Evaluation of in-field efficacy of dietary ferric tyrosine on performance, intestinal health and meat quality of broiler chickens exposed to natural Campylobacter jejuni challenge

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ABSTRACT

Campylobacter is an important pathogen commonly found in chickens that can cause severe acute gastroenteritis in humans. Despite intensive efforts to inhibit food-borne transmission of *Campylobacter* no effective strategy exists to reduce *Campylobacter* loads in farmed broilers. This study examined the capacity of a novel feed additive to lower *Campylobacter jejuni* populations 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 replicates of sixteen chicks per pen. Three groups were fed the basal diets further supplemented with TYPLEX™ chelate (ferric tyrosine) at various concentrations (0.02, 0.05 and 0.20 g/kg, groups T_2-T_4, respectively). Control group (T_1) was fed basal diets in mash form that did not contain added ferric tyrosine. Feed and water were provided *ad libitum*. At 20 days of age, broilers were exposed to natural *C. jejuni* challenge by introducing contaminated litter from a commercial farm. At day 25, pen litter samples analysed positive for *C. jejuni*, and the infection intensity was homogeneous among pens. At the end of the study *C. jejuni* counts in bird caeca were significantly reduced, by 2 $\log_{10}$ in the T_4 group, compared to the T_1 Control and T_3 groups ($p = 0.004$). During this study, a natural infection with *Eimeria tenella* occurred at days 26-29. For animal welfare 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_4 group, but in 5 of 6 pens in the T_1 Control group. In addition, autopsies showed that the T_4 group had the highest 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 age all birds were slaughtered and samples collected for further analysis. Birds in the T_4 group tended to exhibit improved weight gain and feed efficiency, a result that warrants further

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

KEYWORDS

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

1. INTRODUCTION

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 public concerns about antimicrobial resistance, linked to increased risks for human health and food safety (Founou *et al.*, 2017; Santini *et al.*, 2010; Thanner *et al.*, 2016; Vender *et al.*, 2017). The European Union banned the use of antibiotics as growth promoters in 2006 and has since set strict restrictions for their therapeutic use (Lagha *et al.*, 2017). In the USA, Canada and Denmark, significant sections of food production industries have turned their attention towards novel and more natural methods of husbandry without antibiotic use, in order to address consumer concerns related to the misuse of antibiotics and to meet consumer demand for more natural, organic food products (Gaucher *et al.*, 2015).

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™ 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 identify regional spikes in the risk of human infection (Allard *et al.*, 2010; Bi *et al.*, 2008; Fleury *et al.*, 2006; Hearnden *et al.*; Kovats *et al.*, 2005). Therefore, it is important to assess the efficacy of ferric tyrosine under natural infection conditions and different geographical variations of climate. The effects of ferric tyrosine on chicken health, growth performance, *Campylobacter* counts, and meat quality were also evaluated. During this study, a natural infection with *Eimeria tenella* (*E. tenella)* allowed us the opportunity to examine ferric tyrosine efficacy against *E. tenella* in addition to *C. jejuni*.

2. MATERIALS AND METHODS

2.1. Animals, grouping and housing

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

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

2.2. Feeding treatments

Control group (T_1) was fed basal diets in mash form; (starter feed, 1-21 days; grower feed, 22-42 days), without added iron. The basal diets of the other groups were supplemented with ferric tyrosine at 0.02 g/kg feed (T_2), 0.05 g/kg (T_3) or 0.20 g/kg feed (T_4). The ferric tyrosine, brand name TYPLEX™ chelate (Akeso Biomedical Inc., Waltham, USA) is an iron chelate (III) with L-tyrosine (4-hydroxyphenylalanine). All diets were formulated to meet or exceed NRC (1994) recommendations and then analysed (AOAC, 2007) for crude protein, ether extract, dry matter, iron and ash (Suppl. Table 1). Coloured tracers (Micro-Tracers Inc, San Francisco) were initially added to the ferric tyrosine at 10% w/w, to enable visual confirmation of ferric tyrosine content 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). Diets did not contain any added iron compounds, coccidiostats or antibiotic growth promoters.

