The effect of natural antimicrobials on the *Campylobacter coli* T6SS$^{+/-}$ during *in vitro* infection assays and on their ability to adhere to chicken skin and carcasses

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Abstract

Reducing the *Campylobacter* load on poultry carcasses represents a major tasks for the industry as its ability to reduce their presence is of major interest aiming to increase consumer safety. This study investigated the ability of a mixture of natural antimicrobials (A3001) to reduce the adherence of the T6SS$^{+/-}$ *C. coli* isolates (NC1^{hcp-}, NC2 $^{hcp-}$ and NC3 $^{hcp+}$) to chicken neck skin and whole carcasses. Overall, the antimicrobial mixture induced a significant reduction in the capability of our *C. coli* isolates to colonize the chicken skin ($p<0.05$) and carcasses ($p<0.0001$) but with a greater effect (≈3 log reduction) on the NC3 isolate. Using the HCT-8 *in vitro* infection model we also show that at a concentration of 0.5% A3001, the impact on the NC3 isolate is accompanied by the downregulation of the *hcp* gene ($p=0.0001$), and indicator of the T6SS presence. The results described herein also indicated that these isolates are highly resistant to H_2O_2, up to 20mM, suggesting a high resilience to environmental stresses. In summary our study shows that natural antimicrobials can reduce the ability of T6SS positive chicken *C. coli* isolates to adhere to chicken skin or to the whole carcass and to infect epithelial cells *in vitro* and could be considered a potential intervention at processor level.

Keywords: *Campylobacter coli*; infectivity; natural antimicrobials; chicken skin; chicken carcasses

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1. Introduction

Campylobacter spp., are a spiral shaped, Gram – negative, oxidase positive and microaerophilic bacteria that possesses a single polar flagellum (Igwaran & Okoh, 2019). This microorganism does not usually inhabit the environment, but it is rather distributed in warm-blooded animals, mainly in birds (Corrigan, Fay, Corcionivoschi, & Murphy, 2017; Han, et al., 2019). *Campylobacter coli* and *Campylobacter jejuni* are common commensal inhabitants of the gut microbiota in numerous wild and domesticated animal species, being especially prevalent in poultry (Liaw, et al., 2019). Bacteria from the genus *Campylobacter* is the major cause of gastroenteritis worldwide and is one of the most widespread causative agents of infectious diseases of the last century (A. B. Karki, Wells, & Fakhr, 2019; Man, 2011; Sheppard, et al., 2010). The major risk to human health is posed by *Campylobacter jejuni*, then *Campylobacter coli*, and *Campylobacter fetus*, causing severe infections, followed by sepsis and complicated with Guillain Barre either Miller Fisher syndromes (Liaw, et al., 2019). *C. coli* is not as known as *C. jejuni* as a human pathogen and it represents only 10% of the detected infections with the pathogenicity of *C. coli* being almost impossible to be separated from that of *C. jejuni* (Gillespie, et al., 2002).

It is estimated that circa 70% of raw chicken from the UK sold via supermarkets is contaminated with *Campylobacter* spp. (Liaw, et al., 2019). Humans typically

85 become infected by eating poorly cooked meat, unpasteurized dairy products or the
86 use of contaminated water sources. Occasionally occurs on coming into contact with
87 poultry (Ikeda & Karlyshev, 2012; A. B. Karki, et al., 2019). Thus, controlling
88 colonization with *C. jejuni* and *C. coli* is an important critical point in food processing,
89 catering and retailing. To avoid such economic impacts which are more than 2.4
90 billion euros/year registered in the European Union member states, it is necessary
91 to develop new approaches to control the food raw materials and finished products
92 (Cody, Maiden, Strachan, & McCarthy, 2019; Koolman, Whyte, Burgess, & Bolton,
93 2016).
94 A number of different molecular mechanisms are involved in pathogenesis which in
95 turn are required for the occurrence of infection. For example, when colonizing the
96 human gut, campylobacters communicate with the epithelial cells of the
97 gastrointestinal tract. That phenomenon happens due to the attachment of
98 *Campylobacter* spp. through the binding of adhesins directly to eukaryotic cell
99 ligands and extracellular matrix constituents. Understanding the primary phases of
100 infection, which involves incubation, motility, adhesion, invasion, and chemotaxis is
101 crucial to understanding a successful infection (Xu, Abdul-Wakeel, Gunther, &
102 Sommers, 2019). Other infectivity mechanisms of *Campylobacter* include capsular
103 polysaccharides, CmeABC (efflux pump) and quorum sensing regulating system
104 (luxS) involved in antimicrobial resistance and the Type 6 secretion system (T6SS)
105 which is acting as a firing nano-crossbow inducing bacterial and cell death

106 (Francetic, 2018; Klancnik, et al., 2019). The identification of the gene *hcp* from
107 samples can be used as an indicator of the presence of the T6SS mechanism
108 (Corcionivoschi, et al., 2015). Despite the apparently impactful molecular
109 mechanisms, there is still a gap of knowledge that requires further elucidation and
110 more exhaustive studies. The most important survival steps for pathogens within the
111 host involves overcoming aerobic, oxidative and nitrosative stress (Gundogdu, et al.,
112 2011). Therefore, exposing *Campylobacter* strains to oxidative stress factors such
113 as H_2O_2, bile salts, different pH levels and novel commercial antimicrobials may
114 represent a key answer in reducing their infectivity (Gundogdu, et al., 2011). *C. jejuni*
115 strains exhibit elevated resistance to H_2O_2 and aerobic stress compared with *C. coli*
116 strains, which may be explained due to genetic variation (Gundogdu, et al., 2016).
117 The current threat of development of antimicrobial resistance in bacteria is
118 associated with the unjustified and inappropriate widespread use of antibacterial
119 substances in animal and human health, as well as inadequate measures to control
120 the spread of infectious diseases. The actual and future consequences regarding
121 the overuse of antibiotics and acquired bacterial resistance suggests the
122 development of novel antimicrobial strategies is required. Modern biotechnology
123 offers promising natural alternatives to antibiotics. Plant phenolic metabolites have
124 known antimicrobial properties that can change the pH, modify efflux pumps and
125 influence membrane permeability of microbes (Lewis & Ausubel, 2006; Sima, et al.,
126 2018; Srivastava, Chandra, Nautiyal, & Kalra, 2014; A. Stratakos, et al., 2019) and

