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Factors affecting the species of *Campylobacter* colonising chickens reared for meat.

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Abbreviated running title*: Campylobacter* spp. colonising chickens.

Abstract

Aim: To investigate factors influencing *Campylobacter* spp. colonisation of broiler chickens.

Methods and Results: Campylobacters were isolated from caeca from 319 flocks of two different breeds (199 Cobb and 120 Hubbard), reared as standard (199), freedom-food/corn fed (57), free-range (47) or organic (16). . The standard category exclusively used Cobb birds

slaughtered at 38-41 days. The Freedom Food/corn-fed and free range Hubbard birds were slaughtered at 49-56 days and the organic flocks at 70 days. Campylobacters were picked at random from direct plates. Both breed of chicken (Hubbard) and age at slaughter were independently associated with increased likelihood of colonisation by *C. coli* rather than *C. jejuni*, but breed could not be separated from other aspects of husbandry with the data available. **Conclusions**: Chickens are frequently colonised by *C. jejuni* and *C. coli* and most human infections originate from poultry. In most developed countries approximately 90% of human infections are caused by *C. jejuni*, but fewer than 10% by *C. coli*. This might be due to *C. coli* being less pathogenic than *C. jejuni* to humans, and/or to chicken meat carrying fewer *C. coli* than *C. jejuni*. More investigations are needed into these aspects before it can be concluded that slaughtering older birds from slower-growing breeds would reduce the risk of human *Campylobacter* disease.

Significance and impact of the study: Meat from certain breeds of poultry are predominantly colonised by *C. coli* rather than *C. jejuni*. More research is needed to understand the impact this may have on the number and severity of human campylobacter infections.

Keywords: Breed; broilers; free-range; organic**;** age at slaughter; *Campylobacter jejuni;* *Campylobacter coli.*

Introduction

Campylobacter spp. are widely regarded as the most common cause of bacterial gastroenteritis in industrialised countries, including Europe (Ketley 1997; EFSA 2011; Marotta *et al.* 2015; Seliwiorstow *et al.* 2016; EFSA, 2019). The number of confirmed cases of human campylobacteriosis reported in the European Union (EU) has stayed relatively constant since 2005, with over 246,000 (about 65 per 100,000 population), in both 2017 and 2018 (EFSA &

ECDC 2018; EFSA 2019). Systems for reporting campylobacteriosis vary between different EU member countries (EFSA & ECDC 2018). Many cases are not reported, and as many as 9 million people are estimated to suffer from campylobacteriosis annually in the EU (Havelaar *et al.* 2013). The cost of campylobacteriosis for the member countries of the European Union is between 500 and 5000 million euros per year (EFSA, 2011; Robyn *et al.* 2015). *C. jejuni* and *C. coli* are the most frequently reported species in human cases of *Campylobacter* infection (WHO 2018), causing approximately 90 % and 10 % of cases, respectively (Gillespie et al. 2002; Nielsen et al. 2006; EFSA & ECDC 2018; EFSA 2019; Table 1). The situation is similar in other developed and developing countries (WHO 2018).

The sources of human *Campylobacter* infection vary but a significant proportion comes from poultry (EFSA 2010; Cody *et al.* 2019) where these bacteria colonise the intestine, producing few, if any adverse symptoms in the birds (Corry and Atabay 2001). The mean EU *Campylobacter* prevalence in broiler flocks was 71% in 2018, while 37.5% of raw broiler meat samples were reported positive, however, the proportion of chicken flocks colonised by *Campylobacter* sp. at slaughter varies widely, depending on the member state (Norway, Sweden and Finland have low proportions) and the time of year (high in summer and lower in winter) (EFSA 2019). Table 1 summarises the latest EU data on the proportion of human cases infected with *C. jejuni* or *C. coli* and compares them with the species isolated from broiler flocks and broiler meat (EFSA, 2019). Previous studies undertaken in England have found that 98 % of *Campylobacter*-positive samples from raw poultry meat contained *C. jejuni* and only 2 % *C. coli* (Jorgensen *et al.* 2002). Näther *et al.* (2009) found that of 146 intensively-reared flocks, 64 tested positive for *Campylobacter* spp, and, of the positive flocks, 66% were colonised by *C. jejuni* and 33% by *C. coli*. The association of campylobacters with poultry in developing countries is similar. (Kottawatta *et al.* 2017; Mageto *et al.* 2018).

