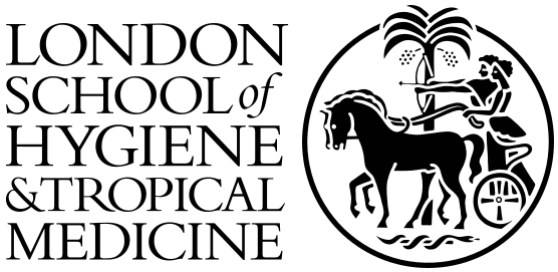

The *in vitro* and *in vivo* effect of Carvacrol in preventing *Campylobacter* infection, colonisation and improve chicken broilers productivity

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

Abstract

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

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

Introduction

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

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

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

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

Material and Methods

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).

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

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

16S rRNA amplification and sequencing

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

qPCR for quantification of lactic acid bacteria

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

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

Mucin mRNA analysis

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

Histology

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

Biofilm assay

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

200 mg/ml and 300 mg/ml carvacrol diluted in ethanol and added to the growth medium in polystyrene flatbottomed 6 well plates and incubated for 48 hours at 42°C. One milliliter of a 1% crystal violet solution was added and the wells were incubated at room temperature for 60 min. Unbound crystal violet was washed off with water and the plates were dried at 37°C. Bound crystal violet was dissolved in 20% acetone in ethanol for 10 min and was then poured into cuvettes, and the *A*590 was measured.

Infection of chicken primary intestinal cells

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

TBARS

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

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

Gas Chromatography (GC) for fatty acids analysis

For GC analysis of fatty acids 1 g of meat sample was mixed with 3 ml methanol and 0.7 ml 10NKOH and incubated at 56°C oven overnight. An internal standard (Tridecanoic acid) was added to check recovery. The sample was allowed to cool before adding 0.58ml 24N H_2SO_4 followed by 90 minutes incubation, with occasional mixing. Once cooled 3 ml of hexane was added and the sample was mixed. The extract was run for 91 minutes to ensure all FAMEs (fatty acid methyl esters) were recovered. These were then identified and analysed accordingly using the GC (Varian 3800 GC).

Results

Effects of carvacrol on cell invasion and biofilm formation in vitro

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

Carvacrol effect on the chicken gastrointestinal compartments

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

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

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

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

Carvacrol induces changes in chicken caecum microbiota

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

Carvacrol improves production parameters at slaughter

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

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

Lipid oxidation and fatty acid composition of broiler thigh muscle

Across the carvacrol treatments the TBARS values decreased significantly only at 21 days as shown in figure 4 (Panel G and H). Treatment T1 showed a 19.18% decrease (p=0.2), at treatment T2 the TBRAS were reduced with 57.38% (p=0.02) and with 22.57% at T3 (p=0.09). At Day 35 compared to the control group, an 8.68% increase in total ω3 fatty acids, 9.34% increase in ω6, 13.77% increase in ω7 and 8.43% in ω9. The total mono-unsaturated fatty acids (MUFA) at Day 35 showed an increase of 8.55% over control and the poly-unsaturated fatty acids (PUFA) increased by 9.24% compared to the control. The SFA increases in T2 (1 mg/g muscle) and T3 (1.46 mg/g) muscle are not significant and probably not biologically relevant. However, there was an increase by 2.11 mg/g muscle in UFA at T2 and by 0.6 mg/g muscle at T3 with no statistical significance as described in Supplementary Table 1.

Discussion

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 Storia 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 from, biofilm .

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

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

The high content of polyunsaturated fatty acids (PUFA) makes poultry meat less susceptible to oxidative deterioration (Luna and others 2010). In our study we found that PUFA was 5.93% in birds fed 120 mg/kg feed of carvacrol, suggesting an increase in meat quality and subsequently in shelf life. The reduced feed intakes observed during the trial could be explained by the enhanced release of satiety hormones, an effect previously described in rats (Yang and others 2013). The consumer will benefit from having a product with increased ω7 concentration as it has been previously shown that it may be useful in the treatment of hypertriglyceridemia with the beneficial added effects of decreasing LDL and hs-CRP and raising HDL (Bernstein and others 2014).

