

## Intestinal colonization of broiler chickens by *Campylobacter* spp. in an experimental infection study

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### SUMMARY

Consumption of poultry meat is considered as one of the main sources of human campylobacteriosis, and there is clearly a need for new surveillance and control measures based on quantitative data on *Campylobacter* spp. colonization dynamics in broiler chickens. We conducted four experimental infection trials, using four isolators during each infection trial to evaluate colonization of individual broiler chickens by *Campylobacter jejuni* over time. Individual and pooled faecal samples were obtained at days 4, 7 and 12 post-inoculation (p.i.) and caecal samples at day 12 p.i. There were large differences between broiler chickens in the number of *C. jejuni* in caecal and faecal material. Faecal samples of *C. jejuni* ranged from 4·0 to 9·4 log c.f.u./g and from 4·8 to 9·3 log c.f.u./g in the caeca. Faecal c.f.u./g decreased with time p.i. Most variation in c.f.u. for faecal and caecal samples was attributed to broiler chickens and a minor part to isolators, whereas infection trials did not affect the total variance. The results showed that pooled samples within isolators had lower c.f.u./g compared to the arithmetic mean of the individual samples. There was a significant correlation between faecal c.f.u./g at days 4 and 7 p.i., days 7 and 12 p.i. and for caecal and faecal c.f.u./g at day 12 p.i.

**Key words:** *Campylobacter*, caecal, faecal, flocks, poultry, variation.

### INTRODUCTION

*Campylobacter* spp. is the leading cause of bacterial gastroenteritis in the world, causing 2·4 million cases

annually in the United States [1]. In 2011, 220209 cases were reported in the European Union (EU) [2], and the total annual cost of campylobacteriosis in the EU is estimated to be €2·4 billion [3]. Campylobacteriosis is largely perceived to be a foodborne disease with poultry meat as the primary infection source, and the incidence of campylobacteriosis in humans correlates with the prevalence of *Campylobacter* spp. in chickens [4]. It is an international priority to eliminate *Campylobacter* spp. from

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broiler chickens to ensure better food safety [5, 6]. Several approaches have been taken to reduce *Campylobacter* spp. flock prevalence and level of colonization. These include increased biosecurity [7–9], competitive exclusion, antibacterial agents, or phage therapy [10, 11], poultry vaccines [12–16], testing and scheduling [17], and improving the genetic resistance to *Campylobacter* spp. colonization of broiler chickens [18].

Risk assessment models have been developed to determine which strategies are the most efficient in reducing *Campylobacter* spp. flock prevalence and the number of cases of campylobacteriosis [8, 19]. However, at present there is no consensus regarding the most appropriate way of sampling a broiler chicken flock to provide data that can be used in risk assessment models. Usually, a large (10–25) number of caecal samples are taken at the slaughterhouse or faecal samples are collected at the farm and pooled for analysis. This can produce misleading results if colony-forming units (c.f.u.s) differ significantly between individuals. In fact, studies have shown that there is large individual variation in the number of *Campylobacter* spp. in the caeca of broiler chicken flocks collected at slaughter plants [20, 21]. Although the dynamics of *Campylobacter* spp. in broiler chickens are not fully understood, chicken lineage and time of colonization seems to influence variability in colonization of the chick intestine [18, 22–24]. Furthermore, it is unclear if faecal samples at the farm level are a good predictor of the caecal load at the slaughter plant [20]. Improvements in the understanding of *Campylobacter* spp. dynamics in broiler chickens are therefore needed to provide concentration distributions of *Campylobacter* spp. in faecal and caecal samples for risk assessment models [25, 26]. Such data are also essential to effectively evaluate control measures, such as testing and scheduling [27].

To increase our understanding of the dynamics of *Campylobacter* spp. in broiler chickens, we studied the number of *C. jejuni* in broiler chickens inoculated under controlled experimental conditions and addressed the effect of pooling samples. The aims were to (1) estimate the variation in the number of *C. jejuni* in faecal and caecal samples over time in a conventional chicken broiler breed (Ross 308) inoculated with a fixed dose of *C. jejuni*, (2) compare the number of *C. jejuni* in pooled samples with the arithmetic mean of individual samples, (3) evaluate any correlation between faecal loads of *C. jejuni* at days 4, 7 and 12 post-inoculation (p.i.), and with caecal loads at day 12 p.i.

