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**The *in vitro* and *in vivo* effect of Carvacrol in preventing *Campylobacter* infection, colonisation and improve chicken broilers productivity**

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Running head: The *in vitro* and *in vivo* effect of carvacrol

26

## Abstract

27

28 The current trend in reducing the antibiotic usage in animal production imposes  
29 urgency in identification of novel biocides., The essential oil carvacrol for example  
30 changes the morphology of the cell and acts against a variety of targets within the  
31 bacterial membranes and cytoplasm and our *in vitro* results show that it reduces  
32 adhesion and invasion of chicken intestinal primary cells and also biofilm formation.  
33 A trial was conducted to evaluate the effects of dietary supplementation of carvacrol  
34 at 4 concentrations (0, 120, 200, and 300 mg/kg of diet) on *Lactobacillus* spp., *E.*  
35 *coli*, *Campylobacter* spp. and broilers performance. Each of the 4 diets was fed to 3  
36 replicates / trial of 50 chicks each from day 0 to 35. Our results show that carvacrol  
37 linearly decreased feed intake, feed conversion rates (FCR) and increased body  
38 weight (BW) at all levels of supplementation. Plate count analysis showed that  
39 *Campylobacter* spp., was only detected at 35 days in the treatment groups  
40 compared with the control group where the colonisation occurred at 21 days. The  
41 absence of *Campylobacter* spp., at 21 days in the treatment groups was associated  
42 with a significant increase in the relative abundance of *Lactobacillus* spp. Also,  
43 carvacrol was demonstrated to have a significant effect on *Eschericia coli* numbers  
44 in the caecum of the treatment groups, at all supplementation levels. In conclusion  
45 this study shows for the first time that at different concentrations of carvacrol can  
46 delay *Campylobacter* spp., colonisation of chicken broilers, by inducing changes in  
47 gut microflora, and demonstrates promise as an alternative to the use of antibiotics.

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49 **Key words:** *Campylobacter*, Carvacrol, infection, colonisation, biofilm

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## Introduction

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54 Since 2006 ban of antibiotics research has taken place to identify alternative  
55 substances which can be used to not only treat animal diseases but also to reduce  
56 the presence of pathogenic bacteria posing a threat to human health. Plant derived  
57 antimicrobials, also known as PDAs, are a suitable alternative to antibiotics as they  
58 do not cause resistance and side effects (Juneja and others 2012). The  
59 effectiveness of essential oils, like carvacrol, against pathogenic bacteria is  
60 expressed by the effects on outer and inner membrane integrity, virulence gene  
61 expression, biofilm formation all regulated through quorum sensing activities (Bassler  
62 2002). A major source of carvacrol is oregano oil, but it is also produced through  
63 biotechnological synthesis by genetically modified microorganisms. Its mechanism of  
64 action is not well studied (Lambert and others 2001), however it has been suggested  
65 that carvacrol disintegrates the outer membrane of pathogenic bacteria, increases  
66 permeability to ATP and depolarise the membranes (Xu and others 2008). Additional  
67 effects also show that it has a beneficial effect against chemically-induced colon  
68 carcinogenesis in rats (Sivaranjani and others 2016).

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70 Food-borne pathogens including *Campylobacter* spp., and *E. coli* are a concern for  
71 the poultry industry. *Campylobacter jejuni*, a microaerophilic bacterium, is well known  
72 for its ability to cause severe gastroenteritis and life-threatening diseases in humans  
73 and is considered a commensal in poultry (Crushell and others 2004). The main  
74 source of infections in humans is considered to be the consumption of improperly  
75 cooked chicken meat. The positive effect of carvacrol, *in vitro*, against  
76 *Campylobacter* spp., has been shown at concentrations of 7.8-800 µg/ml (Aslim and

77 Yucel 2008) but the direct effect on virulence has only been described in INT-407  
78 cells and using *C. jejuni* 108, a human isolate (van Alphen and others 2012). Based  
79 on its antimicrobial properties, using carvacrol to modify the microbiota and reduce  
80 the presence of *Campylobacter* spp., in broilers caecum has gained increasing  
81 interest (Ozogul and others 2015). Meat quality can benefit from the inclusion of  
82 oregano oil in broiler diets and it has been reported that carvacrol can inhibit lipid  
83 oxidation in meat at concentrations of 50-100 mg/kg feed (Luna and others 2010).  
84 However, the industry is reluctant in relation to its applicability due to the fact that the  
85 literature lacks information in this area (Lillehoj and others 2011). Recent data shows  
86 that inclusion of encapsulated carvacrol, thymol, and limonene (up to 100 mg/kg) can  
87 improve performance as well as apparent ileal digestibility of nutrients in broilers  
88 (Hafeez and others 2015).

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90 The present manuscript describes the effect of carvacrol feeding on the  
91 microbiological composition of the caecal content in naturally colonized chicken  
92 broilers and investigates the dose effect of carvacrol on *Campylobacter* spp., *E. coli*  
93 and *Lactobacillus* with focus on key poultry performance indicators as well as meat  
94 quality.

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## Material and Methods

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### *Broilers, diet and experimental design*

This study was carried out using a total of 600 Ross-308 male chicken broilers divided in 4 treatments (Control, T1, T2 and T3) with pens containing 50 birds/pen. The 4 treatments were fed with 120, 200 and 300 mg/kg feed of carvacrol. (Sigma, UK).

### *Analysis of poultry growth and performance*

The performance parameters investigated were: body weight, feed intake, feed conversion ratio and broiler mortality rate. In order to analyse the economic efficiency of growth we have also calculated the European Broiler Index (EBI) and European Production Efficiency Factor (EPEF) (Broiler Management Manual Ross-308 and Home page address: [www.aviagen.com](http://www.aviagen.com)).

### *Plate count enumeration of Campylobacter spp., and E. coli in broilers caeca*

For *Campylobacter* the enumeration method was based on those described in the British Standard BS EN ISO 10272:2006 and the enumeration of *E. coli* was based on the British Standard BS EN ISO16649-2:2001.

### *DNA and RNA extraction*

124 Caecal DNA was extracted using the QIAamp DNA Stool Mini Kit according to the  
125 manufacturer's instructions. Total RNA was isolated from the caecum, large and  
126 small intestine using Qiagen RNA extraction kit according to the manufacturer's  
127 protocol.

