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Sandilands, V; Whyte, F; Williams, LK; Wilkinson, TS; Sparks, NHC; Humphrey, TJ

Published in:
British Poultry Science

DOI:
10.1080/00071668.2018.1523538

First published: 21/09/2018

Submitted to British Poultry Science – short communication

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

Accepted for publication 28 July 2018

Abstract

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

Previous work studying poultry colonised with various organisms (e.g. *Campylobacter* and *Salmonella* spp.) have used oral gavaging techniques (Arsi *et al*, 2015; Upadhyaya *et al*, 2015; Saint-Cyr *et al*, 2017). However, this is invasive, stressful and carries a risk of injury to the birds as well as not reproducing the conditions under which chickens would normally ingest the organism. In order to improve and refine techniques, this study aimed to determine if introducing *Campylobacter*-seeded litter to floor pens housing broiler chickens would result in reliable colonisation.

Materials and Methods

Phase 1: litter seeding

Used poultry litter (a mixture of wood shavings, broiler excreta, spilt feed and water) was collected from a previous trial at Scotland's Rural College (SRUC), Ayr. The litter was weighed and sterilised by drying in an oven at 80°C until a constant weight was obtained, and then divided into 400 g batches in six trays (approximately 38 x 28 x 8 cm). Each dried litter tray was then reconstituted with 1 l of deionised water.

A *Campylobacter* strain that had been isolated previously from caecal samples taken from a commercial chicken farm and stored at -80°C in bead cryopreservation vials (Technical Service Consultants, UK) was used. The strain was resuscitated on Blood Agar No. 2 with Horse Blood (BA) plates (Oxoid, UK). These cultures were used to prepare lawn plates on further BA plates, incubated for 40-48 hrs at 41.5°C microaerobically (CampyGen, Oxoid, UK). The lawn plates were harvested by adding 5 ml Mueller-Hinton (MH) broth (Sigma-Aldrich, UK), gently detaching the culture with a sterile spreader and decanting to a container. The suspension was then adjusted with further MH broth to optical density$_{600}$ of 0.19 – 0.21 (approximately 1.5 x 10^5 cfu ml^{-1}). The litter trays were seeded with a mixture of 20 ml MH broth, 10 ml *Campylobacter* spp. suspension and 10 g dried hen faeces (dried in the same way as the litter), by applying evenly to the surface of the litter.

The litter trays were incubated at 21°C. At 0, 3, 6 and 24 h after seeding, a different quarter of each tray was sampled. A sterile pot was filled with litter collected from the top 1 cm of the selected quadrant. Subsequently, a 5 g portion of the collected litter was mixed with 45 ml of MH broth. A swab was used to streak each litter/broth mix sample onto two modified Charcoal Cefoperazone Deoxycholate agar (mCCDA, Oxoid, UK) plates, which were incubated microaerobically at 41.5°C for 24 h.

Phase 2: colonising birds

Forty-three Ross 308 male broiler chicks (*Gallus gallus domesticus*) were housed from day old in a single litter-floor pen with a brooding heat lamp. The ambient air temperature was 32°C on arrival, and was gradually reduced to 21°C at 21 days of age. At 7 days of age, all chicks were wing tagged and weighed, and the 13 lightest chicks (mean weight 170 g) were removed from further study. The remaining 30 chicks (mean weight 208 g, range 187-239 g) were distributed to three pens of ten chicks each, according to weight in order to reduce variation within pen (i.e. ten lightest chicks to pen 1, ten middle weight chicks to pen 2, ten heaviest chicks to pen 3) with 0.66 m^2 total floor space per bird. Chicks were reared until 28 days of age. Birds were fed a standard commercial starter crumb from arrival to 14 days of age, and then a grower pellet from 14-28 days of age. Food and water were provided *ad libitum* from a circular food hopper and bell drinker.

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

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

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--Insert Table 1 approximately here--

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

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

With each liver sample, 5 g was removed from the bag, dipped in 70% ethanol and flamed using a Bunsen burner to surface sterilise. The liver was placed in a stomacher bag with 45 ml of saline, and samples were homogenised in a Colworth stomacher for 1 min or until an even homogenate was created. A 2 ml sample of each homogenate was placed in a universal and 20 ml of modified Exeter enrichment broth (Mattick et al., 2003) was added to produce a minimal headspace, lids were tightly capped and the enrichments were aerobically incubated at 42°C for 24 hours. After incubation, a 10 µl loopful of the enrichment was plated onto mCCDA, plates were incubated at 42°C for 24 hours microaerobically as described above. Results were interpreted as presence or absence of *Campylobacter* spp. depending on growth. Colonies were picked on duplicate BA plates, one incubated aerobically and one microaerobically at 42°C for 24 hours. If there was any growth on the aerobic plate the results were discounted. Colonies from the microaerobic plate were stored on cyrobeads at -80 ^{o}C.