## MATERIAL AND METHODS

### Experimental birds

The experimental inoculation of the broiler chickens was performed at the National Veterinary Institute (Aarhus, Denmark) according to the Danish legislation for animal welfare and use of experimental animals and approved by the Supervisory Authority on Animal Testing (2010/561–1803). Conventional broiler chickens (Ross 308) of mixed sex were obtained from a Danish hatchery (DanHatch A/S). Chicks were transferred directly from the hatchery to the experimental unit, where they were housed in isolators (Montair Andersen B.V. HM 1500, The Netherlands). All chicks were tested free of *Campylobacter* spp. at placement and before inoculation. The chickens were killed by decapitation, and each chicken was sampled and examined individually at slaughter.

### Experimental design

The placebo group described in the present study was part of a larger vaccine study [28] and, due to the design of this study received 0.1 ml Alhydrogel (2% solution) adjuvant intramuscularly 17 days before *C. jejuni* challenge. In order for an Alhydrogel adjuvant to increase specific immunity against an antigen, in this case *C. jejuni*, the antigen must be mixed with the adjuvant and injected as a mixed suspension and thus it is highly unlikely that the chickens of the placebo group had any specific immunity against *C. jejuni*.

The broiler chickens used were housed in isolators and all handling of the chickens was done through the isolator gloves attached to the isolators. Four identical infection trials were performed in 2011, where only the flock of broiler chickens used differed between trials. During each infection trial four identical isolators were used with an average of nine birds per isolator. In total 134 broiler chickens were inoculated during the study period.

Faecal and caecal samples were taken from all broiler chickens in each of the four isolators during each of four infection trials except in trial 4, where samples were only obtained from three isolators. Samples were collected individually from each broiler chicken at each time-point and kept separately in tubes and stored on ice until c.f.u. determination was done. All birds were marked, which ensured that samples were only taken once from each bird. Faecal droppings were sampled by gentle anal stimulation directly into a sterile Falcon tube avoiding any cross-contamination.

Faecal samples were collected on days 4, 7 and 12 p.i., and caecal samples were collected at slaughter on day 12 p.i. In addition to the individual samples of each broiler chicken one pooled caecal or faecal sample was prepared for each isolator and was made out of 1 g from each individual sample within each isolator. The number of *C. jejuni* (c.f.u./g) of individual and pooled caecal and faecal samples was subsequently established. The number of *C. jejuni* (c.f.u./g) of the individual faecal samples was only determined in samples from trials 2, 3 and 4.

### Challenge with *C. jejuni*

On day 31 post-hatch, all birds were inoculated with  $(1.7 \pm 0.5) \times 10^4$  (mean  $\pm$  s.e.) c.f.u./ml of *C. jejuni* in 0.5 ml 0.9% saline solution. The broiler chickens were inoculated individually by crop instillation, using a 1-ml syringe with an attached flexible tube (diameter 3 mm, length 10 cm).

### Preparation of inoculum

The *C. jejuni* strain used in this study was a broiler strain (DVI-SC181), which belongs to the most common serotype (Penner serotype 2) and flaA type (1/1) [29]. This strain originated from a collection of *Campylobacter* spp. isolates obtained from faecal samples collected at the time of slaughter in Denmark [29]. Bacterial inoculum was prepared from cultures grown on Blood agar plates [blood agar Base No. 2, Oxoid, UK, supplemented with 5% (v/v) calf blood], and incubated at 42 °C for 48 h under microaerobic conditions. Subsequently the bacteria were prepared by shaking of bacterial material in 0.9% saline solutions at 4 °C. Before inoculation the bacterial suspension was adjusted to an optical density of approximately  $OD_{620} = 0.6$  and diluted to the desired concentration (c.f.u./ml). The actual inoculation dose was determined by direct counting of bacteria before and after inoculation.

### Bacterial culture and counting

Quantification of *C. jejuni* followed the Nordic standard protocol for enumeration of thermotolerant *Campylobacter* spp. except that a 0.9% saline solution was used for dilution series [30]. The content of each sample was mixed thoroughly by vortexing to ensure homogeneity before 1 g of caecal or faecal material was weighed and diluted 1:10 in 0.9% saline dilution series. The pooled samples were made out of 1 g

from each individual sample within each isolator. Subsequently dilution series were streaked onto *Campylobacter* spp. selective Abeyta-Hunt-Bark agar plates with 1% triphenyltetrazolium chloride. The plates were incubated microaerobically at 42 °C for 48 h before being enumerated.