128

### 129 *16S rRNA amplification and sequencing*

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131 16S metagenomic sequencing library preparation was constructed using Illumina  
132 guidelines (Illumina, U.S.A). The 16S ribosomal primers used were V3  
133 (tcgtcggcagcgtcagatgtgtataagagacagcctacgggnggcwgcag) and V4  
134 (gtctcgtgggctcggagatgtgtataagagacaggactachvgggtatctaacc) (Klindworth and others  
135 2013). A second PCR step was performed to attach dual indices and Illumina  
136 sequencing adapters using the Nextera XT Index kit (Table 2). Sequencing was  
137 performed on an Illumina MiSeq using a v3 150 bp paired-end kit. Initial data quality  
138 was assessed in FastQC (S 2010). Data was uploaded onto BaseSpace and  
139 analysed using the Qiime preprocessing and visualization apps (Caporaso and  
140 others 2010).

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### 142 *qPCR for quantification of lactic acid bacteria*

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144 The relative abundance of intestinal *Lactobacillus* in DNA isolated from broiler  
145 caecum was measured by qPCR on a 7900 Fast Real-Time System. The PCR  
146 reactions were set using SYBR Green Master mix (Applied Biosystems) and  
147 bacterial 16S group-specific primers (All *Lactobacillus* Forward 5'-

148 AGGGTGAAGTCGTAACAAGTAGCC-3' and All *Lactobacillus* Reverse 5'-  
149 CCACCTTCCTCCGGTYYGTCA – 3').

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### 151 *Mucin mRNA analysis*

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153 The RT-PCR was carried as previously described (Smirnov and others 2005).  
154 Briefly, chicken mucin primers F 5'-GCTGATTGTCACTCACGCCTT-3', R 5'-  
155 ATCTGCCTGAATCACAGGTGC-3') and primers from the *Gallus gallus* 18S  
156 ribosomal RNA gene F: 5'-CGATGCTCTTAACTGAGTGT-3' and R: 5'-  
157 CAGCTTTGCAACCATACTC-3' were used.

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### 159 *Histology*

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161 Gastrointestinal tract samples (colon, small intestine and cecum) were placed in  
162 Carnoy's solution, at 4°C until processing. Following fixation the tissue samples were  
163 stained with hematoxylin (7 min) and eosin (3 min). The stained slides were  
164 dehydrated (70%IMS-1 min, 95% IMS-2 min, 100% IMS-2 min), cleared in xylene  
165 (30 min) and mounted in DPX medium. Slides were analysed under a brightfield  
166 microscope (Leica DMLB). Images were acquired using a Leica DFC300x camera  
167 and the IM50 imaging software (Pircalabioru and others 2016).

168

### 169 *Biofilm assay*

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171 The biofilm assay was performed as previously described (Reuter and others 2010).  
172 Briefly, *C. jejuni* RC039 was grown in Mueller Hinton medium containing 120 mg/ml,

173 200 mg/ml and 300 mg/ml carvacrol diluted in ethanol and added to the growth  
174 medium in polystyrene flatbottomed 6 well plates and incubated for 48 hours at  
175 42°C. One milliliter of a 1% crystal violet solution was added and the wells were  
176 incubated at room temperature for 60 min. Unbound crystal violet was washed off  
177 with water and the plates were dried at 37°C. Bound crystal violet was dissolved in  
178 20% acetone in ethanol for 10 min and was then poured into cuvettes, and the A590  
179 was measured.

180

### 181 *Infection of chicken primary intestinal cells*

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183 The gentamicin protection assay (Corcionivoschi and others 2009) was used to  
184 determine the effect of carvacrol on the virulence of *C. jejuni* RC039. Briefly, chicken  
185 intestinal primary cells were isolated as previously described (Byrne and others  
186 2007). Plate grown *C. jejuni* RC039 was washed and re-suspended in tissue culture  
187 medium at an OD<sub>600</sub> of 0.4. Cells were washed with PBS, and 2 ml of fresh culture  
188 medium containing DMSO or DMSO + carvacrol was added to each well (120mg/ml,  
189 200mg/ml and 300mg/ml) (Qiu and others 2010). The error bars represent standard  
190 deviations for three separate wells. The significance of differences in adhesion and  
191 invasion between samples was determined using the Student *t* test. A P-value of  
192 <0.05 was defined as significant.

193

### 194 *TBARS*

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196 Lipid oxidation was evaluated by determining the thiobarbituric acid reactive  
197 substances as previously described (Cherian and others 2002). The meat sample (5



198 g) was homogenized with 15 ml of deionized distilled water for 10 seconds. To the  
199 meat homogenate butylated hydroxyanisole (50  $\mu$ l, 10%) and thiobarbituric  
200 acid/trichloroacetic acid (TBA/TCA, 2 ml) were added. The absorbance of the  
201 resulting supernatant solution was determined at 531 nm against a blank containing  
202 1 ml of double distilled water (DDW) and 2 ml of TBA/TCA solution. The amounts of  
203 TBARS were expressed as milligrams of malondialdehyde per kilogram of meat.

204

#### 205 *Gas Chromatography (GC) for fatty acids analysis*

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207 For GC analysis of fatty acids 1 g of meat sample was mixed with 3 ml methanol and  
208 0.7 ml 10NKOH and incubated at 56°C oven overnight. An internal standard  
209 (Tridecanoic acid) was added to check recovery. The sample was allowed to cool  
210 before adding 0.58ml 24N H<sub>2</sub>SO<sub>4</sub> followed by 90 minutes incubation, with occasional  
211 mixing. Once cooled 3 ml of hexane was added and the sample was mixed. The  
212 extract was run for 91 minutes to ensure all FAMES (fatty acid methyl esters) were  
213 recovered. These were then identified and analysed accordingly using the GC  
214 (Varian 3800 GC).

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## Results

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### 219 *Effects of carvacrol on cell invasion and biofilm formation in vitro*

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221 In order to reduce pathogen colonisation of the broiler gastrointestinal tract, any  
222 antimicrobial used will have to reduce the capacity of this bacterium to adhere to and  
223 invade the gastrointestinal mucosa. Therefore we have first investigated, *in vitro*, the  
224 efficacy of carvacrol (120, 200 and 300 mg/kg feed) in preventing the colonisation  
225 and infection of a *C. jejuni* chicken isolate to infect chicken primary intestinal cells.  
226 Our results show for the first time (Figure 1, Panels A and B) that following  
227 gentamicin protection assay both the adhesion ( $p < 0.0001$ ) and invasion ( $p < 0.0001$ )  
228 of *C. jejuni* RC039 to chicken intestinal primary cells were reduced significantly when  
229 inoculated in the presence of carvacrol. Moreover carvacrol also significantly  
230 reduced the ability of *C. jejuni* RC039 to form biofilms (Figure 1, Panel C).

