in AWG was also translated into 5.7% improvement in FCR of all birds
263 fed TYPLEX[®] Chelate (T2, T3 and T4) supplemented diets compared to control
264 group (T1). The average feed intake (AFI) over the entire trial period was lower (P <
265 0.05) for T3 (0.05g/kg TYPLEX[®] Chelate) compared to T1 control. This shift in AFI
266 resulted in significantly lower (P < 0.05) FCR for birds in T3 group compared to

267 control. The overall growth performance depicted similar trends and showed 2.9 and
268 4.9% linear improvement ($P < 0.05$) in AWG in birds fed T2 and T3 (0.02 and
269 0.20g/kg TYPLEX[®] Chelate), respectively and 4.1 % lower ($P < 0.05$) FCR for birds
270 fed all TYPLEX[®] Chelate diets (T2, T3 and T4) compared to control (T1). The
271 overall AWG and FCR values were positively correlated ($r^2 = 0.693, 0.822$
272 respectively; $P < 0.05$) whereas, AFI showed no correlation ($r^2 = 0.03$; $P > 0.05$) with
273 the inclusion level of TYPLEX[®] Chelate in the diet. The birds remained healthy
274 through out the experimental period, and the percentage mortality for treatment 1 to 4
275 was 6.0, 6.5, 3.5 and 6.5% respectively. Mortality was not associated with treatment
276 ($P > 0.05$).

277 ***Effect of TYPLEX[®] Chelate on caecal C. jejuni and E. coli colonization.***
278 Microbiological analyses of caecal samples showed a 0.8, 2.1 and 2.1 log₁₀ cfu/g
279 linear reduction ($P < 0.05$) in *C. jejuni* colonization in TYPLEX[®] Chelate
280 supplemented T2, T3 and T4 treatment groups respectively compared to T1 control
281 (Table 5). Similarly, 0.6, 0.8 and 1.2 log₁₀ cfu/g linear reduction ($P < 0.05$) in *E. coli*
282 counts was recorded in birds that received 0.02, 0.05 and 0.20 g/kg TYPLEX[®]
283 Chelate (T2, T3 and T₄) compared with T1 control (Table 5). The *Campylobacter* spp.
284 and *E. coli* counts were positively correlated with the inclusion level of TYPLEX[®]
285 Chelate ($r^2 = 0.931, 0.952$, respectively; $P < 0.05$).

286 ***Effect of dietary TYPLEX[®] Chelate on caecal VFA concentrations.***
287 Individual and total VFA concentrations in the caecal digesta are presented in Table 6.
288 Total VFA concentrations increased by 31 and 26% ($P < 0.05$) in birds fed 0.02 and
289 0.05 g TYPLEX[®] Chelate /kg feed, respectively, compared to the control group. This
290 increase was due to a general increase in most acids. Thus, feeding birds diets
291 containing 0.02 and 0.05 g TYPLEX[®] Chelate/kg feed increased ($P < 0.05$) the

292 concentration of acetic acid by 26 and 24 %, respectively, compared to the non-
293 supplemented T1 control. No additional increase in acetic acid concentration was
294 observed when the inclusion rate of TYPLEX[®] Chelate was further increased from
295 0.05 to 0.20 g/kg feed. Propionic acid concentrations were also consistently higher in
296 birds fed TYPLEX[®] Chelate diets, but the differences were not significant (P > 0.05)
297 when compared with the T1 control. Differences in the concentration of n-butyric acid
298 were more profound and the concentration linearly increased (P < 0.05) by 75, 66 and
299 41% in birds T2, T3 and T4 fed diets containing 0.02 and 0.05 and 0.20g TYPLEX[®]
300 Chelate /kg feed, respectively, compared with T1control . Also, n-valeric, *iso*-butyric
301 and *iso*-valeric acids were produced in very small quantities, but followed the same
302 trend, with significantly greater concentrations in birds fed T3 diets containing 0.05g
303 TYPLEX[®] Chelate/kg feed compared to T1 control.

304

DISCUSSION

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

312 It is known that MOMP binds to multiple host cell membranes by promoting biofilm
313 formation and auto-aggregation. It is the biofilm-forming ability of *C. jejuni* that
314 enables the organism to survive in the environment and enter the food chain (Joshua
315 et al., 2006). Based on our *in vitro* results it is likely that TYPLEX[®] Chelate inhibits
316 FlaA and MOMP-mediated adhesion of *C. jejuni* as evidenced by the reduction in

317 biofilm formation. The ability of *C. jejuni* to attach to surfaces and grow in biofilms,
318 where they are protected from antibiotics, biocides, and other chemical or physical
319 challenges, is a key factor in persistence of infection in humans (Costerton et al.,
320 1999; Stewart, 2002; Stoodley et al., 2004). As the TYPLEX[®] Chelate inhibits
321 biofilm formation it can therefore be helpful in farming and food processing to reduce
322 cross-contamination of food products. This strategy offers an antibiotic-free method
323 which will not encourage the emergence of resistance in pathogenic bacteria within
324 the host organism. The inhibitory effect of TYPLEX[®] Chelate on biofilm formation
325 showed the same pattern in starter and grower diets.

