ORIGINAL RESEARCH ARTICLE

*Campylobacter* growth rates in four different matrices: broiler caecal material, live birds, Bolton broth, and brain heart infusion broth

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**Background:** The objective of this study was to characterise *Campylobacter* growth in enrichment broths (Bolton broth, brain heart infusion broth), caecal material (*in vitro*), and in the naturally infected live broilers (*in vivo*) in terms of mean lag periods and generation times as well as maximum growth rates and population (cell concentration) achieved.

**Methods:** Bolton and brain heart infusion broths and recovered caecal material were inoculated with 10 poultry strains of *Campylobacter* (eight *Campylobacter jejuni* and two *Campylobacter coli*), incubated under microaerobic conditions, and *Campylobacter* concentrations determined periodically using the ISO 10272:2006 method. Caeca from 10 flocks, infected at first thinning, were used to characterise *Campylobacter* growth in the live birds. Mean generation times (G) (early lag to exponential phase) were calculated using the formula: \( G = t/3.3 \log b/B \). Mean lag times and \( \mu_{\text{max}} \) were calculated using the Micro Fit© Software (Version 1.0, Institute of Food Research). Statistical comparison was performed using GENSTAT ver. 14.1 (VSN International Ltd., Hemel, Hempstead, UK).

**Results:** The mean lag periods in Bolton broth, brain heart infusion broth, caecal material, and in the live bird were estimated to be 6.6, 6.7, 12.6, and 31.3 h, respectively. The corresponding mean generation times were 2.1, 2.2, 3.1, and 6.7 h, respectively; maximum growth rates were 0.7, 0.8, 0.4, and 2 generations h\(^{-1}\) and the maximum populations obtained in each matrix were 9.6, 9.9, 7.8, and 7.4 log\(_{10}\) CFU/g, respectively.

**Conclusion:** This study provides data on the growth of *Campylobacter* in a range of laboratory media, caecal contents, and in broilers which may be used to develop predictive models and/or inform science-based control strategies such as the maximum time between flock testing and slaughter, logistical slaughter, and single-stage depopulation of broiler units.

Keywords: *Campylobacter*; Growth rates *in vivo* and *in vitro*; flock thinning; foodborne pathogen

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flocks (6, 7), data on growth within the birds is scarce (5). Moreover, while research has identified the caeca, paired finger-like projections from the proximal colon at its junction with the small intestine, as the primary source of infection within birds (8), information on Campylobacter growth in caecal material and how this compares with growth in laboratory media is lacking.

Such data are important. For example, if Campylobacter grow rapidly in the birds, testing 4 days pre-slaughter (the time required to obtain a culture-based result) might result in false negatives and heavily contaminated flocks cross-contaminating birds slaughtered later in the day. Furthermore, predictive models, such as ComBase, used to estimate Campylobacter growth and model scenarios, such as the likely Campylobacter carriage levels if the birds are infected during first thinning, are based on broth cultures and comparative data on growth in caecal contents and the live birds is required to validate these models.

Such research is also timely, as the European Commission has recently published draft legislation amending Regulation (EC) No. 2073/2005 and proposing processing hygiene criteria (PHC) for the poultry sector. Under this legislation, 10 g of neck flap from 15 randomly selected birds per flock will be pooled to give 5 g of final samples. Within a moving window of 50 samples, no more than 5 may exceed the limit of $10^3$ CFU/g (9). As there is a direct relationship between the caecal concentration of Campylobacter and carcass contamination levels (10), each processor will have to decide if specific interventions are required to achieve this target based on pre-slaughter flock test data and a prediction of the likely increase in Campylobacter caecal concentration in the intervening period between testing and slaughter.

The objective of this study was therefore to provide the Campylobacter growth data required, including estimating mean lag periods and generation times as well as maximum growth rates and population (cell concentration) achieved.