231

### 232 *Carvacrol effect on the chicken gastrointestinal compartments*

233

234 Next we investigated the effect of carvacrol on the integrity and development of the  
235 intestinal surfaces directly involved in bacterial colonisation and nutrient absorption.  
236 The hystologic analysis at slaughter indicate an increase in small intestinal villus  
237 height in all carvacrol groups (Figure 1). Similar investigations performed on tissue  
238 harvested from the large intestine also revealed healthier epithelial surfaces in the  
239 experimental groups compared to the control (Figure 1, Panel G and H). Changes  
240 were observed in the caecum, however clear erythrocyte infiltrations (as indicated by  
241 the yellow arrow in Figure 1, Panel I were observed in the control group and absent

242 in the experimental (Figure 1, Panel J). In order to investigate if the increase in  
243 epithelial surface in the experimental groups was associated with increased mucus  
244 production we have investigated the presence of mucin mRNA (Figure 1, Panel D).  
245 The expression of mucin mRNA increased gradually and significantly in the  
246 experimental groups compared to the control in both large and small intestine. In the  
247 caecum similar increases were observed in mRNA expression; however significance  
248 was only detected in experimental group T3. These results suggest that carvacrol  
249 can increase the epithelial surface and the production of the inner mucus layer.

250

251 *Carvacrol delays Campylobacter spp., detection in naturally colonized chicken*  
252 *broilers*

253

254 Our results show (Figure 2, Panel D) that during the starter (0-10 days) and grower  
255 periods (11-21 days) the relative abundance of *Lactobacillus* spp. in broilers caecal  
256 content is significantly increased compared to the control group, and that the *E. coli*  
257 presence (Figure 2, Panel C by plate count) is significantly reduced in all three  
258 experimental groups compared to the control. This increase in *Lactobacillus*  
259 presence is also associated with lack of *Campylobacter* spp., detection at 10 and 21  
260 days in all the experimental groups (Figure 2, Panel A and B). The presence of  
261 *Campylobacter* spp., in the treatment groups (T1, T2 and T3) only occurs at day 35  
262 when the abundance of *Lactobacillus* sp. decreases below the levels of the control  
263 group. Our results suggest that carvacrol can stimulate the increase in abundance of  
264 probiotic bacteria in broilers caecum and reduce *Campylobacter* spp., presence up  
265 to 31% at levels of supplementation of 120 mg/kg feed.

266

267 *Carvacrol induces changes in chicken caecum microbiota*

268

269 The chicken caecum microbiome was assessed at Day 10, 21 and 35. The major  
270 phyla were the Firmicutes (65.49%), Proteobacteria (28.24%) and Bacteroidetes  
271 (6.13%). In Day 10 Carvacrol samples, T1 (89.9%), T2 (83.7%) and T3 (82.7%)  
272 displayed a higher percentage of Firmicutes when compared to Day 10 control  
273 sample (65.5%). Analysis of the Day 21 samples displays the presence of the three  
274 major Phyla, however the percentage of Bacteroidetes has increased in all Day 21  
275 samples; C (47.2%), T1 (39.4%), T2 (34.9%) and T3 (55.8%). At Day 35 taxonomic  
276 analysis at the class level further identified differences between the samples. Further  
277 investigation of the Firmicutes identified a higher percentage of Bacilli within the  
278 Carvacrol samples. Further analysis of the Proteobacteria distribution identified the  
279 presence of Epsilonproteobacteria in Day 35 control and Carvacrol samples (T1, T2  
280 and T3). The Day 35 control samples contained *Campylobacter* spp., at 10.52%.  
281 This was higher than Day 35 Carvacrol samples T1 (6.43%) and T2 (7.85%),  
282 however the Day 35 Carvacrol T3 percentage *Campylobacter* spp., was noted to be  
283 higher (13.86%) than the respective Day 35 control sample.

284

285 *Carvacrol improves production parameters at slaughter*

286

287 The feed intake (Figure 4, Panels A and B) for the experimental broilers in group T1  
288 was slightly higher (+1.58%) compared to the control but the increase was not  
289 statistically significant. The feed conversion rates were also reduced by 6.7% in  
290 experimental group T1 (NS), by 24.8% ( $p=0.04$ ) at T2 and by 17.5% ( $p=0.09$ ) at T3  
291 (Figure 4, Panels C and D). As shown in Figure 4, Panels E and F at Day 35

292 (slaughter) a 5.45% increase in body weight was recorded for experimental group T1  
293 (p=0.02), a 5.10% increase for T2 (p=0.03) and 4.08% increase for T3 (p=0.02). The  
294 experimental group T2 reduced its feed intake by 12.9% (p=0.006) and T3 by 6.29%  
295 (p=0.04) compared to the control at 35 Days.

296

#### 297 *Lipid oxidation and fatty acid composition of broiler thigh muscle*

298

299 Across the carvacrol treatments the TBARS values decreased significantly only at 21  
300 days as shown in figure 4 (Panel G and H). Treatment T1 showed a 19.18%  
301 decrease (p=0.2), at treatment T2 the TBARS were reduced with 57.38% (p=0.02)  
302 and with 22.57% at T3 (p=0.09). At Day 35 compared to the control group, an 8.68%  
303 increase in total  $\omega$ 3 fatty acids, 9.34% increase in  $\omega$ 6, 13.77% increase in  $\omega$ 7 and  
304 8.43% in  $\omega$ 9. The total mono-unsaturated fatty acids (MUFA) at Day 35 showed an  
305 increase of 8.55% over control and the poly-unsaturated fatty acids (PUFA)  
306 increased by 9.24% compared to the control. The SFA increases in T2 (1 mg/g  
307 muscle) and T3 (1.46 mg/g) muscle are not significant and probably not biologically  
308 relevant. However, there was an increase by 2.11 mg/g muscle in UFA at T2 and by  
309 0.6 mg/g muscle at T3 with no statistical significance as described in Supplementary  
310 Table 1.

