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Reliably colonising broiler chickens with *Campylobacter* spp. using a litter-based method

Authors: Victoria Sandilands[1*], Fraser Whyte[1], Lisa K. Williams[2], Thomas S. Wilkinson[2], Nicholas H.C. Sparks[1], Thomas J. Humphrey[2]

*Corresponding author

Affiliations: [1] Monogastric Science Research Centre, Animal & Veterinary Science Group, SRUC, Ayr, UK

[2] Microbiology and Infectious Disease, Swansea University Medical School, Swansea University, Swansea UK

Email addresses:

Victoria Sandilands Vicky.Sandilands@sruc.ac.uk; Fraser Whyte Fraser.Whyte@sruc.ac.uk; Lisa Williams L.K.Williams@swansea.ac.uk; Thomas Wilkinson T.S.Wilkinson@swansea.ac.uk; Nicholas Sparks Nick.Sparks@sruc.ac.uk; Thomas Humphrey T.J.Humphrey@swansea.ac.uk

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23 **Abstract**

24 1. Chicken-associated *Campylobacter* spp. are the cause of most food poisoning
25 cases in Europe. In order to study the host-pathogen interactions, a reliable
26 and reproducible method of colonising chickens with the bacteria is required.
27 2. This study aimed to identify a more appropriate and less invasive method of
28 colonisation (cf. gavaging) by seeding bedding material (litter) that
29 commercial chickens are kept on with a mixture of *Campylobacter* spp., broth
30 and faeces.
31 3. The first phase of the study tested the longevity of *Campylobacter* spp.
32 recovery in seeded litter over 24 h: significantly more *Campylobacter* spp. was
33 recovered at 0 or 3 h post-seeding than at 6 and 24 h post-seeding, indicating
34 that the pathogen can survive to detectable levels for at least 3 h in this
35 environment.
36 4. In the second phase, three groups of 10 broiler chickens (negative for
37 *Campylobacter* spp. prior to exposure) were exposed at 21 days of age to one
38 of three different *Campylobacter jejuni* and *C. coli* mixes (A, B, C), using the
39 method above. At 28 days of age, birds were euthanised by overdose of
40 barbiturate or cervical dislocation, and livers and caeca removed for
41 *Campylobacte* spp. assessment.
42 5. All liver and 28/30 caeca samples tested positive for *Campylobacte* spp., with
43 mix A and C giving higher counts in the caeca than mix B. The method of
44 euthanasia did not affect *Campylobacter spp.* counts.
45 6. In conclusion, a successful method for reliably colonising broiler chickens
46 with *Campylobacter* spp. has been developed which negates the need for
47 gavaging and is more representative of how contamination occurs in the field.

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Keywords: Contamination, food-borne illness, seeding, gavage, liver, caeca

Introduction

Campylobacter are Gram-negative microaerophilic bacteria that are frequently present in the digestive tract of pigs, cattle and poultry, and can be often found post-slaughter on and in poultry muscle and organs such as liver. *Campylobacter* is thought to cause approximately nine million cases of food-borne illness per year in the European Union, with an estimated loss of productivity of ~ €2.4 billion (European Food Safety Authority, 2014). Chicken meat is responsible for about 80% of *Campylobacter* spp. food-borne illness in the UK (Food Standards Agency, 2017) and, despite much research, carriage rates in chickens and human cases remain high. *Campylobacter* spp. has been shown to have an impact on the health and welfare of broiler chickens in both commercial (Bull *et al*, 2008; Rushton *et al*, 2009) and experimental studies (Williams *et al*, 2013). *Campylobacter* spp. colonisation has been linked to poor flock performance and economic losses (Smith *et al*, 2016). The human health threat from *Campylobacter*-positive chicken is not only from surface contamination, as edible tissues are also positive (Berndtson *et al*, 1992; Scherer *et al*, 2006; Whyte *et al*, 2006; Luber and Bartelt, 2007) and contaminated chicken liver is a major vehicle for human infection (Inns *et al*, 2010; Little *et al*, 2010), as is undercooked chicken meat.

In order to understand how *Campylobacter* spp. in broiler chickens is affected by husbandry practices (such as catching birds for slaughter or in-feed interventions), it is important to be able to study reliably-colonised chickens in a research environment.