326 The birds in the current study were challenged at day 21 because *Campylobacter* spp.
327 is rarely detected in commercial flocks of less than 3 weeks of age, regardless of
328 production methods (Kazwala et al., 1990), species (Allen et al., 2011; Umar et al.,
329 2016) and biosecurity measures (Allain et al., 2014). The lag phase in colonization of
330 poultry, even in the presence of positive birds suggests that a biological mechanism of
331 colonisation resistance may be present in young birds due to maternal antibodies
332 (Cawthraw and Newell, 2010). The relatively dry litter in the poultry house may also
333 limit the ability of *Campylobacter* to survive in the small volume of excreta produced
334 by birds in the first few weeks of life (Sparks, 2016). However, when the flock is
335 infected, the majority of birds become colonized within 4 to 7 days after infection of
336 the first bird (Sahin et al., 2015), and the overall prevalence rises as high as 100% at
337 slaughter age (Barrios et al., 2006), without any apparent clinical manifestations in the
338 chicken host (Kaino et al., 1988).

339 The incidence of human campylobacteriosis is often associated with consumption of
340 poultry products contaminated with *C. jejuni* and this, in turn, is linked to the number
341 of *C. jejuni* present in the caeca of the bird (Wagenaar et al, 2006; Neal-McKinney et

342 al., 2014). As few as 500 cells of *C. jejuni* can cause infection (Black et al., 1988).
343 There is no evidence for a “safe” level of *Campylobacter* contamination, as the
344 minimum infectious dose will always be strain-specific. In general, it is considered
345 that the risk of *Campylobacteriosis* increases as the number of *Campylobacter* on the
346 bird increases (EFSA, 2009). Therefore, it is possible to reduce the incidence of
347 human infection by lowering the number of *C. jejuni* in birds bound for the food
348 supply. In this study, use of TYPLEX[®] Chelate in broiler diets at 0.05 and 0.20 g/kg
349 feed reduced the counts of caecal *C. jejuni* by up to 2 log₁₀ cfu/g sample the counts of
350 caecal *C. jejuni* in broilers aged 42 days. According to food safety risk analysis
351 studies, this level of reduction in colonization may be able to reduce the public health
352 risk associated with human campylobacteriosis (Rosenquist et al., 2003; EFSA, 2011).
353 One of the mechanisms known to reduce *Enterobacteriaceae* (Gram-negative
354 bacteria) in the intestinal microflora is the bacteriostatic effect of VFA’s in the caeca
355 (Van Der et al., 2000). The current study showed that feeding broilers with diets
356 supplemented with 0.02 and 0.05g TYPLEX[®] chelate/kg feed resulted in increased (P
357 < 0.05) caecal concentrations of total VFA, mainly due to higher production of acetic
358 and *n*-butyric acid. High concentrations of VFA are indicative of fermentations by
359 obligate anaerobic bacteria, reported to be an important source of energy for
360 enterocytes and vital for intestinal health (Sunkara et al., 2012). It has been reported
361 that increased concentrations of VFAs lower the intestinal pH, which is associated
362 with suppression of pathogens (Kubena et al., 2001, Rehman et al., 2007). VFAs not
363 only affect host functions but also serve as a carbon source for the endogenous
364 bacteria and at high concentrations can exhibit toxic effects on bacteria. Chickens are
365 omnivores, and the diversity of microbial communities in their intestinal tracts is
366 related to their life-style. It has been proposed that the different nutritional

367 requirements for maintaining homeostasis of the microbiota communities in the gut
368 would be due to different fermentable substrates available in the terminal ilea (Fang et
369 al., 2012). Among the bacterial fermentation end-products in the chicken caecum,
370 butyrate is of particular importance because of its nutritional properties for epithelial
371 cells and pathogen inhibitory effects in the gut (Sun and O’Riordan, 2013). In the
372 current study, the reduction in the proportion of acetic, butyric and valeric acids in the
373 challenged unsupplemented control group (T1) could be due to *C. jejuni*. It has been
374 reported that *Campylobacter* colonization reduces butyrate, iso-butyrate, valerate, and
375 iso-valerate in the caecum (Awad et al., 2016).