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## Discussion

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The most recent report from the European Food Safety Authority (EFSA) places *Campylobacter* spp., as the most commonly reported human gastrointestinal pathogen in European Union with 214,000 cases and 56 deaths recorded in 2013 (Authority 2015). This manuscript describes for the first time the effect of Carvacrol in preventing adhesion and invasion of chicken intestinal primary cells and also new data on chicken broiler microbiota composition, growth performance and *Campylobacter* spp., presence in a farm set up using naturally colonized broilers.

It is known that essential oils such as Carvacrol act by increasing the membrane permeability of Gram-negative bacteria, causing structural and functional changes leading to outer membrane disintegration (La Stora and others 2011). The structural and functional integrity of *C. jejuni* outer membrane structures have been previously described as crucial for this pathogen to efficiently attach and adhere to gut epithelial cells (Corcionivoschi and others 2012). The ability of *C. jejuni* to colonise or to infect the epithelium is highly dependent on the genetic specificity of each strain (Ragimbeau and others 2014). In order to reduce this variability we have used *C. jejuni* RC039, a highly virulent chicken isolate recently described as positive for the newly identified Type Six Secretion System (T6SS) (Corcionivoschi and others 2015). Carvacrol was proven to efficiently reduce the pathogenicity of this isolate when tested on chicken intestinal primary cells. Moreover, because the outer membrane structures are involved in the ability of *C. jejuni* to create biofilm (Naito and others 2010) we have shown that carvacrol has a negative effect on the ability of *C. jejuni* RC039 to form, biofilm .

338

339 As described above it is clear that Carvacrol can reduce the attachment of *C. jejuni*  
340 to chicken intestinal cells (K. Arsi and Donoghue 2014), however if this is also the  
341 case of an *in vivo* scenario on naturally colonized chicken broilers is still under  
342 debate. It has been suggested that probiotic bacteria are very efficient in reducing *C.*  
343 *jejuni* colonisation of the gastrointestinal compartments in chicken broilers, however  
344 in this case the probiotic strains were introduced in the diets and the authors have  
345 not characterised the microbiota composition in the caecum (Cean and others 2015).  
346 We have shown that up to Day 21 Carvacrol was able to increase the presence of  
347 probiotic bacteria which correlates with no *C. jejuni* presence in the experimental  
348 groups.

349

350 Dietary evaluation of essential oils has been indicated to reduce the gut lesions and  
351 improve villus height and crypt depth in the small intestine of broiler chickens fed  
352 with 120-240 mg/kg tymol and carvacrol. It has been suggested that these essential  
353 oils improve intestinal integrity and modulate immune responses in *Clostridium*  
354 *perfringens* challenged chicken broilers (Du and others 2016). Also, an increased  
355 villus height is associated with an increased digestive and absorptive function of the  
356 gut due to increased absorptive surface area, enzyme expression and nutrient  
357 transport system (Amat and others 1996). We are now showing, *in vivo*, that  
358 carvacrol supplementation through feed, improves the expression of mucin mRNA  
359 expression in all the essential gut compartments providing a possible explanation for  
360 the increase in production parameters.

361

362 The high content of polyunsaturated fatty acids (PUFA) makes poultry meat less  
363 susceptible to oxidative deterioration (Luna and others 2010). In our study we found  
364 that PUFA was 5.93% in birds fed 120 mg/kg feed of carvacrol, suggesting an  
365 increase in meat quality and subsequently in shelf life. The reduced feed intakes  
366 observed during the trial could be explained by the enhanced release of satiety  
367 hormones, an effect previously described in rats (Yang and others 2013). The  
368 consumer will benefit from having a product with increased  $\omega$ 7 concentration as it  
369 has been previously shown that it may be useful in the treatment of  
370 hypertriglyceridemia with the beneficial added effects of decreasing LDL and hs-CRP  
371 and raising HDL (Bernstein and others 2014).

372

373 We have demonstrated that carvacrol prevented the infection of chicken primary  
374 intestinal cells *in vitro* and it is also able to prevent campylobacters to form biofilm.  
375 Our plate count data also indicates that carvacrol affects *Campylobacter* spp.,  
376 colonisation *in vivo* and our study indicates the efficient concentrations. Finally, our  
377 results indicate that, at farm level, inclusion of carvacrol can improve poultry health,  
378 feed efficiency, meat quality and delay colonization of foodborne pathogenic bacteria  
379 in broiler chickens

380

### 381 **Acknowledgements**

382 The authors would like to thank the staff at animal research facility at the Agri-Food  
383 and Biosciences Institute for their contribution during the course of the project.