In contrast, *C. coli* rather than *C. jejuni* is commonly isolated from pigs (Madden *et al.* 2007; Sheppard *et al.* 2009), so contaminated pork and pork products may account for a proportion of the *C. coli* infections seen in humans. Gillespie *et al.* (Gillespie *et al.* 2002) found that patients with *C. coli* infection were more likely to have eaten liver pâté, a predominantly pork-based product, than were patients with *C. jejuni* infection. However, chicken meat contaminated with *C. coli* may still play a part, as high numbers of this species have previously been isolated from both free-range (43 %) and organic (92 %) flocks (El-Shibiny *et al.* 2005). Undercooked chicken livers have been implicated in a number of *Campylobacter* outbreaks and sporadic infections in the UK (Forbes et al. 2009; Little et al. 2010; Strachan et al. 2013).

For standard rearing, modern poultry breeds are selected to grow rapidly in closed poultry houses in order to reduce costs and meet market-demand as soon as possible. However, intensive rearing can cause problems, including weak legs due to their rapid weight gain, and foot problems associated with poor litter quality (Bessei et al. 2006; Knowles et al. 2008; Granquist et al. 2019). Also, concern among consumers with respect to welfare has encouraged the use of alternative, more welfare-friendly, rearing systems, such as the RSPCA "Freedom Food" standard (<rspcaassured.org.uk/farm-animal-welfare/>) which include low stocking density, perches and other environmental enrichment, and access to the outside ('free range'), or provision of organic feed in addition to outside access ('organic'). These rearing systems are called 'extensive', in contrast to the more common 'intensive' system used for rearing broilers.

The 'freedom-food' and 'corn-fed' chickens studied in our survey were reared indoors, but were a different breed (Hubbard) and grew more slowly than the standard intensively-reared birds. Hubbard chickens were also used for organically-fed and free-range birds. Extensively reared birds have a lower stocking density, grow more slowly, and are reared for 56 - 80 days, compared to the 32 - 42 days required for intensively-reared broilers. Intensively-reared birds

100 are most often colonized by campylobacters at around 3 weeks of age, while organic and free-
101 range chickens are colonised earlier, often coinciding with the time at which they are allowed
102 out of their brooding houses (Allen *et al.* 2011). Caecal contents are considered better than
103 faeces or samples from other parts of the chicken intestine for monitoring the true prevalence
104 of *Campylobacter* colonisation (Vidal 2012; Allain *et al.* 2014). Numbers of campylobacters
105 in caecal contents at slaughter (≈$\log_{10}$ 6.5 cfu per g) do not differ significantly between
106 intensively- and extensively-reared birds (Allen *et al.* 2011; Williams *et al.* 2013). Intensively-
107 reared chicken meat is still the most widely consumed in the UK, with organic and free-range
108 chicken meat comprising <1% and about 4.5% respectively
109 (https://www.statista.com/statistics/299050/organic-poultry-numbers-in-the-united-kingdom-
110 uk/).
111 In this study we looked at the species of *Campylobacter* isolated from chicken caeca at
112 slaughter and its relation to breed of flock, rearing-regime and age at slaughter.
113
114 **Materials and methods**
115
116 **Collection of samples**
117 Flocks (319) were sampled from three UK poultry processing plants (A, B and C) between
118 December 2003 and October 2008. Flocks were defined as all birds originating from the same
119 house/shed on a farm. The flocks comprised two different breeds: Cobb (199 flocks) and
120 Hubbard (120 flocks). The Cobb flocks were all reared intensively as standard birds. Abattoirs
121 A and C processed only intensively-reared Cobb flocks (82 and 69 flocks respectively), while
122 Abattoir B processed 48 Cobb flocks and 120 Hubbard flocks. Of the 120 Hubbard flocks, 16
123 were reared as organic, 47 were reared as free range, while 57 were reared intensively according
124 to the Freedom-Food or Freedom Food (Corn-Fed) specifications. The age of the flocks at

slaughter varied from 38-41 days for the standard (Cobb) flocks, 49-56 days for the free range, corn-fed and freedom foods (Hubbard) flocks and 70 days for the organic flocks.

