1	Evaluation of in-field efficacy of dietary ferric tyrosine on performance,							
2	intestinal health and meat quality of broiler chickens exposed to natural							
3	Campylobacter jejuni challenge							
4								
5	Ioannis Skoufos <sup>a</sup> , Athina Tzora <sup>a</sup> , Ilias Giannenas <sup>b</sup> , Eleftherios Bonos <sup>a,c</sup> ,							
6	Anastasios Tsinas <sup>a</sup> , Elinor McCartney <sup>d</sup> , Hannah Lester <sup>d</sup> , Efterpi Christaki <sup>b</sup> , Panagiota Florou-							
7	Paneri <sup>b</sup> , Jafar Mahdavi <sup>e*</sup> , Panos Soultanas <sup>e*</sup>							
8								
9	<sup>a</sup> Department of Agricultural Production, School of Agricultural Production, Food Technology and							
10	Nutrition, Technological Educational Institute of Epirus, Kostakioi Artas, 47100, Arta, Greece.							
11	<sup>b</sup> Laboratory of Nutrition, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle							
12	University of Thessaloniki, 54124, Thessaloniki, Greece.							
13	<sup>c</sup> Research Institute of Animal Science / Hellenic Agricultural Organization - DIMITER, Paralimni							
14	Giannitsa, 58100, Pella, Greece.							
15	<sup>d</sup> Pen & Tec Consulting S.L.U., Pl. Ausias March 1, 4 <sup>a</sup> Planta, D01, 08195, Sant Cugat 11 del							
16	Vallès, Barcelona, Spain.							
17	<sup>e</sup> School of Chemistry, Centre for Biomolecular Sciences, University Park, University of							
18	Nottingham, Nottingham, NG7 2RD, UK.							
19								
20	*Joint Corresponding Authors							
21	Jafar.Mahdavi@nottingham.ac.uk							
22	Panos.Soultanas@nottingham.ac.uk							
23								

### 24 ABSTRACT

*Campylobacter* is an important pathogen commonly found in chickens that can cause severe acute 25 gastroenteritis in humans. Despite intensive efforts to inhibit food-borne transmission of 26 *Campylobacter* no effective strategy exists to reduce *Campylobacter* loads in farmed broilers. This 27 study examined the capacity of a novel feed additive to lower *Campylobacter jejuni* populations 28 29 and to improve growth efficiency of broiler chickens. A total of 384 male one-day-old broiler chicks were used in a 42-day trial. Birds were randomly allocated into four treatments with six 30 replicates of sixteen chicks per pen. Three groups were fed the basal diets further supplemented 31 with TYPLEX<sup>TM</sup> chelate (ferric tyrosine) at various concentrations (0.02, 0.05 and 0.20 g/kg, 32 groups  $T_2$ - $T_4$ , respectively). Control group ( $T_1$ ) was fed basal diets in mash form that did not 33 contain added ferric tyrosine. Feed and water were provided *ad libitum*. At 20 days of age, broilers 34 were exposed to natural *C. jejuni* challenge by introducing contaminated litter from a commercial 35 farm. At day 25, pen litter samples analysed positive for *C. jejuni*, and the infection intensity was 36 homogeneous among pens. At the end of the study C. *jejuni* counts in bird caeca were significantly 37 reduced, by 2  $\log_{10}$  in the T<sub>4</sub> group, compared to the T<sub>1</sub> Control and T<sub>3</sub> groups (p = 0.004). During 38 this study, a natural infection with *Eimeria tenella* occurred at days 26-29. For animal welfare 39 40 reasons all birds were treated with an anti-coccidial drug as recommended, for two consecutive days. At day 42, diarrhoea was observed on the litter in only 1 of 6 pens in the T<sub>4</sub> group, but in 5 41 of 6 pens in the  $T_1$  Control group. In addition, autopsies showed that the  $T_4$  group had the highest 42 43 percentage of birds with normal intestinal tracts. The  $T_1$  group had the lowest percentage of birds with infection-free tracts, and higher incidence of coccidiosis and bloody diarrhoea. At 42 days of 44 age all birds were slaughtered and samples collected for further analysis. Birds in the T<sub>4</sub> group 45 46 tended to exhibit improved weight gain and feed efficiency, a result that warrants further

- 47 investigation. Collectively, our data suggest that addition of ferric tyrosine at 0.20 g/kg exerts a
- 48 protective effect against *C. jejuni* and coccidiosis.
- 49

# 50 KEYWORDS

51 Ferric tyrosine; broiler chickens; gut microbiota; *Campylobacter jejuni*; coccidiosis

52

#### 54 **1. INTRODUCTION**

55 Antibiotics have been used extensively in diets of livestock to prevent disease and/or increase production efficiency. However, there is global pressure to limit their use, due to growing 56 public concerns about antimicrobial resistance, linked to increased risks for human health and food 57 safety (Founou et al., 2017; Santini et al., 2010; Thanner et al., 2016; Vender et al., 2017). The 58 European Union banned the use of antibiotics as growth promoters in 2006 and has since set strict 59 restrictions for their therapeutic use (Lagha et al., 2017). In the USA, Canada and Denmark, 60 significant sections of food production industries have turned their attention towards novel and 61 more natural methods of husbandry without antibiotic use, in order to address consumer concerns 62 63 related to the misuse of antibiotics and to meet consumer demand for more natural, organic food products (Gaucher et al., 2015). 64

Thus, there is an urgent need for alternatives to antibiotic growth promoters that can protect farm animals and limit the establishment and growth of bacterial pathogens in their gastrointestinal tracts. Bacterial pathogens can colonise the gut of susceptible animal species causing subclinical or clinical disease, with severe economic consequences, especially under intensive farming conditions (Hermans *et al.*, 2011; Jorgenesn *et al.*, 2011; Humphrey *et al.*, 2014). Moreover, many pathogens can survive food processing and so contaminate meat, milk and eggs in retail outlets posing serious health hazards for human consumers (Hermans *et al.*, 2011).

*Campylobacter* is one of the commonest bacterial causes of human gastroenteritis worldwide (Fitzerald, 2015), along with other pathogens such as *Salmonella* and *Escherichia coli* (Chaveerach *et al.*, 2004; Hermans *et al.*, 2011; Santini *et al.*, 2010). In the USA, campylobacteriosis is in nearly half (46%) of laboratory-confirmed cases of bacterial gastroenteritis (Thormar *et al.*, 2006). Chickens can be healthy and asymptomatic when harbouring

high numbers of *Campylobacter* in the intestinal content and especially in the caeca, up to  $10^8 - 10^9$  colony forming units (CFU) per gram (Hermans *et al.*, 2011; Thibodeau *et al.*, 2015). In some cases, *Campylobacter* infection can cause symptomatic disease in broiler chickens, with increased mortality and lower overall performance (Humphrey *et al.*, 2014). Chicken meat can be contaminated by *Campylobacter* during harvest/slaughter and/or processing (Hermans *et al.*, 2011). The reduction of *Campylobacter* infection in chicken flocks and processed chicken products would considerably lower the risk for human consumers (Thormar *et al.*, 2006).