127 their effect in reducing bacterial infectivity via the inactivation of the T6SS has been
128 previously reported (Sima, et al., 2018).
129 The role of natural antimicrobial mixtures in preventing *Campylobacter jejuni* and
130 *Campylobacter coli* was previously investigated only in regards to their ability to
131 colonise the chicken gut but the effect in preventing contamination at slaughter or to
132 increase product safety at retail level was not investigated so far. Undertaking such
133 work was considered essential because it will improve our knowledge in regards to
134 the ability of these natural antimicrobials to be used as a carcass wash to decrease
135 the presence of campylobacters on the final processed product and to increase in
136 this way consumer confidence (Corcionivoschi, et al., 2015; Sima, et al., 2018). As
137 a consequence we have designed this study to characterize the phenotype and
138 infectivity of three new *C. coli* chicken neck skin isolates since it has been reported
139 that 56.1% of the *C. coli* isolates are positive the T6SS infectivity factor compared to
140 *C. jejuni* where only 28.8% of the isolates were detected as positive (Corcionivoschi,
141 et al., 2015). Moreover, the aim of our study was to investigate the possible role of
142 natural antimicrobials in reducing the adherence of *C. coli* isolates to chicken skin
143 and whole carcass, *ex vivo*, and also to explore their anti-virulent potential by using
144 an *in vitro* infection model on epithelial cells. In order to achieve knowledge for further
145 development this study investigates the effect of one of our antimicrobial mixtures
146 both *ex vivo* and *in vitro* using *C. coli* chicken isolates from commercially sourced
147 chickens.

2. Materials and Methods

2.1. Microbiology and antimicrobials

The *C. coli* NC1, NC2, NC3 and RC018 strains were grown in Mueller-Hinton media purchased from Thermo Fisher Scientific Ltd, Basingstoke UK, and all reagents supplied by Sigma-Aldrich Ltd, Gillingham, UK unless otherwise stated. The *C. coli* RC018 was isolated from chicken cecum in one of our previous studies (Corcionivoschi, et al., 2015). All incubations were performed microaerobically (85% N_2, 10% CO_2 and 5% O_2, all v/v) in a Don Whitley MACS 500 workstation (Don Whitley Scientific, Shipley, UK) at 41.5°C. A mixture of antimicrobials containing contains lactic acid, E330 citric acid and citrus extract was used. The antimicrobial product (Auranta 3001) was obtained from Envirotech Innovative Products Ltd.

2.2. Isolation and identification of the *C. coli* isolates

Samples of retail chicken were prepared as previously described (Madden, Moran, Scates, McBride, & Kelly, 2011). Briefly, we have used a stomacher bag and buffer peptone water (225 ml) to emulsify 25g of skin and flesh sample in a Seward 400 blender (Seward Ltd, Worthing, UK). The emulsified sample (25ml) was transferred into a container with 225 ml of BBW (Bolton broth). The BS EN ISO10272-1:2006

was followed as previously described (Moran, Kelly, & Madden, 2009). The 225ml BB were first incubated for 4h at 37°C, followed by a second incubation step of 24h at 41.5°C. The resulting culture was plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) and incubated at 41.5°C until single colonies were countable. In order to confirm that the resulting colonies represent a typical *Campylobacter* colony the motility and oxidase tests were performed. DNA was extracted from each individual isolate using half of a 10µl loopful in 1 ml of SET buffer (150 mmol l−1 NaCl, 15 mmol l−1 EDTA, 10 mmol l−1 Tris–HCl, pH 8.0). Long-term stocks (−80°C) were prepared in 1 ml of NB plus (nutrient broth plus) containing 10% (v/v) glycerol. *C. Coli* RC018 was used as a control strain in the infectivity assays.

2.3. PCR detection of *hcp* and *gltA* genes

To detect the *hcp* multiplex PCR analysis was used as previously described (Corcionivoschi, et al., 2015) using the *gltA* as a control housekeeping gene. For amplification of *gltA* the primers *gltAF* (gcccaaagcccatcaagcgga) and *gltAR* (gcgctttggggtcatgcaca) and for the amplification of the *hcp* gene primers *hcpF* (caagcggtgcatctactgaa) and *hcpR* (taagctttgccctctctcca) were used. *C. jejuni* NCTC 12502 served as the hcp^{+} control.

2.4. Multiplex PCR for identification of *C. coli*

Isolate speciation was done accordingly to a previously published multiplex PCR assay (Wang, et al., 2002). Briefly, each multiplex PCR tube contained 200 µM deoxynucleoside triphosphate; 2.5µl of 10X DreamTaq Green Buffer, 0.5µM *C. jejuni* primers CJF (ACTTCTTTATTGCTTGCTGC) and CJR (GCCACAACAAGTAAAGAAGC), 0.5µM *C. lari* primers CLF (TAGAGAGATAGCAAAAGAGA) and CLR (TACACATAATAATCCCACCC), 1µM *C. coli* primers CCF (GTAAAACCAAAGCTTATCGTG) and CCR (TCCAGCAATGTGTGCAATG) , 1µM *C. fetus* primers CFF (GCAAATATAAATGTAAGCGGAGAG) and CFR (TGCAGCGGCCCCACCTAT); 2µM *C. upsaliensis* primers CUF (AATTGAAACTCTTGCTATCC) and CUR (TCATACATTTTACCCGAGCT); 0.2µM 23S rRNA primers 23SF (TATACCGGTAAGGAGTGCTGGAG) and 23SR (ATCAATTAACCTTCGAGCACCG), 0.75U of DreamTaq DNA Polymerase (Thermofisher Scientific) and 4.0µl of sample DNA. The volume was adjusted with sterile distilled water to give 25µl. DNA amplification was carried out in a Techne thermocycler using an initial denaturation step at 95°C for 6min followed by 30 cycles of amplification (denaturation at 95°C for 0.5min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), ending with a final extension at 72°C for 7 min. PCR product was visualised on a 2% agarose gel using a 100bp DNA Ladder (Promega G2101).