### Methods and materials

#### Inoculation study – Campylobacter isolates

Challenge studies were undertaken to investigate and compare Campylobacter growth in laboratory-based broths and caecal material. Poultry isolates (10) (eight Campylobacter jejuni, two Campylobacter coli) were used in the study (Table 1). Of these, six were obtained from the culture collection at Teagasc Food Research Centre (Ashtown) and four from the Animal Health Veterinary Laboratory in Surrey, UK.

#### Preparation of caecal material

Two thousand caeca from a random selection of flocks were collected by staff at the broiler processing plant between January and February 2014, immediately following slaughter and evisceration. Samples were delivered to the laboratory in Teagasc Food Research Centre, Ashtown, on the same day and processed within 24 h. Briefly, the contents were removed aseptically and pooled to create sample sets of 10 g, 20 g, and 50 g final samples. Within a moving window of 50 samples, no more than 5 may exceed the limit of $10^3$ CFU/g (9). As there is a direct relationship between the caecal concentration of Campylobacter and carcass contamination levels (10), each processor will have to decide if specific interventions are required to achieve this target based on pre-slaughter flock test data and a prediction of the likely increase in Campylobacter caecal concentration in the intervening period between testing and slaughter.

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### Table 1. The strains used to inoculate caecal contents

| Strain identity | Isolated from | Species and sequence type/clonal complex (when available) |
|-----------------|---------------|---------------------------------------------------------|
| CJ1             | Broiler Farm, UK | Campylobacter jejuni                                   |
| CJ2             | Broiler Farm, UK | Campylobacter jejuni                                   |
| CC1             | Broiler Farm, UK | Campylobacter coli                                     |
| CC2             | Broiler Farm, UK | Campylobacter coli                                     |
| LK115           | Broiler Farm, Ireland – Caeca | Campylobacter jejuni, ST814/cc-661                      |
| ST45            | Caeca, Ireland  | Campylobacter jejuni, ST45/cc-45                       |
| LK016           | Caeca, Ireland  | Campylobacter jejuni, ST257/cc-257                     |
| LK014           | Caeca, Ireland  | Campylobacter jejuni, ST6764/cc-257                   |
| LK253           | Caeca, Ireland  | Campylobacter jejuni, ST6763/cc-661                   |
| 11168           | Human clinical strain | Campylobacter jejuni typed, national collection        |
cefoperazone and amphotericin (SR0155E, CCDA selective supplement, Oxoid, Cambridge, UK). The remaining broths containing caecal contents were enriched by incubating under microaerobic conditions using Anaero Jars (AG0025A, Fannin, Dublin) with atmosphere generation Kits, Campygen (CN025A, Oxoid, Cambridge, UK) at 37°C for 5 h followed by 42°C for 48 h. After incubation, samples were plated out on mCCDA. All plates were examined to ensure absence of Campylobacter in the irradiated samples.

Preparation of inocula

Cultures were prepared from frozen stocks by aseptically placing one bead (TSC, Lancashire, UK) of each isolate in 25 ml Hunts broth containing 0.65 g nutrient broth (CM0001B, Oxoid, Cambridge, UK) and 0.15 g Yeast extract (CM0019B, Oxoid, Cambridge, UK), 5% Lyased Horse Blood (SR048C, Lennox, Dublin), and 0.4% Campylobacter growth supplement (SR0232E, Oxoid, Cambridge, UK). The inoculated broths were incubated under microaerobic conditions at 42°C for 48 h. After incubation, broths were vortexed for 30 s followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was suspended in 25 ml phosphate-buffered saline (P4417, Sigma-Alrich Arklow, Wicklow, Ireland) and vortexed. Cell suspensions were diluted to 10^{-5} in 9 ml Maximum Recovery Diluent (MRD, CM0733B Oxoid, Cambridge, UK). A 1 ml volume of the suspensions were then transferred to 99 ml Hunts broth to give a final cell concentration of 3 log_{10} CFU/ml for spiking of the caeca. Plate counts were carried to confirm spiking concentrations.