### Statistics

A mixed linear model was used to estimate the contribution of infection trial, isolator and broiler chicken (residual) to the variation seen in the c.f.u./g found in individual faecal and caecal samples. Based on the estimated variances for trial, isolator and broiler chicken in the mixed linear model, the percentage of total variance that was due to the infection trial, isolator and broiler chicken was calculated. The data obtained at the different time-points were analysed separately. For each day of sampling (days 4, 7 and 12 p.i., respectively), the data from all infection trials were included in the model. At day 12 p.i., the data from faecal and caecal samples were analysed separately. C.f.u. data was log-transformed ( $\log$  c.f.u./g) to normalize the data. In the mixed model, the effect of the infection trial and the isolator within infection trial were both assumed to be normally distributed ( $N(0, \sigma^2)$ ). Distributions of the number of *C. jejuni* in individual faecal samples at different sampling time-points (days 4, 7 and 12 p.i., respectively) were diagrammed as box plots. A non-parametric ANOVA (Kruskal–Wallis test) based on ranks was used to test for the effect of time on c.f.u./g in the faecal samples and Dunn multiple comparisons was used to compare time-points. The *P* values were compared to the Bonferroni-corrected significance level. Individual faecal and caecal c.f.u./g between time-points is shown as a scatter plot with the regression line. Correlation analysis (Pearson) was used to evaluate the relationship between caecal and faecal c.f.u./g of *C. jejuni* at different time-points. C.f.u. of the pooled caecal samples was compared to the arithmetic mean of the individual caecal samples from each isolator. When comparing pooled and individual samples, it is important to differentiate between the geometric and arithmetic mean. The mean of *n* concentrations *C*, expressed in c.f.u./g,  $\Sigma C/n$ , is the arithmetic mean, which can be expressed in logs as  $\log(\Sigma C/n)$ . If the *n* concentrations are given as  $\log(C)$  in  $\log$  c.f.u./g, the mean  $\Sigma(\log(C))/n$  is the geometric mean, expressed in log units. When comparing a pooled sample with individual samples, as in the present study, the

Table 1. Colony-forming units (c.f.u.) of *C. jejuni* in faecal and caecal material collected from broiler chickens at different time-points post-inoculation (p.i.) with *C. jejuni*. The table shows geometric mean  $\pm$  s.d. c.f.u. for individual samples and c.f.u. of pooled samples (log c.f.u./g faecal or caecal content). All samples used to establish individual and pooled c.f.u./g are paired. *N* is based on number of samples obtained from each isolator

