



# Parallel Sequencing Reveals *Campylobacter* spp. in Commercial Meat Chickens Less than 8 Days Old

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**ABSTRACT** *Campylobacter* from contaminated poultry meat is a major source of human gastroenteritis worldwide. To date, attempts to control this zoonotic infection with on-farm biosecurity measures have been inconsistent in outcome. A cornerstone of these efforts has been the detection of chicken infection with microbiological culture, where *Campylobacter* is generally not detectable until birds are at least 21 days old. Using parallel sequence-based bacterial 16S profiling analysis and targeted sequencing of the *porA* gene, *Campylobacter* was identified at very low levels in all commercial flocks at less than 8 days old that were tested from the United Kingdom, Switzerland, and France. These young chicks exhibited a much greater diversity of *porA* types than older birds testing positive for *Campylobacter* by culture or quantitative PCR (qPCR). This suggests that as the bacteria multiply sufficiently to be detected by culture methods, one or two variants, as indicated by *porA* type, dominate the infection. The findings that (i) most young chicks carry some *Campylobacter* and (ii) not all flocks become *Campylobacter* positive by culture suggest that efforts to control infection, and therefore avoid contamination of poultry meat, should concentrate on how to limit *Campylobacter* to low levels by the prevention of the overgrowth of single strains.

**IMPORTANCE** Our results demonstrate the presence of *Campylobacter* DNA among fecal samples from a range of commercially reared meat chicks that are less than 8 days of age, consistent across 3 European countries. The recently developed, sensitive detection method indicates that infection occurs on commercial farms much earlier and more widely than previously thought, which opens up new opportunities to control *Campylobacter* contamination at the start of the food chain and reduce the unacceptably high levels of human disease.

**KEYWORDS** broiler chickens, *Campylobacter*

The consumption of raw or undercooked poultry meat contaminated with the bacterium *Campylobacter* is one of the major causes of gastroenteritis within human populations in high-income countries (1, 2). Current strategies for reducing infection in poultry have primarily focused on increasing on-farm biosecurity to prevent infection of chicken flocks during the growing period. These approaches have been largely ineffective as an intervention, and rates of infection have remained high with ~70% of European Union flocks being contaminated (2, 3). Detecting the presence of *Campylobacter* within a commercially reared broiler flock currently relies on bacteriological culture or quantitative PCR (qPCR), which generally identify *Campylobacter*-positive flocks at >2 weeks of age (4–6). These findings have led to the widespread assumption that chickens are

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*Campylobacter*-free before this age, but it is not known whether they are genuinely free from infection or whether they are infected below levels detectable using these methods. To address this question, we applied different methods for detecting *Campylobacter* in chickens: (i) microbiological culture or qPCR, (ii) 16S bacterial profiling analysis, and (iii) parallel sequencing of the *Campylobacter* *porA* gene (7). The culture methods used standard growth media, and the 16S bacterial profiling analysis was conducted with an average sequencing depth of 65,000 reads per sample. The *porA* gene was chosen because it encodes a surface-exposed major outer membrane protein (MOMP) in *Campylobacter* and has been successfully included in typing schemes to assess genetic diversity (8). Parallel sequencing of a fragment of the short variable region of the gene (*porAf2*) was used since this efficiently identifies multiple strains within samples (7). Based upon extensive use of the *porA* locus in this context, it is known that while a *porA* variant shows general association with a *Campylobacter* species, clonal complex, or even sequence type, there is evidence that *porA* variants may also be exchanged between *C. jejuni* and *C. coli*. Since it is not possible to reliably distinguish between *Campylobacter* species, at least for *C. jejuni* and *C. coli*, which cause the majority of human disease, using fragments of this gene, we therefore refer to “*Campylobacter*” in this study. The species-specific qPCR results from flocks tested in this study showed that *C. jejuni* was detected in all of the *Campylobacter*-positive flocks at their endpoint, and *C. coli* was additionally detected in three flocks, indicative of mixed infection. The 34 commercial broiler (meat) flocks sampled for this study were from the United Kingdom, France, and Switzerland. The flocks were chosen to represent a range of different housing and management conditions in order to determine whether or not *Campylobacter* DNA could be detected from young chicks in a variety of circumstances. The *Campylobacter* status of each of the flocks shortly before slaughter was determined by standard culture or qPCR—henceforth referred to as *Campylobacter* endpoint culture/qPCR positive or negative for clarity. With a range of collaborating partners, and adhering to commercial practices across different farms, companies and countries, there was some variation in sample time points, for example, with some flock types being slaughtered later than others. We limited variation as much as possible by using centrally assembled sample kits, by limiting DNA storage and extraction to two methods, consistent by country, and by replicating each sample time point and flock type. Although we did not identify any substantive differences in DNA storage and extraction method, we make only limited comparisons between the different countries. Our aim was instead to identify the presence or absence of *Campylobacter* among a diverse set of samples from young chicks.

## RESULTS

**Detection of *Campylobacter* DNA among chicks <8 days of age.** Detection of *Campylobacter* DNA, by both *porAf2* and bacterial 16S rRNA gene (region 3 to 4) targets were compared among 78 fecal samples from chicks <7 days of age, for a subset of 16 U.K. flocks (Table 1). *Campylobacter* DNA was detected in 58/78 (74.4%) of fecal samples tested by *porAf2* and from at least one bird in all of the flocks tested. Using the bacterial 16S rRNA gene sequencing approach, *Campylobacter* DNA was detected in 39/78 (50%) of fecal samples from at least one bird in 14/16 (87.5%) of the flocks tested.

The remainder of the flocks tested in this study were subject to *porAf2* parallel sequencing only. In total, *porAf2* variants were identified among 139/169 (82.2%) fecal samples tested from chicks <8 days of age and from at least one chick among all of the 34 flocks tested in the study (Table 1 and Fig. 1a and b). Variants of *porAf2* were detected among 9 of 10 samples from two flocks of 7-day-old chicks that were processed individually at the DNA extraction and PCR setup stages. Nontemplate controls for DNA extraction and PCRs were routinely negative. No *Campylobacter* DNA was detected among 60 samples tested from high-biosecurity specific-pathogen-free (SPF) chickens tested at 3 to 49 days of age.

**Comparison of *porAf2* type detected directly from fecal samples by parallel sequencing and cultured *Campylobacter* isolates.** *Campylobacter* isolates were cultured from 28 samples within 7 flocks, from birds aged 25 to 32 days, from U.K.

**TABLE 1** Presence/absence of *Campylobacter* DNA among fecal samples from U.K. broiler chicks <7 days of age, detected by parallel sequencing of the 16S rRNA gene or *porAf2* targets<sup>a</sup>

Company	Farm/flock	Campylobacter presence/absence in young chicks < 7 days tested by:										End-point flock Campylobacter status shortly before slaughter, tested by culture/qPCR*
		16S					porAf2					
		1	2	3	4	5	1	2	3	4	5	
A	Farm 1A** F1											Negative <sup>a</sup>
	Farm 1B** F2											Negative <sup>a</sup>
	F3											Negative <sup>a</sup>
	Farm 1C** F4											Negative <sup>a</sup>
	F5											Positive <sup>a</sup>
	F6											Positive <sup>a</sup>
	Farm 2 F7											Positive <sup>a</sup>
	F8											Positive <sup>a</sup>
B	Farm 3A** F9											Positive <sup>b</sup>
	F10					NT					NT	Positive <sup>b</sup>
	Farm 3B** F11											Positive <sup>b</sup>
	F12											Positive <sup>b</sup>
	Farm 4 F13											Positive <sup>b</sup>
	F14					NT					NT	Positive <sup>b</sup>
	Farm 5 F15											Positive <sup>b</sup>
	F16											Positive <sup>b</sup>

<sup>a</sup>Fresh fecal samples were collected from up to 5 birds selected at random at each time point. The 16S rRNA gene and *porAf2* targets were amplified from DNA extracted directly from individual samples by PCR, and the number of variants was detected by parallel sequencing. 16S rRNA gene analysis at high depth was used to look for the presence of *Campylobacter* among the total bacterial profile, while amplification of the *porAf2* target was used to enrich for *Campylobacter*-specific DNA relative to the other microbiota.