Inoculation of broiler caecal material and broths

Separate sterilised caecal samples (100 g) were then inoculated with 1 ml of each Campylobacter isolate and mixed thoroughly to give final concentrations of approximately 1 log_{10} CFU/g. Plate counts were performed to confirm and samples were then incubated at 42°C under microaerophilic conditions.

Preparation of inocula for broths

For broth inoculation, the above procedure for the preparation of inocula for caeca was followed with just a dilution difference; the broths were diluted five times to 10^{-5} in 9 ml MRD and then transferred to 99 ml of either Bolton broth (CM0983, Oxoid, Cambridge, UK) or brain heart infusion broth (CM1135B, Oxoid, Cambridge, UK) to provide an inoculum with approximately 1 log_{10} CFU/ml. Plate counts were performed to confirm this concentration.

Sampling time points

Each isolate was tested at the following times: (h): 0, 3, 6, 9, 15, 18, 21, 24, 27, 30, 33, 39, 42, 45, 48, 51, 54, 57, 63, 69, 72, 75, 78, 81, 84, 87, 90, and 93 through the use of alternating caecal samples. For example; caecal sample 1 was inoculated with the relevant isolate at 9 am and tested at the following times: (h): 0, 3, 6, 9, 24, 27, 30, 33, 48, 51, 54, 57, 72, 75, 78, and 81. Caecal sample 2 was then inoculated with the same isolate at 6 pm and tested: (h) 0, 15, 18, 21, 24, 39, 42, 45, 48, 63, 66, 69, 72, 84, 87, 90, and 93. All isolates were tested in duplicate and repeated on three separate occasions.

At each time point, 1 g or 1 ml of sample was placed in 9 ml MRD, vortexed for 30 s, diluted, and plated out on mCCDA, as previously described.

Farm Sampling – Sample Collection

Broiler farms (8) were sampled for this study between February and August 2014 to determine the Campylobacter growth profile in naturally contaminated broilers. The farms tested had flock sizes ranging from 25,000 to 33,000 birds with farm size on each site varying from one to six houses. A total of 10 caecal samples were aseptically collected from 15 flocks on these farms on day 28, at first thinning (partial depopulation at 35 days) and at time of final depopulation (final thinning at 42 days). On day 28, the caeca were aseptically removed by the company’s veterinarian. At first and final thinning, caeca were collected from the slaughter plant immediately following evisceration. All samples were transported immediately in a cool box at approximately 2°C to the laboratory and analysed within 24 h.

Isolation of Campylobacter spp. from caeca

Samples were both direct plated and enriched as described previously. Briefly, 1 g of caecal material was added to 9 ml of Bolton broth and vortexed. Serial dilutions were prepared in MRD, and 100 μL volumes were plated out on mCCDA. The remaining broths containing caecal contents were enriched by incubating under microaerobic conditions as above at 37°C for 5 h followed by 42°C for 48 h. After incubation, samples were plated out on mCCDA.

Campylobacter Identification

All presumptive Campylobacter isolates were confirmed initially by Gram staining (3% w/v KOH, Sigma-Aldrich, Arklow, Wicklow, Ireland) and a series of biochemical tests (Oxidase test) (Oxoid, Cambridge, UK) and the L-ALA test (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) followed by streaking on Campy Food ID chromogenic agar (Biomerieux, Durham, NC).
**Statistical Analysis**

*Campylobacter* counts in all matrices were converted to log10 CFU/g. Generation times (G) (early lag to exponential phase) were calculated using the formula: G = t/3.3 logb/B, where t = time interval in h, b = number of bacteria at the end of the time interval, and B = number of bacteria at the beginning of the time interval (5). Lag times and $\mu_{\text{max}}$ were calculated using the Micro Fit Software (Version 1.0, Institute of Food Research) and graphs from this software used to calculate stationary, exponential, and decline phase information. Micro Fit is a 32-bit application which is designed to give a graphical representation of microbiological data and fit a growth model to the data to obtain parameters (13). Statistical comparison of all parameters was performed in GENSTAT by Anova ver. 14.1 (VSN International Ltd., Hemel, Hempshead, UK) by comparing strain, medium, and interaction of strain and medium. Significance was determined at the 5% ($P < 0.05$ level).