Approaches to limit or eliminate gastrointestinal colonization include hygienic and biosecurity practices, vaccination, treatment of drinking water, and use of feed additives (Chaveerach *et al.*, 2004; Hermans *et al.*, 2011; Thibodeau *et al.*, 2015; Thormar *et al.*, 2006). In spite of these endeavours, campylobacteriosis remains today a serious health hazard. It is, therefore, important to develop novel strategies to inhibit *Campylobacter* colonisation and/or growth in the chicken gastrointestinal tract, in order to limit contamination of poultry products (Hermans *et al.*, 2011; Thibodeau *et al.*, 2015; Thormar *et al.*, 2006).

A novel approach is the use of chelated iron complexes with specific effects against *Campylobacter* and other pathogenic bacteria. In one such study carried out in Scotland, an iron chelate with the amino acid L-tyrosine (TYPLEX<sup>TM</sup> chelate) protected broilers intentionally infected with litter seeded with *C. jejuni* strains that were previously isolated from local farms (Khattak *et al.*, 2018; Currie *et al.*, 2018).

The aim of the present study was to evaluate the efficacy of ferric tyrosine in broiler diets using a more natural *C. jejuni* infection model in Greece, selected in contrast to Scotland as a different geographical and climatic area, and to assess whether ferric tyrosine affects the growth efficiency of broiler chickens. Incidents of *Campylobacter* infection exhibit strong seasonal, geographical and climate variations (Weisent *et al.*, 2014) and temporal models of

campylobacteriosis have been produced in Europe, Canada, Australia and New Zealand in order to 101 identify regional spikes in the risk of human infection (Allard et al., 2010; Bi et al., 2008; Fleury 102 et al., 2006; Hearnden et al.; Kovats et al., 2005). Therefore, it is important to assess the efficacy 103 104 of ferric tyrosine under natural infection conditions and different geographical variations of climate. The effects of ferric tyrosine on chicken health, growth performance, Campylobacter 105 106 counts, and meat quality were also evaluated. During this study, a natural infection with Eimeria 107 tenella (E. tenella) allowed us the opportunity to examine ferric tyrosine efficacy against E. tenella 108 in addition to C. jejuni.

109

## 111 2. MATERIALS AND METHODS

## 112 2.1. Animals, grouping and housing

The trial protocol was approved by the Institutional Committee for Animal Use and Ethics 113 of the Technological Institute of Epirus, Department of Agriculture Technology, Division of 114 Animal Production. Throughout the trial, the birds were handled in compliance with local laws and 115 regulations (Presidential Degree 56/2013 on harmonization of the Directive 2010/63/EU) on the 116 protection of animals used for scientific purposes and in accordance to the principles and guidelines 117 for poultry welfare (NRC, 1996). Three hundred and eighty-four (384) male broilers (Ross-308) 118 were randomly allocated into 4 groups with 6 replicate pens of 16 chicks and reared for 42 days in 119 a commercial farm in Arta (39°09'38"N; 20°59'07"E), Epirus, Greece. 120

Birds were housed in floor pens and bedded on rice hull litter. The stocking density was 16 121 birds per m<sup>2</sup>. Commercial husbandry practices were employed throughout the trial: natural and 122 artificial light was provided for 23 hours/day for the first 2 days, 16 hours/day from day 3 to day 123 14, 21 hours from day 15 to slaughter at day 42, ambient temperature and humidity were controlled 124 (initial temperature 33°C, gradually decreased by 3°C per week and then kept constant at 20-22°C; 125 humidity 55-65%). All birds were vaccinated against Marek disease after hatching; and against 126 Newcastle Disease, Infectious Bronchitis and Gumboro during the second week of their life. Feed 127 and drinking water were offered *ad libitum*. All birds were weighed at the time of their placing into 128 the poultry house and then every week until slaughter age. Pen feed consumption and 129 mortality/culls were recorded daily. Average pen weight gain (AWG), average feed intake (AFI) 130 and feed conversion ratio (FCR, feed:gain) were calculated for 0-21, 21-42 and 0-42 days on trial. 131 132

133 2.2. Feeding treatments

Control group  $(T_1)$  was fed basal diets in mash form; (starter feed, 1-21 days; grower feed, 134 22-42 days), without added iron. The basal diets of the other groups were supplemented with ferric 135 tyrosine at 0.02 g/kg feed (T<sub>2</sub>), 0.05 g/kg (T<sub>3</sub>) or 0.20 g/kg feed (T<sub>4</sub>). The ferric tyrosine, brand 136 name TYPLEX<sup>TM</sup> chelate (Akeso Biomedical Inc., Waltham, USA) is an iron chelate (III) with L-137 tyrosine (4-hydroxyphenylalanine). All diets were formulated to meet or exceed NRC (1994) 138 recommendations and then analysed (AOAC, 2007) for crude protein, ether extract, dry matter, 139 iron and ash (Suppl. Table 1). Coloured tracers (Micro-Tracers Inc, San Francisco) were initially 140 added to the ferric tyrosine at 10% w/w, to enable visual confirmation of ferric tyrosine content 141 142 and uniform mixing in feed samples. Proximate analyses of feed samples acted as a double check on feed homogeneity and confirmed that feed nutrients were within the expected ranges (Table 1). 143 Diets did not contain any added iron compounds, coccidiostats or antibiotic growth promoters. 144

145

## 146 2.3. Challenge protocol

In commercial broiler farming *Campylobacter* is usually undetectable in the first 2-3 weeks 147 of young broilers and there is a lag phase before infection can be detected. The reasons for this lag 148 phase are not known but have been attributed to the possible presence of maternal antibodies, 149 150 antibiotic feed additives and the development of the intestine as well its microbial flora (Newell and Wagenaar, 2000; Sahin et al., 2003). However, once the first bird in a flock becomes colonized, 151 infection spreads very rapidly throughout the entire shed in just few days. Therefore, at 20 days 152 153 of age broilers were exposed to natural *C. jejuni* challenge by means of contaminated litter, from 154 commercial broilers, sourced from a local farm that tested positive for C. *jejuni* at 44 days of age. A previous study in Scotland used litter artificially contaminated with C. jejuni (Khattak et al., 155 156 2018) but in this study our main intention was to use a completely natural mode of infection from a different geographical region in order to evaluate campylobacter replication behaviour in 157

158 commercial units. The infecting inoculum was prepared by mixing thoroughly 6 kg of contaminated litter to ensure an even distribution throughout and using 200 g to contaminate each 159 pen. C. jejuni is highly infectious and it has been shown before that even a single bird infected with 160 low levels of *C. jejuni* is sufficient to infect a whole flock of broiler chickens (Stern *et al.*, 2001) 161 with the contamination spreading across the environment and persisting for many weeks (Herman 162 163 et al., 2003; Johnsen et al., 2006). Furthermore, pens were randomised to avoid any experimental bias. Thereafter, pens were examined daily for diarrhoea and fecal oocysts per gram (OPG). On 164 the last day of the trial (day 42) pens were observed for diarrhoea in the litter, after which chickens 165 166 were slaughtered under commercial conditions.

167

#### 168 2.4. Sampling and analysis

From each replicate pen 6 birds were randomly selected and further processed. Post-mortem analyses of the intestinal tracts were performed in these birds and intestinal coccidiosis scoring was carried out as described in Johnson and Reid, 1970. At days 25 and 42, caeca were collected for microbiological analyses. Breasts and thighs were removed from the carcass, weighed and then stored for chemical analyses. Chemical content and meat quality were evaluated using FoodScan technology and a taste panel assessed organoleptic properties of the meat.