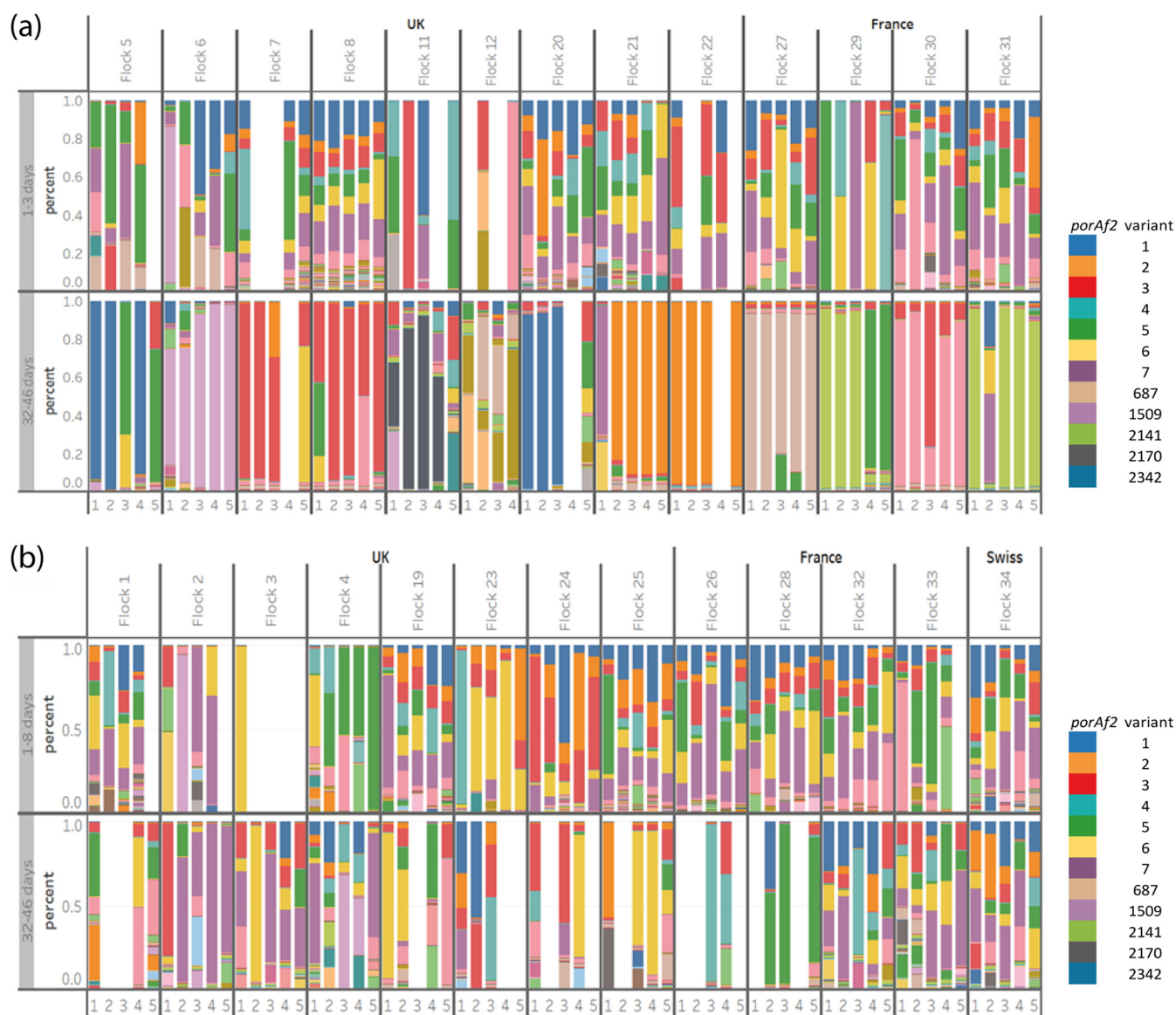
\*Flocks were tested for *Campylobacter* by conventional culture (<sup>a</sup>) or qPCR (<sup>b</sup>), using samples collected from the second sample date when birds were aged 28 to 35 days.

\*\*These flocks were present on farms 1 and 3 in separate rotations and distanced by time.

company A. The *porAf2* type of a single colony pick per bird/sample was compared with the *porAf2* variants detected by parallel sequencing from the same samples. The *Campylobacter porAf2* type detected by conventional culture matched those that were predominant in the same sample and detected by parallel sequencing in 18/28 (64.3%) of samples tested (Fig. 2). For nine samples, the predominant *porAf2* type detected by the parallel sequencing method was not the same as the *porAf2* type detected by culture using a single colony pick. Almost all (27/28) of the cultured *porAf2* types were detected in the same sample by parallel sequencing, at a percentage of the total *Campylobacter porAf2* types recovered, ranging from 0.01% to 46.99%. For example, *porAf2* type 2866 was identified among *Campylobacter* isolates cultured from four of five samples from flock 20 (Fig. 2), but it was present at ~0.1% of *porAf2* variants detected from the fecal samples by direct parallel sequencing (data not shown). The *porAf2* variant 2866 was rarely identified among samples from the wider study, being identified on only four other occasions among single samples from four different flocks at <0.01% of the variants. The only exception where a cultured *porAf2* variant was not detected by parallel sequencing from the same sample was also *porAf2* type 2866 (Fig. 2), from sample 5/flock 20.

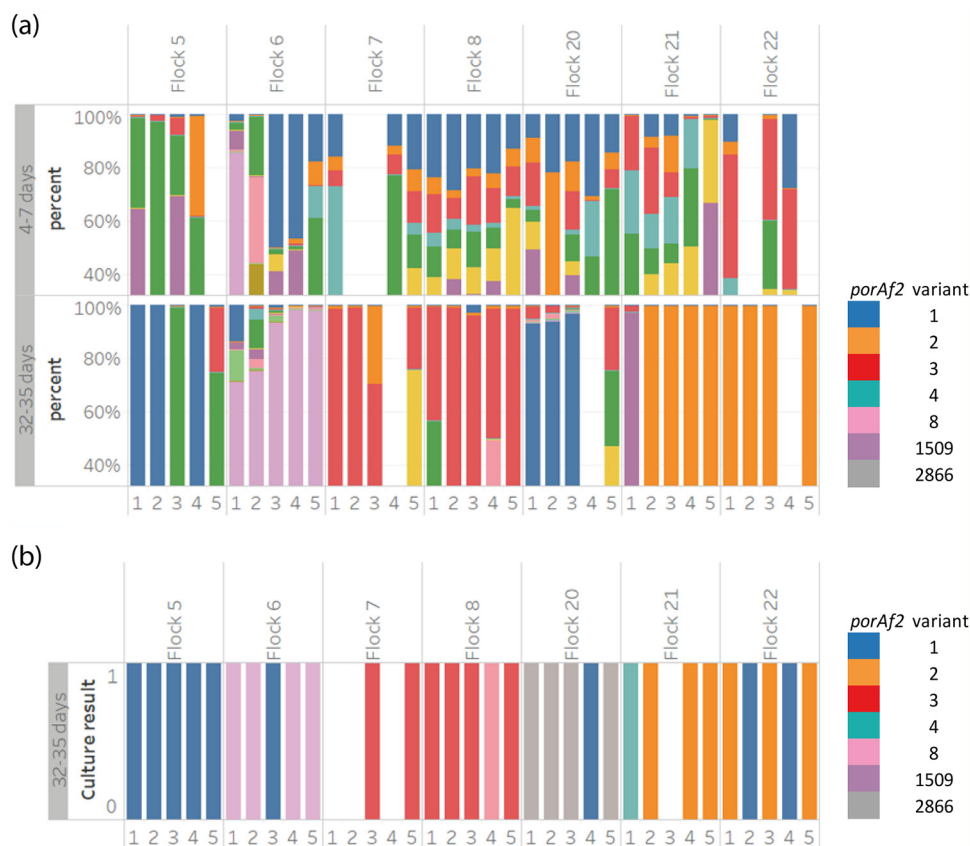
**Diversity of *porAf2* variants recovered from the study.** A total of 1,098 *porAf2* types (1,066 in samples from 25 U.K. flocks; 353 from 8 French flocks; 560 from 1 Swiss flock), corresponding to 746 peptide sequences, were identified among the flocks tested in the study (see Fig. S2 in the supplemental material). The *porAf2* variants were manually checked on a sequencing alignment, with approximately 80% sequence similarity between the most disparate and up to 100% similarity to those identified among cultured *Campylobacter* isolates. Point mutations and indels were spread along the length of the short variable region fragment that was sequenced.