**Results**

The mean lag period, generation time, maximum growth rate, and maximum concentration obtained in Bolton broth were 6.6, 2.1, 0.7 generations h⁻¹, and 9.6 log₁₀ CFU/ml, respectively (Table 2). Statistically similar ($P > 0.05$) values were obtained in brain heart infusion broth at 6.7, 2.2, 0.8 generations h⁻¹, and 9.9 log₁₀ CFU/ml, respectively. In contrast, the mean lag period and generation times in caecal contents (*in vitro*) were significantly ($P < 0.05$) longer at 12.6 and 3.1 h, respectively. The mean lag period and generation time were also significantly ($P < 0.05$) longer (31.3 and 6.7 h, respectively) in the broilers (*in vivo*) when compared with the other growth media (also Table 2). The maximum growth rate and concentrations achieved in caecal contents were 0.4 generations h⁻¹ and 7.8 log₁₀ CFU/g, respectively. The corresponding figures for *Campylobacter* growth in the broilers were 2.0 generations h⁻¹ and 7.4 log₁₀ CFU/g, respectively. While the former was statistically significant ($P < 0.05$), the latter was not. No significant differences were observed between the two species, *C. jejuni* and *C. coli*, in the inoculated broths or caecal material.

**Discussion**

This study observed mean generation times of 2.1 (126 min) and 2.2 h (132 min) in Bolton and brain heart infusion broths incubated at 42°C, respectively, over a period of time covering the early lag and exponential phases of growth. The mean $\mu_{\text{max}}$ (exponential phase) were 0.7 and 0.8 generations h⁻¹, respectively. These are similar to the values reported previously (14) for *C. jejuni* F38011 (0.7 generations h⁻¹) and *C. jejuni* 02-833L (0.5 generations h⁻¹) in Muller-Hinton broth incubated at 37°C, and for *C. jejuni* (NCTC 11168) grown in Brucella broth; 0.61 generations h⁻¹ at 37°C and 0.72 generations h⁻¹ at 42°C (15). Interestingly, the mean generation time observed in broilers, 6.7 h, was also similar to the 4.9 h reported by a previous Irish study (5), although the study design (enumerating *Campylobacter* counts at first and second thinning and dividing by the time period in between) was very different.

*Campylobacter* grew well in the irradiated caecal materials in this study, achieving a mean generation time of 3.1 h, a max of 0.4 generation h⁻¹, and a maximum concentration of 7.8 log₁₀ CFU/g. The latter is similar to the 7.8–8.2 log₁₀ CFU/g reported (16) in the caeca of French broilers and the 8 log₁₀ CFU/g obtained in Swedish birds (17) but considerably higher than the 5.5–6.6 log₁₀ CFU/g found in Irish broilers (5). High caecal numbers were not unexpected as it is well established that *Campylobacter* grow well in caecal material (17–19), not least because of the microaerobic environment, nutrient availability, and the pH, typically 6.8 (20). Several studies have reported a positive correlation between the *Campylobacter* concentration in the caeca and mean carcass counts (10, 21). Thus, as the

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**Table 2.** Growth parameters in Bolton broth, brain heart infusion broth, irradiated caecal contents, and in the birds (*in vivo*)