Infection trial	Isolator	Log c.f.u./g of faecal content						Log c.f.u./g of caecal content			
		Days p.i.		Days p.i.		Days p.i.		Days p.i.			
		4	7	7	12	12	12	Individual	Pooled	<i>n</i>	
1	1		8.0		7.8		8.0	9	8.5 $\pm$ 0.4	8.6	9
	2		7.3		7.0		7.5	9	8.2 $\pm$ 0.7	8.5	9
	3		7.5		7.5		7.9	10	8.4 $\pm$ 0.6	8.8	10
	4		6.9		7.3		7.9	9	8.1 $\pm$ 0.3	8.2	9
2	1	7.7 $\pm$ 0.5	7.9	7.4 $\pm$ 1.3	7.6	7.2 $\pm$ 0.6	7.4	8	7.9 $\pm$ 0.8	8.3	8
	2	7.1 $\pm$ 0.9	6.7	7.6 $\pm$ 0.8	8.0	7.2 $\pm$ 1.1	8.3	9	8.2 $\pm$ 0.7	8.5	9
	3	7.1 $\pm$ 1.0	7.6	7.1 $\pm$ 0.9	7.2	6.6 $\pm$ 0.6	7.3	8	7.5 $\pm$ 0.6	7.8	8
	4	7.4 $\pm$ 0.6	7.7	6.5 $\pm$ 0.8	7.2	6.6 $\pm$ 1.1	7.2	8	8.1 $\pm$ 0.4	8.3	7
3	1	6.6 $\pm$ 0.4	7.5	7.1 $\pm$ 0.7	7.5	7.2 $\pm$ 0.5	7.3	8	8.2 $\pm$ 0.5	8.4	8
	2	7.1 $\pm$ 0.8	7.7	6.9 $\pm$ 0.4	7.3	7.5 $\pm$ 0.5	7.7	6	8.4 $\pm$ 0.3	8.5	5
	3	8.2 $\pm$ 0.9	7.9	7.8 $\pm$ 0.6	7.8	7.3 $\pm$ 0.2	7.3	4	8.5 $\pm$ 0.3	8.5	4
	4	*									
4	1	7.5 $\pm$ 0.5	7.9	7.6 $\pm$ 0.5	7.9	7.3 $\pm$ 0.6	7.4	11	7.9 $\pm$ 0.3	7.9	11
	2	7.3 $\pm$ 0.7	7.4	7.2 $\pm$ 1.0	7.6	7.4 $\pm$ 0.6	7.9	13	8.7 $\pm$ 0.3	8.5	13
	3	7.9 $\pm$ 0.5	8.0	7.5 $\pm$ 0.3	7.7	7.0 $\pm$ 1.1	7.6	10	7.2 $\pm$ 1.0	7.8	12
	4	7.4 $\pm$ 0.4	7.4	6.7 $\pm$ 0.7	7.4	5.7 $\pm$ 1.1	6.6	12	6.3 $\pm$ 1.1	7.4	12

\* Birds from this isolator were not included due to functional breakdown of the isolator. Grey areas indicate that individual samples were not taken during this infection trial.

concentration in the pooled sample is expected to be the arithmetic mean of the individual samples.

## RESULTS

### Concentrations of *C. jejuni* at different time-points

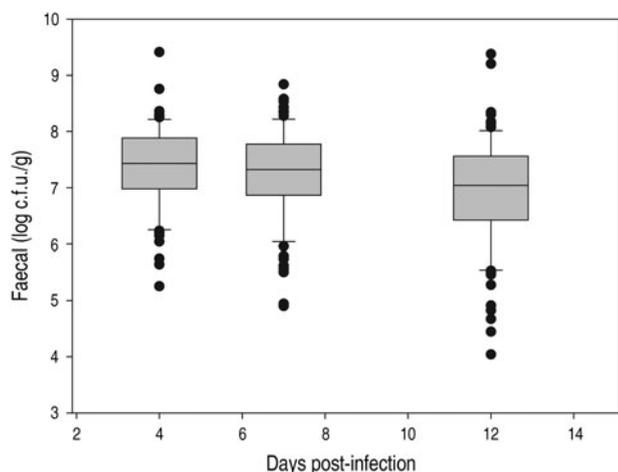
There was a large difference in the gut content of *C. jejuni* between individual broiler chickens. In the individual faecal samples the number of *C. jejuni* ranged from 4.0 to 9.4 log c.f.u./g and for the caecal samples from 4.8 to 9.3 log c.f.u./g. The mean log concentration (geometric mean) of *C. jejuni* for individual faecal samples within isolators ranged from 5.7 to 8.2 log c.f.u./g and for the caecal samples from 6.3 to 8.7 log c.f.u./g (Table 1). The mean log concentration of *C. jejuni* detected in the caecal contents of all the broiler chickens was 7.9 log c.f.u./g. When comparing the faecal c.f.u./g of *C. jejuni* at days 4, 7 and 12 p.i. of each individual broiler chicken there was a slight decrease in c.f.u./g over time, with mean concentration (geometric mean) decreasing from 7.4 on day 4 p.i. to 6.9 log c.f.u./g on day 12 p.i.

(Fig. 1). ANOVA analysis indicated that there was a significant effect of time on faecal c.f.u./g ( $P = 0.003$ ) and individual comparisons showed that faecal c.f.u./g at day 12 p.i. were significantly lower than at day 4 p.i. ( $P < 0.001$ ), whereas neither c.f.u./g at days 4 and 7 ( $P = 0.180$ ) or days 7 and 12 ( $P = 0.041$ ) were significantly different compared to the Bonferroni-corrected significance level ( $P = 0.017$ ).