2.3. Challenge protocol

In commercial broiler farming *Campylobacter* is usually undetectable in the first 2-3 weeks of young broilers and there is a lag phase before infection can be detected. The reasons for this lag phase are not known but have been attributed to the possible presence of maternal antibodies, 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, infection spreads very rapidly throughout the entire shed in just few days. Therefore, at 20 days of age broilers were exposed to natural *C. jejuni* challenge by means of contaminated litter, from 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.*, 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

158 commercial units. The infecting inoculum was prepared by mixing thoroughly 6 kg of
159 contaminated litter to ensure an even distribution throughout and using 200 g to contaminate each
160 pen. *C. jejuni* is highly infectious and it has been shown before that even a single bird infected with
161 low levels of *C. jejuni* is sufficient to infect a whole flock of broiler chickens (Stern *et al.*, 2001)
162 with the contamination spreading across the environment and persisting for many weeks (Herman
163 *et al.*, 2003; Johnsen *et al.*, 2006). Furthermore, pens were randomised to avoid any experimental
164 bias. Thereafter, pens were examined daily for diarrhoea and fecal oocysts per gram (OPG). On
165 the last day of the trial (day 42) pens were observed for diarrhoea in the litter, after which chickens
166 were slaughtered under commercial conditions.
167
168 ***2.4. Sampling and analysis***
169 From each replicate pen 6 birds were randomly selected and further processed. Post-mortem
170 analyses of the intestinal tracts were performed in these birds and intestinal coccidiosis scoring was
171 carried out as described in Johnson and Reid, 1970. At days 25 and 42, caeca were collected for
172 microbiological analyses. Breasts and thighs were removed from the carcass, weighed and then
173 stored for chemical analyses. Chemical content and meat quality were evaluated using FoodScan
174 technology and a taste panel assessed organoleptic properties of the meat.
175
176 ***2.5. Microbiological analysis***
177 Faecal swabs, caecal and litter samples were taken on day 25 and 42, respectively, for PCR
178 amplification (Suppl. Table 2) to confirm the presence of *C. jejuni* (Suppl. Fig. 1). In addition, litter
179 samples from days 25 and 42 and caecal samples from day 42 and were collected and analysed for
180 *C. jejuni* analysis by conventional culture (Suppl. Fig. 2, 3 and 4). The caeca of two birds per pen
181 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
183 gr, was weighed into sterile Universal bottles, diluted with 4 ml sterile Maximum Recovery Diluent
184 (MRD, Oxoid Basingstoke, UK), and mixed thoroughly. This constituted the 1:2 dilution (w/v).
185 Further eight serial dilutions of 1:30 were made in MRD and 10 µl of each dilution were inoculated
186 on CCDA and *Brilliance* CampyCount Agar (Oxoid, Basingstoke, UK). Plates were incubated
187 microaerophilically at 42°C for 48 hr and then assessed for the presence or absence of
188 thermotolerant *Campylobacter* species. Plates of an appropriate dilution were selected and colonies
189 enumerated.

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

197 Coccidial OPGs were also determined in excreta samples taken from each subgroup daily
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
200 days. OPGs were also determined in excreta samples from each subgroup at the end of the trial at
201 the birds that had bloody diarrhoea. Samples collected from each subgroup were placed in separate
202 airtight plastic bags, homogenized thoroughly by a domestic mixer, and kept refrigerated until
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
205 McMaster chambers (Hodgson, 1970).

206

207 ***2.6. DNA extraction***

208 In a PCR tube (300 µl; Starlab PCR Product), 5-10 random colonies were dissolved into
209 100 µl TE 10:1. The DNA was denatured by boiling for 10 min. The tube was centrifuged at 20,000
210 g (4ºC) for 5 min. The samples were diluted 1:10 in TE 10:1, recommended by the EURL-AR
211 (Denis *et al.*, 1999; Van de Giessen *et al.*, 1998; Vandamme *et al.*, 1997).