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73 Previous work studying poultry colonised with various organisms (e.g.
74 *Campylobacter* and *Salmonella* spp.) have used oral gavaging techniques (Arsi *et al*,
75 2015; Upadhyaya *et al*, 2015; Saint-Cyr *et al*, 2017). However, this is invasive,
76 stressful and carries a risk of injury to the birds as well as not reproducing the
77 conditions under which chickens would normally ingest the organism. In order to
78 improve and refine techniques, this study aimed to determine if introducing
79 *Campylobacter*-seeded litter to floor pens housing broiler chickens would result in
80 reliable colonisation.
81
82 **Materials and Methods**
83 Phase 1: litter seeding
84 Used poultry litter (a mixture of wood shavings, broiler excreta, spilt feed and water)
85 was collected from a previous trial at Scotland's Rural College (SRUC), Ayr. The
86 litter was weighed and sterilised by drying in an oven at 80°C until a constant weight
87 was obtained, and then divided into 400 g batches in six trays (approximately 38 x 28
88 x 8 cm). Each dried litter tray was then reconstituted with 1 l of deionised water.
89
90 A *Campylobacter* strain that had been isolated previously from caecal samples taken
91 from a commercial chicken farm and stored at -80°C in bead cryopreservation vials
92 (Technical Service Consultants, UK) was used. The strain was resuscitated on Blood
93 Agar No. 2 with Horse Blood (BA) plates (Oxoid, UK). These cultures were used to
94 prepare lawn plates on further BA plates, incubated for 40-48 hrs at 41.5°C
95 microaerobically (CampyGen, Oxoid, UK). The lawn plates were harvested by adding
96 5 ml Mueller-Hinton (MH) broth (Sigma-Aldrich, UK), gently detaching the culture
97 with a sterile spreader and decanting to a container. The suspension was then adjusted

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98 with further MH broth to optical density$_{600}$ of 0.19 – 0.21 (approximately 1.5 x 10^5

99 cfu ml^{-1}). The litter trays were seeded with a mixture of 20 ml MH broth, 10 ml

100 *Campylobacter* spp. suspension and 10 g dried hen faeces (dried in the same way as

101 the litter), by applying evenly to the surface of the litter.

102

103 The litter trays were incubated at 21°C. At 0, 3, 6 and 24 h after seeding, a different

104 quarter of each tray was sampled. A sterile pot was filled with litter collected from the

105 top 1 cm of the selected quadrant. Subsequently, a 5 g portion of the collected litter

106 was mixed with 45 ml of MH broth. A swab was used to streak each litter/broth mix

107 sample onto two modified Charcoal Cefoperazone Deoxycholate agar (mCCDA,

108 Oxoid, UK) plates, which were incubated microaerobically at 41.5°C for 24 h.

109

110 Phase 2: colonising birds

111 Forty-three Ross 308 male broiler chicks (*Gallus gallus domesticus*) were housed

112 from day old in a single litter-floor pen with a brooding heat lamp. The ambient air

113 temperature was 32°C on arrival, and was gradually reduced to 21°C at 21 days of

114 age. At 7 days of age, all chicks were wing tagged and weighed, and the 13 lightest

115 chicks (mean weight 170 g) were removed from further study. The remaining 30

116 chicks (mean weight 208 g, range 187-239 g) were distributed to three pens of ten

117 chicks each, according to weight in order to reduce variation within pen (i.e. ten

118 lightest chicks to pen 1, ten middle weight chicks to pen 2, ten heaviest chicks to pen

119 3) with 0.66 m^2 total floor space per bird. Chicks were reared until 28 days of age.

120 Birds were fed a standard commercial starter crumb from arrival to 14 days of age,

121 and then a grower pellet from 14-28 days of age. Food and water were provided *ad*

122 *libitum* from a circular food hopper and bell drinker.

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123

124 At 21 days of age, one litter sample per pen and one cloacal swab per bird were
125 collected to check for the presence of *Campylobacter* spp. All samples were
126 processed on the day of collection. For litter samples, a sterile pot was filled with
127 litter collected from the top 1 cm of the pen. For cloacal swabs, a viscose-tipped
128 Amies charcoal transport swab (12 cm long, Deltalab, Spain) was gently inserted 2
129 cm into the vent of each bird and rotated back and forth for approximately 5 sec, then
130 sealed inside the integrated holder. Litter samples were processed as described
131 previously using MH broth, and plated in duplicate onto mCCDA plates. Each cloacal
132 sample was streak plated onto mCCDA plates in duplicate. All plates were incubated
133 for 40-48 h at 41.5°C microaerobically as before.