We evaluated the contributors to the variance in c.f.u. over time (Table 2). In the caecal and faecal samples, most variation was attributed to the broiler chicken (residual), and a minor part to the isolator, whereas the infection trial did not affect the total variance. For the faecal samples the total variance increased slightly with time, but the proportion of the different levels remained the same at the different time-points.

### C.f.u./g of *C. jejuni* in pooled samples vs. individual samples

The pooled caecal samples showed slightly lower *C. jejuni* numbers compared to the arithmetic mean



**Fig. 1.** Colony-forming units (c.f.u.)/g of individual faecal samples from each broiler chicken at days 4, 7 and 12 post-inoculation ( $n = 97$ ). The boundary of the box closest to zero indicates the 25th percentile, the line within the box indicates the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles and black dots the outliers.

**Table 2.** Variance estimates (percentage) of the various levels in the infection trials using quantitative data from faecal and caecal samples

	Faecal			Caecal
	Days p.i.			Days p.i.
	4	7	12	12
Infection trial	0%	0%	0%	4%
Isolator	20%	8%	23%	40%
Residual	80%	92%	77%	56%
Total	0.51	0.66	0.87	0.72

p.i., Post-inoculation.

of the individual samples, in 13 isolators out of a total of 15, during four infection trials (Fig. 2). Pooled and individual faecal samples from each isolator group were only evaluated in infection trials 2, 3 and 4 and showed that the pooled faecal samples taken at day 12 p.i. were lower than or equal to the arithmetic mean of the individual samples in eight out of a total of 11 isolators during three infection trials (results not shown). The pooled sample means were therefore lower than or equal to the arithmetic mean of the individual samples in 21/26 trials, which makes it highly unlikely that this is by chance alone.

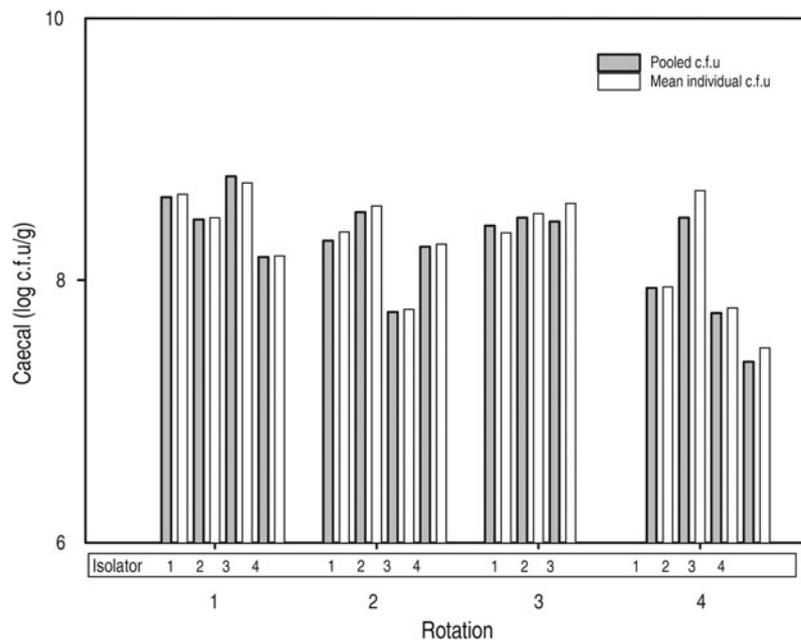
### Correlation of c.f.u./g of faecal and caecal samples

The collection of faecal and caecal samples from each individual at multiple time-points allowed us to compare the number of *C. jejuni* of faecal samples at different time-points and also faecal with caecal samples (Fig. 3). There was a significant correlation between faecal c.f.u./g at days 4 and 7 p.i. [ $r = 0.3$ , 95% confidence interval (CI) 0.11–0.47] and days 7 and 12 p.i. ( $r = 0.2$ , 95% CI 0.02–0.40). Similarly, a significant correlation was found between faecal and caecal c.f.u./g at day 12 p.i. ( $r = 0.7$ , 95% CI 0.5–0.8).