175

#### 176 2.5. Microbiological analysis

Faecal swabs, caecal and litter samples were taken on day 25 and 42, respectively, for PCR amplification (Suppl. Table 2) to confirm the presence of *C. jejuni* (Suppl. Fig. 1). In addition, litter samples from days 25 and 42 and caecal samples from day 42 and were collected and analysed for *C. jejuni* analysis by conventional culture (Suppl. Fig. 2, 3 and 4). The caeca of two birds per pen were sampled. A sterile scalpel was used to cut off the blind end of both caecal sacks from each

182 sampled chicken. For each sample, 1 gr of content from each caecal sack (left and right), in total 2 gr, was weighed into sterile Universal bottles, diluted with 4 ml sterile Maximum Recovery Diluent 183 (MRD, Oxoid Basingstoke, UK), and mixed thoroughly. This constituted the 1:2 dilution (w/v). 184 Further eight serial dilutions of 1:30 were made in MRD and 10 µl of each dilution were inoculated 185 on CCDA and Brilliance CampyCount Agar (Oxoid, Basingstoke, UK). Plates were incubated 186 microaerophilically at 42°C for 48 hr and then assessed for the presence or absence of 187 thermotolerant Campylobacter species. Plates of an appropriate dilution were selected and colonies 188 189 enumerated.

190 As a confirmatory measurement, two colonies from each presumptively positive plate were selected and sub-cultured onto paired blood agar plates (Oxoid, Basingstoke, UK). These plates 191 192 were incubated at 37°C for 48 hr, one plate aerobically, one plate microaerophilically. The presence of *Campylobacter* was indicated by a lack of growth aerobically and colonies with *Campylobacter* 193 morphology that grow microaerophilically. In addition to this, Gram stains were carried out on all 194 presumptively positive samples. As a further step, oxidase strips (Oxoid, Basingstoke, UK) were 195 196 used to confirm that samples were oxidase positive (Corry et al., 1995; Cowan and Steel, 1965).

Coccidial OPGs were also determined in excreta samples taken from each subgroup daily 197 198 for the first and second day that blood presence was noticed in faeces. Sampling was carried out 199 by collecting randomly 50 g samples of excreta, two times per day from each cage for 2 consecutive days. OPGs were also determined in excreta samples from each subgroup at the end of the trial at 200 201 the birds that had bloody diarrhoea. Samples collected from each subgroup were placed in separate airtight plastic bags, homogenized thoroughly by a domestic mixer, and kept refrigerated until 202 203 assessed for total oocyst counts. Homogenized samples were ten-fold diluted with water to be 204 further diluted with saturated NaCl solution at a ratio of 1:10. OPGs were determined using McMaster chambers (Hodgson, 1970).

10

206

## 207 2.6. DNA extraction

In a PCR tube (300 μl; Starlab PCR Product), 5-10 random colonies were dissolved into
100 μl TE 10:1. The DNA was denatured by boiling for 10 min. The tube was centrifuged at 20,000
g (4°C) for 5 min. The samples were diluted 1:10 in TE 10:1, recommended by the EURL-AR
(Denis *et al.*, 1999; Van de Giessen *et al.*, 1998; Vandamme *et al.*, 1997).

212

## 213 2.7. PCR protocol

Colony PCR: Speciation of *Campylobacter* strains is important for strain characterization and for selecting the right interpretative criteria for the correct categorization of the antimicrobial susceptibility profile. The primer sets in this multiplex PCR protocol target the identification of *C*. *jejuni* and *Campylobacter coli* based on the amplification of the two genes, *mapAC. jejuni* and *ceuE C. coli* (Suppl. Table 2). In addition, a 16S primer set has been included as quality assurance of the DNA-preparation and analysis (internal control), DTU food (National Food Institute) recommended by the EURL-AR.

Briefly, PCR was carried out using the Phusion® High-Fidelity PCR Master Mix (New England Biolabs) in a total volume of 25  $\mu$ l containing 0.5 U (1.0 U/50  $\mu$ l) of Phusion DNA polymerase, 500 nM of each primer, 200  $\mu$ M of each dNTP (Deoxyribonucleotide mix, 10 mM each), 5  $\mu$ l of 5xPCR buffer with 3% v/v DMSO (New England Biolabs) and 10-100 ng DNA template. The PCR amplification conditions were: initiation step for 3 min at 95°C, denaturation step for 30 sec at 98°C, annealing for 15 sec at 60°C, extension step for 1min at 72°C, (for 30 cycles) and a final extension for 5 min at 72°C.

Agarose gel electrophoresis: 1.5% agarose gel electrophoresis was used to analyse the PCR
products. The prepared gel was stained with 5% 10 μg/ml Gel Red (Biotium) and run in 1xTris

Boric EDTA buffer (TBE buffer, Sigma) at 100 V for 45 min. The gel was visualized using an
ultraviolet trans-illuminator to detect the gel red labeled DNA. Then 2-log (0.1-10.0 kb) DNA
ladder mixes (New England Biolabs, USA) were used to estimate the size of PCR product.

233

## 234 2.8. Meat chemical analysis

The breast and thigh meat samples collected at day 42 were analysed for moisture, crude protein and fat content, by near infra-red spectroscopy using a FoodScanTM Lab (FOSS, Denmark) in transmittance mode. Initially samples were thawed at room temperature (20° C), the breast (*Pectoralis major*) and the thigh (*Biceps femoris*) meat was carefully separated from the skin and the bones, minced (Cutter K35, Electrolux) and then 200 g of the minced meat was placed in the sample tray of the FoodScan. Contents of fat, moisture and protein, were determined by the reference method AOAC 2007.04 for meat and meat products (Anderson, 2007; AOAC, 2007).

242

## 243 2.9. Meat sensory attributes estimation using a panel test

Before the test, 12 frozen carcasses per treatment (2 carcasses per pen selected at random) 244 were removed from the freezer and held at 4° C for 2 days for thawing. Then the carcasses were 245 246 cut up. Breast muscles were separated from the bone and cut to stripes (10 x 2 x 2 cm). Thighs were removed from the carcass and cut to smaller pieces. The breast pieces of each group were put 247 into separate grill baskets and then cooked at the same time for 12 min. The cooked pieces from 248 249 each group were placed in a large plate, were assigned a random letter and then were presented to the panel test members at the same time for scoring. This process was repeated for the thigh meat 250 251 pieces.

The sensory panel consisted of 14 participants (both males and females; ages from 22 to 65 years). The participants were asked to record their degree of liking the appearance, tenderness, juiciness and overall preference of the cooked pieces, as described by Smith *et al.*, (2012). Panelists
were given water, an unsalted snack and napkins before each new sample.

Each parameter was set up on a hedonic scale from 1 (negative perception) to 9 (positive perception) (AMSA, 2015). Panelists were also asked to provide additional comments if they chose to. Each participant provided scores for all samples from all treatment groups.