134

135 On the same day, subsequent to the initial sample collection, three trays of dried
136 reconstituted litter were seeded with ten *Campylobacter* strains, identified either as
137 mix A, B or C (Table 1), which were added to pen 1, 2, or 3 respectively, using the
138 method described in Part 1. Each mix used five strains common among all three
139 mixes (1-5) and five unique strains (A6-A10, B6-B10, and C6-C10). Mix A used
140 known strains that have been previously used in colonisation studies, mix B used
141 systemic isolates that were previously isolated from the liver of commercial broiler
142 chickens, and mix C used non-systemic isolates that were previously isolated from the
143 gut of commercial broiler chickens, but were unique in genotype to the systemic
144 isolates. The trays were sunk into the existing floor litter of the pens, close to the
145 feeder tubes, and some broiler feed was sprinkled on top to encourage foraging at the
146 seeded litter and subsequent ingestion of the bacteria.

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148 **--Insert Table 1 approximately here--**

149

150 On day 28, all 30 birds were humanely killed, half by manual cervical dislocation and
151 the other half by overdose of barbiturate (pentobarbital sodium administered IV at 1
152 ml kg^{-1} body weight) to assess if either method affected the recovery of
153 *Campylobacter spp*. The caecum and a sample of the liver were aseptically removed
154 and placed into separate sterile bags with the *Campylobacter* mix (A, B or C) noted.
155 Samples were stored on ice in a polystyrene box until processing 2 hours later.

156

157 A 1 g sample of caecal contents were removed from the caecum and placed into a
158 universal, to which 9 ml of saline was added and vortexed to mix. Subsequent
159 dilutions (1:10) were performed using saline in a microtitre plate, 100 µl of the -2, -4
160 and -6 dilutions were spread plated onto mCCDA, then plates were incubated at 42°C
161 for 24 h microaerobically as described above. Numbers of suspect colonies were
162 counted to yield cfu g^{-1} and a subset confirmed as *Campylobacter* spp. using growth
163 on duplicate BA plates, one incubated aerobically and one microaerobically at 42 °C
164 for 24 h. If there was any growth on the aerobic plate the results were discounted.
165 Colonies from the microaerobic plate were stored on cyrobeads at -80 °C.

166

167 With each liver sample, 5 g was removed from the bag, dipped in 70% ethanol and
168 flamed using a Bunsen burner to surface sterilise. The liver was placed in a stomacher
169 bag with 45 ml of saline, and samples were homogenised in a Colworth stomacher for
170 1 min or until an even homogenate was created. A 2 ml sample of each homogenate
171 was placed in a universal and 20 ml of modified Exeter enrichment broth (Mattick et
172 al., 2003) was added to produce a minimal headspace, lids were tightly capped and

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173 the enrichments were aerobically incubated at 42°C for 24 hours. After incubation, a
174 10 µl loopful of the enrichment was plated onto mCCDA, plates were incubated at
175 42°C for 24 hours microaerobically as described above. Results were interpreted as
176 presence or absence of *Campylobacter* spp. depending on growth. Colonies were
177 picked on duplicate BA plates, one incubated aerobically and one microaerobically at
178 42°C for 24 hours. If there was any growth on the aerobic plate the results were
179 discounted. Colonies from the microaerobic plate were stored on cyrobeads at -80 ^{o}C.
180

181 Ethical note

182 The study was conducted in the UK under a Home Office licence (PPL 60/4505) and
183 was approved by SRUC's Animal Welfare and Ethical Review Body. The study
184 fulfils the EU requirements on the protection of animals used for scientific purposes
185 (European Commission, 2010).
186

187 Statistical analysis

188 Data were analysed using Genstat (Release 16.1, 2013). For litter seeding data, $\log_{10}$
189 of counts (cfu g-^{1}) were calculated and analysed by one-way ANOVA for the time
190 effect (degrees of freedom (d.f.) = 3) on counts, with 'tray' designated as the block.
191 Binary data for liver samples (presence of *Campylobacter* = yes/no) were generated,
192 but because all samples gave the same result, no statistical test was undertaken. For
193 caecal data, $\log_{10}$ of counts (cfu g-^{1}) were calculated and analysed by two-way
194 ANOVA to examine the effect of *Campylobacter* strain mix (d.f.) = 2), kill method
195 (d.f. = 1), and their interaction (d.f. = 2).
196

197 **Results**

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198 Phase 1: litter seeding

199 Samples from all six trays had a measurable amount of *Campylobacter* spp. growth at
200 0, 3 and 6 h after seeding. No *Campylobacter* spp. were recovered from samples
201 taken 24 hours after seeding (Table 2). Significantly ($P<0.001$) more *Campylobacter*
202 spp. was recovered from litter samples taken at 0 or 3 hours than at 6 and 24 hours
203 after seeding.