Four flocks were selected at random by the processing plant operatives on each sampling day and at least four pairs of caeca were collected from each flock. All caeca were transported to the laboratory on ice, where they were refrigerated, if necessary, prior to analysis. Care was taken to make sure that the caeca were not frozen, which could have inactivated campylobacters, and analysis was carried out within 24 h.

Detection and isolation of *Campylobacter*

All caeca from all the flocks were examined by plating to determine whether or not the flocks were colonised by *Campylobacter*. One caecum from each pair of caeca was placed in a sterile Petri dish and a swab of caecal content was spread directly onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA), (Oxoid, Basingstoke, UK, CM739 with SR155 supplement). Plates were incubated microaerobically in an atmosphere comprising 5 - 6 % oxygen, 3 - 7 % carbon dioxide and 7 % hydrogen in a balance of nitrogen, at 41.5 °C for 24 - 48 h. Flocks which were not fully positive, or negative for *Campylobacter* (i.e. where some or all plates contained few or no *Campylobacter* colonies*)* were not further studied. Plates from *Campylobacter*-colonised flocks contained high numbers of colonies that all looked similar. In most cases two colonies were picked at random, but due to limited resources, in some instances only one colony per sample was picked. The colonies were subcultured onto duplicate plates of Columbia Blood agar (CBA) with 5 % (v/v) defibrinated horse blood (Oxoid, PB0122). One set of plates was incubated aerobically and the other microaerobically at 41.5 °C for 48 h. Colonies that had grown under microaerobic but not aerobic conditions were confirmed as *Campylobacter* spp. by a positive oxidase test and the confirmed *Campylobacter* isolates were stored using cryobeads (Microbank®) at -80 °C prior to further examination.

Speciation of *Campylobacter* isolates

Stock beads were plated onto CBA (Columbia Blood Agar, Oxoid, pre-poured plates) and incubated in a microaerobic atmosphere at 37 ºC for 48 h. A DNA template was prepared by suspending a 10 µl loop of culture in 500 µl dH_2O and heating at 100 ºC for 10 min. PCR was carried out according to a modified version of Wang et al. (2002), involving three primer sets (Table 2) designed to identify simultaneously the *hipO* gene from *C. jejuni*, the *glyA* gene from *C. coli* and 23S rRNA from *Campylobacter* spp. Each PCR reaction contained 25 µl HotStar Taq Master Mix (Qiagen, Manchester, UK), 4 µl $MgCl_2$ (25 mM), 4 µl primer mix (from stock mix containing 5 µl *C. jejuni* primers, 10 µl *C. coli* primers, 2 µl 23S rRNA primers and 43 µl nuclease-free water), 1 µl template DNA and 16 µl nuclease-free water to make a final volume of 50 µl. Amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research) under the conditions specified by Wang *et al.* (Wang *et al.* 2002), with the following modification: an initial denaturation step was carried out at 95 °C for 15 min. The PCR products were analysed by gel electrophoresis through 2 % (w/v) agarose, containing 1 µl ml^{-1} ethidium bromide, in 1 x TAE buffer. The DNA bands were visualised by means of an ultra-violet transilluminator (BioDoc-It™ Imaging System, UPV). Five µl Hyperladder™ I (Bioline) was used as a molecular marker. Isolates were confirmed as *Campylobacter* sp. if a band was present at 650 bp (23S rRNA). An isolate was determined as *C. jejuni* or *C. coli* if a band was present at 323 bp (*hipO*) or 126 bp (*glyA*) respectively.

Analysis of results

As all colonies looked similar, the first (or only) colony picked was regarded as a random sample. Results from the first or only isolate picked were first tested for association between the species of *Campylobacter* isolated and breed and rearing regime by chi-square tests.