The number of different *porAf2* types recovered from an individual fecal sample ranged from 9 to 358. Rarefaction curves demonstrated that the sequencing depth



**FIG 1** The prevalence of *Campylobacter porAf2* types among fecal samples collected from 34 commercially reared broiler flocks from the United Kingdom, France, and Switzerland (transformed data). (a and b) Results are shown for flocks that tested (a) *Campylobacter* positive and (b) *Campylobacter* negative at 32 to 46 days by culture/qPCR. Flocks that were tested at 28 to 31 days of age are not shown, together with flock 14, which had low sequencing depth. Each color represents a different *porAf2* type; common variants are given in the legend.

was sufficient to recover most of the flock diversity from samples from young chick <8 days of age, with a maximum of approximately 450 *porAf2* variants recovered from both endpoint *Campylobacter* culture/qPCR negative and positive flocks (Fig. 3a). For birds >28 days of age, 100 to 300 *porAf2* variants accounted for most of the flock diversity among endpoint *Campylobacter* culture/qPCR positive flocks, and approximately 200 *porAf2* variants accounted for most of the diversity among endpoint culture/qPCR-negative flocks (Fig. 3b). There were four endpoint culture/qPCR positive U.K. flocks from company B where upwards of 350 *porAf2* variants did not appear to capture the full diversity. Similarly, there were six more flocks that were endpoint culture/qPCR negative where either recovery of the *porAf2* variants was relatively low, below  $n = 250$ , or in the case of one French and one Swiss flock, upward of 350 *porAf2* variants did not appear to capture the full diversity in these samples. No systematic difference in *porAf2* population structure related to DNA extraction method was noted (Fig. S3).

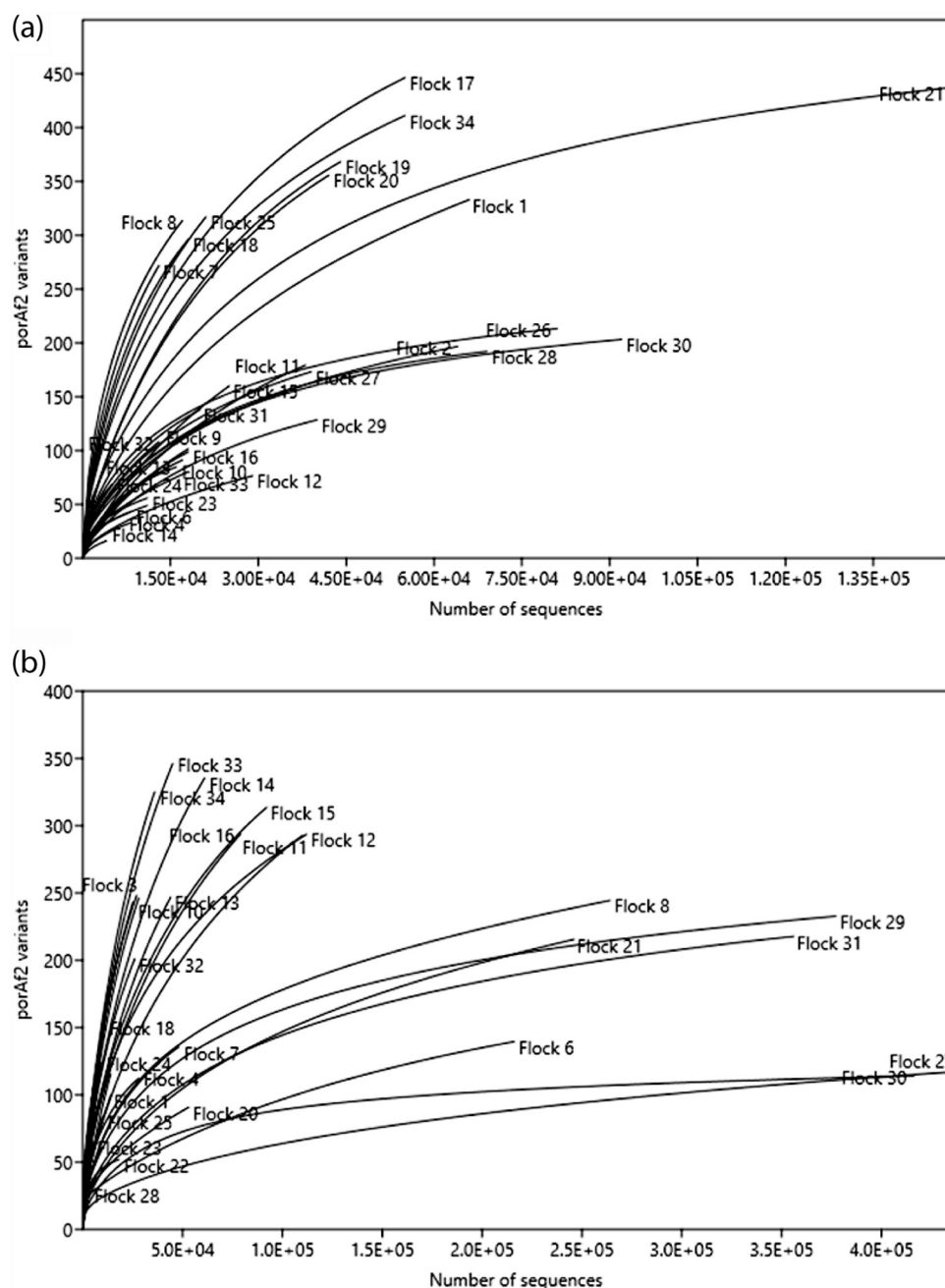


**FIG 2** (a and b) Comparison of *Campylobacter* *porAf2* types identified among samples using (a) parallel sequencing and (b) conventional culture among a subset of flocks with a single colony pick per bird/sample. Each color represents a different *porAf2* type; common variants are given in the legend.

**Comparison of *porAf2* population structure between endpoint *Campylobacter* culture/qPCR negative and positive flocks.** There was no significant difference in the average number of *porAf2* types recovered from young broiler flocks that tested *Campylobacter* endpoint culture/qPCR negative at 28 to 46 days, compared to flocks that tested *Campylobacter* endpoint culture/qPCR positive by standard methods (transformed data,  $n = 33$  flocks, Mann-Whitney U test,  $P = 0.6713$ ) or among flocks tested at early and later time points (transformed data, Wilcoxon rank-sum exact test,  $n = 33$  flocks,  $P = 0.618$ ). For *porAf2* types that became predominant within flocks, 76.1% (16/21) were identified among younger birds from the same flock, and often (11/21, 52.4%) at a higher proportion of the total *porAf2* sequenced compared to young birds from other flocks (Wilcoxon rank-sum exact test,  $n = 13$  flocks,  $P = 0.001$  within countries and  $P = 0.002$  between countries (Table 2).

Simpson's and Shannon's diversity indices were used to compare the patterns of diversity within *porAf2* of flocks sampled at earlier and later time points (Fig. 4). For *Campylobacter* endpoint culture/qPCR positive flocks, greater diversity was evident for samples from young birds, <8 days of age, compared to birds tested at 35 to 46 days of age from the same flock ( $n = 13$  flocks, Simpson's diversity  $P < 0.001$ , Shannon's H diversity  $P = 0.002$ ), but this was not true for endpoint culture/qPCR negative flocks ( $n = 13$  flocks, Simpson's diversity  $P = 0.787$ , Shannon's H diversity  $P = 0.68$ ). Aitchison distances, calculated between pairs of samples, demonstrated that there was no apparent relationship between *porAf2* type population and whether or not the flock became *Campylobacter* endpoint culture/qPCR positive (Fig. 5a). The pattern of *porAf2* sequences was, however, clearly correlated with age at time of sampling (Fig. 5b) and flock (Fig. 5c).