| Matrix                      | Initial concentration (log₁₀ CFU/g) | Generation time (h) | μmax (generations h⁻¹) | Maximum concentration observed (log₁₀ CFU/g) |
|-----------------------------|------------------------------------|---------------------|-----------------------|-------------------------------------------|
|                             | Mean | Range          | Mean | Range | Mean | Range | Mean | Range          |
| Bolton broth                | 1.0  | 6.6<sup>a</sup> | 2.3–9.0 | 2.4<sup>a</sup> | 1.0–3.5 | 0.7<sup>a</sup> | 0.1–1.1 | 9.6<sup>a</sup> log₁₀ CFU/ml | 7.5–10.7 CFU/ml |
| Brain Heart Infusion broth  | 1.0  | 6.7<sup>a</sup> | 3.1–8.6 | 2.2<sup>a</sup> | 1.0–3.6 | 0.8<sup>a</sup> | 0.3–4.4 | 9.9<sup>a</sup> log₁₀ CFU/ml | 6.2–10.8 CFU/ml |
| Caecal (*in vitro*)         | 1.0  | 12.6<sup>b</sup> | 6.5–20.5 | 3.1<sup>b</sup> | 1.2–5.1 | 0.4<sup>b</sup> | 0.1–0.9 | 7.8<sup>b</sup> log₁₀ CFU/g | 5.3–9.9 CFU/ml |
| Broilers (*in vivo*)        | Unknown | 31.3<sup>c</sup> | NA | 6.7<sup>c</sup> | NA | 2.0<sup>c</sup> | NA | 7.4<sup>c</sup> log₁₀ CFU/g | NA |

NA = not applicable.

<sup>a</sup>Generation time (early lag and exponential phase).

<sup>b</sup>Numbers with the same superscript letter are not significantly different ($P < 0.05$).
European Commission introduces PHC for the poultry sector based on Campylobacter skin flap counts, data on caecal concentrations will become an important resource for predicting the likelihood of achieving compliance. Interestingly, all studies reported Campylobacter caecal concentrations above 5 log_{10} CFU/g, the count at which the associated carcasses should be considered to be high risk (22, 23).

The maximum concentration of Campylobacter achieved in the laboratory broths (9.6–9.9 log_{10} CFU/ml) were significantly higher than those observed in the caecal inoculation studies (7.8 log_{10} CFU/g) and in the birds (7.4 log_{10} CFU/g). Moreover, the mean generation times and mean μmax in the broths were significantly (P < 0.05) different to those observed in caecal material and in the broilers. The differences between the laboratory broth and caecal/bird Campylobacter growth parameters may be attributed to the optimised growth conditions in the former and, at least in the case of the in vivo studies, the lack of competing microflora (24). Regardless, these differences call into question the appropriateness of using broth-based models for estimating Campylobacter growth in poultry.

It was concluded that Campylobacter grow well in vitro (broths and in caecal material) and in vivo, reaching concentrations in excess of 7 log_{10} CFU/g. However, the significant differences between key growth parameters suggest new models are required if a predictive approach is to be applied to inform the need for risk management practices such as logistic slaughter to achieve compliance with the proposed European Commission Campylobacter criteria. Moreover, the data provided in this study will contribute to the development of such predictive tools.

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Conflict of interest and funding
No conflict of interest declared.