259

## 260 2.10. Statistical analysis

The basic study design was RCB (random complete block design), and the pen was 261 262 considered the experimental unit for all parameters. The basic statistical model employed was ANOVA. Significant differences were declared at  $p \leq 0.05$ , while near significant trends were 263 considered for 0.05 . Means were separated by Tukey's Test. IBM SPSS (Version 20)264 265 was used as the statistical program. To assess if Campylobacter infection intensity was homogeneous between pens, the distribution of Campylobacter counts obtained from the litter 266 samples taken on days 25 and 42 were assessed. If Campylobacter counts were randomly 267 distributed among pens, the counts obtained should follow a Poisson distribution, where variance 268 equals the mean. If variance exceeds the mean this indicates overdispersion and demonstrates that 269 270 the counts are not homogenous. The distribution of *Campylobacter* spp. counts from the litter were 271 assessed for overdispersion by multiplying the variance to mean ratio by the number of degrees of 272 freedom, and comparing the results with the chi-square distribution (Bliss and Fisher, 1953). 273 Overdispersion was confirmed when p < 0.05. The same analysis was applied to the caecal Campylobacter counts obtained on day 42. 274

Incidence data (coccidia, diarrhoea, bloody diarrhoea) and the sensory panel data were
analysed using binary logistic regression using the generalized linear model function in R (RStudio,

Version 3.3.3 (The R Foundation for Statistical Computing, 2017)), specifying the family as binomial, linked to logit transformation. *p*-values of  $\leq 0.05$  were considered statistically significant, whereas values of 0.05 were declared a near-significant trend. The Hosmer-Lemeshowtest (Hosmer and Lemeshow, 2005) was used to assess overall model fit using the'ResourceSelection' package (Lele, 2009).To test if there was a significant difference (*p*<0.05) in the proportion of birds in eachtreatment group with normal intestinal pathology, a binomial test was performed using the prop.test

284 function in RStudio.

285

- 287 **3. RESULTS**
- 288 3.1. Performance data

Performance parameters measured from 0 to 42 days on trial indicated that the dietary supplementation of ferric tyrosine did not have any significant effects on body weight, weight gain, feed intake and feed efficiency (Table 2). General health was good with low mortality until the end of the trial (Suppl. Table 3).

293

## 294 3.2. Campylobacter

295 On day 25 (5 days after introduction of contaminated litter) C. jejuni was isolated from pen litter samples (Suppl. Fig. 2). All pens were infected with C. *jejuni* and the counts were evenly 296 distributed among the pens. However, C. jejuni counts were significantly lower in T<sub>4</sub> birds 297 298 compared to those in T1, T<sub>2</sub> and T<sub>3</sub> (p = 0.007). At the end of the study (day 42), C. jejuni counts 299 in the litter did not differ significantly between groups, but lower contamination was observed in 300 the  $T_4$  group (Suppl. Fig. 3). C. *jejuni* counts in bird caeca on day 42 were significantly lower in the T<sub>4</sub> group, compared to the T<sub>1</sub> Control, T<sub>2</sub> and T<sub>3</sub> groups (p = 0.004, Fig. 1 and Suppl. Fig. 4). 301 None of the pens were negative either at day 25 or 42 and the counts were evenly distributed among 302 the pens while at day 42 all birds were infected (Suppl. Fig. 2, 3, and 4) and no negative counts 303 were observed, suggesting that all birds were exposed initially to a similar level of infection at day 304 305 20.

306

307 3.3. Health, coccidiosis and diarrhoea

An *E. tenella* infection occurred during the trial, most probably due to the commercial litter introduced at 20 days on trial, and to the absence of coccidiostats in the diet. Clinical and postmortem examinations were carried out to examine abnormalities in the birds' intestines (Johnson, 311 J. et al., 1970; Tsiouris et al., 2013; Tsiouris et al., 2015). The infection was detected in the ceca and was identified by accumulation of blood in the ceca, bloody droppings, pathologoanatomic 312 severe lesions and large numbers of OPG. Post mortem exams showing caecal cores with 313 accumulations of clotted blood further supported the presence of *E. tenella* infection (Suppl. Fig. 314 5 and 6). Intestinal smears were evaluated under microscopy to establish the presence of OPG after 315 316 post mortem examination. On day 21, two birds died from  $T_3$  group died from *E. tenella*. The intestines of these two birds were examined for coccidial oocysts, E. coli and Clostridium 317 perfringens. E. tenella was isolated and large numbers oocysts were microscopically observed in 318 319 both caecal samples. On days 28 and 29, for animal welfare reasons, all birds on trial were treated with an anti-coccidial drug against *E. tenella* (Baycox: 25 mg Toltrazuril /ml solution, 1 L/1000 L 320 drinking water for 48 hours). The incidence of coccidia was significantly lower in T<sub>4</sub> birds 321 compared to control group (p = 0.005, Fig. 2), and a near significant reduction was observed in T<sub>3</sub> 322 birds compared to  $T_1$  (p = 0.07, Fig. 2). A near significant reduction in the incidence of bloody 323 diarrhoea was observed in T<sub>3</sub> and T<sub>4</sub> birds compared to T<sub>1</sub> birds (p = 0.06, Fig. 2) and the incidence 324 of diarrhoea was significantly lower in  $T_3 \& T_4$  birds compared to the control birds (p = 0.05 and 325 p = 0.024, respectively, Fig. 2). 326

Diarrheal scores of all pens were checked from day 24 to the end of the study (day 42). From Day 26 to 36, the incidence of diarrhoea was 100% (6/6) in all pens from all treatment groups. However, the average diarrhoea score was lower in the birds fed the  $T_2$ ,  $T_3$  and  $T_4$  diets compared to the control (Suppl. Table 4). Furthermore, on day 42 100% (6/6) of pens from the  $T_1$  group had diarrhoea compared to 17% (1/6).

On day 42, the intestines of 37 birds per treatment group were examined post mortem. Significantly more birds fed the T<sub>4</sub> diet had normal intestinal tracts compared to the control birds (p = 0.007,Suppl. Table 5). 335

## 336 *3.3. Meat proximate analysis (FoodScan)*

337 In the breast meat samples, the  $T_4$  group tended to have a higher protein content compared to the

- 338 T<sub>3</sub> group (p = 0.087, Suppl. Table 6). In the thigh meat samples, the T<sub>3</sub> group had significantly
- lower fat (p = 0.006) compared to groups T<sub>1</sub> and T<sub>4</sub>, and significantly higher moisture (p = 0.001)
- compared to groups  $T_1$ ,  $T_2$  and  $T_4$ . The  $T_4$  group had significantly lower (p = 0.002) protein content
- $341 \quad \text{ compared to groups } T_1, \, T_2 \text{ and } T_3.$
- 342

## 343 *3.4. Meat sensory attributes (Panel Test)*

A sensory panel of 14 members recorded their degree of liking of cooked breast and thigh meat. Regarding breast meat, the T<sub>3</sub> group had significantly better scores in tenderness (p = 0.002) and juiciness (p = 0.008) compared to T<sub>1</sub> and T<sub>4</sub> (Fig. 3). T<sub>2</sub> and T<sub>3</sub> groups had significantly better scores in "*like overall*" than T<sub>4</sub> (Fig. 3). No significant differences (p > 0.05) in sensory parameters were noted for cooked thigh meat.