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212 **2.5. *Hcp* gene expression assay in the presence of Auranta 3001**

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214 Total RNA was isolated from bacteria exposed to Auranta 3001 at a concentration
215 of 0.5% for and 3 h by using the RNeasy®Plus Mini Kit (Qiagen, United Kingdom).
216 The RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis
217 Kit (Roche, United Kingdom) according to the manufacturer's protocol. The mRNA
218 levels were determined by quantitative RT-PCR using QuantiNovaSYBR® Green
219 PCR Kit (Qiagen, United Kingdom) on a LightCycler® 96 (Roche, United Kingdom).
220 For the hcp gene the primers used were 5′ CAAGCGGTGCATCTACTCAA 3′ and 5′
221 TAAGCTTTGCCCTCTCTCCA 3′ and for the 16S rRNA gene the primers were 5′
222 ATCTAATGGCTTAACCATTAAAC 3′, 5′ GGACGGTAACTAGTTTAGTATT 3′. The
223 conditions for genes rRNA 16S consisted of incubating for 10 min at 95°C followed
224 by 45 cycles of 95°C for 10s, 55°C for 30s, and 72°C for 10s. A total of 5µl of SYBR
225 Green master mixture was used in each reaction along with 0.5µl of 10µM primer
226 mixture, 3µl of molecular grade water, and 1µl of DNA sample. For *hcp* (2 min at
227 95°C, followed by 40 cycles of 95°C for 5s, 60°C for 10s, and a final extension at
228 72°C for 5min), a total of 5µl of SYBR Green master mixture was used in each
229 reaction along with 0.8µl of 20µM primer mixture, 7.4µl of molecular grade water,
230 and 1µl of DNA sample. Relative quantity of the mRNA was calculated using the ΔCt

method. rARN 16S gene was used as an endogenous control since it was transcribed in equal rates in both treated and untreated cells.

2.6. Motility

The motility of *C. coli* isolates NC1, NC2, NC3 and RC018 isolates was measured after the two strains were exposed to Auranta 3001 for 2h at a concentration 0.5%. In short, 5µl of culture (grown on blood agar for 48h and recovered in 1ml brain heart infusion – BHI – broth) was inoculated into the center of a 20ml semi-solid BHI plate (0.4% agar). The radius of the zone of visible growth was measured after 48h of incubation under microaerophilic conditions at 41.5°C. The experiment was carried out in triplicate, on three different days. The results are expressed as percentage decrease compared to the control.

2.7. Resistance to antibiotics

C. coli strains were grown microaerophilically at 42°C for 48h on blood agar plates. Cells were harvested from the surface of a blood agar plate by flooding the plate with Mueller Hinton broth (MHB) (Oxoid, UK) and scraping the colonies off the plate using a sterile spreader. A 100µl aliquot of this suspension was added to the surface of a blood agar plate and spread over the surface of the agar using a sterile spreader.

252 The plates were allowed to dry (<15 min) before adding an M. I. C. (minimum
253 inhibitory concentration) Evaluator™ antibiotic strip (Oxoid, UK) containing
254 ciprofloxacin, tetracycline and erythromycin. Immediately after adding the strip the
255 agar plates were incubated at 42°C for 48h microaerophilically. The results were
256 interpreted and MICs assigned according to the manufacturer's instructions. The
257 experiment was carried out on two separate occasions.
258
259 **2.8. Exposure to H_2O_2**
260
261 The MIC of H_2O_2 was determined for each C. coli strain by the broth dilution method.
262 *C. coli* were harvested from a 48h culture on blood agar plates as described above.
263 The resulting suspension (in BHI containing 10% foetal bovine serum) was adjusted
264 to an OD600 of 0.4 using the same medium. A 100 mM solution of H_2O_2 was
265 prepared in BHI containing 10% foetal bovine serum. A 1:2 dilution series of this
266 solution down to 0.312 mM was prepared in the same medium. A 100µl aliquot of
267 each dilution was added to the wells of a 96-well plate and 100µl of the bacterial
268 suspension was added to each well to give a series of solutions containing *C. coli* in
269 50 mM to 0.156 mM H_2O_2. The growth medium without inoculation was used as a
270 negative control and the growth medium without any added H_2O_2, but with the *C. coli*
271 inoculum added, was used as a positive control. The plate was incubated for 48h at
272 42°C in an Omega Fluostar plate reader in an atmosphere of 5% CO2. After 48h the

OD at 600nm (optical density measured at 600nm) was recorded and any wells showing an increase in OD were interpreted as positive for growth. The minimum inhibitory concentration was assigned at the lowest concentration where no growth had occurred.. After 48h incubation at 42°C, in a microaerophilic atmosphere, the plates were examined for evidence of growth. The lowest concentration where no growth was evident on the plate was assigned as the MBC.

2.9. Antimicrobial and chicken skin samples preparation

The novel antimicrobial (Auranta 3001) was supplied by Auranta – Envirotech Innovative Products Ltd and contains lactic and citric acid. The antimicrobial also contains: glycerine-based emulsifying agent, sodium chloride, sodium hydroxide, citrus extract (6%), oregano extract (1%), grape seed extract (2%). For the preparation of the rinse suspension, concentrations of 0.25, 0.5, and 1% were used and with water only for the control. The chicken neck skin samples were procured commercially and separated from underlying muscles using a sterile scalpel, with the skin portions were exposed to UV light for 5 min to kill the background microbial flora before the application. The neck and breast skin (in one piece) was removed and stored at –20°C until the day of experiment. A total of 240 skin samples were used for 3 trials. For each trial, 80 neck and breast skin samples were randomly allocated to 4 treatments (0, 0.25, 0.5, 1). Each skin sample was inoculated with 100