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

Acknowledgements

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

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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

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

Figure captions

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

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

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

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

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

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

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

Panel A describes the effect of Carvacrol on broiler feed intake profile from 0-35 days and Panel B the percentage change in feed intake relative to the control group. The feed conversion rates (FCR) are shown in Panel C and the percentage change over control in Panel D. The body weight profiles between the experimental groups are indicated in panel E and F. Lipid oxidation is presented in Panel G at 21 and 35 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

Fatty acid composition of meat samples

Specifications	Total SFA	Total UFA	Total MUFA	Total PUFA	Total ω3	Total ω6	Total ω7	Total ω9
mg/g muscle								
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
Fold increase over control								
T1 vs C	0.913380155	0.940667878	0.930147642	0.947599657	0.941176471	0.948069241	1.025948104	0.921745152
T2 vc C	1.054385017	1.039721822	1.098904588	1.002464638	1.014939309	1.000847355	1.27744511	1.084314404
T3 vs C	1.079056366	1.011340142	1.085569138	1.092477497	1.086834734	1.093451156	1.137724551	1.084314404
Percentage increase over control								
T1 vs C	-8.661984513	-5.933212205	-6.985235752	-5.240034291	-5.882352941	-5.193075899	2.594810379	-7.825484765
T2 vc C	5.438501711	3.972182194	9.890458803	0.246463781	1.493930906	0.084735504	27.74451098	8.431440443
T3 vs C	7.905636593	1.13401416	8.556913796	9.247749679	8.683473389	9.345115603	13.77245509	8.431440443

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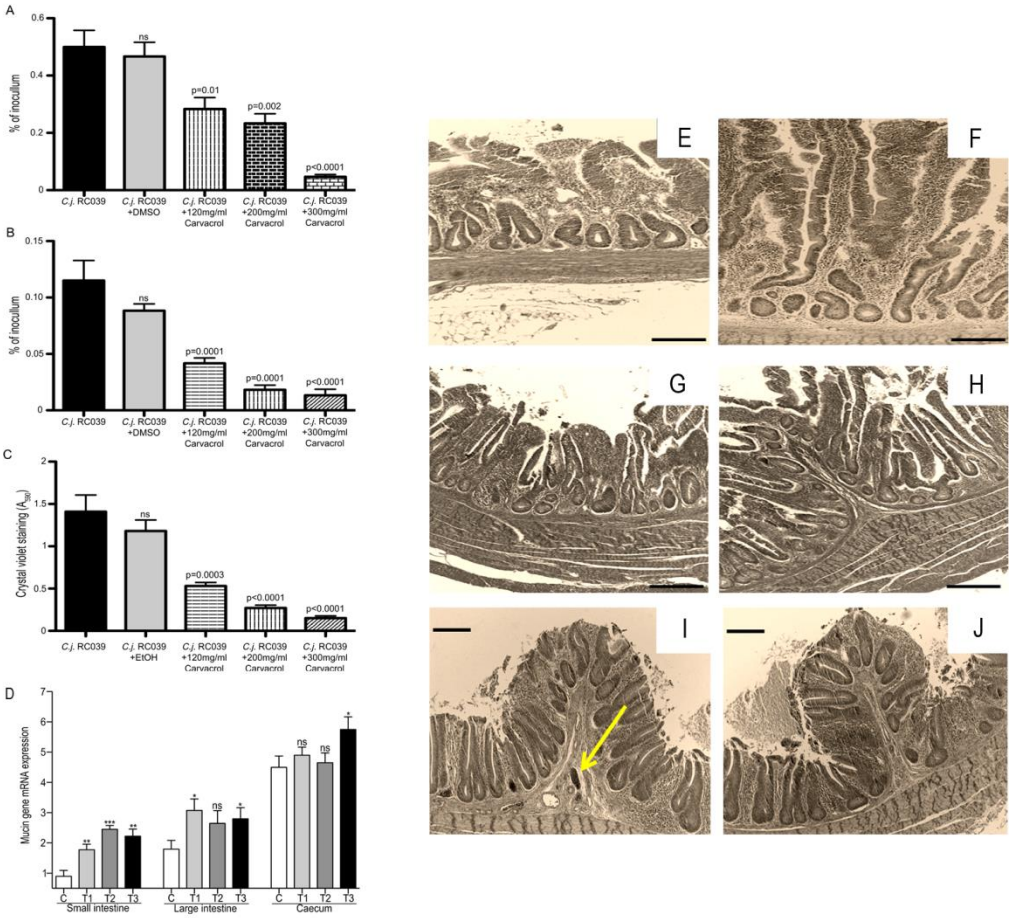
544 Figure 1