349

#### 351 4. DISCUSSION

352 Considerable global efforts are being made to prevent human campylobacteriosis by nonantibiotic means, due to public concerns about over-reliance of antibiotics in farming with 353 inconclusive results (Gracia et al., 2016; Guyard-Nicodeme et al., 2016; Hermans et al., 2011; Zhu 354 355 *et al.*, 2006). Despite intensive efforts during the last decades, effective and reliable methods to 356 stop or limit *Campylobacter* colonization in poultry do not exist. Plant essential oils and short-357 chain fatty acids, feed acidification and combinations of lactic acid bacteria with fermented low pH feed at most delayed only the onset of *Campylobacter* colonization and reduced moderately 358 fecal counts (Gracia et al., 2016; Guyard-Nicodeme et al., 2016; Hermans et al., 2011). In this 359 360 study, ferric tyrosine, a non-antibiotic feed additive (Khattak et al., 2018; Currie et al., 2018), was 361 evaluated for possible benefits in chicken nutrition, welfare, zootechnical parameters, meat quality and for efficacy in reducing natural *Campylobacter* colonization in the chicken intestinal tract. 362

363 Campylobacter colonization of chicken intestinal tracts is usually commensal and without noticeable effects in performance parameters, although there are reports that Campylobacter can 364 be detrimental for the birds in some instances (Humphrey et al., 2014). Ferric tyrosine 365 supplementation resulted in significant reduction of C. *jejuni* contamination of pen litter 5 days 366 after a natural challenge introduced via infected litter. By the last day of the trial, the groups 367 supplemented with ferric tyrosine, especially the T<sub>4</sub> group (0.20 g ferric tyrosine/kg feed), had 368 lower diarrheal scores (Suppl. Table 4), lower C. jejuni caecal counts and a lower percentage of 369 birds with abnormal intestinal tracts (related to post-mortem evidence of coccidiosis, diarrhea or 370 bloody diarrhea), (Fig. 2 and Suppl. Fig. 5 and 6). Faecal *C. jejuni* contamination is one of the main 371 ways of diffusion through the food chain (Santini *et al.*, 2010). Under practical farming conditions 372 it is very difficult to avoid contact between chickens and Campylobacter (Hermans et al., 2011). 373

For this reason, even a partial reduction of contamination of the environment and the carcasses is very important when considering the risks of human campylobacteriosis (Hermans *et al.*, 2011). For example, it has been reported that the incidence of disease in humans could be reduced by 48%, 85% and 96%, if carcass contamination by *Campylobacter* can be reduced by 1, 2 or 3 log<sub>10</sub> CFU, respectively (Messens *et al.*, 2007). Consistent with previous studies (Khattak *et al.*, 2018; Currie *et al.*, 2018), here we show that ferric tyrosine has the potential to be efficacious in the prevention or reduction of the infection of poultry with *Campylobacter* (Fig. 1).

During the experimental trial, an unexpected coccidial infection was diagnosed, possibly 381 382 due to the absence of coccidiostats in the diets. Birds' symptoms (bloody diarrhea) and following tests (post mortem exams and microscopy of intestinal smears) implicated E. tenella as the main 383 pathogen. It was noticed that the groups supplemented with increased levels of ferric tyrosine had 384 lower incidence of diarrhea in the pens and of abnormal digestive tract and bloody diarrhea (in 385 post-mortem examination), suggesting a possible protective effect against the parasite (Suppl. Fig. 386 5 and 6). During a coccidial infection, inflammatory cytokines produced by the immune system 387 can stimulate a number of cell types, including primed host macrophages, to synthesize large 388 quantities of NO by an induced NO synthase (iNOS) (Liew and Cox, 1991). NO has strong oxidant 389 390 properties and can react with intracellular iron-containing compounds, becoming toxic to both the coccidian and the cells infected by the parasite (Allen, 1997). Ferric tyrosine may be acting via this 391 392 pathway but its precise molecular mode of action in conferring protection against coccidiosis is 393 currently unknown.

Ferric tyrosine did not adversely affect meat quality as all breast and thigh meat samples had chemical compositions and sensory characteristics within the expected and acceptable range for the consumer, although minor differences were noticed mainly in the thigh meat. It is possible, that the protective effect of ferric tyrosine against *C. jejuni* challenge resulted in a healthier gut microbiome with beneficial effects on nutrient absorption and metabolism that affected meat tissue
formation (Giannenas *et al.*, 2015; Rincker *et al.*, 2004).

400	L-Tyrosine (4-hydroxyphenylalanine), is an essential amino acid used in the synthesis of
401	proteins (Chinevere et al., 2002; EFSA, 2013; NCBI, 2017). As such it is ubiquitous in the natural
402	environment, and in animal proteins, including chicken and turkey meat. L-tyrosine is approved
403	for use as a feed additive in the EU (EFSA, 2013). In the EU, food animal diets may be
404	supplemented with up to 0.5% tyrosine, equivalent to 5.0 g/kg feed. In the current study, dietary
405	supplementation with ferric tyrosine at 0.20 g/kg feed (T <sub>4</sub> ), resulted in a tendency to improve the
406	FCR, demonstrating value as a non-antibiotic alternative to support poultry health. This will be a
407	significant factor to investigate further, particularly as there is evidence that lower slaughtering
408	mass after C. jejuni infection may be due to the reduction in the feed efficiency, even though no
409	differences were observed in the average daily feed intake between control and infected birds
410	(Awad <i>et al.</i> , 2015).

411

#### 412 **5. CONCLUSION**

In this trial, ferric tyrosine was evaluated as a feed additive for broiler chickens to prevent 413 natural Campylobacter colonization and to support growth performance. Ferric tyrosine did not 414 adversely affect growth performance and exerted a significant inhibitory effect against C. jejuni 415 colonization in the gastrointestinal tract, limiting intestinal damage and lowering C. jejuni loads in 416 the chicken intestine and faeces. During the study, natural infection with E. tenella gave us the 417 opportunity to discover that ferric tyrosine also ameliorates the negative health effects of 418 coccidiosis in broilers. The data from this study indicate that ferric tyrosine seem to be a promising 419 420 feed additive for the poultry industry.

## 422 ACKNOWLEDGEMENTS

423 This research was financed by Akeso Biomedical, Inc., USA. Akeso Biomedical helped in the

424 design of the study but had no involvement in the collection and analysis of the data, in the writing

425 of the report and in the decision to submit the article for publication. P.S. and J.M. are shareholders,

426 licensors and consultants of Akeso Biomedical.