### DISCUSSION

The present study showed large variation in the number of *C. jejuni* in the caecal and faecal samples as has been found in other studies (e.g. [20]). The mean (geometric) number of *C. jejuni* detected in the caecal content of the broiler chickens was 7.9 log c.f.u./g in the present study. This is slightly higher, but still within the range reported in other studies [20, 31, 32]. Faecal content was slightly lower than the caecal content, with mean (geometric) concentration decreasing from 7.4 on day 4 to 6.9 log c.f.u./g at day 12 p.i. In contrast to earlier studies the broiler chickens in the present study were inoculated with the same dose of *C. jejuni* and at the same age. This approach is more likely to give accurate estimates of *Campylobacter* spp. colonization of broiler chickens, but care should be taken when extrapolating results obtained under laboratory conditions to field conditions. The results, however, confirm that colonization differs widely between individual broiler chickens and support the concern raised by Hansson *et al.* [20], that limited sampling of broiler chickens for quantification of *Campylobacter* spp. might not be representative of large broiler chicken flocks.

The design of our trials allowed us to determine which factors contributed to the variance of *C. jejuni* numbers in the individual faecal and caecal samples. Most of the variation found in c.f.u. of *C. jejuni* could be attributed to differences between broiler chickens and less to differences between isolators and infection trials. The total variation increased slightly with time in the faecal samples, with the same factors attributing proportionally to the total variance. Similar temporal changes have been found in other studies comparing *Campylobacter* spp. in carcass rinse samples [33]. In the present study, the broiler chickens were inoculated with the same dose of



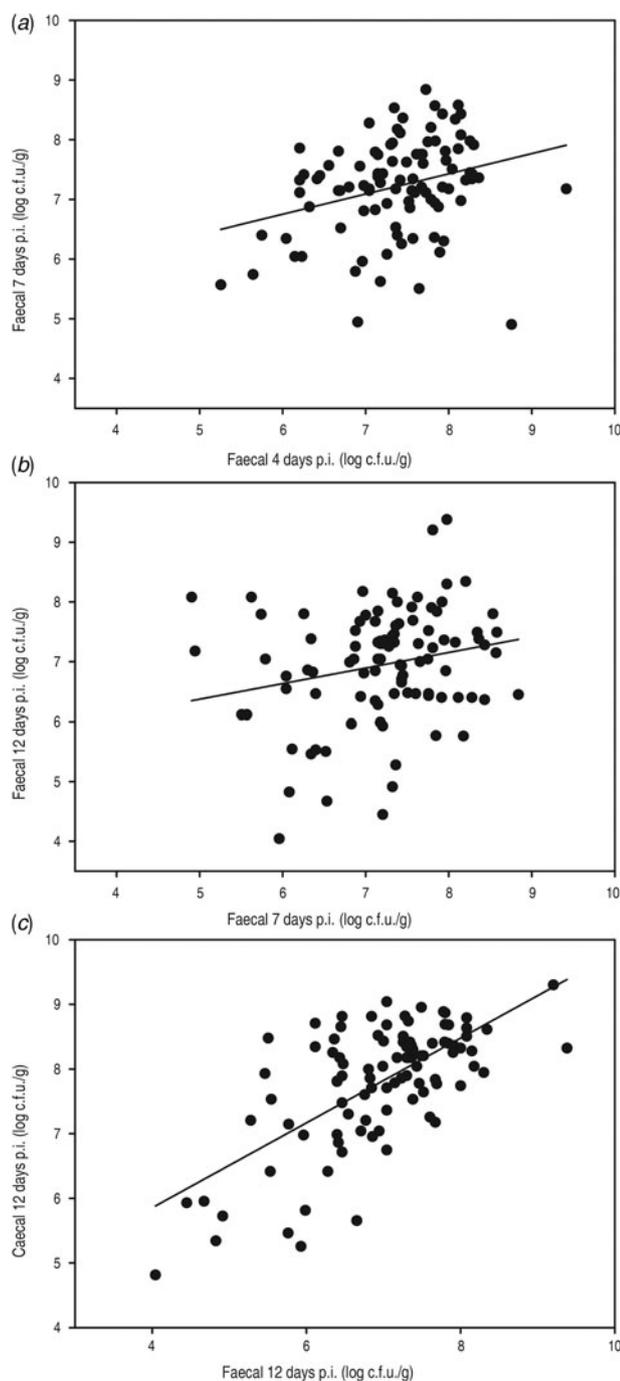
**Fig. 2.** Colony-forming units (c.f.u.)/g of pooled caecal samples (grey bars) from each isolator and the mean c.f.u./g of the individual caecal samples (open bars) obtained from each isolator. One pooled caecal sample was prepared for each isolator and was made out of 1 g from each individual sample within each isolator. Samples were taken at slaughter from each of four isolators during each of four infection trials except in trial 4 where samples were only obtained from three isolators.