294 µL (~7 $\log_{10}$ cfu/sample) with cultures of NC1, NC2, NC3 and RC018 strains of *C.*
295 *coli* and allowed to adhere for 3 hours. Inoculation was followed by washing with 0,
296 0.25, 0.5, 1 100ml solution of Auranta with gentle shaking for 2 min. The skin
297 samples were drip dried for 5 min and analysed immediately.
298
299 **2.10. Preparation of the carcasses, inoculum and spraying solutions**
300
301 The chicken carcasses were purchased commercially at 1.8kg each. The
302 experiments were performed in three replications (n = 3) on three independent days.
303 The 36 carcasses were stored at 4˚C until the next day and exposed to UV light for
304 5 min to kill the background microbial flora before the application. The *C. coli* isolates
305 were grown as described above diluted to concentration of 2–4 x 10^8 CFU/ml. All
306 carcasses were inoculated with 1ml of the 2–4 x 10^8 CFU/ml *C. coli* NC1, NC2, NC3
307 and RC018 suspension which was spread on the carcass. After a drying period of
308 25min at room temperature both the carcasses were treated with 0.5% Auranta in
309 sterile distilled water. Sterile distilled water was used as control solution. Application
310 of the solutions was performed with a manual spray gun equipped with a 0.5mm
311 nozzle. The chicken carcasses were evenly sprayed for 30s with 5 ml 0.5% Auranta
312 3001 or sterile distilled water as control solution from a distance of 20cm. Untreated
313 carcasses were used as positive controls. After 2min of exposure enumeration of
314 bacteria was performed as described above.

315

316 **2.11. *In vitro* infection assays**

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318 The gentamicin protection assay was used to test the ability of *C. coli* chicken neck
319 skin isolates by comparison with the highly virulent strain *C. coli* RC018 to adhere
320 and invade human intestinal epithelial cells (Corcionivoschi, et al., 2009). Briefly,
321 HCT-8 cells were grown (60% confluence) for 15 to 18h in six-well tissue culture
322 plates at a concentration of 1×10^5 cells per well. Plate grown *C. coli* RC018 wild
323 type and *C. coli* chicken skin isolates were washed and re-suspended in tissue
324 culture medium at an OD_{600} of 0.4. For some experiments the bacterial isolates were
325 incubated for 3h in the presence of Auranta 3001 in order to assess the impact on
326 pathogenicity. The HCT-8 cells were washed with PBS, and 2ml of fresh culture
327 medium was added to each well. Bacteria were added to give a multiplicity of
328 infection of 10. Tissue culture plates were centrifuged at 250 × g for 5min and
329 incubated for 3h at 37°C in 10% CO_2. To quantify the number of cell-associated
330 bacteria, infected monolayers were washed at least three times with PBS and treated
331 with 0.1% Triton X-100 in PBS at 37°C for 30min. Tenfold dilutions of the contents
332 of each well were plated on Mueller Hinton agar and colonies were enumerated after
333 3 days of incubation. Invasion efficiency was calculated as the average of the total
334 number of CFU/total initial inoculum. *C. coli* RC018 passaged in RPMI 1640 (without
335 cells) was also tested for the ability to adhere to and invade HCT-8 cells. The

experiments were conducted on three separate occasions. Results for a representative experiment are presented. 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.

2.12. Statistical analysis

Data are presented as mean ± standard deviation (SD). All experiments represent at least three biological replicates performed in triplicate in each experiment. Statistical analyses were performed using Prism software (GraphPad Software). Variables were compared using Student's *t* test. We have defined as significant any P value which is ≤ 0.05.

3. Results

3.1. Speciation of *C. coli* isolates and identification of T6SS gene marker

All three poultry isolates were firstly identified as *C. coli* via the amplification of the 23S rRNA and of the 126pb *glyA* gene. Conventional multiplex PCR, with end point horizontal agarose-gel electrophoresis, was used to confirm which of the isolates were negative or positive for the T6SS, based on the *hcp* gene detection. Similar results were observed in the case of *C. coli* 11366 control strain (Fig 1, panel A). As shown in figure 1 panel B only the isolate NC3 was found to be positive for the T6SS system due to the 463bp *hcp* gene amplification. In addition, campylobacter coli isolates NC1, NC2, and NC3 were positively confirmed for *glt* gene presence by using *gltA* (encoding citrate synthase) which served as a control housekeeping gene (Fig 1, panel B).

3.2. Motility

Next we have analyzed the motility of the *C. coli* NC1, NC2 and NC3 isolates as this represents a major factor in bacterial adherence and consequently will play an important role in *Campylobacter* ability to adhere and colonise organic surfaces. Our results show that overall the three isolates are less motile when compared to the

cecum isolate RC018. The results are presented as percentage of the control strain (RC018) motility. We show that the NC2 and NC3 *C. coli* isolates resembled between 80-90% of the control strain motility weather the NC1 strain only between 50-60% (Fig 2).

3.3. Natural antimicrobials are efficient against T6SS $^{+/-}$ *C. coli* adherence spiked neck skins

The *ex vivo* experiment involved testing the ability of the antimicrobial mixture to remove the bacterial cells following spiking of chicken neck skins as described in material and methods (Fig 3). Our data shows that exposure of the spiked skins to 0.25, 0.5, and 1% of A3001 led to a significant decrease ($p<0.05$) in *C. coli* counts at all concentrations after 2 minutes of exposure and wash. All the tested doses of antimicrobial suspension (0.25, 0.5, and 1%) significantly reduced *C. jejuni* counts (>10^4 cfu/sample) compared to the controls. Only NC3 at 0.5 and 1% reached about 3.5 log cycles decrease, as depicted in figure 3. This data shows that our T6SS positive *C. coli* strains are more susceptible to antimicrobial was removal, however the positive effect was observed for all isolates.

3.4. The effect of natural antimicrobials on whole chicken carcass impacts on *C. coli* isolates adherence

In order to investigate the effect on the whole chicken carcass we have next tested the effect of antimicrobial spraying against adherence of the *C. coli* isolates as described in material and methods In this experiment, following bacterial inoculation, the carcasses were sprayed with 0.5% A3001 as described in material and methods. The results obtained confirm the effects of the neck skin experiment showing a significant reduction ($p=0.0001$ for NC1 and NC2, $p=0.0002$ for RC018) in attachment to the carcass after antimicrobial spraying (Fig 4). The most significant decrease was observed in the case of the T6SS positive NC3 isolate ($p<0.0001$) similarly to the results described in figure 3. Overall this data shows that mixtures of natural antimicrobials can be efficient in reducing the pathogen load on whole chicken carcass when used in a spray form for only 2 minutes.