427

## 429 **REFERENCES**

- Allard, R., Plante, C., Garnier, C., Kosatsky, T., (2010). The reported incidence of
  campylobacteriosis modelled as a function of earlier temperatures and numbers of cases,
  Montreal, Canada, 1990-2006. Int. J. Biometereol. 55, 353-360.
- 433 Allen, P.C., (1997). Poultry Sci. 76:810-813.
- AMSA, (2015). Research guidelines for cookerey, sensory evaluation, and intrumental tenderness
   measurement of meat. American Meat Science Association, Champaign, IL, USA.
- Anderson, S., (2007). Determination of fat, moisture, and protein in meat and meat products by
  using the FOSS FoodScan near-infrared spectrophotometer with FOSS artificial neural
  network calibration model and associated database: collaborative study. J. AOAC Int. 90,
  1073-1083.
- 440 AOAC, (2007). Official Methods of Analysis, 18th ed. Association of Analytical Chemists, AOAC
  441 International, Arlington, Virginia, USA.
- 442 Awad WA, Molnár A, Aschenbach JR, Ghareeb K, Khayal B, Hess C, Liebhart D, Dublecz
- K, Hess M., (2015). Campylobacter infection in chickens modulates the intestinal epithelial
  barrier function. Innate Immun. 21(2):151-60.
- Bi, P., Cameron, A.S., Zhan, g.Y., Parton, K.A., (2008). Weather and notified *Campylobacter*infections in temperate and sub-tropical regions of Australia: an ecological study. J. Infect.
  57, 317-323.
- Bliss, C.I., Fisher, R.A., (1953). Fitting the negative binomial distribution to biological data, with
  a note on the efficient fitting of the negative binomial. Biometrics, 9, 176.
- 450 CDC, (2014). Campylobacter. CDC, Centers for Diseae Control and Prevention.
- Chaveerach, P., Kauzenkamp, D.A., Lipman, L.J.A., Van Knapen, F., (2004). Effect of organic
  acids in drinking water for young broilers on Campylobacter infection, volatile fatty acid
  production, gut microflora and histologial cell changes. Poult. Sci. 83, 330-334.
- Chinevere, T.D., Sawyer, R.D., Creer, A.R., Conlee, R.K., Parcell, A.C., (2002). Effects of Ltyrosine and carbohydrate ingestion on endurance exercise performance. J. Appl. Physiol.
  93, 1590-1597.
- 457 Corry, J.E.L., Post, D.E., Colin, P., Laisney, M.J., (1995). Culture media for the isolation of
  458 Campylobacters. Int. J. Food Microbiol. 26, 43-76.

- Cowan, S.T., Steel, K.J., (1965). Characters of Gram-negative bacteria, in: Barrow, G., Feltham,
  R. (Eds.), Cowan and Steel's Manual for the Identification of Medical Bacteria. Cambridge
  University Press, Cambridge, pp. 94-164.
- 462 Currie, D., Green, M., Dufailu, O.A., Pitoulias, M., Soultanas, P., McCartney, E., Lester, H., Van
  463 Den Eede, L., Apajalathi, J., Mahdavi, J., (2018). Dietary supplementation with ferric
  464 tyrosine improves zootechnical performance and reduces caecal Campylobacter spp. load
  465 in broilers. Br. Poultr. Sci. doi: 10.1080/00071668.2018.1507015.
- Denis, M., Soumet, C., Rivoal, K., Ermel, G., Blivet, D., Salvat, G., Colin, P., (1999). Development
  of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett.
  Appl. Microbiol. 29, 406-410.
- 469 EFSA, (2013). Scientific opinion on the safety and efficacy of L-tyrosine for all animal species.
  470 EFSA J. 11, 3310.
- 471 Fitzerald, C., (2015). *Campylobacter*. Clin. Lab. Med. 35, 289-298.
- Fleury, M., Charron, D.F., Holt, J.D., Allen, O.B., Maarouf, A.R., (2006). A time series analysis
  of the relationship of ambient temperature and common bacterial enteric infections in two
  Canadian provinces. Int. J. Biometereol. 50, 385-391.
- Founou, L.L., Founou, R.C., Essack, S.Y., (2017). Antibiotic resistance in the food chain: a
  developing country-perspective. Front Microbiol. 7, 188.
- Gaucher, M.-L., Quessy, S., Letellier, A., Arsenault, J., Boulianne, M., (2015). Impact of a drugfree program on broiler chicken growth performances, gut health, *Clostridium perfringens*and *Campylobacter jejuni* occurrences at the farm level. Poult. Sci. 94, 1791-1801.
- Giannenas, I., Skoufos, I., Bonos, E., Sarakatsianos, I., Tzora, A., Skoufos, S., Christaki, E., Florou
  Paneri, P., (2015). Effect of dietary iron sulfate and iron chelate on growth performance,
  hematlogical traits, intestinal microbial flora of fattening pigs and quality parameters of
  porkmeat. J. Vet. Sci. Med. 3, 11.
- Gracia, M.I., Millan, C., Sanchez, J., Guyard-Nicodeme, M., Mayot, J., Carrer, Y., Csorbai, A.,
  Chemaly, M., Medel, P., (2016). Efficacy of feed additives against *Campylobacter* in live
  broilers during the entire rearing period: Part B Poult. Sci. 95, 886-892.
- Guyard-Nicodeme, M., Keita, A., Quesne, S., Amelot, M., Pezevara, T., Le Berre, B., Sanchez, J.,
  Vesseur, P., Martin, A., Medel, P., Chemaly, M., (2016). Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period. Poult. Sci. 95, 298-305.

- Hearnden, M., Skelly, C., Eyles, R., Weinstein, P., (2003). The regionality of campylobacteriosis
  seasonality in New Zealand. Int. J. Environ. Health Res. 13, 337-348.
- Herman, L., Heyndrickx, M., Grijspeerdt, K., Vandekerchove, D., Rollier, I. and De Zutter, L.
  (2003). Routes for *Campylobacter* contamination of poultry meat: Epidemiological study
  from hatchery to slaughterhouse. Epidemiology and Infection, 131, 1169-80.
- Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F.,
  Rasschaert, G., Heyndrickx, M., Pasmans, F., (2011). Campylobacter control in poultry by
  current intervention measures ineffective: urgent need for intensified fundamental research.
  Vet. Microbiol. 152, 219-228.
- Hodgson, J. N. (1970). Coccidiosis: oocyst-counting technique for coccidiostat evaluation. *Experiment. Parasitol.* 28, 99-102.
- Hosmer, D.W., and Lemeshow, S., (2005). Applied Logistic Regression, Second Edition Print
   ISBN:9780471356325 |Online ISBN:9780471722144 |DOI:10.1002/0471722146
- Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., (2014).
   *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affect
   birds welfare. MBio 5, UNSP e01364-01314.
- Johnsen, G., Kruse, H. & Hofshagen, M. (2006) Genetic diversity and description of transmission
   routes for *Campylobacter* on broiler farms by amplified-fragment length polymorphism.
   Journal of Applied Microbiology, 101, 1130-39.
- Johnson, J., Reid, W.M. (1910). Anticoccidial drugs: Lesion scoring techniques in battery and
  floor-pen experiments with chickens. Experimental Parasitology. 28(1): 30-36.
- Jorgensen, F., Ellis-Iversen, J., Rushton, S., Bull, S.A., Harris, S.A., Bryan, S.J., Gonzalez, A.,
   Humphrey, T.J., (2011). Influence of season and geography on *Campylobacter jejuni* and
- 513 *C. coli* subtypes in housed broiler flocks reared in Great Britain. Appl. Environ. Microbiol.
  514 77, 3741-3748.
- Khattak, F., Paschalis, V., Green, M., Houdijk, J., Soultanas, P., Mahdavi, J., (2018). TYPLEX, a
  novel feed additive, inhibits *Campylobacter jejuni* biofilm formation and caecal
  colonization in broiler chicken. Poult. Sci., 97, 1391-1399.
- Kovats, R., Edwards, S., Charron, D., Cowden, J., D'Souza, R., Ebi, K., Gauci, C., Gerner-Smidt,
  P., Hajat, S., Hales, S., Hernadez Pezzi, G., Kriz, B., Kutsar, K., McKeown, P., Mellou, K.,