204

205 **--Insert Table 2 approximately here--**

206

207 Phase 2: colonising birds

208 At day 21, all litter and cloacal swabs were found to be negative for *Campylobacter*
209 spp. At 28 days of age, birds weighed on average 1653-1782 g (SD: 76-157 g), and
210 birds killed by cervical dislocation were on average 20 g lighter (mean ± SD: 1696 ±
211 160.6 g) than those killed by overdose of barbiturate (1716 ± 125.6 g).

212

213 *Campylobacter* spp. were detected in all 30 liver samples using enrichment culture;
214 thus, there was no effect of strain mix on recovery. On further identification, the
215 strains were found to be *Campylobacter jejuni* multilocus sequence type 257 (n=22)
216 and *C. coli* multilocus sequence type 828 (n=8). All birds exposed to mixes A and B
217 had livers that contained *C. jejuni* multilocus sequence type 257 whereas mix C had
218 eight birds with livers that contained *C. jejuni* multilocus sequence type 257 and two
219 birds with livers containing *C. coli* multilocus sequence type 828.

220

221 *Campylobacter* spp. were detected in the caeca of 28/30 birds. Both negative results
222 came from birds exposed to mix B (one culled by overdose of barbiturate, one killed

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223 by cervical dislocation). *Campylobacter* spp. counts from the caeca were
224 significantly affected by the strain mix (P <0.001), but not by the cull method
225 (P=0.308), nor was there an interaction between strain mix and cull method (P=0.711;
226 Table 3). Excluding the two birds from strain mix B where no *Campylobacter* spp.
227 counts were obtained did not greatly alter the results (i.e. effect of strain mix:
228 P=0.002, effect of cull method: P=0.308, interaction: P=0.745).
229
230 **--Insert Table 3 approximately here—**
231
232 **Discussion**
233 Litter seeding with a mixture of *Campylobacter* spp., broth and chicken faeces was
234 successful in that measurable amounts of *Campylobacter* spp. were recovered up to 3
235 h after seeding. Recovered *Campylobacter* spp. at 6 h was significantly lower than at
236 0 and 3 h, and did not differ from 24 h (where counts were always zero), suggesting
237 that the organism is viable in this environment for less than 6 h, but at least for 3 h.
238 This is important, as the organism must survive long enough for some birds to ingest
239 it via foraging in the seeded litter.
240
241 When three different *Campylobacter* spp. mixtures were presented using litter
242 seeding to naïve broiler chickens, the method worked successfully in that all liver
243 samples and 93% of caeca samples tested positive for at least one of the relevant
244 *Campylobacter* strains seven days after bird exposure. The negative caecal results
245 could have been due to inhibition of *Campylobacter* spp. by other bacterial species or
246 that these strains did not establish themselves in the caecal niche. Nevertheless, these
247 results indicated that the organism survived long enough for at least some birds to

248 ingest it, presumably due to foraging on the seeded trays. Even if only a few chickens
249 ingested the organism in the first instance from the trays, the subsequent production of
250 colonised faeces and frequent foraging behaviour, in which chickens scratch and peck
251 at the floor litter (which has the faeces in it), will recycle the organism until it has
252 spread to birds throughout the pen. This could be confirmed by doing sequential
253 sampling of birds on seeded litter, as opposed to sampling all birds at one time point
254 (as was done in this experiment), and investigating variation in organism counts over
255 time. However, previous studies have shown that inoculating just a few birds in a
256 group leads to successful colonisation of the organism in the naïve birds (Shanker *et*
257 *al*, 1990; Line *et al*, 1998). Foraging behaviour tends to decline with increasing age
258 in broilers (Bessei, 1992; Wallenbeck *et al*, 2016), so colonisation rate using litter
259 seeding may be affected by bird age.
260
261 It was notable that the two caeca in which *Campylobacter* spp. Were not detected
262 came from mix B, so this may be a less reliable mix compared to mix A or C. Neither
263 mix A nor C had a greater count of *Campylobacter* spp. in the caeca, so they may be
264 equally suitable for use, depending on whether or not the point of study is to
265 investigate effects of mainly *C. jejuni* strains, which predominated in mix A, but were
266 roughly equal with *C. coli* in mix C.
267
268 The method of killing did not affect the mean counts of *Campylobacter* spp. in the
269 caeca, which indicated that either method can be used without affecting data. This is
270 important, as studies carried out on *e.g*. commercial broiler farms are more likely to
271 use cervical dislocation as a method of killing, as opposed to using controlled
272 medicines.