For samples from which two isolates had been obtained, the dependence of the species isolated (both colonies *C. coli* versus both colonies *C. jejuni*) on breed and age at slaughter (mean-centred days) was further examined by logistic regression analyses. Additionally, multinomial logistic regression was used to include the isolation of one colony of each species. All regressions were tested for goodness of fit by the chi-square method of Hosmer and Lemeshow (Hosmer and Lemeshow 1989). Calculations were done with SAS version 9.4.

Results

Speciation of isolates

A higher proportion of standard (Cobb) flocks was sampled than non-standard (Hubbard) in all years except for 2008 (Table 3). Isolates (584 were speciated, 403 of which were *C. jejuni*, 178 *C. coli* and three of which were *Campylobacter* species other than *C. jejuni* or *C. coli)*. Overall, *C. jejuni* was the first isolate identified from 72 % of flocks while *C. coli* was the first identified isolate from 28 % of flocks.

Species of *Campylobacter* in relation to flock type

C. jejuni was more prevalent in Cobb birds reared as standard than in Hubbard birds reared as either free-range (16 flocks), freedom food/corn-fed (57 flocks) or organic (47 flocks) (Table 4; Figure 1). Based on the first isolate speciated, there was a significant association between the breed of the chicken flock and the species of *Campylobacter* colonising the flock (Chi-square test; $P < 0.001$). Omitting flocks where only one isolate was identified, both *C. jejuni* and *C. coli* were identified from 21 flocks when a second isolate from 121 standard and 102 Hubbard flocks was examined (Table 5). For these 223 flocks there was a significant association between breed and species of *Campylobacter* colonising the flock (Chi-square test; $P < 0.001$). All the

Hubbard flocks were markedly older at slaughter than the Cobb flocks, and it was clear that there was a correlation between age at slaughter and breed of chicken. These factors were further investigated by logistic regression analysis on data from abattoir B only. The outcomes modelled were: both colonies *C. jejuni versus* both colonies *C. coli*.

Both breed and age at slaughter were independently associated with outcome. For age at slaughter, the Odds Ratio (OR, 95% Confidence Interval) = 1.116 (1.072, 1.162). For Cobb versus Hubbard, OR = 0.232 (0.081, 0.667). Owing to the evident correlation between breed and age at slaughter, the effect of the latter was confirmed by analysing each breed separately with statistically significant results: for Cobb flocks, OR = 1.163 (1.071, 1.261); for Hubbard flocks, OR = 1.097 (1.048, 1.148). Colonisation by *C. coli* was favoured by later age at slaughter and by breed being Hubbard.

Additionally, multinomial logistic regression was used to include the identification of one colony of each species (mixed colonisation). This showed that age at slaughter had a significant effect also when the outcome = one colony of *C. col*i + one colony of *C. jejuni* was compared with the outcome = two colonies of *C. jejuni*, OR=0.89 (0.85, 0.93)), but not when compared with two colonies of *C. coli* OR=0.99 (0.95, 1.040). Goodness of fit was satisfactory for all the regression models (Hosmer and Lemeshow 1989), and no significant interaction between factors was detected.

Discussion

Our study examined *Campylobacter*-colonised chickens at slaughter in order to investigate the factor(s) influencing the species (*C. jejuni, C. coli* or a mixture of the two species). These factors included the strain of chicken (Cobb or Hubbard), rearing regime (intensive, extensive and diet) and age at slaughter. Significant associations were found between both the strain of chicken (Hubbard more likely than Cobb birds to be colonised with *C. coli*) and age at slaughter

(older birds more likely to be colonised with *C. coli*). Both the breed of chicken and the age at slaughter were independently associated with an increasing likelihood of birds becoming colonised by *C. coli* rather than *C. jejuni*, but breed could not be separated from other aspects of husbandry using the data available.