212

213 ***2.7. PCR protocol***

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

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

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

230 Boric EDTA buffer (TBE buffer, Sigma) at 100 V for 45 min. The gel was visualized using an
231 ultraviolet trans-illuminator to detect the gel red labeled DNA. Then 2-log (0.1-10.0 kb) DNA
232 ladder mixes (New England Biolabs, USA) were used to estimate the size of PCR product.
233
234 ***2.8. Meat chemical analysis***
235 The breast and thigh meat samples collected at day 42 were analysed for moisture, crude
236 protein and fat content, by near infra-red spectroscopy using a FoodScanTM Lab (FOSS, Denmark)
237 in transmittance mode. Initially samples were thawed at room temperature (20° C), the breast
238 (*Pectoralis major*) and the thigh (*Biceps femoris*) meat was carefully separated from the skin and
239 the bones, minced (Cutter K35, Electrolux) and then 200 g of the minced meat was placed in the
240 sample tray of the FoodScan. Contents of fat, moisture and protein, were determined by the
241 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***
244 Before the test, 12 frozen carcasses per treatment (2 carcasses per pen selected at random)
245 were removed from the freezer and held at 4° C for 2 days for thawing. Then the carcasses were
246 cut up. Breast muscles were separated from the bone and cut to stripes (10 x 2 x 2 cm). Thighs
247 were removed from the carcass and cut to smaller pieces. The breast pieces of each group were put
248 into separate grill baskets and then cooked at the same time for 12 min. The cooked pieces from
249 each group were placed in a large plate, were assigned a random letter and then were presented to
250 the panel test members at the same time for scoring. This process was repeated for the thigh meat
251 pieces.
252 The sensory panel consisted of 14 participants (both males and females; ages from 22 to 65
253 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.

2.10. Statistical analysis

The basic study design was RCB (random complete block design), and the pen was 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 considered for $0.05 < p \leq 0.10$. Means were separated by Tukey's Test. IBM SPSS (Version 20) 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 samples taken on days 25 and 42 were assessed. If *Campylobacter* counts were randomly distributed among pens, the counts obtained should follow a Poisson distribution, where variance equals the mean. If variance exceeds the mean this indicates overdispersion and demonstrates that the counts are not homogenous. The distribution of *Campylobacter* spp. counts from the litter were assessed for overdispersion by multiplying the variance to mean ratio by the number of degrees of freedom, and comparing the results with the chi-square distribution (Bliss and Fisher, 1953). Overdispersion was confirmed when $p < 0.05$. The same analysis was applied to the caecal *Campylobacter* counts obtained on day 42.

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 ≤0.05 were considered statistically significant, whereas values of $0.05 < p \leq 0.10$ were declared a near-significant trend. The Hosmer-Lemeshow test (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 each treatment group with normal intestinal pathology, a binomial test was performed using the prop.test function in RStudio.

3. RESULTS

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).

3.2. Campylobacter

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 distributed among the pens. However, *C. jejuni* counts were significantly lower in T_4 birds compared to those in T1, T_2 and T_3 ($p = 0.007$). At the end of the study (day 42), *C. jejuni* counts in the litter did not differ significantly between groups, but lower contamination was observed in the T_4 group (Suppl. Fig. 3). *C. jejuni* counts in bird caeca on day 42 were significantly lower in the T_4 group, compared to the T_1 Control, T_2 and T_3 groups ($p = 0.004$, Fig. 1 and Suppl. Fig. 4). None of the pens were negative either at day 25 or 42 and the counts were evenly distributed among the pens while at day 42 all birds were infected (Suppl. Fig. 2, 3, and 4) and no negative counts were observed, suggesting that all birds were exposed initially to a similar level of infection at day 20.

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 post-mortem 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
312 and was identified by accumulation of blood in the ceca, bloody droppings, pathologoanatomic
313 severe lesions and large numbers of OPG. Post mortem exams showing caecal cores with
314 accumulations of clotted blood further supported the presence of *E. tenella* infection (Suppl. Fig.
315 5 and 6). Intestinal smears were evaluated under microscopy to establish the presence of OPG after
316 post mortem examination. On day 21, two birds died from T_3 group died from *E. tenella.* The
317 intestines of these two birds were examined for coccidial oocysts, *E. coli* and *Clostridium*
318 *perfringens*. *E. tenella* was isolated and large numbers oocysts were microscopically observed in
319 both caecal samples. On days 28 and 29, for animal welfare reasons, all birds on trial were treated
320 with an anti-coccidial drug against *E. tenella* (Baycox: 25 mg Toltrazuril /ml solution, 1 L/1000 L
321 drinking water for 48 hours). The incidence of coccidia was significantly lower in T_4 birds
322 compared to control group ($p = 0.005$, Fig. 2), and a near significant reduction was observed in T_3
323 birds compared to T_1 ($p = 0.07$, Fig. 2). A near significant reduction in the incidence of bloody
324 diarrhoea was observed in T_3 and T_4 birds compared to T_1 birds ($p = 0.06$, Fig. 2) and the incidence
325 of diarrhoea was significantly lower in T_3 & T_4 birds compared to the control birds ($p = 0.05$ and
326 $p = 0.024$, respectively, Fig. 2).