3.5. *In vitro* adhesion and invasion of *C. coli* isolates and the effect of antimicrobial mixtures

Next we have investigated, using an *in vitro* model of infection, the potential of *C. coli* strains to adhere and invade human HCT-8 cell line (Fig 5, panel A and B). The *C. coli* NC3 strain revealed significantly elevated levels ($p \leq 0.005$) of adherence and invasiveness compared to the control RC018 strain, as well to NC1 and NC2 strains. The NC1 and NC2 strains exhibit slightly reduced adherent potential with a small

414 increase in invasiveness efficacy towards HCT-8 cells compared with the RC018
415 strain. Nevertheless, these variations were not statistically significant. The exposure
416 of the three isolates and the control strain to 0.5% Auranta 3001 led to a significant
417 reduction in their abilities to reduce both the adhesion and invasion to HCT-8
418 epithelial cells. The most significant reduction in invasion, following exposure to
419 Auranta 3001, was recorder in the case of the T6SS positive train (NC3) (Fig 5, panel
420 B, p=0.0001) probably as a result of the significant reduction in *hcp* expression (Fig
421 6).
422
423 **3.6. Resistance to antibiotics**
424
425 In the present study, four *C. coli* strains (NC1, NC2, NC3 and RC018) were tested
426 with M.I.C. Evaluator™ (M.I.C.E. ™) strip containing ciprofloxacin (32–0.002 µg/ml),
427 tetracycline (256–0.015µg/ml) and erythromycin (256–0.015 µg/ml) µg/ml
428 concentrations. The minimum inhibitory concentrations (MIC) are presented in table
429 1. The results revealed that *C. coli* RC018 and NC1 strains exhibit a similar minimal
430 inhibitory concentration to ciprofloxacin (0.12), tetracycline (0.25) and erythromycin
431 (0.50). *C. coli* NC2 showed increased tetracycline (<256) resistance compared to
432 other tested strains. Moreover, NC2 showed a slight increase of ciprofloxacin (0.25)
433 and erythromycin (1.00) MIC values towards *C. coli* NC1 and RC018 strains.

However, *hcp*$^{+}$ C. coli NC3 strain also had elevated ciprofloxacin MIC in contrast to NC1, NC2 and the control RC018.

Table 1. Antimicrobial susceptibility to antibiotics of *C. coli* poultry isolates

Minimum Inhibitory Concentration (μg/ml)			
C. coli	Ciprofloxacin	Tetracycline	Erythromycin
NC1	0.12	0.25	0.50
NC2	0.25	>256	1.00
NC3	8.00	0.50	0.25
RC018	0.12	0.25	0.50

3.7. Resistance and growth rates under H_2O_2 exposure

Resistance to H_2O_2 was tested at different concentrations ranged from 50mM to 0.156mM concentrations. The MIC concentration was assigned at the lowest concentration where no growth has occurred, whilst MBC was assigned at the decreased concentration that caused bacterial death after 48 h of incubation. All four *C. coli* strains showed an increased sensitivity to hydrogen peroxide stress (Table 2). The minimal inhibitory concentration (MIC) for NC1 and NC2 isolates was 10mM, while for NC3 and RC018 it was 20mM of H_2O_2. The minimal bactericidal

449 concentration (MBC) for NC1 and NC2 isolates was 10 mM and for NC3, RC018 it
450 was 20mM. Higher H_2O_2 concentrations of 50, 25mM resulted in bacterial death in
451 all of *C. coli* isolates.

452

453 Table 2. Resistance and growth rate under hydrogen peroxide exposure

454

Minimum inhibitory and bactericidal concentrations of H_2O_2 (mM)			
C. coli	Origin	MIC	MBC
NC1	poultry	10	10
NC2	poultry	10	10
NC3	poultry	20	20
RC018	poultry	20	20

455

4. Discussion

Campylobacter species can be found in a variety of environments, including water, soil and other animal habitats (farms). The rise of antibiotic resistance notwithstanding, many natural substances (peptides) derived from insect venoms, animals and plants can contain rich sources of antimicrobial substances to combat infectious pathogens (Patra, Amasheh, & Aschenbach, 2019; Perumal Samy, Stiles, Franco, Sethi, & Lim, 2017; Wencewicz, 2016). These biologically active compounds have evolved as the components of the origin organism's defense system. Specifically, it was found that some plant constituents can trigger the secretion of antimicrobial peptides from the intestinal epithelium in animals producing an additional protective barrier (Patra, et al., 2019). Research critical to the poultry and dairy industries has been produced regarding the genetic identification, tracking and classification of antibiotic-resistant bacteria (Drame, et al., 2020; Yang, et al., 2019). The strategy of supplementation of animal feed with biologically active substances (essential oils, phenolics, organic acids, peptides, etc.) showed pronounced antimicrobial activity against a wide range of microorganisms and is becoming more and more popular as well as more efficient(Aziz & Karboune, 2018; Sima, et al., 2018). The novel area of natural antimicrobial formulations enriched with organic acids and plant extracts presents promising candidates for successful pathogen control (Singh, Smith, & Bailey, 2015).

We hypothesized that mixtures of natural antimicrobials could reduce the presence of highly virulent *C. coli* counts on poultry products and could be used as an effective antimicrobial treatment to improve food safety since it has been previously shown that genes involved in pathogenicity and adherence to cells and surfaces can downregulated by natural antimicrobials (Sima, et al., 2018). One might ask, why focus on *C. coli*, and we believe that given the fact that it has been reported that the prevalence of T6SS-positive *C. coli* strains is regularly detected in immunocompromised patients (Agnetti, et al., 2019) the need to reduce its presence on poultry products becomes a necessity. We have previously shown that the T6SS system is important in adherence and invasion abilities of *C. jejuni* and *C. coli* and also our previous data shows that natural antimicrobials can downregulate T6SS related genes, *hcp*, leading to a decrease in infectivity (Corcionivoschi, et al., 2015; Sima, et al., 2018).