Our observation that carriage of *C. coli* increases with the age of the birds is supported by the study of El-Shibiny *et al.* (El-Shibiny *et al.* 2007) who monitored *Campylobacter* species and campylobacter-specific phages in two Ross (a breed which we did not study) broiler flocks, in the United Kingdom, one reared as organic for 73 days, and a similar flock raised as free-range on a second farm for 56 days. They found that *C. jejuni* was the dominant species in both flocks until approximately 35 days of age, after which *C. coli* became the dominant species until slaughter. Studying the phages present, indicated that phages were not responsible for selecting the strains of *Campylobacter* colonising the birds. The same research group (El-Shibiney *et al.* 2007) carried out an *in vitro* experiment to investigate whether a particular strain of *C. coli* was antagonistic to a single strain of *C. jejuni*. Results showed that each strain multiplied readily in the presence of the other, but with a low initial ratio of *C. jejuni* to *C. coli*, the *C. jejuni* exhibited a premature decline phase. Laboratory studies using Ross broilers, colonised with the *C. jejuni* strain, showed that the *C. coli* strain outnumbered the *C. jejuni* strain only when the birds were 35 days old or more. Similar results were found when three other *C. jejuni* strains were tested. Although there are several other studies that indicate that chickens slaughtered later in their lives are more frequently colonised with *C. coli*, some (e.g. Cui *et al.* 2005) used an enrichment step, rather than direct plating**,** to detect *Campylobacter*, which could alter the proportion of each species present. Work by Denis et al. (2008) with commercial flocks of undefined poultry strains failed to observe a relationship between *C. coli* colonisation and organic or free-range rearing. Similarly, Colles *et al.* (Colles *et al.* 2010) found most campylobacters from 80- to 81-day-old chickens were *C. jejuni*. They took swabs from

the anal area of live free-range “Hubbard crossbreed” birds at 80 days of age on farm, and carcass rinse samples from the same flocks the following day at the abattoir. These sampling techniques risk contamination from litter and the abattoir environment respectively. Of 222 colonies from 25 live birds they found 81% *C. jejuni* and 19 % *C. coli*, while, of 250 colonies taken from 25 carcasses at the abattoir, they found 62% *C. jejuni* and 37% *C. coli*. .

Our finding that the proportion of *C. coli* to *C. jejuni* colonising the chicken intestine increases with age, concurs with results from several other studies, but our observation that the breed of chicken also influences the predominating species of *Campylobacter*, is new. The increasing proportion of *C. coli* colonizing chickens during the rearing period is of interest because *C. coli* causes only about 10% of human *Campylobacter* cases while *C. jejuni* causes 90%. Thus, meat from older birds may be less hazardous when consumed than meat from younger birds. Alternatively, the proportion of *C. coli* to *C. jejuni* cases might merely reflect the fact that most chickens are slaughtered and consumed at a young age, when *C. jejuni* predominates. Currently there is no evidence that *C. coli* from chickens is less pathogenic for humans than *C. jejuni* from chickens, but appropriate non-pathogenic strain/s of *C. coli* might be suitable for competitive exclusion strategies to reduce the numbers of *C. jejuni* on poultry meat (see O’Kane and Connerton, 2017). Further investigation of the effect of breed on the *Campylobacter* species predominating at slaughter might enable selection of breeds colonized by *C. coli* at a younger age.

Both the breed of chicken and the age at slaughter were independently associated with an increasing proportion of birds being colonised by *C. coli* rather than *C. jejuni*. As *C. coli* causes a lower number of human infections, slaughtering chickens from slower-growing breeds at an older age might reduce numbers of *Campylobacter* infections in the human population. This might be due to *C. coli* being less pathogenic than *C. jejuni* to humans, and/or to chicken meat carrying fewer *C. coli* than *C. jejuni*. There is some evidence that *C. jejuni* strains carry a

greater number of virulence genes (Lapierre et al. 2016). Also. the fact that Guillain-Barré syndrome, a rare and severe disease in humans, sometimes follows a *C. jejuni*, but not a *C. coli* infection (Jasti et al. 2016), indicates that *C. coli* may be less pathogenic. However, meat from these birds would be more expensive than from younger and faster-growing birds. Alternatively, it might be possible to select breeds which become colonised with *C. coli* at an earlier age, and/or to inoculate the chickens with a known low-pathogenic strain of *C. coli*. This would yield cheaper meat. . More investigations are needed into these aspects before it can be concluded that slaughtering older birds from slower-growing breeds would reduce the risk of human *Campylobacter* disease.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Fig. 1. Percentage of flocks with *C. jejuni* (dark grey) and *C. coli* (light grey) isolates in Cobb and Hubbard breeds of chicken.