**FIG 3** Rarefaction curves for *Campylobacter porAf2* types isolated from commercial broiler flocks from the United Kingdom, France, and Switzerland. Results represent pooled sequence counts for up to 5 birds/flock and are grouped by bird age. (a) Young birds, 1 to 8 days; (b) older birds, 28 to 46 days.

**Basic comparison of *porAf2* variants recovered from countries.** Of the 1,098 *porAf2* types identified from the study as a whole, 439 (40.0%) were unique to U.K. flocks and 32 (2.9%) were unique to French flocks. None of the 1,098 *porAf2* types were unique to the Swiss flock, with all types identified among U.K. flocks and 254 types found in French flocks. Of the 1,098 7 (0.64%) *porAf2* types (*porAf2* types 1 to 7) were identified in all 34 flocks, accounting for 49.36% (2,315,725/4,691,464) of the total sequences recovered across the whole study.

**Proportion of *Campylobacter* detected among the bacterial 16S profile.** *Campylobacter* 16S rRNA gene variants were recovered in samples from birds less than 8 days of age between 0 and 6 times (average 0.95), with an average sequencing depth

**TABLE 2** The predominant *porAf2* types identified within a flock among birds aged 28 to 46 days, compared to their prevalence among birds 1 to 8 days of age<sup>a</sup>

		Average prevalence in young birds (1–7 days):		
Flock	Predominant <i>porAf2</i> type(s) <sup>b</sup> (% of birds)	Same flock (% of birds)	Different flock: U.K. (% of birds)	Different flock: France (% of birds)
U.K. flocks				
Flock 5	1 (75)	0.122 (100)	0.124 (100)	0.376 (100)
	5 (50)	7.606 (100)	0.109 (96)	0.351 (100)
Flock 6	1509 (100)	6.877 (100)	<0.001 (75)	Not detected
Flock 7	3 (100)	1.532 (100)	0.112 (100)	0.245 (100)
	6 (33)	1.500 (100)	0.119 (100)	0.328 (100)
Flock 8	3 (100)	2.209 (100)	0.111 (100)	0.245 (100)
Flock 11	2170 (75)	Not detected	<0.001 (8)	<0.001 (14)
Flock 12	2366 (33)	Not detected	<0.001 (8)	<0.001 (14)
	687 (33)	<0.001 (33)	0.003 (67)	0.001 (86)
	2328 (33)	Not detected	<0.001 (33)	<0.001 (72)
Flock 20	2342 (60)	Not detected	<0.001 (13)	<0.001 (14)
Flock 21	7 (20)	3.59 (100)	0.190 (100)	0.581 (100)
	2 (80)	1.407 (100)	0.040 (96)	0.638 (86)
Flock 22	2 (80)	1.471 (100)	0.055 (96)	0.638 (86)
French flocks				
Flock 27	687 (100)	0.007 (100)	0.003 (67)	0.001 (86)
Flock 29	2141 (60)	0.043 (80)	0.001 (38)	0.001 (86)
		4.031 (80)	0.107 (96)	0.34 (100)
Flock 30	8 (80)	6.64 (100)	0.048 (92)	0.127 (100)
	3 (20)	1.479 (100)	0.108 (100)	0.228 (100)
Flock 31	2141 (80)	Not detected	0.001 (38)	0.002 (86)
	7 (20)	7.105 (100)	0.218 (100)	0.608 (100)

<sup>a</sup>The prevalence of a *porAf2* type was compared between young birds from the same flock and young birds from other flocks, based upon total sequence data recovered per sample.

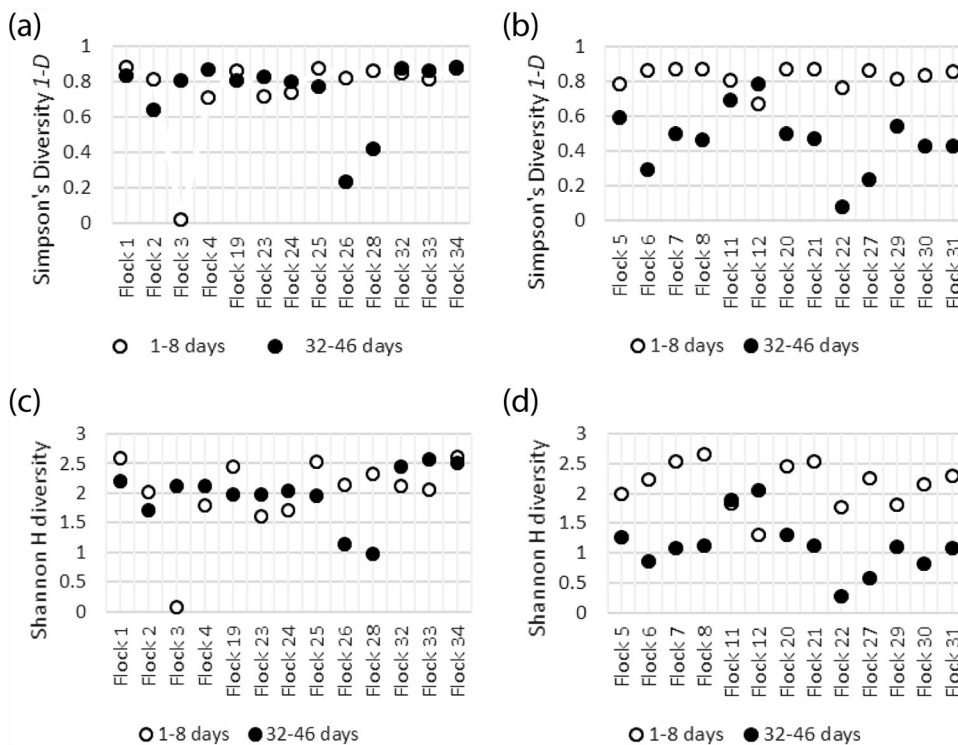
<sup>b</sup>Predominant *porAf2* types were defined as being greater than 20% or, most often, greater than 75% of the total sequences recovered from a sample. The percentage of birds within a flock from which the specific *porAf2* type was identified is shown in parentheses.

of 65,000 reads per sample (Fig. S1). *Campylobacter* 16S rRNA gene variants were recovered in samples from birds 28 to 35 days of age between 0 and 543 times (average, 12.1), with an average sequencing depth of 49,000 reads per sample.

The proportion of *Campylobacter* detected among the bacterial 16S profile of young commercial bird samples was extremely small, ranging from 0.0001 to 0.012% of the total 16S rRNA gene reads (Table 3), and was similar, regardless of whether or not the flocks subsequently tested *Campylobacter* culture or qPCR positive at 28 to 35 days of age. However, a small subset of samples (5/151 samples; 3.3%) from 35-day-old chickens across three flocks that tested *Campylobacter* endpoint culture/qPCR positive at 35 days had greater than 0.1% *Campylobacter* abundance. This gave an average proportion of *Campylobacter* sequences among the bacterial 16S profile that was >100-fold larger, at 0.1377% of the sequencing reads ( $\pm 0.490\%$  standard deviation) (Wilcoxon rank-sum exact test,  $n = 6$  flocks,  $P = 0.03$ ). With flocks that never became endpoint culture/qPCR positive, the proportion of *Campylobacter* sequences/total 16S rRNA gene reads did not differ significantly between young and older birds from the same flock (Wilcoxon rank-sum exact test,  $n = 4$  flocks,  $P = 0.375$ ).