- 520 Menne, B., O'Brien, S., van Pelt, W., Schmid, H., (2005). Climate variability and 521 campylobacter infection: an international study. Int. J. Biometereol. 49, 207-214.
- Lagha, A.B., Haas, B., Gottschalk, M., Grenier, D., (2017). Antimicrobial potential of bacteriocins
  in poultry and swine production. Vet. Res. 48, 22.
- Lele, S.R., (2009). A new method for estimation of resource selection probability function. Journal
   of Wildlife Management 73, 122–127.
- Liew, F.Y., Cox, F.E.G., (1991). Nonspecific defence mechanism: the role of nitric oxide. Immun.
  Today 12:A17-21.
- Messens, W., Hartnett, E., Gellynck, X., Viaene, J., Halet, D., Herman, L., Grijspeerdt, K., (2007).
  Quantitative risk assessment of human campylobacteriosis through the consumption of
  chicken meat in Belgium, XVIII European Symposium on the Quality of Poultry Meat and
  The XII European Symposium on the Quality of Eggs and Egg products, Prague, Czech
  Republic, pp. 167-168.
- NCBI, (2017). L-tyrosine. CID=6057. National Center for Biotechnology Information. PubChem
  Compound Database.
- Newell, D.G., Wagenaar, J.A., (2000). Poultry infections and their control at the farm level.
   *Campylobacter* 2<sup>nd</sup> Washington, DC; American Society for Microbioloogy, 497-509.
   Editors: Nachamkin, I., Blaser, M.J.
- 538 NRC, (1994). Nutrient Requirements of Poultry, 9<sup>th</sup> Rev. National Academy Press, Washington,
  539 USA.
- 540 NRC, (1996). Guide for the care and use of laboratory animals. National Academy Press,
  541 Washington, DC.
- 542 Rincker, M.J., Hill, G.M., Link, J.E., Rowntree, J.E., (2004). Effects of dietary iron
  543 supplementation on growth performance, hematological status, and whole-body mineral
  544 concentrations of nursery pigs. J. Anim. Sci. 82, 3189-3197.
- Sahin, O., Huang, L.N., Zhang, Q., (2003). Effect of *Campylobacter*-specific maternal antibodies
  on *Campylobacter jejuni* colonization in young chickens. Appl. Environ. Microbiol., 69,
  5372-5379.
- Santini, C., Baffoni, L., Gagia, F., Granata, M., Gasbarri, R., Di Gioia, D., Biavatti, B., (2010).
  Characterization of probiotic strains: An application as feed additives in poultry against *Campylobacter jejuni*. Int. J. Food Microbiol. 141, S98-S108.

- Smith, D.P., Northcutt, J.K., Steinberg, E.L., (2012). Meat quality and sensory attributes of a
  conventional and a Label Rouge-type broiler strain obtained at retail. Poult. Sci. 91, 14891495.
- Stern, N.J., Cox, N.A., Bailey, J.S., Berrang, M.E., Musgrove, M.T., (2001). Comparison of
  mucosal competitive exclusion and competitive treatment to reduce Salmonella and
  Campylobacter spp. colonization in broiler chickens. Poul. Sci. 80, 156-160.
- Thanner, S., Drissner, D., Walsh, F., (2016). Antimicrobial resistance in agriculture. MBio 7,
  302227-302215.
- Thibodeau, A., Fravalo, P., Yergeau, E., Arsenault, J., Lahaye, L., Letellier, A., (2015). Chicken
  caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by a
  non-antibiotic feed additive. PLOS One 10, e0131978.
- Thormar, H., Hilmarsson, H., Bergsson, G., (2006). Stable concentrated emulsions of the 1monoglyceride of capric acid (monocaprin) with microbicidal activities against the foodborne bacteria *Campylobacter jejuni*, *Salmonella spp.*, and *Escherichia coli*. Appl. Environ.
  Microb. 72, 522-526.
- Tsiouris, V., Georgopoulou, I., Batzios, Chr., Pappaioannou, N., Diakou, A., Petridou, E.,
  Ducatelle, R. & Fortomaris, P., (2013). The role of an attenuated anticoccidial vaccine on
  the intestinal ecosystem and on the pathogenesis of experimental necrotic enteritis in broiler
  chickens. Avian Pathology, 42, 163-170.
- Tsiouris, V., Georgopoulou, I., Batzios, Chr., Pappaioannou, N., Ducatelle, R. & Fortomaris, P.,
  (2015). High stocking density as a predisposing factor for necrotic enteritis in broiler
  chicks. Avian Pathology, 44, 59-66.
- 573 Van de Giessen, A., Tilburg, J., Ritmeester, W., Van de Plas, J., (1998). Reduction of
  574 Campylobacter infection in boiler flocks by application of hygiene measures. Epidemiol.
  575 Infect. 121, 57-66.
- Vandamme, P., Van Doom, L.J., Al Rashid, S.T., Quint, W.G.V., Van der Plas, J., Chan, V.L., On,
  S.L.W., (1997). *Campylobacter hyoilei* Alderton *et al.*, 1995 and *Campylobacter coli* Veron
  and Cahatelain 1973 are subjective synonyms. Int. J. Syst. Bacteriol. 47, 1055-1060.
- Vender, H., Henningsen, M.L., Begg, S.L., (2017). Antimicrobial resistance in healthcare,
  agriculture and the environment: the biochemistry behind the headlines. Essays Biochem.
  61, 1-10.

- Weisent, J., Seaver, W., Odoi, A., Rohrbach, B., (2014). The importance of climatic factors and
  ouliers in predicting regional monthly campylobacteriosis risk in Georgia, USA. Int. J.
  Biometereol. 58, 1865-1878.
- Zhu, J., Zhang, Y., Hua, X., Hou, J., Jiang, Y., (2006). Antibiotic resistance in *Campylobacter*.
  Rev. Med. Microbiol. 17, 107-121.

587

5	58	39
5	59	90

Table 1. Composition and calculated analyses of basal diets.