In order to make our results relevant to the direct user we have investigated the effect of natural antimicrobials on the adherence to chicken skin and whole carcass using fresh *C. coli* isolates with and without the novel T6SS system. The new isolates were confirmed by PCR analysis revealing the presence of *C. coli* strains through the amplification of the 23S and only one strain (NC3) displayed the T6SS system, evident from the amplification of the *hcp* gene (463 bp). This T6SS positive strains

498 are more and more relevant and this was reported in a study conducted in Northern
499 Ireland, where it was reported that 56.1 % of *C. coli* retailed chicken isolates carry a
500 functional T6SS mechanism (Corcionivoschi, et al., 2015).
501
502 Furthermore, our results provide insight into the pathogenic features of four *C. coli*
503 poultry isolates. The phenotypic assay results revealed that *C. coli* isolates (NC3)
504 exposed to stressed environmental conditions displayed significantly elevated levels
505 of adhesion and invasion *in-vitro* in comparison to the RC018 reference strain. In
506 addition, the exposure of each isolate to a natural antimicrobial mixture (Auranta
507 3001) caused a significant alleviation of their potential to decrease adhesion and
508 invasion to HCT-8 epithelial cells. Recent elegant work describes the diminishing
509 effects of commercial antimicrobials (Auranta 3001) on the *C. coli* RC013 and *C.*
510 *jejuni* RC039 chicken isolates motility and invasion rate towards the HCT-8 cell line
511 (Sima, et al., 2018). This study suggested that antimicrobial mixture may intervene
512 within the host-cells metabolic reactions, thereafter impairing the bacterial capacity
513 to infect the cells (Sima, et al., 2018).
514
515 Overall, the swarming motility data of NC1, NC2 and NC3 *C. coli* indicated that these
516 isolates were less motile than the cecum isolated strain (RC018). This could be an
517 important observation as motility represents an important factor involved in the ability
518 of *C. coli* to adhere to organic surfaces. It has been previously indicated that the *C.*

519 *coli* OR12 (T6SS-positive) strain was able to grow and survive aerobic conditions on
520 blood supplemented agar unlike *C. coli* RM2228 (T6SS-positive) which cannot
521 (O'Kane & Connerton, 2017). During the adherence/invasion process bacterial will
522 face exposure to increased stresses frequencies were observed in *C. coli* strains
523 more than in *C. jejuni* strains (Anand B. Karki, Marasini, Oakey, Mar, & Fakhr, 2018).
524 Strains with increase capacity to resist to oxidative stress will be more able to adhere
525 and infect mammalian cells since H_2O_2 is produced as a result of *Campylobacter*
526 infection (Corcionivoschi, et al., 2012). Our study shows that our *C. coli* isolates can
527 withstand H_2O_2 concentrations of up to 20mM since it has been shown before that
528 5mM concentrations can be lethal (Corcionivoschi, et al., 2012). One hypothesis
529 which can be drawn from these results is that that certain phenotypic, including
530 resistance to stress, are strongly expressed in biologically relevant niches.
531
532 Over the last forty years, a void in the area of the development of new classes of
533 antibiotics has occurred since linezolid and daptomycin last discovery was reported
534 from the 1980s (Durand, Raoult, & Dubourg, 2019). Understanding such peril for
535 humans and animals, it is necessary to establish alternative novel solutions to treat
536 bacterial pathogens by replacing antibiotics. This is necessary to overcome the
537 emerging and intensifying antimicrobial resistance. Various authors reported
538 multidrug-resistant *C. coli* isolates from poultry sources exhibiting great resistance
539 to tetracycline, ciprofloxacin, ampicillin, erythromycin (Kottawatta, et al., 2017; Wei,

et al., 2014). Alternatively, citric acid, reuterin and commercially available antimicrobial mixtures presented very efficient antimicrobial activity towards *C. coli* and other food-related pathogens acting through direct or indirect anti-infectivity activity (Asare, et al., 2020; Beier, et al., 2018; Pinkerton, et al., 2019; Sima, et al., 2018; A. C. Stratakos, et al., 2020). In our study we clearly show that mixtures of natural antimicrobials can significantly reduce the ability of *C. coli* to adhere to chicken skin, *ex vivo*, with a greater affinity for T6SS positive strains since as it has been also previously reported (Liaw, et al., 2019; Sima, et al., 2018). Moreover the results we have obtained on chicken neck skin have also been replicated by using the antimicrobial as spray on the whole chicken carcass. The efficacy of natural antimicrobials as spray on chicken carcasses have been described before having the potential to be implemented at industrial scale (Bertram, Kehrenberg, Seinige, & Krischek, 2019a, 2019b).

5. Conclusions

The demand for antibiotic-free food products is becoming ever more increased requiring elimination of bacterial pathogens at different stages of food production using interventions that exclude the usage of antibiotic based products. The chicken skins and carcasses represents a source of such pathogens due to their biological and biochemical characteristics that facilitates survival bacteria including newly

561 characterized highly virulent strains (T6SS positive). Our results showed that our
562 T6SS positive isolate had reduced ability to attach to chicken carcasses during
563 washing with mixtures of natural antimicrobials. This technology requires further
564 development in an industrial setup but could potentially represent an alternative to
565 the current chlorination and lactic acid treatment of chicken carcasses method for
566 elimination of bacterial pathogens that pose a threat to human health.

567

568 **Author Contributions**

569

570 Conceptualization, Nicolae Corcionivoschi; Data curation, Patrick Ward and Nicolae
571 Corcionivoschi; Formal analysis, Igori Balta, Laurette Pinkerton, Mark Linton, Carmel
572 Kelly, Ioan Pet, Lavinia Stef; Adina Horablaga. Funding acquisition, Nicolae
573 Corcionivoschi; Investigation, Ozan Gundogdu; Methodology, Mark Linton, Carmel
574 Kelly; Project administration, Patrick Ward and Nicolae Corcionivoschi; Resources,
575 Lavinia Stef; Writing – original draft, Igori Balta, Ozan Gundogdu and Nicolae
576 Corcionivoschi; Writing – review & editing, Mark Linton, Igori Balta, Ozan Gundogdu
577 and Nicolae Corcionivoschi.