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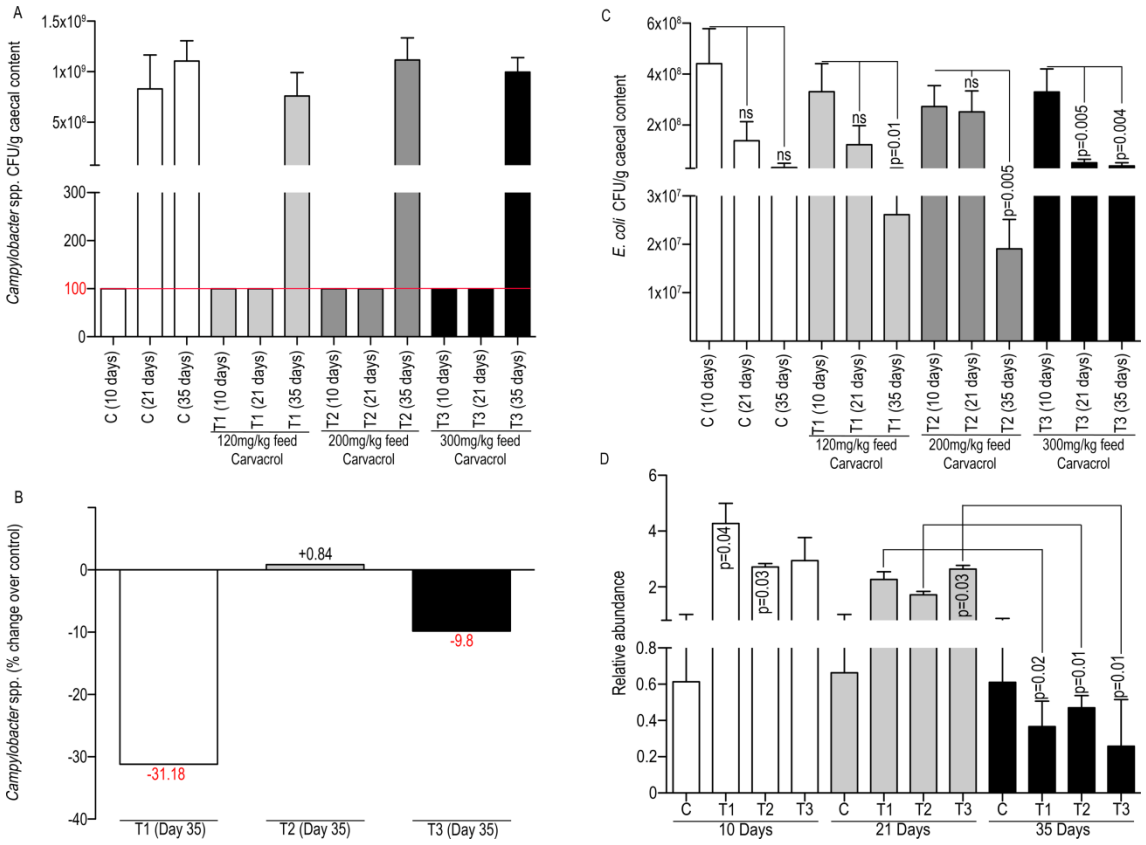
547 Figure 2



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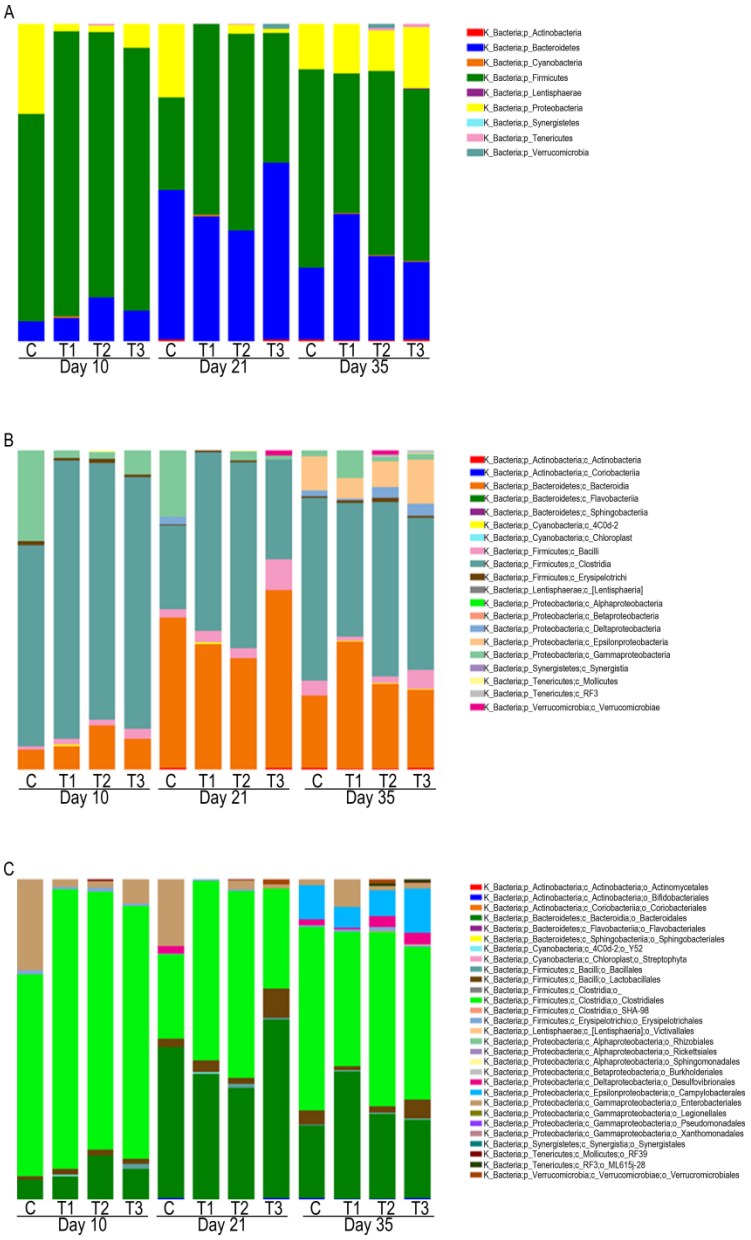
552 Figure 3