## DISCUSSION

The *porAf2* and 16S rRNA gene sequencing approaches show that *Campylobacter* DNA is widely present in commercial broiler flocks in birds as young as 1 to 8 days of age, long before it is possible to detect *Campylobacter* using conventional culture or qPCR methods. The very low abundance (0.0001 to 0.012%) of the bacterial 16S profile attributable to *Campylobacter* provided a possible explanation for why these chicks are

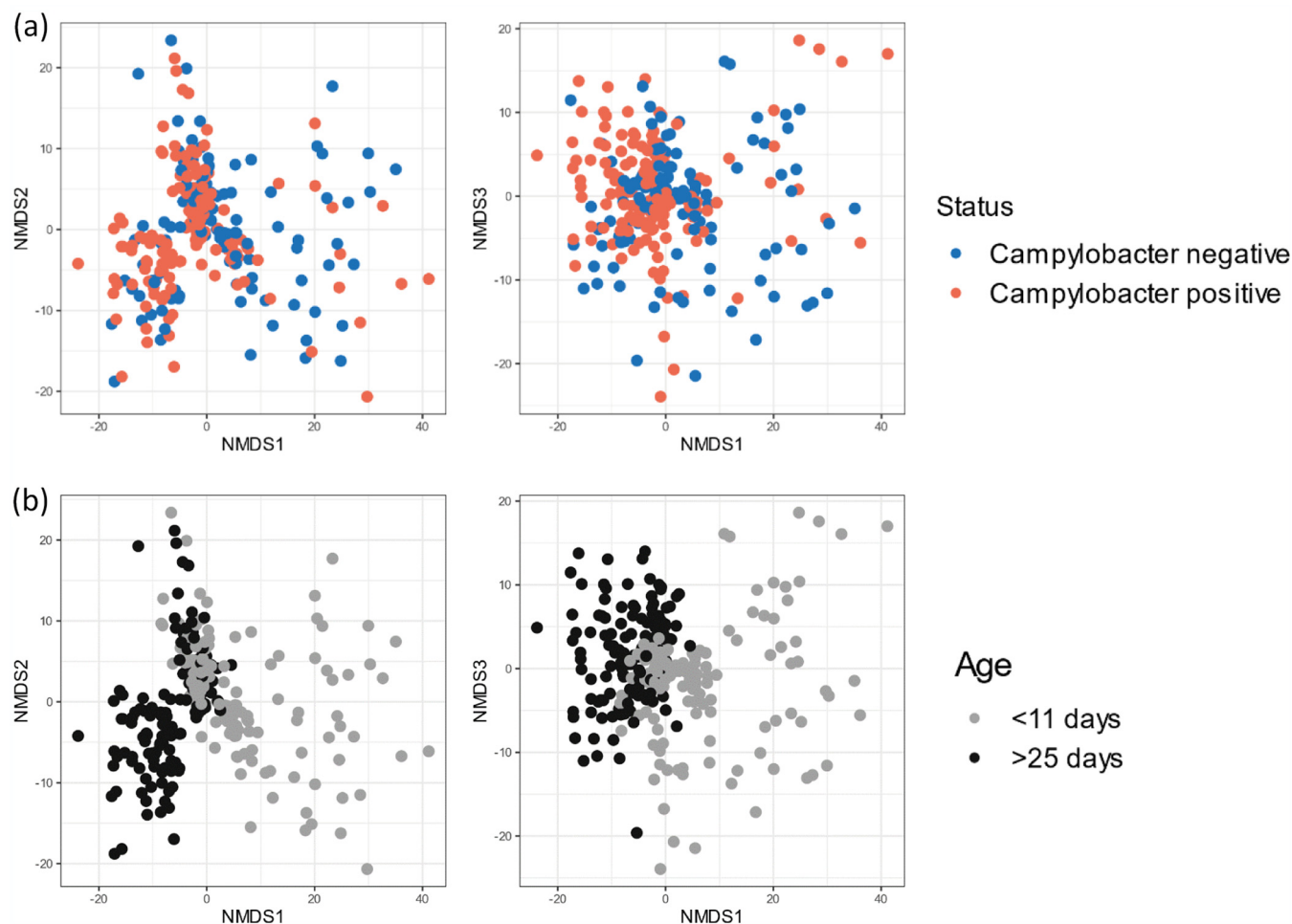


**FIG 4** (a to d) Diversity of *Campylobacter porAf2* populations in *Campylobacter* endpoint culture/qPCR-negative flocks (a and c) and *Campylobacter* endpoint culture/qPCR-positive flocks (b and d) using interpolated and extrapolated data. Inverse Simpson's diversity 1-D (a and b) and Shannon H diversity (c and d).

negative using these methods. With a developing microbiome, the already small proportion of *Campylobacter* may be vanishingly small in terms of biomass and thus below the threshold of detection by culture/qPCR, but not by the more sensitive parallel sequencing approach. That *Campylobacter* can be detected at an early age, however, is consistent with results from studies of cultured *Campylobacter* from 0.75% of 2,000 hatchery tray liners, representing pooled samples of 50 day-of-hatch chicks per tray liner (9). Other molecular studies have identified amplifiable *Campylobacter* DNA among hatchery samples and the reproductive tract of broiler breeders (10, 11). Vertical transfer of *Campylobacter* from parent flocks by direct or indirect routes provides a possible explanation for such early infection, but this idea remains controversial (6, 12–15).

Variants of *porAf2* were identified from at least one fecal sample tested from young chicks <8 days old from each of the commercially reared flocks, irrespective of management regime or DNA extraction method and storage, but not from chickens reared under SPF conditions. A second sequencing-based method of detection using the 16S target also identified *Campylobacter* DNA among samples from young chicks, from all except two of the commercial flocks tested. Specific amplification of *Campylobacter* DNA using the *porAf2* fragment giving added sensitivity of detection, compared to the broad 16S bacterial profile in which *Campylobacter* sequences were often rare, may explain the variation in results. Although 16S sequencing analysis pipelines typically include filters to remove very rare sequences, this was not appropriate in this case, where the purpose of the experiment was to detect the presence of predetermined rare sequences (i.e., *Campylobacter*). Instead, we treat these reads as informative because no samples had a high enough level of *Campylobacter* 16S sequence to plausibly suggest them as a significant source of cross-sample contamination, either in the laboratory or in data processing. It cannot be certain why so many *porAf2* variants were detected by parallel sequencing among samples from older birds that tested negative by culture or qPCR. The correlation





**FIG 5** Ordination plots showing Aitchison distances between pairs of samples using nonmetric multidimensional scaling (NMDS). (a to c) The effects of (a) *Campylobacter* culture/qPCR endpoint status, (b) flock age at time of sampling, and (c) individual flock are shown.

between low or high *porAf2* variant diversity and culture/qPCR positive or negative result was consistent, however, with the same *porAf2* variants predominant among individuals within a flock but different from those predominant in other flocks. Our results suggest that predominance of a single *porAf2* type is linked with a higher proportional representation of *Campylobacter* among the microbiome in at least some birds from a culture/qPCR positive flock, potentially making it more easily detectable. *Campylobacter* has long proven to be difficult to culture in the laboratory and has been recognized to persist in a viable nonculturable state, with cells retaining an ability to resuscitate under favorable conditions (16). Even when *Campylobacter* can be cultured, there is evidence of different species or variants being preferentially selected, which could also be a contributing factor (7, 17).

While unable to determine the viability of all *Campylobacter porAf2* variants, culture of isolates from endpoint samples for a subset of flocks demonstrated the viability of some at this stage. Each of the *porA* variants were manually checked by alignment with those derived by culture, and no other species were identified by nucleotide BLAST search using the NCBI database. Although it is possible that the diversity of *porAf2* types may be overestimated if there is cross-reaction among other nonrelated species, or by cross-contamination during the sequencing process and technical pipeline, we have found no evidence of this (18).