376 Furthermore, translocation of *E. coli* to the liver, spleen and caecum increases in birds
377 infected with *C. jejuni* (Awad et al., 2016). Results from some of the epidemiological
378 studies also reported an increase of *E. coli* in chicken carcasses that were infected
379 with *Campylobacter* (Duffy et al., 2014). The results from this study suggest that
380 *Campylobacter* infection may have an influence on the development of other
381 microbial populations, such as *E. coli*, illustrated by the data on TYPLEX[®] Chelate,
382 used at higher doses. At 0.20 g/kg feed, TYPLEX[®] Chelate not only caused a 2.1
383 log₁₀ cfu/g reduction in caecal *C. jejuni* colonization but also resulted in 1 2 log₁₀
384 cfu/g reduction in *E. coli* populations. In a previous study, we found that inclusion of
385 TYPLEX[®] Chelate in broiler diets without coccidiostats caused a significant
386 reduction in coccidial oocyst counts and elevated the microbial dominance at 52-54%
387 Guanine + Cytosine in comparison with non-supplemented diets, indicative of high-
388 performing healthy birds (Khattak et al., 1997). These results support the notion that
389 TYPLEX[®] Chelate may reduce the activities of pathogenic bacteria but favour
390 beneficial bacteria and thus enhance growth performance. In addition, the assessment
391 of minimum inhibitory concentrations (MIC) of TYPLEX[®] Chelate to *C. jejuni*, *E.*

392 *coli*, and *Salmonella enterica* showed that TYPLEX[®] Chelate did not inhibit the
393 growth of these pathogens (personal communications with Dr Juha Apajalahti;
394 Alimetrics Group Ltd). The MIC values were higher than 400mg/l for *C. jejuni* and
395 higher than 200mg/l for *E. coli* and *Salmonella*, indicating that none of the enteric
396 pathogens tested were inhibited by TYPLEX[®] Chelate at concentrations relevant for
397 animal feed applications. It is possible that the TYPLEX[®] Chelate does not kill these
398 pathogens but reduces their ability to adhere to the gut mucosa and thus lower their
399 chances to compete with beneficial bacteria.

400 The addition of TYPLEX[®] Chelate to the diet consistently improved broiler AWG
401 and FCR compared to non-supplemented control birds. It has been reported that *C.*
402 *jejuni* can cause a significant decrease in weight gain of poultry (Awad et al., 2014a)
403 and that there is a highly significant association between *Campylobacter* positivity
404 and poorer FCR (Sparks, 2016). *Campylobacter* infection is found to significantly
405 downregulate the gene expression of the sodium/glucose cotransporter (SGLT-1),
406 peptide transporter (PepT-1), glucose transporter (GLUT-2), cationic amino acid
407 transporter (CAT-2), excitatory amino acid transporter (EAAT-3) and the L-type
408 amino acid transporter (y⁺LAT-2) in different parts of the gut (Awad et al., 2014b).
409 Such decreased nutrient absorption not only explains the negative effect of
410 *Campylobacter* colonization on body weight but this could be crucial for the
411 persistence of *Campylobacter* itself. To colonize and invade, *C. jejuni* bacteria require
412 adequate nutrients, mainly amino acids and Ca²⁺ (Fang et al., 2012; Awad et al.,
413 2015). Therefore, reduction in the intestinal uptake of nutrients by an avian host may
414 increase carbon and nitrogen sources that are essential for bacterial growth (Guccione
415 et al., 2008). The present study suggests that TYPLEX[®] Chelate may also promote
416 growth performance by enhancing intestinal nutrient absorption and reducing

417 colonization of *C. jejuni*. However, in addition to on-farm environmental conditions
418 and host immune response (Lin, 2009), any negative effects on broiler body weights
419 due to *Campylobacter* could also be strain-specific as different isolates of *C. jejuni*
420 can have different colonization potential (Hermans et al., 2011).

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

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636

637 **Table 1.** Composition of basal diets

Ingredients (% unless otherwise stated)	Starter diets (d 0-21)	Grower diets (d 21-42)
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 66G 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
Fibre	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
Analysed 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

638 *Premix provided per kg: vitamin A, 2400 IU; vitamin D3, 1000 IU; vitamin E,
639 10,000 IU; vitamin K3, 600 mg/kg; vitamin B1, 400 mg/kg; vitamin B2, 1,400
640 mg/kg; pantothenic acid, 3,000 mg/kg; nicotinic acid, 10,000 mg/kg; vitamin B6,
641 1,000 mg/kg; vitamin B12, 3,000 ug/kg; folic acid: 200 mg/kg; biotin: 40 mg/kg;
642 copper, 2,000 mg/kg; zinc, 16,000 mg/kg; manganese, 20,000 mg/kg; iodine , 200
643 mg/kg; selenium, 40 mg/kg; choline choride, 500 g: No added iron in premix.