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## References

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- 389 Amat C., Planas J.M. and Moreto M. Kinetics of hexose uptake by the small and large intestine of the  
390 chicken. *The American journal of physiology* 1996; 271:R1085-9.
- 391 Aslim B. and Yucel N. In vitro antimicrobial activity of essential oil from endemic *Origanum*  
392 *minutiflorum* on ciprofloxacin-resistant *Campylobacter* spp. *Food Chemistry* 2008; 107:602-  
393 606.
- 394 Authority E.F.S. Trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013.  
395 *EFSA Journal* 2015.
- 396 Bassler B.L. Small talk. Cell-to-cell communication in bacteria. *Cell* 2002; 109:421-4.
- 397 Bernstein A.M., Roizen M.F. and Martinez L. Purified palmitoleic acid for the reduction of high-  
398 sensitivity C-reactive protein and serum lipids: a double-blinded, randomized, placebo  
399 controlled study. *Journal of clinical lipidology* 2014; 8:612-7.
- 400 Broiler Management Manual Ross-308 and Home page address: [www.aviagen.com](http://www.aviagen.com) p.-.
- 401 Byrne C.M., Clyne M. and Bourke B. *Campylobacter jejuni* adhere to and invade chicken intestinal  
402 epithelial cells in vitro. *Microbiology* 2007; 153:561-9.
- 403 Caporaso J.G., Kuczynski J., Stombaugh J., et al. . QIIME allows analysis of high-throughput  
404 community sequencing data. *Nature methods* 2010; 7:335-6.
- 405 Cean A., Stef L., Simiz E., et al. . Effect of human isolated probiotic bacteria on preventing  
406 *Campylobacter jejuni* colonization of poultry. *Foodborne pathogens and disease* 2015;  
407 12:122-30.
- 408 Cherian G., Selvaraj R.K., Goeger M.P. and Stitt P.A. Muscle fatty acid composition and thiobarbituric  
409 acid-reactive substances of broilers fed different cultivars of sorghum. *Poultry science* 2002;  
410 81:1415-20.
- 411 Corcionivoschi N., Alvarez L.A., Sharp T.H., et al. . Mucosal reactive oxygen species decrease  
412 virulence by disrupting *Campylobacter jejuni* phosphotyrosine signaling. *Cell host & microbe*  
413 2012; 12:47-59.
- 414 Corcionivoschi N., Clyne M., Lyons A., et al. . *Campylobacter jejuni* cocultured with epithelial cells  
415 reduces surface capsular polysaccharide expression. *Infect Immun* 2009; 77:1959-67.
- 416 Corcionivoschi N., Gundogdu O., Moran L., et al. . Virulence characteristics of hcp (+) *Campylobacter*  
417 *jejuni* and *Campylobacter coli* isolates from retail chicken. *Gut pathogens* 2015; 7:20.
- 418 Crushell E., Harty S., Sharif F. and Bourke B. Enteric *Campylobacter*: purging its secrets? *Pediatr Res*  
419 2004; 55:3-12.
- 420 Du E., Wang W., Gan L., Li Z., Guo S. and Guo Y. Effects of thymol and carvacrol supplementation on  
421 intestinal integrity and immune responses of broiler chickens challenged with *Clostridium*  
422 *perfringens*. *Journal of animal science and biotechnology* 2016; 7:19.
- 423 Hafeez A., Manner K., Schieder C. and Zentek J. Effect of supplementation of phytogenic feed  
424 additives (powdered vs. encapsulated) on performance and nutrient digestibility in broiler  
425 chickens. *Poultry science* 2015.
- 426 Juneja V.K., Dwivedi H.P. and Yan X. Novel natural food antimicrobials. *Annual review of food science*  
427 *and technology* 2012; 3:381-403.
- 428 K. Arsi A.M.d., K. Venkitanarayanan, A. Kollanoor-Johny, A.C. Fanatico, and Donoghue P.J.B.a.D.J. The  
429 Efficacy of the Natural Plant Extracts, Thymol and Carvacrol against *Campylobacter*  
430 Colonization in Broiler Chickens. *Journal of Food safety* 2014; 34:321-325.
- 431 Klindworth A., Pruesse E., Schweer T., et al. . Evaluation of general 16S ribosomal RNA gene PCR  
432 primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids*  
433 *Res* 2013; 41:e1.
- 434 La Storia A., Ercolini D., Marinello F., Di Pasqua R., Villani F. and Mauriello G. Atomic force  
435 microscopy analysis shows surface structure changes in carvacrol-treated bacterial cells.  
436 *Research in microbiology* 2011; 162:164-72.

437 Lambert R.J., Skandamis P.N., Coote P.J. and Nychas G.J. A study of the minimum inhibitory  
438 concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of*  
439 *applied microbiology* 2001; 91:453-62.

440 Lillehoj H.S., Kim D.K., Bravo D.M. and Lee S.H. Effects of dietary plant-derived phytonutrients on the  
441 genome-wide profiles and coccidiosis resistance in the broiler chickens. *BMC proceedings*  
442 2011; 5 Suppl 4:S34.

443 Luna A., Labaque M.C., Zygadlo J.A. and Marin R.H. Effects of thymol and carvacrol feed  
444 supplementation on lipid oxidation in broiler meat. *Poultry science* 2010; 89:366-70.

445 Naito M., Fridrich E., Fields J.A., et al. . Effects of sequential *Campylobacter jejuni* 81-176  
446 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis.  
447 *Journal of bacteriology* 2010; 192:2182-92.

448 Ozogul Y., Kuley E., Ucar Y. and Ozogul F. Antimicrobial Impacts of Essential Oils on Food Borne-  
449 Pathogens. *Recent patents on food, nutrition & agriculture* 2015; 7:53-61.

450 Pircalabioru G., Aviello G., Kubica M., et al. . Defensive Mutualism Rescues NADPH Oxidase  
451 Inactivation in Gut Infection. *Cell host & microbe* 2016; 19:651-63.

452 Qiu J., Feng H., Lu J., et al. . Eugenol reduces the expression of virulence-related exoproteins in  
453 *Staphylococcus aureus*. *Applied and environmental microbiology* 2010; 76:5846-51.

454 Ragimbeau C., Colin S., Devaux A., et al. . Investigating the host specificity of *Campylobacter jejuni*  
455 and *Campylobacter coli* by sequencing gyrase subunit A. *BMC microbiology* 2014; 14:205.

456 Reuter M., Mallett A., Pearson B.M. and van Vliet A.H. Biofilm formation by *Campylobacter jejuni* is  
457 increased under aerobic conditions. *Applied and environmental microbiology* 2010; 76:2122-  
458 8.

459 S A. FastQC. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>, v0112  
460 <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/> 2010.

461 Sivaranjani A., Sivagami G. and Nalini N. Chemopreventive effect of carvacrol on 1,2-  
462 dimethylhydrazine induced experimental colon carcinogenesis. *Journal of cancer research*  
463 *and therapeutics* 2016; 12:755-62.

464 Smirnov A., Perez R., Amit-Romach E., Sklan D. and Uni Z. Mucin dynamics and microbial populations  
465 in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter  
466 supplementation. *The Journal of nutrition* 2005; 135:187-92.

467 van Alphen L.B., Burt S.A., Veenendaal A.K., Bleumink-Pluym N.M. and van Putten J.P. The natural  
468 antimicrobial carvacrol inhibits *Campylobacter jejuni* motility and infection of epithelial cells.  
469 *PloS one* 2012; 7:e45343.

470 Xu J., Zhou F., Ji B.P., Pei R.S. and Xu N. The antibacterial mechanism of carvacrol and thymol against  
471 *Escherichia coli*. *Letters in applied microbiology* 2008; 47:174-9.

472 Yang Z.H., Takeo J. and Katayama M. Oral administration of omega-7 palmitoleic acid induces satiety  
473 and the release of appetite-related hormones in male rats. *Appetite* 2013; 65:1-7.

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Table 1. Chemical composition of basal diet

Item	Starter 0-10 days	Grower (11-24 days)	Finisher (25-35) days
Wheat	54.623	57.553	61.300
Full fat soya	12.000	12.000	12.000
Brazilian GM hipro	25.000	21.000	17.000
Lime bulk	0.717	0.700	0.500
DCP bulk (18.1% p)	1.654	2.000	2.150
Salt bulk	0.200	0.200	0.200
Sod.bi-carbonate	0.199	0.166	0.162
DL methionine	0.487	0.435	0.378
L-lysine	0.373	0.318	0.281
Threonine	0.247	0.128	0.029
Vitamin+mineral premix	0.500	0.500	0.500
Soyabean oil	4.000	5.000	5.500
Calculated composition (%)			
ME Kcal/kg	2999	3081	3133.8
CP	23.12	21.53	20.04
Lys	1.45	1.308	1.17
Met+Cys	1.089	0.996	0.91
Ca	0.97	0.906	0.85
AvP	0.49	0.41	0.409

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483 Table 2. Samples used in study and corresponding I7 and I5 index primer used in this study.