Ethical note

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

Statistical analysis

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

Results

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Phase 1: litter seeding

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

--Insert Table 2 approximately here--

Phase 2: colonising birds

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

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

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

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by cervical dislocation). *Campylobacter* spp. counts from the caeca were significantly affected by the strain mix (P <0.001), but not by the cull method (P=0.308), nor was there an interaction between strain mix and cull method (P=0.711; Table 3). Excluding the two birds from strain mix B where no *Campylobacter* spp. counts were obtained did not greatly alter the results (i.e. effect of strain mix: P=0.002, effect of cull method: P=0.308, interaction: P=0.745).

--Insert Table 3 approximately here—

Discussion

Litter seeding with a mixture of *Campylobacter* spp., broth and chicken faeces was successful in that measurable amounts of *Campylobacter* spp. were recovered up to 3 h after seeding. Recovered *Campylobacter* spp. at 6 h was significantly lower than at 0 and 3 h, and did not differ from 24 h (where counts were always zero), suggesting that the organism is viable in this environment for less than 6 h, but at least for 3 h. This is important, as the organism must survive long enough for some birds to ingest it via foraging in the seeded litter.

When three different *Campylobacter* spp. mixtures were presented using litter seeding to naïve broiler chickens, the method worked successfully in that all liver samples and 93% of caeca samples tested positive for at least one of the relevant *Campylobacter* strains seven days after bird exposure. The negative caecal results could have been due to inhibition of *Campylobacter* spp. by other bacterial species or that these strains did not establish themselves in the caecal niche. Nevertheless, these results indicated that the organism survived long enough for at least some birds to

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ingest it, presumably due to foraging on the seeded trays. Even if only a few chickens ingested the organism in the first instance from the trays, the subsequent production of colonised faeces and frequent foraging behaviour, in which chickens scratch and peck at the floor litter (which has the faeces in it), will recycle the organism until it has spread to birds throughout the pen. This could be confirmed by doing sequential sampling of birds on seeded litter, as opposed to sampling all birds at one time point (as was done in this experiment), and investigating variation in organism counts over time. However, previous studies have shown that inoculating just a few birds in a group leads to successful colonisation of the organism in the naïve birds (Shanker *et al*, 1990; Line *et al*, 1998). Foraging behaviour tends to decline with increasing age in broilers (Bessei, 1992; Wallenbeck *et al*, 2016), so colonisation rate using litter seeding may be affected by bird age.

It was notable that the two caeca in which *Campylobacter* spp. Were not detected came from mix B, so this may be a less reliable mix compared to mix A or C. Neither mix A nor C had a greater count of *Campylobacter* spp. in the caeca, so they may be equally suitable for use, depending on whether or not the point of study is to investigate effects of mainly *C. jejuni* strains, which predominated in mix A, but were roughly equal with *C. coli* in mix C.

The method of killing did not affect the mean counts of *Campylobacter* spp. in the caeca, which indicated that either method can be used without affecting data. This is important, as studies carried out on *e.g*. commercial broiler farms are more likely to use cervical dislocation as a method of killing, as opposed to using controlled medicines.

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

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

Acknowledgements

Accepted for publication 28 July 2018

We are grateful to the support of stock workers at SRUC for their assistance with the study, and to Jackie Ferrie for assistance in the lab.

Disclosure

No potential conflict of interest was reported by the authors

Funding

This work was funded by the UK's Biotechnology and Biological Sciences Research Council (BBSRC), grant reference BB/M008096/1.

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Accepted for publication 28 July 2018

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 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*

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

Accepted for publication 28 July 2018

Table 2: Mean log_{10} of counts (cfu g^{-1}) of recovered Campylobacter spp. and standard deviation (SD) at 0, 3, 6, and 24 h after seeding litter (n=6). P<0.001 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

Accepted for publication 28 July 2018

Table 3 Mean log_{10} of counts (cfu g^{-1}) of Campylobacter spp. from caeca samples, according to cull method or Campylobacter spp. mix, with standard deviation (SD) shown. N=10 birds per mix; P<0.001 where superscripts differ (by two-way 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		

Accepted for publication 28 July 2018