Sensitive molecular techniques able to detect both low levels of *Campylobacter* and distinguish among species and strains will be essential in tracing the epidemiology of

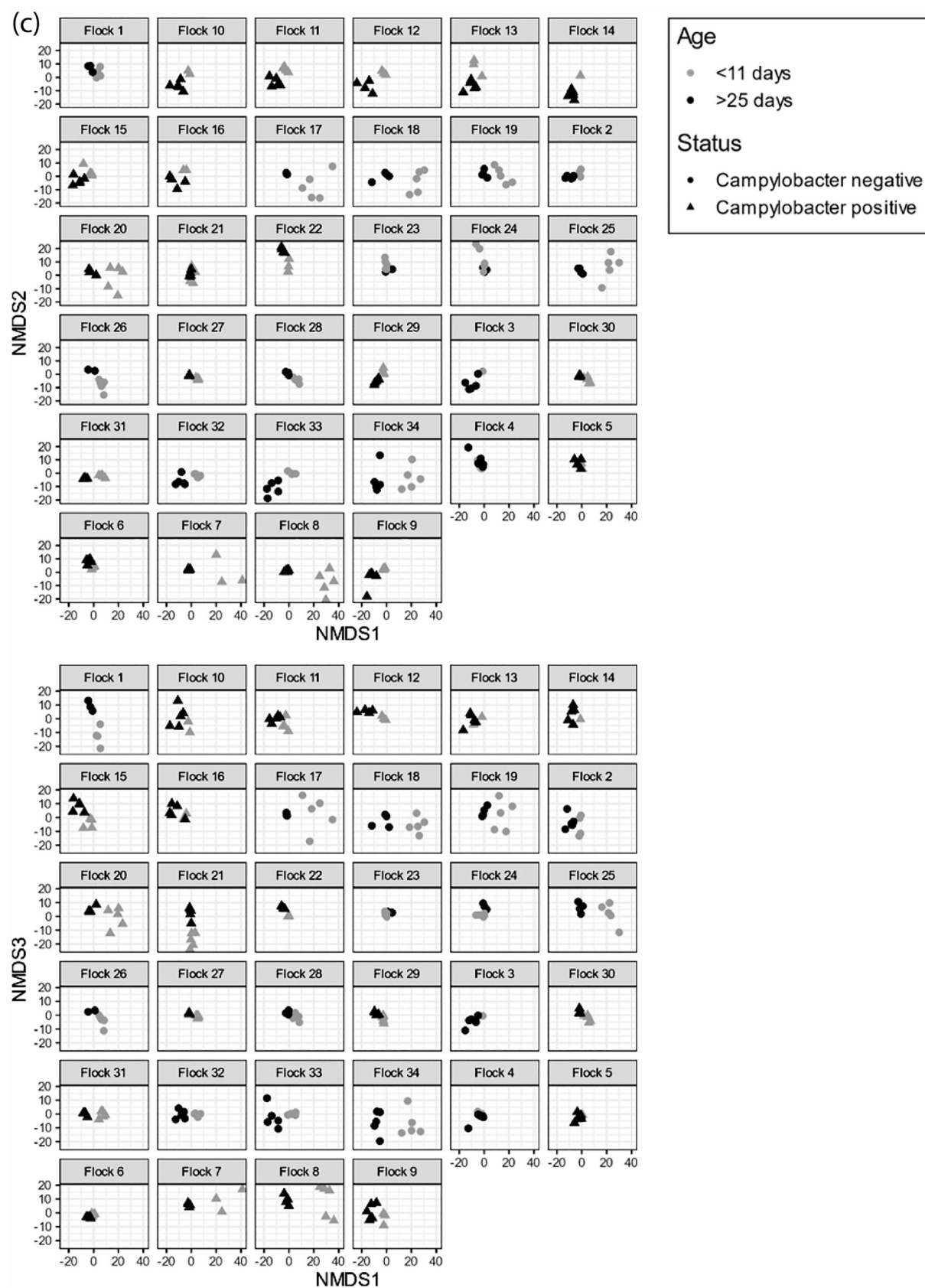


FIG 5 (Continued)

**TABLE 3** Detection of *Campylobacter* DNA<sup>a</sup>

Flock	<i>Campylobacter</i> negative		<i>Campylobacter</i> positive		
	6–7 days (SD)	34–35 days (SD)	6–7 days (SD)	28 days (SD)	34–35 days (SD)
Company A					
Flock 1	0.0022 (0.002)	0.0012 (0.002)			
Flock 2	0.0017 (0.004)	0.0000 (0.000)			
Flock 3	0.0003 (0.001)	0.0016 (0.002)			
Flock 4	0.0081 (0.003)	0.0057 (0.004)			
Flock 5			0.0120 (0.006)		0.6856 (1.305)
Flock 6			0.0099 (0.010)		0.1563 (0.162)
Flock 7			0.0005 (0.001)		0.0012 (0.002)
Flock 8			0.0007 (0.001)		0.0185 (0.028)
Company B					
Flock 9			0.0005 (0.001)	0.0008 (0.001)	
Flock 10			0.0001 (0.000)	0.0002 (0.000)	
Flock 11			0.0014 (0.001)		0.0060 (0.008)
Flock 12			0.0018 (0.001)		0.0507 (0.062)
Flock 13			0.0003 (0.002)	0.0011 (0.001)	
Flock 14			0.0000 (0.000)	0.0008 (0.002)	
Flock 15			0.0012 (0.002)	0.0063 (0.012)	
Flock 16			0.0000 (0.000)	0.0146 (0.021)	
Overall mean	0.0031	0.0021	0.0024	0.0036	0.1377

<sup>a</sup>Detection is defined as the percentage of the total number of 16S rRNA bacterial profile sequences recovered from fecal samples, with results pooled for each of the flocks. The figures shown are the average of samples from 4 to 5 birds, with sample standard deviation from the mean shown in parentheses. Results are shown for a subset of 16 U.K. flocks from 2 companies that were tested at early and late time points during rearing. The results are separated by flocks that were endpoint culture/qPCR negative and positive for *Campylobacter* at 28 to 35 days.

the organism, and techniques will no doubt be refined as the technology continues to develop (12).

A wide range of genotypes were present in all sample types, with rarefaction curves indicating that up to 450 *porAf2* variants may be identified within pooled samples from a flock, irrespective of the endpoint culture/qPCR *Campylobacter* culture status of the flock. Many of the *porAf2* variants were present at very low frequencies (less than 1% of the total). This level of resolution is unachievable by standard culture approaches. In addition, culture induces potential bias where *Campylobacter porAf2* variants differ in their capacity for growth under standard laboratory conditions (5). This was evidenced by the recovery of *porAf2* type 2866 from flock 20 by culture, where *porAf2* type 1 was predominant by parallel sequencing direct from the fecal sample. There was ~80% sequence homology between the most disparate *porAf2* variant nucleotide sequences, which matches the sequence homology when comparing housekeeping loci between *C. jejuni* and *C. coli* species (19). The large number of *porAf2* variants may reflect diversity derived from host immune selection, which is presumed to act on the outer membrane loop of the MOMP protein encoded by the *porA* locus (8). In this study, a subset of *porAf2* variants (types 1 to 7) were common among all of the flocks tested. It is known that particular *Campylobacter* lineages are adapted to chickens and are widespread across the globe (20). However, while different *porAf2* variants identify different *Campylobacter* genotypes, typing methods using more loci would give greater resolution to those *Campylobacter* strains with matching *porAf2* variants. The proportion of each *porAf2* variant among samples with saturated levels of detection were more evenly distributed in young birds, with *Campylobacter* DNA representing a very small amount of the total bacterial population. This contrasted markedly with older flocks that were *Campylobacter* endpoint culture/qPCR positive from 28 days of age, where there were one or two dominant genotypes (7). On average, there was an order of magnitude more *Campylobacter* 16S OTUs detectable among

samples from endpoint culture/qPCR positive flocks, though this was dominated by a small number of birds within three flocks with up to 2.5% *Campylobacter* detectable. These birds may represent “super shedders,” or individuals that are more susceptible to infection within a flock, and may also be the reason that one or two *porAf2* types become dominant among older birds within culture-positive broiler flocks (21). In 76% of cases, the *porAf2* variant that became dominant among older birds was detected among samples from young chicks <8 days old from the same flock, despite only 5 samples being tested per flock at each time point.

The *porAf2* analysis of the young bird samples showed no difference in either the abundance of *Campylobacter* present or in the range of genotypes between flocks that subsequently (i.e., from 28 days) became *Campylobacter* endpoint culture/qPCR positive and those that remained endpoint culture/qPCR negative. This suggests that all young flocks may have similar potential for becoming *Campylobacter* positive but that only some flocks experience the predominance of a limited number of *Campylobacter* variants that causes them to become culture or qPCR positive at slaughter. The observation that *Campylobacter* populations undergo a consistent change in composition with age (as revealed by *porAf2* type structure) supports the view that these populations are subject to *in vivo* selective pressures. These selective pressures may relate to age-associated changes in gut physiology, immune processes, competition with other gut microbes, stress, or other factors.