Sample Name	I7 Index ID	Index	I5 Index ID	Index 2
Day10C	N709	GCTACGCT	S502	CTCTCTAT
Day10T1	N710	CGAGGCTG	S517	GCGTAAGA
Day10T2	N707	CTCTCTAC	S502	CTCTCTAT
Day10T3	N708	CAGAGAGG	S502	CTCTCTAT
Day21C	N711	AAGAGGCA	S517	GCGTAAGA
Day21T1	N712	GTAGAGGA	S517	GCGTAAGA
Day21T2	N701	TAAGGCGA	S502	CTCTCTAT
Day21T3	N702	CGTACTAG	S502	CTCTCTAT
Day35C	N703	AGGCAGAA	S502	CTCTCTAT
Day35T1	N704	TCCTGAGC	S502	CTCTCTAT
Day35T2	N705	GGACTCCT	S502	CTCTCTAT
Day35T3	N706	TAGGCATG	S502	CTCTCTAT

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488 **Figure captions**

489

490 Figure 1. Adhesion, internalization, biofilm formation *in vitro*, and *in vivo* mucin  
491 expression

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493 Panel A shows the adhesion and Panel B the invasion of chicken intestinal primary  
494 cells of *C. jejuni* RC039 in the presence of Carvacrol. Panel C shows the effect on  
495 biofilm formation. Panel D, mucin mRNA expression. Micrographs of epithelial  
496 integrity and villus height of small intestine (Panel E control, F - experimental) large  
497 intestine (Panel G control group, H – experimental) and caecum (Panel I control, J  
498 experimental). Yellow arrow indicates erythrocyte infiltration in the lamina propria in  
499 the control group sections. Bar = 10µm. The experiments were done in triplicate and  
500 on three separate occasions. Significance was assessed by Student's *t* test.  
501 (\*\* $P < 0.05$ ,  $P < 0.005$ , \*\*\* $P < 0.0005$ ).

502

503 Figure 2. *Campylobacter* spp., *Lactobacillus* spp. and *E. coli* quantification in caecal  
504 content at 10, 21 and 35 days.

505

506 Panel A shows the *Campylobacter* spp. counts from the experimental groups (T1, T2  
507 and T3) at 0-35 days. The percentage change over control is presented in Panel B.  
508 The data presented was obtained from 12 broilers/experiment (n=48/each time  
509 point). The *E. coli* counts have shown significant decrease at 35 days in all

510 experimental groups (Panel C).. The *P* values were calculated relative to the count  
511 obtained at 21 days. Error bars represent  $\pm$ S.D. of 12 broilers/experiment (n=48/each  
512 time point). Statistical significance (Student's *t* test) relative to the level of control  
513 group is indicated. The relative abundance of *Lactobacillus spp.* as determined by  
514 qPCR (Panel D) from broilers cecal DNA. Each stacked bar represents the mean  
515 relative abundance; *Eubacteria* 16S was used for normalization. Error bars represent  
516  $\pm$ S.D. of 12 broilers/experiment (n=48/each time point). Statistical significance  
517 (Student's *t* test) relative to the level of control group is indicated.

518

519 Figure 3. Plot bar charts of bacteria classified as phyla (A), class (B) and order (C)  
520 detected from microbiome studies. Percentage distribution for phyla and all the other  
521 levels in Supplementary Fable 1. Data was generated by uploading onto BaseSpace  
522 and analysed using the Qiime preprocessing and visualization apps.

523

524 Figure 4. The effect of Carvacrol on the production parameters of naturally colonized  
525 chicken broilers

526

527 Panel A describes the effect of Carvacrol on broiler feed intake profile from 0-35  
528 days and Panel B the percentage change in feed intake relative to the control group.  
529 The feed conversion rates (FCR) are shown in Panel C and the percentage change  
530 over control in Panel D. The body weight profiles between the experimental groups  
531 are indicated in panel E and F. Lipid oxidation is presented in Panel G at 21 and 35  
532 days for each experimental group and in Panel H the data is expressed as % TBARS

533 inhibition compared to control. Statistical significance (Student's *t* test) relative to the  
534 control group feed intake is indicated.

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Supplementary Table S1

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## Fatty acid composition of meat samples

Specifications	Total SFA	Total UFA	Total MUFA	Total PUFA	Total $\omega$ 3	Total $\omega$ 6	Total $\omega$ 7	Total $\omega$ 9
<b>mg/g muscle</b>								
Control	18.51	53.20	20.99	31.10	3.57	27.53	1.67	19.25
T1	16.90	50.04	19.53	29.47	3.36	26.10	1.71	17.74
T2	19.51	55.31	23.07	31.18	3.62	27.56	2.13	20.87
T3	19.97	53.80	22.79	33.98	3.88	30.11	1.9	20.87
Significance	NS	NS	NS	NS	NS	NS	NS	NS
<b>Fold increase over control</b>								
T1 vs C	0.91338 0155	0.94066 7878	0.93014 7642	0.94759 9657	0.94117 6471	0.94806 9241	1.02594 8104	0.92174 5152
T2 vs C	1.05438 5017	1.03972 1822	1.09890 4588	1.00246 4638	1.01493 9309	1.00084 7355	1.27744 511	1.08431 4404
T3 vs C	1.07905 6366	1.01134 0142	1.08556 9138	1.09247 7497	1.08683 4734	1.09345 1156	1.13772 4551	1.08431 4404
<b>Percentage increase over control</b>								
T1 vs C	- 8.66198 4513	- 5.93321 2205	- 6.98523 5752	- 5.24003 4291	- 5.88235 2941	- 5.19307 5899	- 2.59481 0379	- 7.82548 4765
T2 vs C	5.43850 1711	3.97218 2194	9.89045 8803	0.24646 3781	1.49393 0906	0.08473 5504	27.7445 1098	8.43144 0443
T3 vs C	7.90563 6593	1.13401 416	8.55691 3796	9.24774 9679	8.68347 3389	9.34511 5603	13.7724 5509	8.43144 0443