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

332 On day 42, the intestines of 37 birds per treatment group were examined post mortem.
333 Significantly more birds fed the T_4 diet had normal intestinal tracts compared to the control birds
334 ($p = 0.007$, Suppl. Table 5).

3.3. Meat proximate analysis (FoodScan)

In the breast meat samples, the T_4 group tended to have a higher protein content compared to the T_3 group (p = 0.087, Suppl. Table 6). In the thigh meat samples, the T_3 group had significantly lower fat (p = 0.006) compared to groups T_1 and T_4, 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 compared to groups T_1, T_2 and T_3.

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_3 group had significantly better scores in tenderness (p = 0.002) and juiciness (p = 0.008) compared to T_1 and T_4 (Fig. 3). T_2 and T_3 groups had significantly better scores in "*like overall*" than T_4 (Fig. 3). No significant differences (p > 0.05) in sensory parameters were noted for cooked thigh meat.

4. DISCUSSION

Considerable global efforts are being made to prevent human campylobacteriosis by non-antibiotic means, due to public concerns about over-reliance of antibiotics in farming with inconclusive results (Gracia *et al.*, 2016; Guyard-Nicodeme *et al.*, 2016; Hermans *et al.*, 2011; Zhu *et al.*, 2006). Despite intensive efforts during the last decades, effective and reliable methods to stop or limit *Campylobacter* colonization in poultry do not exist. Plant essential oils and short-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 fecal counts (Gracia *et al.*, 2016; Guyard-Nicodeme *et al.*, 2016; Hermans *et al.*, 2011). In this study, ferric tyrosine, a non-antibiotic feed additive (Khattak *et al.*, 2018; Currie *et al.*, 2018), was 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.

Campylobacter colonization of chicken intestinal tracts is usually commensal and without noticeable effects in performance parameters, although there are reports that *Campylobacter* can be detrimental for the birds in some instances (Humphrey *et al.*, 2014). Ferric tyrosine supplementation resulted in significant reduction of *C. jejuni* contamination of pen litter 5 days after a natural challenge introduced via infected litter. By the last day of the trial, the groups supplemented with ferric tyrosine, especially the T_4 group (0.20 g ferric tyrosine/kg feed), had lower diarrheal scores (Suppl. Table 4), lower *C. jejuni* caecal counts and a lower percentage of birds with abnormal intestinal tracts (related to post-mortem evidence of coccidiosis, diarrhea or bloody diarrhea), (Fig. 2 and Suppl. Fig. 5 and 6). Faecal *C. jejuni* contamination is one of the main ways of diffusion through the food chain (Santini *et al.*, 2010). Under practical farming conditions it is very difficult to avoid contact between chickens and *Campylobacter* (Hermans *et al.*, 2011).

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 $\log_{10}$ 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 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 pathogen. It was noticed that the groups supplemented with increased levels of ferric tyrosine had lower incidence of diarrhea in the pens and of abnormal digestive tract and bloody diarrhea (in post-mortem examination), suggesting a possible protective effect against the parasite (Suppl. Fig. 5 and 6). During a coccidial infection, inflammatory cytokines produced by the immune system can stimulate a number of cell types, including primed host macrophages, to synthesize large quantities of NO by an induced NO synthase (iNOS) (Liew and Cox, 1991). NO has strong oxidant 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 pathway but its precise molecular mode of action in conferring protection against coccidiosis is 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).

L-Tyrosine (4-hydroxyphenylalanine), is an essential amino acid used in the synthesis of proteins (Chinevere *et al.*, 2002; EFSA, 2013; NCBI, 2017). As such it is ubiquitous in the natural environment, and in animal proteins, including chicken and turkey meat. L-tyrosine is approved for use as a feed additive in the EU (EFSA, 2013). In the EU, food animal diets may be supplemented with up to 0.5% tyrosine, equivalent to 5.0 g/kg feed. In the current study, dietary supplementation with ferric tyrosine at 0.20 g/kg feed (T_4), resulted in a tendency to improve the FCR, demonstrating value as a non-antibiotic alternative to support poultry health. This will be a significant factor to investigate further, particularly as there is evidence that lower slaughtering mass after *C. jejuni* infection may be due to the reduction in the feed efficiency, even though no differences were observed in the average daily feed intake between control and infected birds (Awad *et al.*, 2015).