In gradiants $(0)$	<mark>Starter Mash</mark>	Grower Mash	
Ingredients (%)	1-21 days of age	22-42 days of age	
Wheat	<mark>64.0</mark>	<mark>64.7</mark>	
<b>Barley</b>	<mark>-</mark>	<mark>3.0</mark>	
Soybean meal, 48% CP*	<mark>28.0</mark>	<mark>25.0</mark>	
Sodium bicarbonate	<mark>0.13</mark>	<mark>0.22</mark>	
Soy protein concentrate 66%	<mark>2.50</mark>		
Soy oil	<mark>2.00</mark>	<mark>4.00</mark>	
L-lysine HCl	<mark>0.13</mark>	<mark>0.18</mark>	
DL-methionine	<mark>0.12</mark>	<mark>0.16</mark>	
Choline chloride	<mark>0.07</mark>	<mark>0.07</mark>	
Dicalcium phosphate	<mark>0.50</mark>	<mark>0.50</mark>	
Calcium carbonate	<mark>1.90</mark>	<mark>1.50</mark>	
Sodium chloride	<mark>0.16</mark>	<mark>0.16</mark>	
Minerals and vitamins <sup>1</sup>	<mark>0.50</mark>	<mark>0.50</mark>	
Total	<mark>100</mark>	<mark>100</mark>	
Calculated analyses			
<mark>ME Broiler, Kcal/kg</mark>	<mark>3.0</mark>	<mark>3,1</mark>	
Crude protein %	<mark>21.4</mark>	<mark>19.0</mark>	
Crude fibre, %	<mark>3.2</mark>	<mark>3.2</mark>	
Ash, %	<mark>6.0</mark>	<mark>5.4</mark>	
Moisture, %	<mark>12.3</mark>	<mark>12.1</mark>	
Crude fat <mark>%</mark>	<mark>3.5</mark>	<mark>5.3</mark>	
Lysine, %	<mark>1.2</mark>	<mark>1.1</mark>	
Methionine, %	<mark>0.5</mark>	<mark>0.4</mark>	
Methionine + cysteine, %	<mark>0.6</mark>	<mark>0.5</mark>	
Threonine, %	<mark>0.8</mark>	<mark>0.7</mark>	
Tryptophan, %	<mark>0.3</mark>	<mark>0.2</mark>	
Calcium, %	<mark>0.9</mark>	<mark>0.8</mark>	
Sodium, %	<mark>0.1</mark>	0.2	

- 593 mg; Vit. B2: 8 mg; Vit. B6: 5 mg; Vit. B12: 11 mcg; Folic acid: 1.5 mg; Biotin: 150 mcg; Ca
- pantothenate: 25 mg; nicotinic acid: 65 mg; Ethoxyquin: 150 mg; Mn: 60 mg; Zn: 40 mg; I: 0.33
- 595 mg; Cu: 8 mg; Se 0.15 mg; No exogenous Fe was added.
- 596 \*Soybean was used to premix the ferric tyrosine for  $T_2$ - $T_4$  diets.
- 597
- 598

<sup>&</sup>lt;sup>1</sup>Supplies per kg: Vit. A: 12,000 IU; Vit. D3: 2,400 IU; Vit. E: 30 mg; Vit K3: 3 mg; Vit. B1: 2.2

**Table 2.** Effect of dietary addition of ferric tyrosine on broiler performance parameters.

Period: 1-21 days								
<b>Treatment</b>	<mark>BW, 1 d (g)</mark>	<mark>BW, 21 d (g)</mark>	ADG (g)	<mark>ADFI (g)</mark>	FCR (feed/gain)			
$T_1$	45.8	<mark>655</mark>	30.5	53.8	<mark>1.77</mark>			
T <sub>2</sub>	45.9	<mark>653</mark>	30.3	54.0	<mark>1.78</mark>			
T <sub>3</sub>	46.4	<mark>680</mark>	31.7	54.5	<mark>1.73</mark>			
$T_4$	45.8	<mark>659</mark>	30.7	54.9	<mark>1.79</mark>			
<mark>SEM</mark>	<mark>0.220</mark>	<mark>8.18</mark>	<mark>0.41</mark>	<mark>0.36</mark>	<mark>0.020</mark>			
p (value)	0.763	<mark>0.64</mark>	<mark>0.65</mark>	<mark>0.70</mark>	<mark>0.46</mark>			
	Period: 22-42 days							
	<mark>BW, 22 d (g)</mark>	<mark>BW, 42 d (g)</mark>	<mark>ADG (g)</mark>	<mark>ADFI (g)</mark>	FCR (feed/gain)			
T <sub>1</sub>	<mark>655</mark>	<mark>2,142</mark>	70.8	<mark>142</mark>	<mark>1.99</mark>			
T <sub>2</sub>	<mark>653</mark>	<mark>2,136</mark>	70.6	<mark>141</mark>	<mark>1.98</mark>			
T <sub>3</sub>	<mark>680</mark>	<mark>2,173</mark>	71.1	<mark>141</mark>	<mark>1.98</mark>			
<mark>T</mark> 4	<mark>659</mark>	<mark>2,261</mark>	76.3	<mark>142</mark>	<mark>1.86</mark>			
<mark>SEM</mark>	<mark>8.18</mark>	<mark>25.3</mark>	<mark>1.09</mark>	<mark>1.73</mark>	0.02			
<mark>p (value)</mark>	<mark>0.64</mark>	<mark>0.30</mark>	<mark>0.23</mark>	<mark>0.98</mark>	<mark>0.09</mark>			
		Period: 1-4	<mark>12 days</mark>					
	<mark>BW, 1 d (g)</mark>	<mark>BW, 42 d (g)</mark>	ADG (g)	ADFI (g)	FCR (feed/gain)			
T <sub>1</sub>	45.8	<mark>2,142</mark>	51.1	<mark>100</mark>	<mark>1.93</mark>			
$T_2$	45.9	<mark>2,136</mark>	51.0	<mark>99</mark>	<mark>1.92</mark>			
T <sub>3</sub>	46.4	<mark>2,173</mark>	51.9	<mark>100</mark>	<mark>1.90</mark>			
$T_4$	45.8	<mark>2,261</mark>	54.0	<mark>100</mark>	<mark>1.84</mark>			
SEM	<mark>0.22</mark>	<mark>25.3</mark>	<mark>0.62</mark>	1.05	<mark>0.02</mark>			
p (value)	<mark>0.76</mark>	<mark>0.30</mark>	<mark>0.30</mark>	<mark>0.98</mark>	<mark>0.17</mark>			

601

 $N^{\circ}$  replicates = each treatment had 6 pens of 16 male birds/pen;

T<sub>1</sub>; Control; 0 g ferric tyrosine/kg feed, T<sub>2</sub>; 0.02 g ferric tyrosine/kg feed, T<sub>3</sub>; 0.05 g ferric

- 604 tyrosine/kg feed, and T<sub>4</sub>; 0.20 g ferric tyrosine/kg feed
- 605 SEM = Standard error of mean; BW = body weight; ADG = Average daily gain; ADFI = Average
- 606 daily feed intake; FCR = Feed Conversion Ratio (feed/gain)



# Figure 1.

Effect of dietary addition of TYPLEX<sup>TM</sup> on *C. jejuni* infection. The CFU counts ( $\log^{10}$ ) from caecal samples taken at study end (42 days on trial) (mean ± SEM). Replicates; 2 birds per pen, 6 pens per treatment i.e. 12 samples in total, and 3 plate replicates for each sample i.e. a final total of 2 x 6x 3 = 36 replicate samples. Values in the same treatment with no common <sup>abc</sup> superscript differ significantly ( $p \le 0.05$  and ns = no significance; One way ANOVA).



# Figure 2.

Effect of dietary addition of TYPLEX<sup>TM</sup> on the incidence of coccidian, diarrhea and bloody diarrhea at the end of the trial (42 days). N replicates = 144 (6 or 7 birds sacrificed per pen/treatment.



## Figure 3.

Effect of dietary addition of ferric tyrosine on sensory panel scores of cooked breast meat on appearance, tenderness, juiciness and overall. N° replicates: 48 (2 carcasses per pen/treatment) scored by 14 panelists. Results range: From 1 (negative perception) to 9 (positive perception). p < 0.05.