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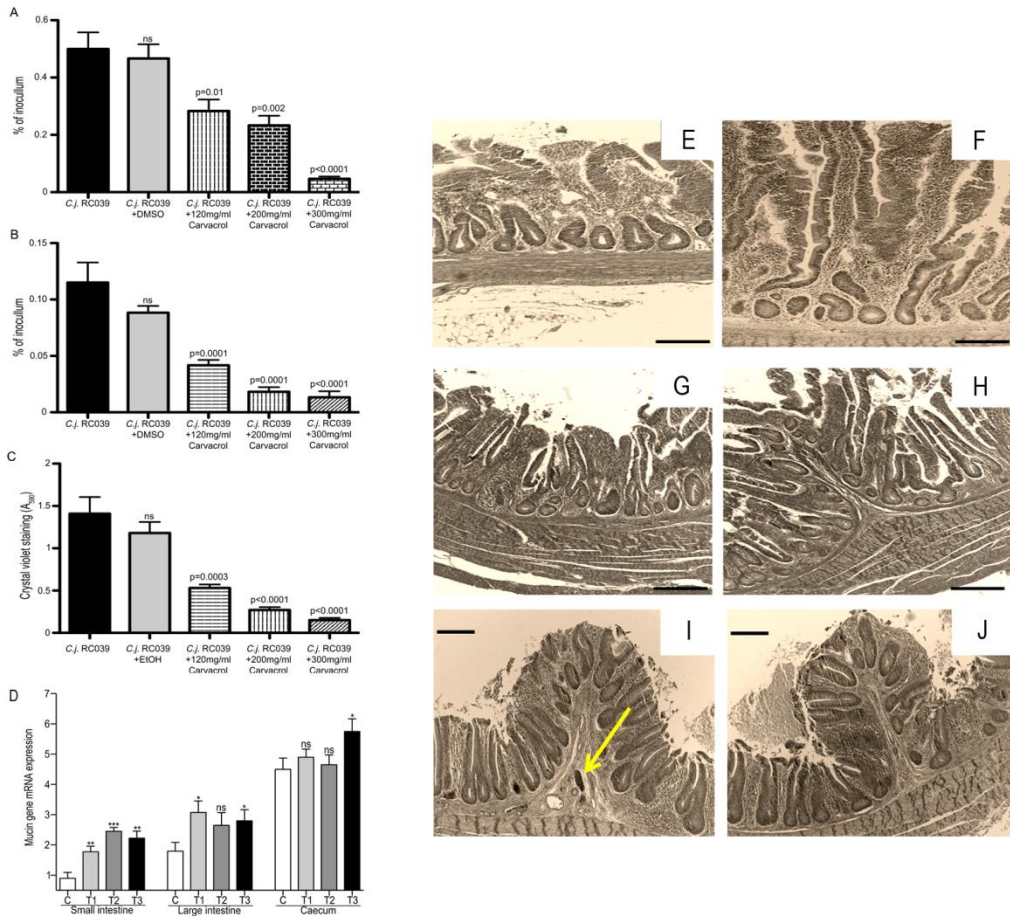
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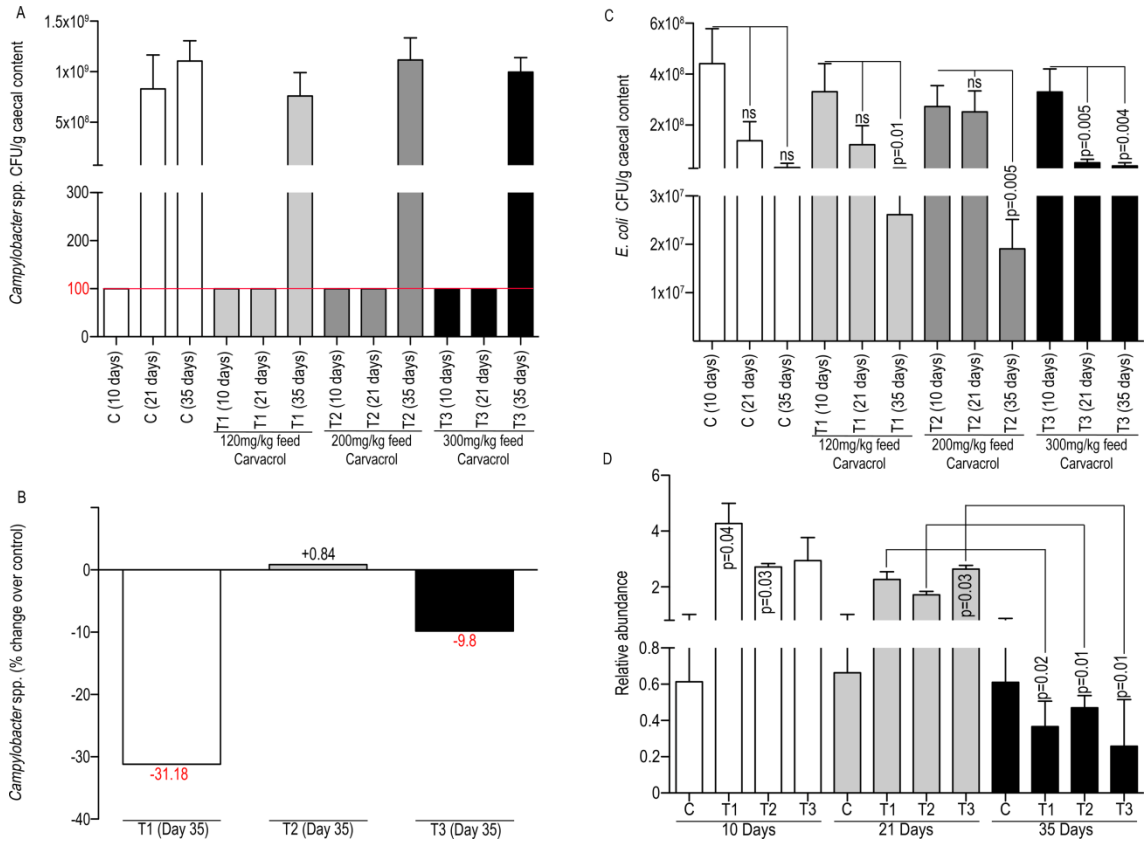
544 Figure 1