5. CONCLUSION

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

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588

Table 1. Composition and calculated analyses of basal diets.

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

[1]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 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 mg; Cu: 8 mg; Se 0.15 mg; No exogenous Fe was added.

*Soybean was used to premix the ferric tyrosine for T_2-T_4 diets.

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

Period: 1-21 days					
Treatment	BW, 1 d (g)	BW, 21 d (g)	ADG (g)	ADFI (g)	FCR (feed/gain)
T_1	45.8	655	30.5	53.8	1.77
T_2	45.9	653	30.3	54.0	1.78
T_3	46.4	680	31.7	54.5	1.73
T_4	45.8	659	30.7	54.9	1.79
SEM	0.220	8.18	0.41	0.36	0.020
p (value)	0.763	0.64	0.65	0.70	0.46
Period: 22-42 days					
	BW, 22 d (g)	BW, 42 d (g)	ADG (g)	ADFI (g)	FCR (feed/gain)
T_1	655	2,142	70.8	142	1.99
T_2	653	2,136	70.6	141	1.98
T_3	680	2,173	71.1	141	1.98
T_4	659	2,261	76.3	142	1.86
SEM	8.18	25.3	1.09	1.73	0.02
p (value)	0.64	0.30	0.23	0.98	0.09
Period: 1-42 days					
	BW, 1 d (g)	BW, 42 d (g)	ADG (g)	ADFI (g)	FCR (feed/gain)
T_1	45.8	2,142	51.1	100	1.93
T_2	45.9	2,136	51.0	99	1.92
T_3	46.4	2,173	51.9	100	1.90
T_4	45.8	2,261	54.0	100	1.84
SEM	0.22	25.3	0.62	1.05	0.02
p (value)	0.76	0.30	0.30	0.98	0.17

Nº replicates = each treatment had 6 pens of 16 male birds/pen;

T_1; Control; 0 g ferric tyrosine/kg feed, T_2; 0.02 g ferric tyrosine/kg feed, T_3; 0.05 g ferric tyrosine/kg feed, and T_4; 0.20 g ferric tyrosine/kg feed

SEM = Standard error of mean; BW = body weight; ADG = Average daily gain; ADFI = Average daily feed intake; FCR = Feed Conversion Ratio (feed/gain)

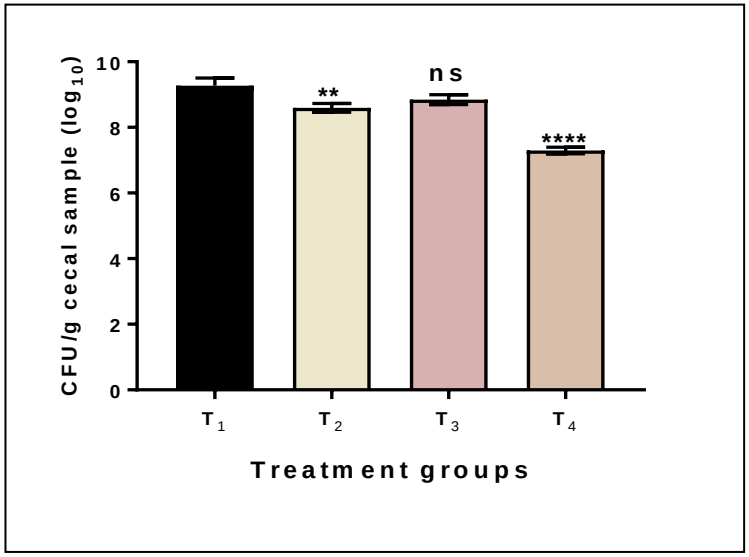

Figure 1.

Effect of dietary addition of TYPLEX™ 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 ^{abc} superscript differ significantly ($p \leq 0.05$ and ns = no significance; One way ANOVA).