The key implication of these observations is that they suggest the need for development in the focus of control methods in commercial poultry meat production. This change would expand the focus of interventions from biosecurity during the growing phase to prevention or mitigation of (i) colonization in the very youngest birds and (ii) *Campylobacter* outgrowth in growing birds. Defining the details of these two processes will be instrumental to reducing *Campylobacter* colonization or, more importantly, carriage at a high level, which represents the greater risk of transfer to humans. First, it is important to determine how so many chicken flocks become contaminated with *Campylobacter* at this young age. Second, given the widespread presence of *Campylobacter* in young flocks, it is important to understand why some maintain low levels of *Campylobacter* throughout their lives and others exhibit the overgrowth of single types that result in *Campylobacter* culture-positive flocks. In addition to the on-farm biosecurity measures that have formed the poultry industry's main line of defense against contamination, it may also be necessary to consider upstream vertical sources of colonization in more detail, as well as the welfare and management of the birds themselves, as key to controlling absolute levels of *Campylobacter* contamination in foods.

## MATERIALS AND METHODS

**Fecal sample collection.** Fresh fecal samples were collected from 2015 to 2018 from 34 commercially grown broiler chicken flocks, representing 15 farms, 5 companies, and 3 countries (Table 4). Samples from company A (United Kingdom) were cultured for *Campylobacter* within 48 h of collection and then stored at  $-80^{\circ}\text{C}$ . Samples from company B (United Kingdom) were frozen at  $-80^{\circ}\text{C}$  within a day of collection and later tested by qPCR. Samples from companies C, D, and E (France and Switzerland) were immediately added to RNAlater (Thermo Fisher Scientific, UK) and stored at  $-20^{\circ}\text{C}$  before long-term storage at  $-80^{\circ}\text{C}$ . Fecal samples collected from 10 SPF chickens at 3, 7, 14, 21, 35, and 49 days of age and stored at  $-80^{\circ}\text{C}$  were employed as negative controls.

**Detection of *Campylobacter* using culture or qPCR.** Samples from company A were cultured on charcoal cefoperazone deoxycholate agar (CCDA) (PO0119; Oxoid, UK) and incubated in a microaerobic atmosphere using the GenBox Microaer system (bioMérieux Ltd., UK) at  $42^{\circ}\text{C}$  for 48 h. Preliminary work for this study, as well as that previously published, has shown that *Campylobacter* culture-positive fecal samples from our flocks are typically dominated by a single *porAf2* variant, and hundreds of colonies would need to be subcultured per sample before rarer *porAf2* variants could be identified (7). Given the volume of samples, a pragmatic approach was therefore taken, with a single presumptive *Campylobacter* colony from each sample subcultured onto Columbia blood agar (CBA) (PB0122; Oxoid, UK) and incubated for another 48 h at  $42^{\circ}\text{C}$ . Identity was confirmed by characteristic appearance and Gram stain, by catalase and oxidase tests, and by DNA sequencing of the *porAf1* fragment (previously referred to as the *porA* fragment) using the Sanger sequencing method (7).

Fecal samples from companies B, C, D, and E were tested for the presence of *Campylobacter* using qPCR. Between 10 and 20 samples were pooled for each flock at each time point, and the DNA was extracted using the protocols described below. Primers and probes detecting portions of the *mapA*

**TABLE 4** Details of the commercial broiler flocks from which fecal samples were tested<sup>a</sup>

Sample source	Flock	Year	1st age (days)	2nd age (days)	<i>Campylobacter</i> status <sup>b</sup> (culture/qPCR)
U.K.					
Company A					
Farm 1A <sup>c</sup>	Flock 1	2018	7	35	Negative
	Flock 25	2018	7	35	Negative
Farm 1B <sup>c</sup>	Flock 2	2018	7	35	Negative
	Flock 3	2018	7	35	Negative
Farm 1C <sup>c</sup>	Flock 4	2017	6	35	Negative
	Flock 5	2017	6	35	Positive
	Flock 6	2017	6	35	Positive
Farm 2	Flock 7	2016	7	35	Positive
	Flock 8	2016	7	35	Positive
Farm 6	Flock 17	2016	3	31	Negative
	Flock 18	2016	3	31	Negative
Farm 7	Flock 19	2017	4	32	Negative
	Flock 20	2017	4	32	Positive
Farm 8	Flock 21	2017	7	35	Positive
	Flock 22	2017	7	35	Positive
Farm 9	Flock 23	2017	7	35	Negative
	Flock 24	2017	7	35	Negative
Company B					
Farm 3A	Flock 9	2015	7	28	Positive
	Flock 10	2015	7	28	Positive
Farm 3B	Flock 11	2015	7	35	Positive
	Flock 12	2015	7	35	Positive
Farm 4	Flock 13	2015	7	28	Positive
	Flock 14	2015	7	28	Positive
Farm 5	Flock 15	2015	7	28	Positive
	Flock 16	2015	7	28	Positive
France					
Company C					
Farm 10	Flock 26	2017	1	42	Negative
	Flock 27	2017	1	46	Positive
Farm 11	Flock 28	2016/7	1	43	Negative
	Flock 29	2017	3	42	Positive
	Flock 30	2017	1	42	Positive
Farm 12	Flock 31	2017	3	46	Positive
Company D					
Farm 13	Flock 32	2016	3	32	Negative
Farm 14	Flock 33	2016/7	3	32	Negative
Switzerland					
Company E					
Farm 15	Flock 34	2016	8	36	Negative

<sup>a</sup>A total of 34 broiler flocks were tested from 3 countries and 5 companies, sampled from 2015 to 2018. Up to 5 fresh fecal samples were tested from different birds at early (<8 days of age) and late (28 to 46 days of age) time points for each flock.

<sup>b</sup>Flocks were tested for *Campylobacter* by conventional culture or qPCR, using samples collected from the second sample date when birds were aged 28 to 46 days.

<sup>c</sup>Flocks from farms 1 and 3 were placed in rotation and separated by time.

(*C. jejuni*) and *ceuE* (*C. coli*) genes, published by Best et al. (22), were used to detect the presence of *C. jejuni* and/or *C. coli*. Positive results were recorded for threshold cycle ( $C_T$ ) values between 20 and 32.

**DNA extraction from fecal samples.** Equipment and surfaces were decontaminated using 2% Virkon and DNAZap PCR DNA degradation solutions (AM9890; Thermo Fisher Scientific, UK) and 70% ethanol before use and between batches. DNA was extracted from fecal samples not stored in RNAlater using the Maxwell 16 LEV blood DNA kit (AS1290; Promega, UK) and automated DNA extraction system. Briefly, 250 mg of sample was added to a tube containing one-quarter volume 0.5-mm zirconia/silica beads (Thistle Scientific, UK) and 600  $\mu$ l lysis buffer. Samples were subjected to a bead beater for 1 min at high speed and then heated at 95°C for 10 min in a water bath. They were centrifuged in a microcentrifuge at 13,200 rpm for 5 min before the supernatant was transferred to fresh tubes containing proteinase K and incubated at 56°C for 20 min. The samples were then loaded onto the Maxwell 16 LEV blood kit cartridges, and DNA extraction was performed using the manufacturer's "blood" protocol.



Fecal samples stored in RNAlater were centrifuged at 13,200 rpm for 10 min, and the supernatant was removed prior to DNA extraction procedures in order to concentrate and retrieve the original sample material. They were then washed in excess cold phosphate-buffered saline (PBS) and centrifuged for another 10 min at 13,200 rpm, and the supernatant was discarded. DNA was then extracted using the Qiagen DNeasy PowerSoil kit (no. 12888-100; Qiagen, UK) following the manufacturer's instructions. DNA prepared using the Maxwell system was diluted 1:10 before use to remove the effects of PCR inhibitors but was used neat for Qiagen extractions.