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547 Figure 2



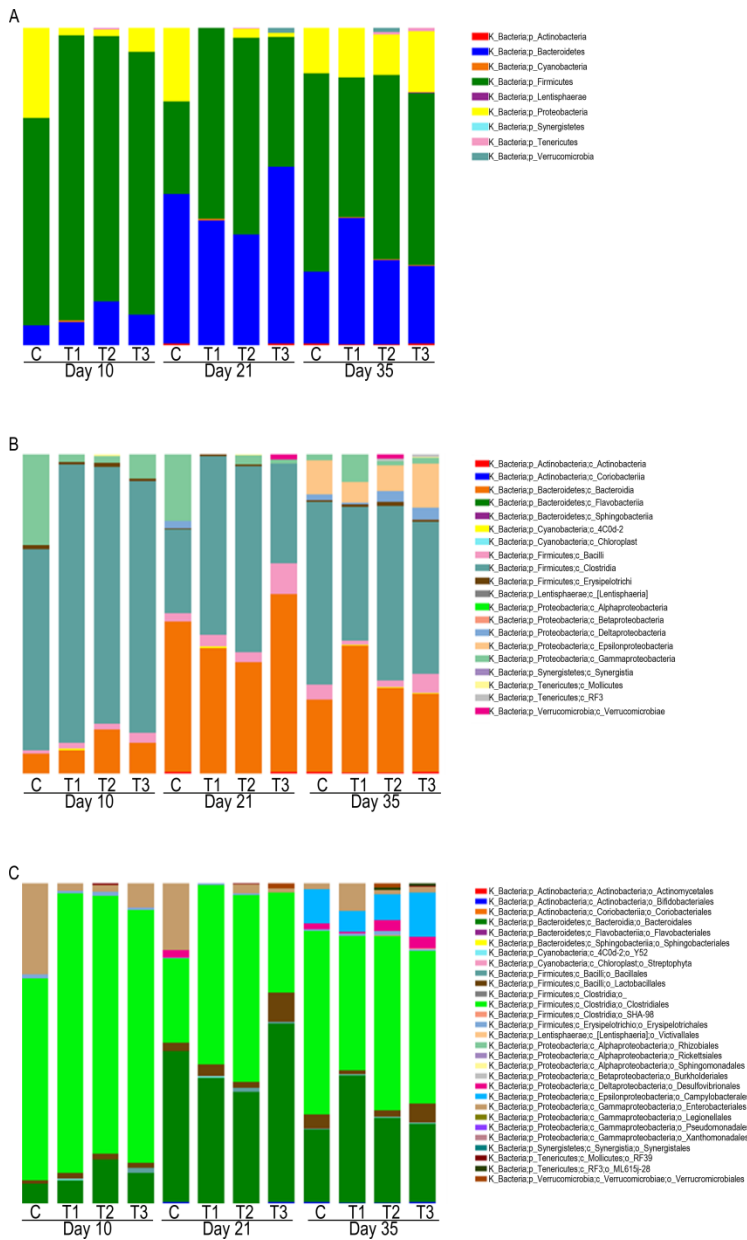
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552 Figure 3



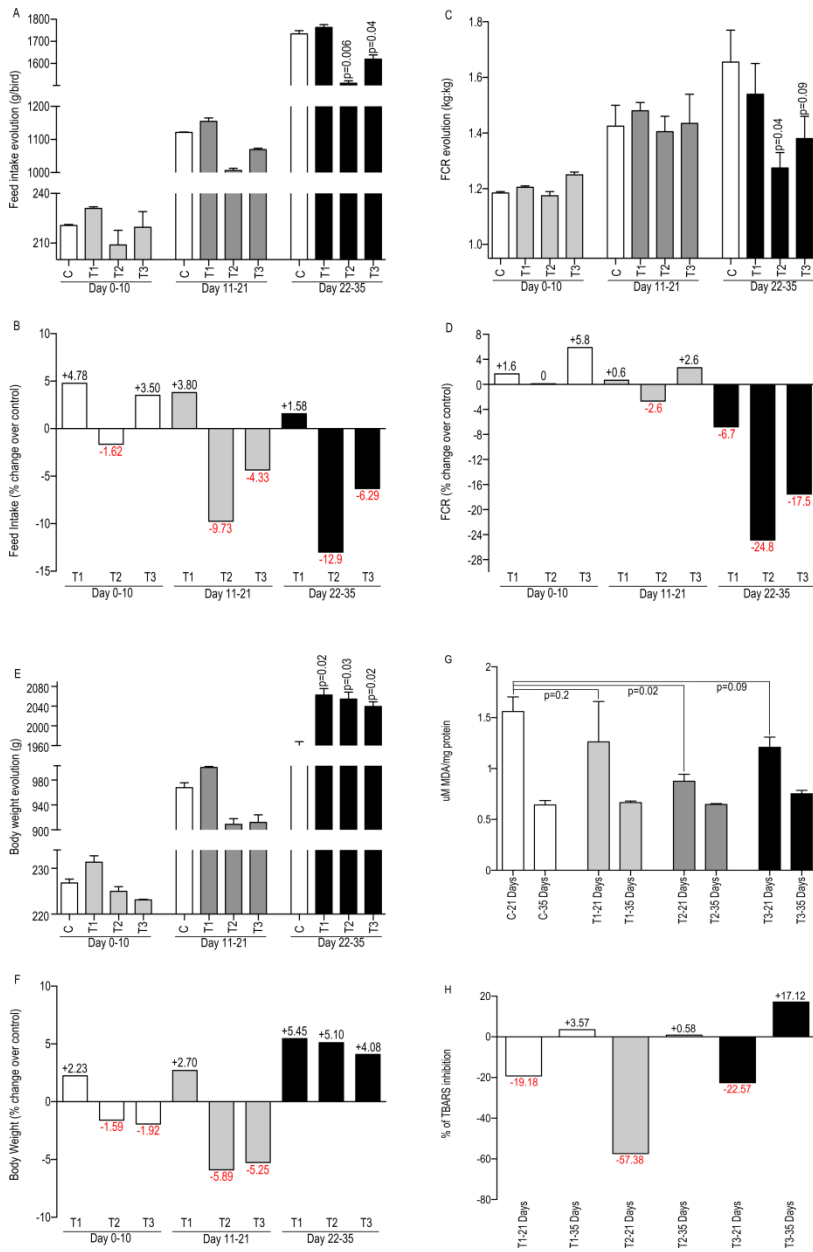
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556 Figure 4

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