644 † Crude protein = Nitrogen x 6.25.

645 **Table 2.** Calculated and analysed value of TYPLEX[®] Chelate in experimental diets.

Treatment	Calculated Value	Analysed Value of TYPLEX [®] Chelate in starter diets (g/kg)	Analysed 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

646

647 **Table 3.** Inhibitory effect of TYPLEX[®] Chelate on *C. jejuni* (NCTC11168) biofilm
 648 formation using plastic beads.
 649

Treatment	Starter diet Log ₁₀ cfu/ml	Grower diet Log ₁₀ cfu/ml
T1	5.608	5.573
T2	5.082	5.194
T3	5.285	4.129
T4	5.123	4.583
SEM	0.040	0.076
P-value's for contrast		
T1 versus T2	<0.001	<0.001
T1 versus T3	<0.001	<0.001
T1 versus T4	<0.001	<0.001
Linear	<0.001	

650 N°. replicates/treatment = 18; cfu = colony forming units.

651 SEM = standard error of the mean.

652 T1= Control ((HCl/KCl) ; T2 = 0.02 g/kg TYPLEX[®] Chelate ;T3 = 0.05 g/kg TYPLEX[®]

653 Chelate; T4 = 0.20 g/kg TYPLEX[®] Chelate.

654 Table 4. Effect of dietary treatments on growth performance of broilers¹

655

Item	Starter phase (d 0-21)			Grower phase (d 21-39)			Overall Performance (d0-39)		
	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									
T1	0.773	1.077	1.400	0.381	2.948	1.611	2.618	4.025	1.544
T2	0.821	1.076	1.314	0.428	2.915	1.579	2.695	3.991	1.495
T3	0.776	1.024	1.327	0.461	2.865	1.533	2.660	3.890	1.471
T4	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									
T1 versus T2	0.002	0.979	0.004	0.027	0.361	0.182	0.02	0.484	0.007
T1 versus T3	0.842	0.003	0.012	<0.001	0.031	0.002	0.181	0.009	<0.001
T1 versus T4	<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

656 ¹All means are average of 10 pens per treatment.657 ² AWG = Average weight gain.658 ³ AFI = Average feed intake.659 ⁴ FCR = Feed conversion ratio.660 T1= Control; T2 = 0.02 g/kg TYPLEX[®] Chelate; T3 = 0.05 g/kg TYPLEX[®] Chelate; T4 = 0.20 g/kg TYPLEX[®] Chelate.661 ⁵Significance level (P ≤ 0.05).

662 **Table 5.** Caecal microbial counts at 42 days of age ¹

Treatment	<i>Campylobacter</i> spp. ¹	<i>E. coli</i> ¹
	log10 cfu/g	log10 cfu/g
T1	5.86	7.83
T2	5.03	7.24
T3	3.81	7.05
T4	3.74	6.64
SEM	0.296	0.191
P-values for contrast ²		
1 vs 2	0.005	0.002
1 vs 3	<0.001	<0.001
1 vs 4	<0.001	<0.001
Linear	<0.001	<0.001

663 ¹All means are average of 20 (2 x culture plates/treatment). *Campylobacter* spp. cultured on
 664 CCDA medium, *E. coli* cultured on chromogenic agar; CFU = colony forming unit; SEM =
 665 standard error of the mean;

666 T1= Control; T2 = 0.02 g/kg TYPLEX[®] Chelate; T3 = 0.05 g/kg TYPLEX[®] Chelate; T4 =
 667 0.20 g/kg TYPLEX[®] Chelate

668 ²Significant level (P≤0.05).

669 **Table 6.** Effect of experimental diets on the concentrations of volatile fatty acid
 670 (VFA; mg/kg) in the caecal content of broilers at 42 d of age.
 671

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 ^b	9567
T ₄	5497	1728	1155	237	256	144 ^{ab}	9018
SEM	387.9	188.9	95.2	30.3	37	18.09	622.4
P values for contrast ¹							
1 versus 2	0.003	<0.001	0.059	0.115	0.965	0.635	<0.001
1 versus 3	0.003	<0.001	0.118	0.015	0.012	0.009	0.002
1 versus 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

672 Means represents 2 birds per pen and 10 pens /treatment.

673 ¹Significance level (P ≤ 0.05).

674 SEM = Standard error of differences of means.

675 [†]Total VFA = sum of all individual volatile fatty acid (VFA).