578 **Funding**

579 This study was supported by a grant awarded to Environtech, Dublin, Ireland.

580 **Conflicts of Interest**

581 The authors declare no conflict of interest. The funders had no role in the design of
582 the study; in the collection, analyses, or interpretation of data; in the writing of the
583 manuscript, or in the decision to publish the results.

584

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746 **Figure legends**

747

748 **Fig.1.** Genotypic identification and the detection of *hcp* and *glt* genes of *C. Coli*
749 chicken isolates. (A) Multiplex PCR identification of C. coli isolates NC1, NC2, and
750 NC3 showing special indicative amplicon from the 23S rRNA gene responsible for
751 the presence of *Campylobacter* spp. (B) PCR analysis of *hcp* and *glt* gene
752 expression in NC1, NC2 and NC3 strains. *C. jejuni* NCTC 12502 served as the hcp
753 positive control (+C). Expected sizes are 463bp (hcp), 142bp (*glt*). Multiplex PCR
754 was conducted using gltAF, gltAR, hcpF and hcpR primers.

755

756 **Fig.2.** Motility of the *C. coli* isolates. Indicates the decrease in percentages of *C. coli*
757 NC1, NC2 and NC3 over the RC018 control strain when exposed to Auranta 3001.
758 *Asterisks* indicate significant differences ($^{*}p < 0.05$, $^{***}p < 0.001$). *Error bars*
759 represent the standard deviation of means from three different experiments, each
760 containing triplicate samples.

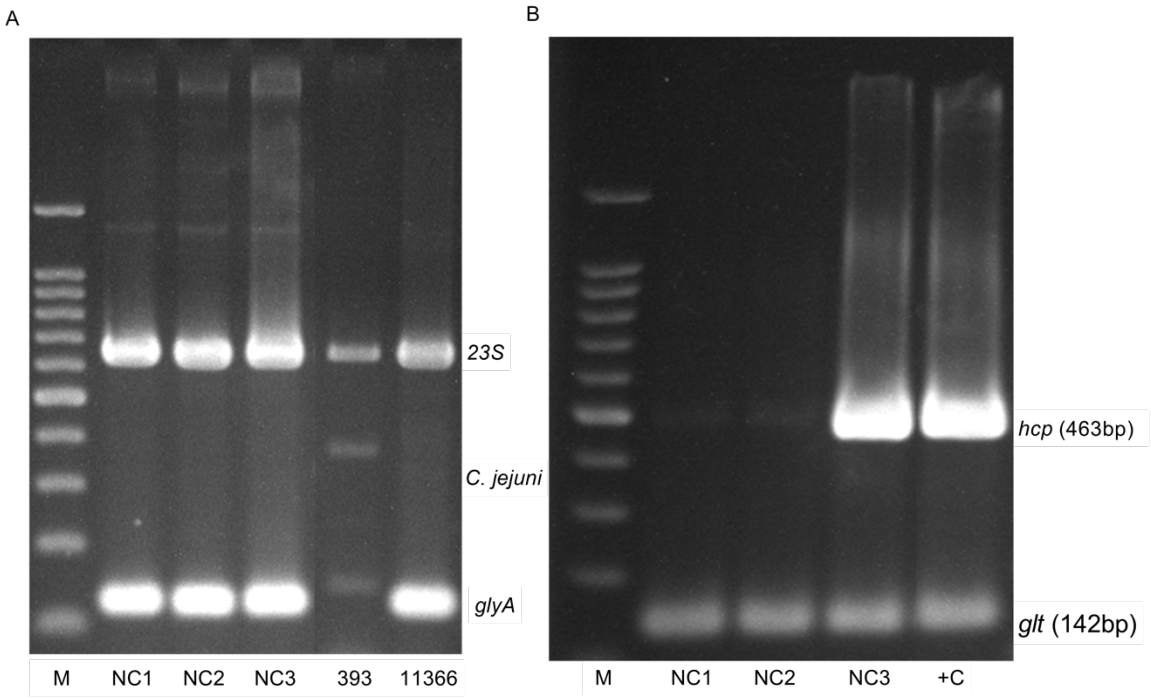
761

762 **Fig.3.** The effect of natural antimicrobials in reducing the ability of *C. coli* to adhere
763 to chicken neck skin *ex vivo*. *Error bars* represent the standard deviation of means
764 from three different experiments, each containing triplicate samples. (ns – not
765 significant). Statistical significance was defined using (Student's *t* test) relative to the
766 level for *C. coli* RC018 strain is indicated.

Fig.4. The effect of antimicrobial spraying in reducing the ability of *C. coli* to adhere to whole chicken carcass. Distilled water wash was used as a control (C). *Error bars* represent the standard deviation of means from three different experiments, each containing triplicate samples. Statistical significance was defined using (Student's *t* test) relative to the level for *C. coli* RC018 strain is indicated.

Fig.5. Adhesion and invasion efficacy of the four chicken isolates to HCT-8 cells. (A) Adhesion to HCT-8 cells without +/- Auranta 3001; (B) Invasion of HCT-8 cells of *C. coli* RC018 and C. coli chicken neck skin isolates NC1, NC2 and NC3 +/- Auranta 3001. The experiments were conducted in triplicate on three separate occasions. Statistical significance was defined using (Student's t test) relative to the level for C. coli RC018 strain is indicated. Asterisks denote a statistically significant probability at ($p \leq 0.005$**) level. The error bars represent standard deviations for three separate wells.

Fig.6. Effect of Auranta 3001 on *C. coli* NC3 after 3 h of exposure to 0.5% Auranta 3001. Asterisks indicate significant differences (Student’s t-test ***$p < 0.01$). Error bars represent the standard deviation of means from three different experiments.