References
1. Sasaki Y, Haruna M, Mori T, Kusakawa M, Murakami M, Tsujiyama Y, et al. Quantitative estimation of Campylobacter cross contamination in carcasses and chicken products at an abattoir. Food Control 2013; 43: 10–17.
2. EFSA Panel on Biological Hazards (BIOHAZ). Scientific opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. EFSA J 2011; 9: 2105.
3. EFSA Panel on Biological Hazards (BIOHAZ). Scientific opinion on quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. EFSA J 2010; 8: 1437.
4. EFSA Panel on Biological Hazards (BIOHAZ). Analysis of the baseline survey on the prevalence of Campylobacter in broiler batches and of Campylobacter and Salmonella on broiler carcasses, in the EU, 2008; part B: analysis of factors associated with Campylobacter colonization of broiler batches and with Campylobacter contamination of broiler carcasses; and investigation of the culture method diagnostic characteristics used to analyse broiler carcass samples. EFSA J 2010; 8: 1522.
5. Koolman L, Whyte P, Bolton DJ. An investigation of broiler caecal Campylobacter counts at first and second thinning. J Appl Microbiol 2014; 117: 876–81.
6. Newell DG. The ecology of Campylobacter jejuni in avian and human hosts and in the environment. Int J Infect Dis 2002; 6: 3516–21.
7. Thakur S, Brake J, Keclara S, Zou M, Sussick E. Farm and environmental distribution of Campylobacter and Salmonella in broiler flocks. Res Vet Sci 2013; 94: 33–42.
8. Clench M, Mathias JR. The Avian cecum: a review. Wilson Bull 1995; 107: 93–121.
9. Commission Regulation (EC) 2073 on Microbiological criteria for foodstuffs of 2005. Publ.L.No 338, 1–26, 22nd December 2005.
10. Reich F, Atanassova V, Haunhorst E, Klein G. The effects of Campylobacter numbers on the contamination of broiler carcasses with Campylobacter. Int J Food Microbiol 2008; 127: 116–20.
11. International Organisation for Standardisation (2006). Microbiology of food and animal feeding stuffs—Horizontal method for detection and enumeration of Campylobacter spp., ISO-10272-1. Geneva, Switzerland: International Organisation for Standardisation.
12. International Organisation for Standardisation (2006). Microbiology of food and animal feeding stuffs. Horizontal method for detection and enumeration of Campylobacter jejuni part 2: Colony-count technique. ISO-10272-2. Geneva, Switzerland: International Organisation for Standardisation.
13. Sobratee N, Mohee R, Driver MFB. Variation of broth composition by addition of broiler litter composting substrate extracts: influence on faecal bacterial growth. J Appl Microbiol 2009; 107: 1287–97.
14. Konkel ME, Christensen JE, Dhillon AS, Lane AB, Hare-Sanford R, Schaberg DM, et al. Campylobacter jejuni strains compete for colonization in broiler chicks. Appl Environ Microbiol 2007; 73: 2297–305.
15. Corcoran AT, Moran AP. Influence of growth conditions on diverse polysaccharide production by Campylobacter jejuni. FEMS Immunol Med Microbiol 2007; 49: 124–32.
16. Hue O, Allain V, Laisney M-J, Le Bouquin S, Lalande F, Petetin I, et al. Campylobacter contamination of broiler caeca and carcasses at the slaughterhouse and correlation with salmonella contamination. Food Microbiol 2011; 28: 862–8.
17. Hansson I, Pudas N, Harbom B, Olsson Engvall E. Within-flock variations of Campylobacter loads in caeca and on carcasses from broilers. Int J Food Microbiol 2010; 141: 51–5.
18. Grant IH, Richardson NJ, Bokkenheuser VD. Broiler chickens as potential source of Campylobacter jejuni infections in humans. J Clin Microbiol 1980; 11: 508–10.
19. Stern NJ, Robach MC. Enumeration of Campylobacter spp. in broiler faeces and in corresponding processed carcasses. J Food Prot 2003; 66: 1557–63.
20. Kril M, Angelovičová M, Mrážová L. Application of probiotics in poultry production. Anim Sci Biotechnol 2012; 45: 55–7.
21. Rosenquist H, Sommer HM, Nielsen NL, Christensen BB. The effect of slaughter operations on the contamination of chicken carcasses with thermo-tolerant Campylobacter. Int J Food Microbiol 2006; 108: 226–32.

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22. Nuata M, Hill A, Rosenquist H, Brynestad S, Fetsch A, Van der Logt P, et al. A comparison of risk assessments on \textit{Campylobacter} in broiler meat. Int J Food Microbiol 2009; 129: 107–23.

23. Food Safety Authority of Ireland (FSAI) (2011). Report of the scientific committee of the food safety authority of Ireland. In recommendations for a practical control programme for \textit{Campylobacter} in the poultry production and slaughter chain. Dublin: FSAI.

24. Al-Zeyara S, Jarvis B, Mackey BM. The inhibitory effect of natural microflora of food on growth of \textit{Listeria monocytogenes} in enrichment broths. Int J Food Microbiol 2011; 145: 98–105.