442 Fig 1

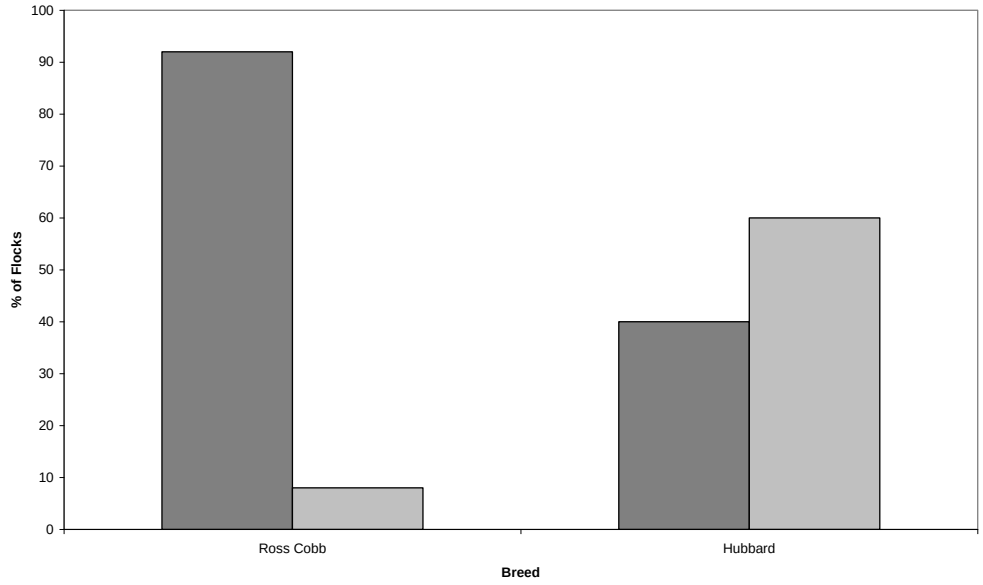

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Table 1 Proportions (%) of *Campylobacter jejuni* and *C. coli* isolates reported in the European Union in 2018*

	C. jejuni	***C. coli***
Human cases	**84**	**10**
Broiler flocks	**63**	**37**
Broiler meat	**76**	**24**

*EFSA (2019)

Table 2 Primer sequences used for speciation of *Campylobacter* isolates*.

Species	Gene	Primer	Sequence (5' - 3')	Amplicon Size (bp)
C. jejuni	*hipO*	CJF	ACT TCT TTA TTG CTT GCT GC	323
		CJR	GCC ACA ACA AGT AAA GAA GC	
C. coli	*glyA*	CCF	GTA AAA CCA AAG CTT ATC GTG	126
		CCR	TCC AGC AAT GTG TGC AAT G	
C. spp.	*23S*	23SF	TAT ACC GGT AAG GAG TGC TGG AG	650
		23SR	ATC AAT TAA CCT TCG AGC ACC G	

*Wang *et al*. (2002)

Table 3 Number of positive flocks investigated by breed and year of study.

Breed	2004	2005	2006	2007	2008
Cobb	89	78	21	7	4
Hubbard	23	16	27	5	49

Table 4 Number and percentage of Hubbard flocks slaughtered at Abattoir B with two *C. jejuni* or two *C. coli* isolates compared to rearing regime.

Rearing Regime	Number of flocks with two *C. jejuni* isolated (%)	Number of flocks with two *C. coli* isolated (%)
Freedom-food/Corn-fed	24 (71)	10 (29)
Free-range	5 (45)	6 (55)
Organic	3 (9)	30 (91)

Table 5 Numbers of flocks of each breed slaughtered in the three abattoirs, where two isolates were speciated, and the first and second isolates speciated were either both *C. jejuni*, or both *C. coli* or one of each species.

Breed	*C. jejuni*	*C. coli*	Mixed	Total
Cobb	107	10	4	121
Hubbard	32	46	4	82