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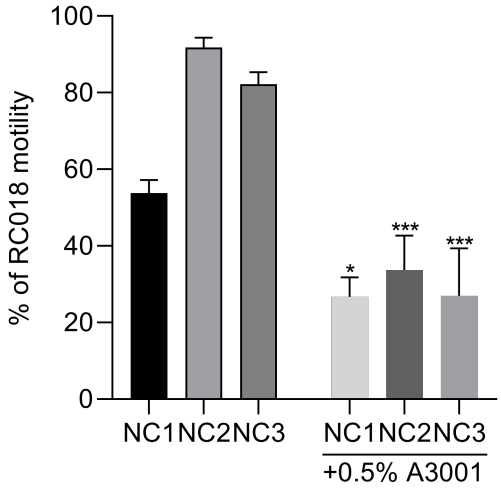

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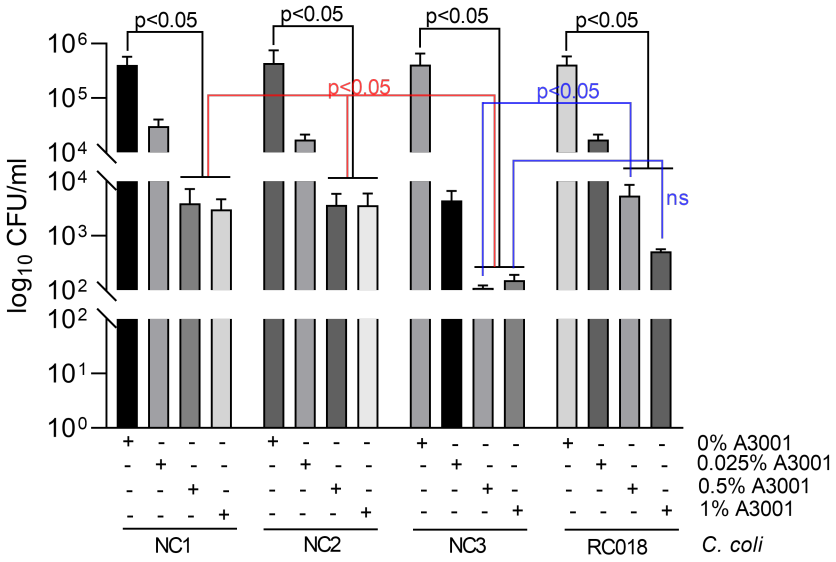

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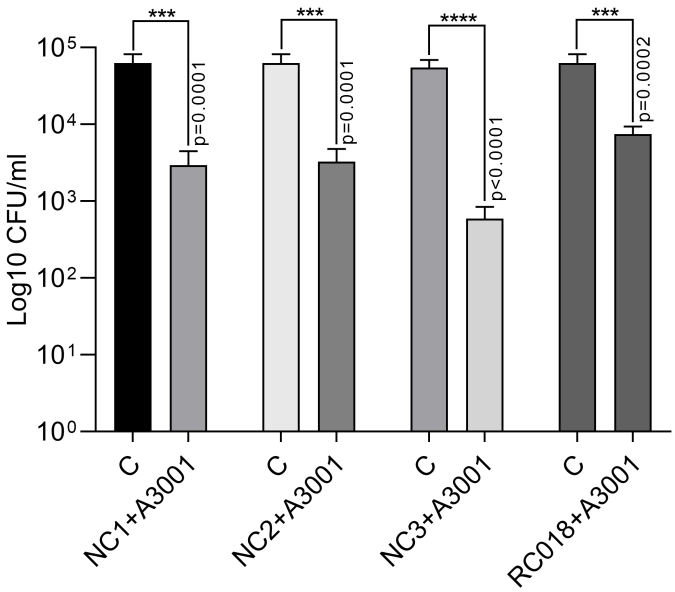

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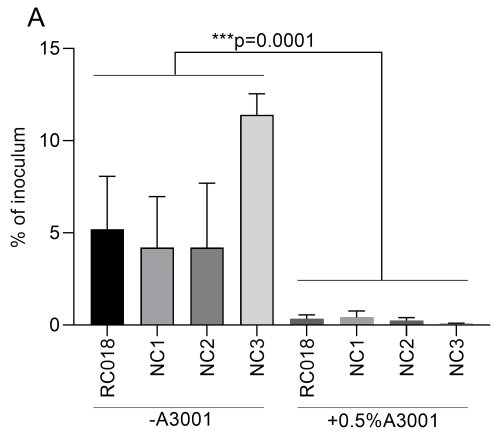

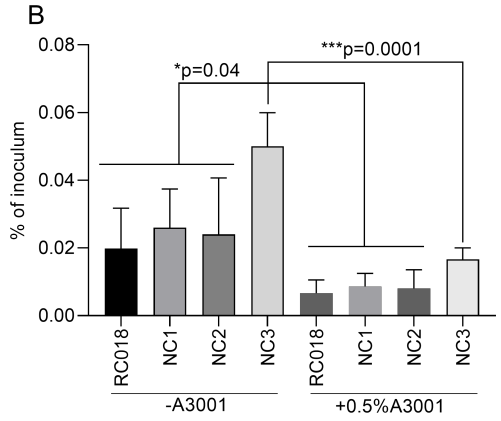

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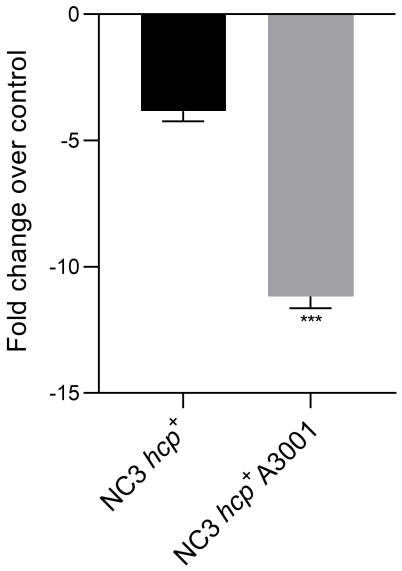

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