**Parallel sequencing for 16S (bacterial profiling) and *porA2* (*Campylobacter*).** Using a standard approach, 25- $\mu$ l PCRs were prepared in triplicate in a PCR UV cabinet in a designated clean room. Equipment was decontaminated before use and between batches using DNAzap (AM98902; Thermo Fisher Scientific, UK), 70% ethanol, and UV light for a minimum of 15 min. Fresh plastic ware and reagents/aliquots and nontemplate controls containing molecular water were used for every set of PCRs.

For a subset of samples, variable regions 3 and 4 of the 16S rRNA gene were sequenced using the following primers: E338 5'-ACTCTACGGGAGGAGCAGT-3' and R806 5'-GGACTACHVGGGTWTCTAAT-3'. For all samples, a short 405- to 473-bp region of the *porA* gene was sequenced using the following primers: MOMP B 5'-CCA CAA TTA TGG TTA GCT TA-3' and MOMP 2R 5'-TGA GAA GTT AAG TTT TGG AGA G-3' (7). The E338 forward primer was tagged with 10 different nucleotide barcodes for each reaction, and the MOMP2R reverse primer was tagged with different 7 nucleotide barcodes for each reaction, enabling reactions to be multiplexed within the same sequencing library. For both sets of PCR, the high-fidelity Phusion Hot Start Flex DNA polymerase enzyme and 5X Phusion HF buffer (New England Biolabs, UK; M0535) were used according to the manufacturer's recommendations. The following thermocycling conditions were used: (i) for 16S, initial denaturation at 98°C for 5 min, 25 cycles of 98°C for 15 s, 50°C for 15 s, 72°C for 90 s, and final extension of 72°C for 10 min; (ii) for *porA2*, initial denaturation at 98°C for 30 s, 35 cycles of 98°C for 10 s, 58°C for 30 s, 72°C for 30 s, and final extension of 72°C for 10 min. Library preparation was performed in a third room, separate from the DNA extraction and PCR setup, using protocols described previously (7). PCR products were sequenced using the Illumina MiSeq platform and 600-cycle MiSeq reagent kit v3 (Illumina, UK; MS-102-3003), giving 300 paired nucleotide reads. Samples were loaded with 10% phiX.

**Quality control.** The processes of DNA extraction, PCR set-up, and sequencing library preparation were performed in separate rooms. All surfaces, including equipment, were decontaminated with DNAzap (Invitrogen, UK) between batches of samples. Samples from young chicks containing small amounts of DNA were handled before samples from older chicks, apart from a pilot study where DNA was extracted and sequenced from both sample types in a mixed arrangement. PCR was performed in a dedicated PCR room, within separate PCR hoods, reserved for master mix preparation and addition of sample DNA. The sequencing reactions were performed across 10 nonsuccessive MiSeq runs and were set up by three members of staff.

A subset of 10 samples, from flocks 1 and 21, aged 7 days, were prepared individually through DNA extraction and PCR processes to control for potential cross-contamination between samples prepared in the same batch. At each stage, only one of the samples was handled in a room at a time, and the process of DNA extraction was performed, or PCR was completed for a particular sample, before the next sample was started. DNA extraction was performed for three samples from flock 1 using a Maxwell 16 robot (a single cartridge used at a time). DNA was extracted for two samples from flock 1 and five samples from flock 21 using the Qiagen DNeasy PowerSoil kit in enclosed tubes.

**Processing of MiSeq data.** Barcoded reads were demultiplexed and terminal primer sequences were trimmed using custom Python scripts. Any reads still containing a copy of either primer sequence after this trimming were identified using cutadapt v1.15 and excluded from further analyses on the assumption that they represented PCR artifacts (e.g., concatemers). The fastq\_eestats2 command in USEARCH v10.0.240 was used to assess the optimal 3' trimming length to remove low-quality sequences, which were 270 bp for our data (23). The DADA2 3.10 software package was used to trim reads, filter to  $\leq 1$  expected errors/read, assign sequence variants (using pooled sequences), merge read pairs, and remove chimeras (using the removeBimeraDenovo function with method = consensus) (24). The counts of *Campylobacter* 16S operational taxonomic units (OTUs) per sample were calculated from a phyloseq object using the subset\_taxa function (25). The *porA2* nucleotide sequences were aligned in MEGA X to confirm that they were coding sequences for correct fragments from *Campylobacter* (26). A small number of sequences containing stop codons were removed from further analyses. Allele numbers for nucleotide and peptide sequences for *porA2* were assigned using the PubMLST *Campylobacter* database (<https://pubmlst.org/organisms/campylobacter-jejunicoli>) (27). The assigned *porA2* alleles are publicly available and can be found by downloading allele sequences that are not within assigned typing schemes, on the typing section of the database. The raw data reads have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number [PRJEB45083](https://www.ebi.ac.uk/ena/record/PRJEB45083).

**Data analyses.** Data were transformed to an even sampling depth, giving proportional frequency of each *porA2* type, using the phyloseq package in R (25). Data from flock 14, aged 7 days, were removed from the analyses at this point, as the sequencing depth was low, between 127 and 281. These data were used for all subsequent analyses, with the exception of Simpson's and Shannon's diversity indices, which were performed on raw and interpolated/extrapolated data (28) and rarefaction analyses performed on the raw data. Bar charts and Table 3 calculations were prepared using Tableau v2019.4 software. Paired Wilcoxon rank-sum exact tests were used to compare (i) the difference in the percentage of bacterial 16S profile represented by *Campylobacter* 16S rRNA gene variants between flocks tested at young and older time points, (ii) the prevalence of a *porA2* type among young birds in a flock before it

became predominant, with the average prevalence among young birds from other flocks, (iii) the number of *porA*2 types identified among flocks tested at young and older times points, and (iv) Simpson's diversity index and Shannon's H diversity index between *Campylobacter* culture-positive and -negative flocks. A Mann-Whitney U test was used to compare the number of *porA*2 types identified among *Campylobacter* culture-positive and -negative flocks. The tests were performed using Paleontological Statistics (PAST) v4.0 software (29).

PAST v4.0 software was also used to calculate rarefaction curves for samples pooled by flock/age (total sequencing data). The iNEXT (interpolation/extrapolation) R package was used to calculate Simpson's and Shannon's diversity indices on raw data, as well as to give interpolated/extrapolated diversity estimates for each sample, allowing them to be compared to each other in a standardized way (28). For Simpson's diversity index, a 1-D value of 1.0 indicated that all members of a population could be distinguished from each other, and a 1-D value of 0 indicated that all members of a population were identical (30). Shannon's diversity index was included, as it is considered to give more weight to rare species (*porA*2 variants) (31). An H value of 0 indicated that all species were the same. H increases with increasing number of species.

As parallel sequencing data are by nature compositional, differences between *Campylobacter porA*2 populations was assessed using a nonmetric multidimensional scaling (NMDS) visualization of the Aitchison distance between samples (32). DADA2-processed MiSeq data (see above) were further analyzed using various R packages, as noted below, with the use of the data.table package (33) for data handling and the ggplot2 package (34) for plotting. First, the data were filtered to remove samples with <500 reads and sequences which occurred in only a single sample. To permit the subsequent (log-based) transform, low nonzero values were then input for zero values using zCompositions::multRepl (35) with the CZM method. The zero-input data were transformed by centered log-ratio (clr) using compositions::clr (36), and the Aitchison distances between samples were calculated (Aitchison distance being the Euclidean distance following clr-transform). NMDS was then performed in three dimensions with vegan::metaMDS (37) using the monoMDS engine.

The Bray-Curtis dissimilarity index was used to assess the effects of using two different DNA extraction kits (38). The ordination function with the NMDS method, together with the phyloseq, ggPlot2, and Pylr packages were used to produce the Bray-Curtis ordination plots in R (25, 34, 39).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

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This study used broiler flocks that were reared commercially by industry partners, in line with standard industry practice, and involved noninvasive sampling of feces. All prevailing local, national, and international regulations and conventions and normal scientific ethical practices were respected. The Swiss samples were approved by the Canton of Bern (BE97/16) and met all cantonal and federal regulations for the treatment of animals.

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