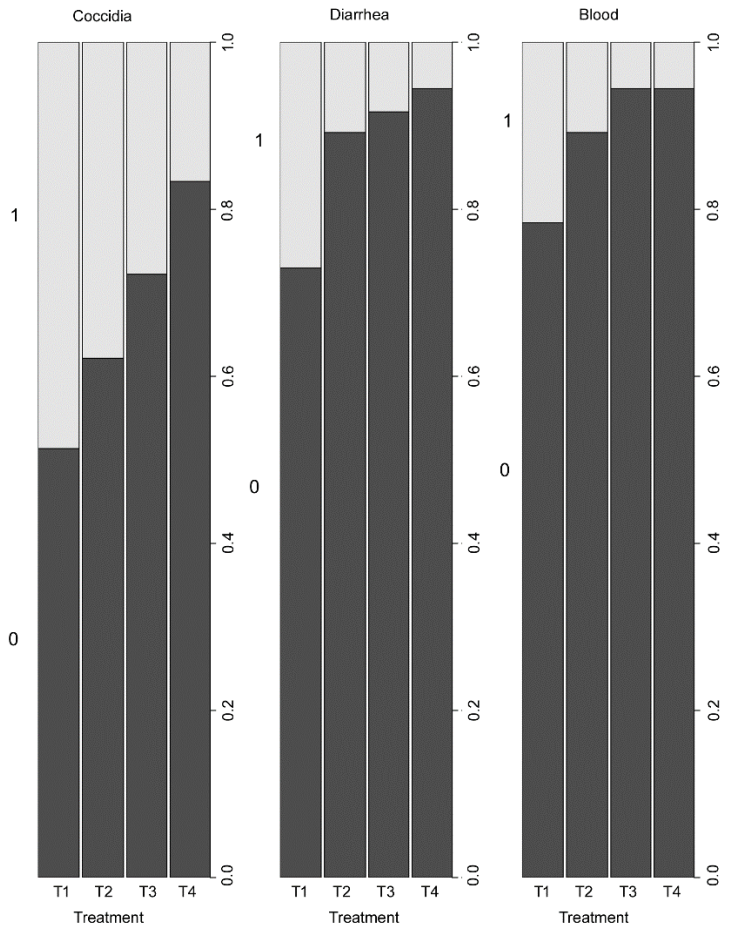

Figure 2.

Effect of dietary addition of TYPLEX^{TM} 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.

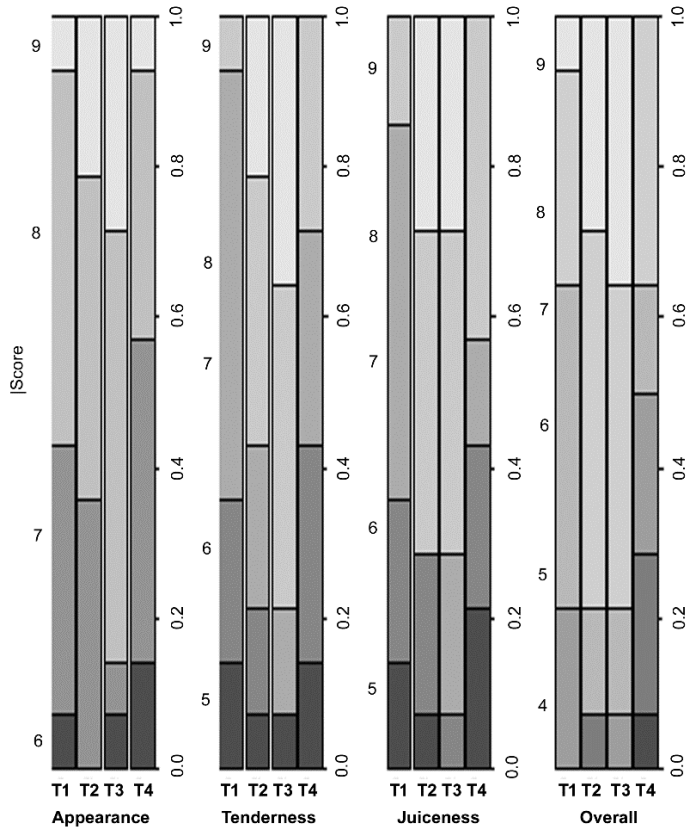

Figure 3.

Effect of dietary addition of ferric tyrosine on sensory panel scores of cooked breast meat on appearance, tenderness, juiciness and overall. Nº replicates: 48 (2 carcasses per pen/treatment) scored by 14 panelists. Results range: From 1 (negative perception) to 9 (positive perception). $p < 0.05$.