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556 Figure 4

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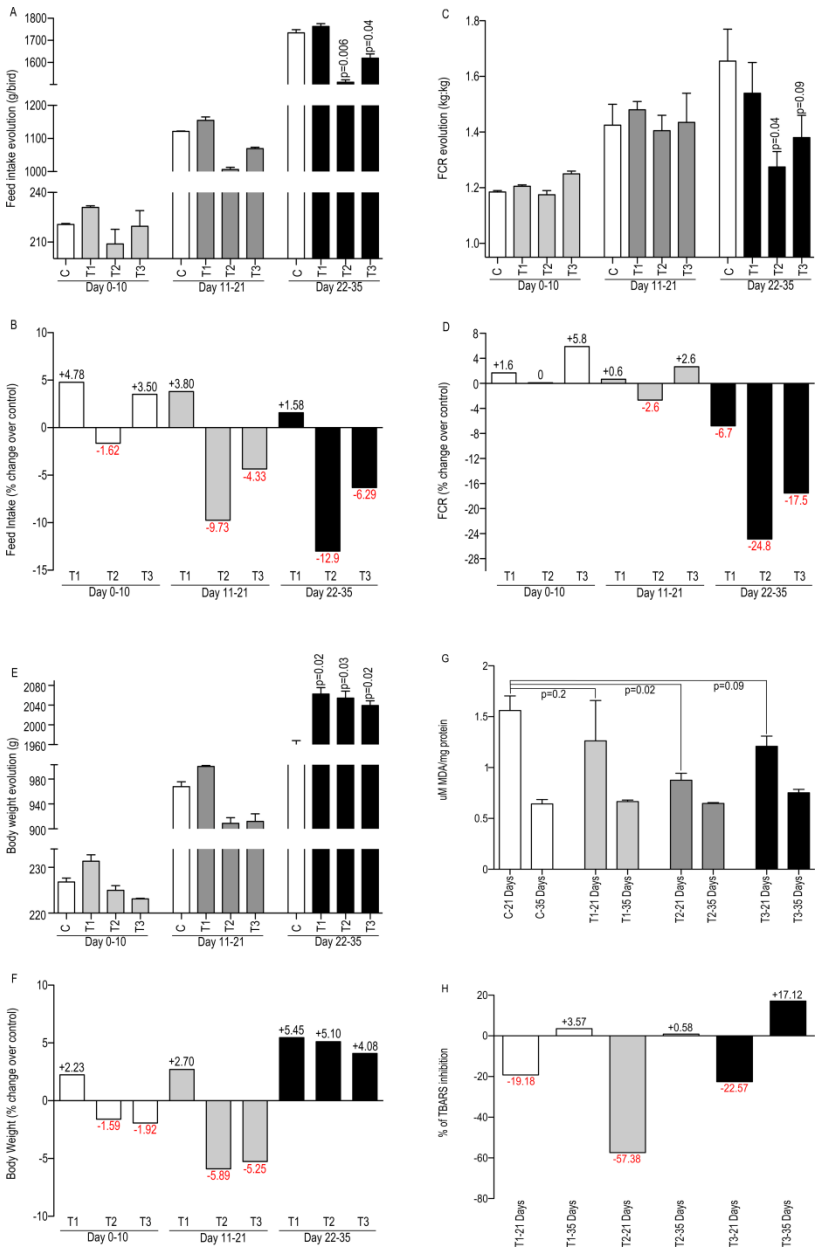

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