*C. jejuni* at the same time. Therefore the variation observed between individuals in our study is not due to the time of infection, but instead suggests that other environmental and genetic factors are involved. Studies have shown that genetics are involved in the *Campylobacter* spp. dynamics in broiler chickens and could thus explain some of the variation observed between broiler chickens [18, 34]. Moreover, the chicken intestinal physiology, most probably the caecal function, may cause an intermittent and fluctuating excretion of *Campylobacter* spp. [7].

In the present study, c.f.u.s from individual and pooled caecal samples were obtained from each isolator. To our knowledge, no other studies have compared c.f.u./g of *C. jejuni* in paired pooled and individual caecal samples. We expected that the number of *C. jejuni* in the pooled samples would be equal to the arithmetic mean of the paired individual caecal samples. In most cases, however, the estimated arithmetic mean of the individual samples was a little higher compared to the c.f.u./g of the pooled samples (<1 log) (Fig. 2). One explanation for the lower c.f.u./g in the pooled samples could be that the mean of a lognormal distribution is usually underestimated when it is based on sample data: on average, the fewer samples taken, the lower the estimate. When

comparing the c.f.u./g of *C. jejuni* in the pooled samples with the geometric mean of the individual caecal samples (Table 1) the latter generally showed lower values, but this is because the geometric mean of lognormal-distributed variables is lower than the arithmetic mean. The results therefore suggest that pooling of samples will generally lead to an underestimate of c.f.u./g compared to the arithmetic mean c.f.u./g of individual samples.

Human risk of campylobacteriosis from broiler chickens results predominantly from meat products with high concentrations of *Campylobacter* spp. [5, 26, 35–37]. It has therefore been suggested that the human incidence of campylobacteriosis can be reduced significantly by targeting control strategies at products with relatively high concentrations of *Campylobacter* spp. Several studies have therefore suggested that ‘testing and scheduling’ might be an efficient control strategy for *Campylobacter* spp. in broiler chicken meat [17, 27]. This strategy entails testing of broiler flocks at the farm shortly before transport to the processing plant. Flocks with high concentrations of *Campylobacter* spp. at the farm can then be diverted from the fresh meat production chain. For this approach to be successful there needs to be a significant correlation between concentrations



**Fig. 3.** Illustration of colony-forming units (c.f.u./g) of individual samples for (a) faecal samples at days 4 and 7 post-inoculation (p.i.), (b) faecal samples at days 7 and 12 p.i. and (c) faecal and caecal samples at day 12 p.i. ( $n = 97$ ). The plotted line is the estimated regression line.

of *Campylobacter* spp. in the faeces and in the meat product [27]. Earlier studies have shown a correlation between the proportion of positive cloacal and caecal samples or the number of bacteria in the caecal content and the number of *Campylobacter* spp. on

carcasses [5, 38, 39]. Our results established that there was a significant correlation between c.f.u./g in individual faecal and caecal samples before slaughter and that caecal values were slightly higher than the faecal values. The significant correlation is supported by other studies [40] and suggests that ‘testing and scheduling’ could be possible with faecal sampling before slaughter. However, if faecal samples are taken earlier there is no or only a weak correlation with c.f.u./g in caecal samples at slaughter. Furthermore, results show that most of the variation in faecal or caecal load found in the present study is indeed due to variation between broiler chickens and not isolators or infection trials. Hence, the usefulness of ‘testing and scheduling’ could prove difficult due to low variance of *Campylobacter* spp. concentrations between flocks and high variance of *Campylobacter* spp. concentrations between broiler chickens within flocks [27].

In summary, the data of the present study provides a better understanding of the dynamics of *Campylobacter* spp. in broiler chickens and highlights the importance of such data for the optimization of new surveillance and control measures.

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#### DECLARATION OF INTEREST

None.

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