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274 Previous studies (Stern *et al*, 1991; Young *et al*, 1999; Dhillon *et al*, 2006; de los
275 Santos *et al*, 2008; Arsi *et al*, 2015) have used oral gavage as a reliable method of
276 introducing *Campylobacter* spp. to chickens. A study of colonisation over time
277 (Stern, 2008) demonstrated that the caeca of broiler chicks were colonised with *C.*
278 *jejuni* within four days of inoculation, and that the numbers generally increased with
279 time up to week four (ranging from 10^6-10^8 cfu g^{-1}), regardless of *C. jejuni* challenge
280 levels (10^4-10^7 cfu). Similarly, McCrea et al. (2006) found that 20-day old broiler
281 chickens inoculated with *C. jejuni* isolates from either squabs, ducks, or chickens by
282 oral gavage had average colonisation rates of 10^6-10^7 cfu g^{-1} 10 days post-inoculation.
283 Here, the litter seeding method gave comparable results seven days post exposure, but
284 with the advantage of refining the method to avoid invasive gavage techniques and to
285 more accurately represent how chickens would pick up the organism naturally in a
286 commercial poultry shed environment.
287

288 In conclusion, this method of litter seeding with different mixtures of *Campylobacter*
289 spp. was successful at colonising 21-day old broilers by 28 days of age, with
290 *Campylobacter* spp. reliably recovered in the liver and caeca (but less so with mix B).
291 It is therefore proposed that this is a suitable technique for colonising broiler chickens
292 for the study of *Campylobacter* spp. in a commercially-relevant manner, without the
293 need to gavage. The method might also be used successfully with other organisms,
294 but this would require further study.
295

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299

300 **Disclosure**

301 No potential conflict of interest was reported by the authors

302

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306
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406 ***Table 1: Mixes A (4.5 x 10^{7} cfu ml^{-1}), B (8.0 x 10^{7} cfu ml^{-1}) and C (7.0 x 10^{7} cfu ml^{-1}) of different Campylobacter strains, with multilocus sequence type shown in***
407
408 ***brackets. All mixes used five common strains (1-5).***

Mix A (pen 1)	Mix B (pen 2)	Mix C (pen 3)
	1. *C. jejuni* 11168 (43)	
	2. *C. jejuni* M1 (137)	
	3. *C. coli* RM 2228 (107)	
	4. *C. coli* (828)	
	5. *C. jejuni* 13126 (21)	
A6. *C. jejuni* 12662 (257)	B6. *C. coli* L4 (828)	C6. *C. coli* I4 (828)
A7. *C. jejuni* DBM1 (344)	B7. *C. jejuni* L8 (464)	C7. *C. coli* C8 (828)
A8. *C. jejuni* 12744 (658)	B8. *C. jejuni* L14 (464)	C8. *C. coli* C15 (828)
A9. *C. jejuni* hen (45)	B9 *C. coli* L24 (828)	C9. *C. jejuni* C24 (353)
A10. *C. jejuni* 3L44 (283)	B10 *C. coli/C. jejuni* L16*	C10. *C. coli/C. jejuni* C20*

409 * typed as both *C. jejuni* and *C. coli*

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410 ***Table 2: Mean log_{10} of counts (cfu g^{-1}) of recovered Campylobacter spp. and***

411 ***standard deviation (SD) at 0, 3, 6, and 24 h after seeding litter (n=6). P<0.001***

412 ***where superscripts differ (by one-way ANOVA).***

Sample time	Mean	SD
0 h	2.22[a]	1.79
3 h	1.85[b]	1.74
6 h	0.85[c]	0.74
24 h	0.00[c]	0.00

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414 ***Table 3 Mean log_{10} of counts (cfu g^{-1}) of Campylobacter spp. from caeca samples,***

415 ***according to cull method or Campylobacter spp. mix, with standard deviation (SD)***

416 ***shown. N=10 birds per mix; P<0.001 where superscripts differ (by two-way***

417 ***ANOVA).***

	Cervical dislocation		**Overdose of barbiturate**		**Overall**	
Mix	Mean	SD	Mean	SD	**Mean**	**SD**
A	6.12	5.68	5.98	5.63	6.06^{a}	5.67
B	5.39	5.51	5.21	5.53	5.31^{b}	5.50
C	5.99	5.82	5.96	5.56	5.97^{a}	5.70
Overall	5.93	5.82	5.83	5.71		

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