Investigation of *Campylobacter* colonization in three Australian commercial free-range broiler farms

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ABSTRACT *Campylobacter* spp. contaminated poultry products are strongly associated with foodborne illnesses worldwide. Development of effective management strategies to reduce contamination by *Campylobacter* spp. requires an improved understanding of the numerous factors that drive these contamination processes. Currently, chicken farms are using more free-range chicken meat production systems in response to consumer preferences. However, *Campylobacter* spp. colonization has rarely been investigated on free-range broiler farms. The present study investigated the temporal and environmental factors influencing *Campylobacter* spp. colonization of free-range broilers as well as potential sources and genetic diversity of *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) in commercial free-range broiler farms. Genetic linkages among the isolates were analyzed using *flaA* amplicon analysis. *Campylobacter coli* was first detected in fecal samples of a commercial free-range broiler flock on day 10 of rearing. Multiple genotypes of *C. jejuni* and *C. coli* were identified in this study. The farm environment was identified as a potential source of *C. jejuni* and *C. coli* colonization of free-range broilers. The dominant *Campylobacter* genotype varied between free-range broiler farms over time, with *C. jejuni* being the most frequently isolated species. These findings enhance the understanding of *C. jejuni* and *C. coli* colonization in free-range broiler farms and could inform the development of more effective intervention strategies to help control this important foodborne pathogen.

Key words: *Campylobacter jejuni*, *Campylobacter coli*, free-range broiler, colonization, genetic diversity

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INTRODUCTION

Campylobacter species are important zoonotic pathogens, with *Campylobacter jejuni* (***C. jejuni***) and *Campylobacter coli* (***C. coli***) being the most 2 common etiological agents of human enteric infections (WHO, 2012). Chickens are commonly considered as natural hosts of *Campylobacter* spp. as the birds can carry large loads of *Campylobacter* bacteria in their intestines without showing any clinical signs (Beery et al., 1988; Hermans et al., 2012).

In commercial farms, *Campylobacter* spp. are often isolated from chickens around 3 wk of rearing (Bull et al., 2006; Yano et al., 2013; Ingresa-Capaccioni et al., 2015; Prachantasena et al., 2016). The production environment of intensive commercial poultry farms is an important source of *Campylobacter* spp. which colonize chickens (Messens et al., 2009; Ellis-Iversen et al., 2012). Recently, free-range production of broilers has increased in response to consumer demands for products produced by nonintensive systems. However, information on sources and routes of transmission of *Campylobacter* spp. in broilers from free-range systems is limited, despite the increasing numbers of these types of free-range farms (Miele, 2011; Naald and Cameron, 2011; Singh and Cowieson, 2013; Walley et al., 2015). Templeton (2014) described the diversity of *C. jejuni* genotypes isolated from cecal contents from Australian intensive and free-range broiler chickens in slaughterhouses but did not investigate colonization and transmission in farms.

The *flaA* gene has proven to be informative in molecular epidemiological studies (Meinersmann et al., 1997; Petersen and On, 2000; Hiett et al., 2007; Singh and Kwon, 2013; Gomes et al., 2016). In the

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present study, we used *flaA* amplicon analysis to investigate the sources, transmission processes, and genetic diversity of *C. jejuni* and *C. coli* isolates in commercial free-range broilers in New South Wales (**NSW**), Australia.

MATERIALS AND METHODS

Farms

Before commencement of this study, appropriate ethics approval was obtained from the Charles Sturt University Animal Care and Ethics Committee (protocol number: 15/057). From May to August 2016, a total of 11 farms were sampled including 8 breeder farms (designated BD–A to BD–H) and 3 free-range broiler farms (designated FB1 to FB3). As illustrated in Figure 1, the 8 breeder farms supplied Ross chicks to the 3 free-range broiler farms. Of the 8 breeder farms, 5 (BD–B, BD–C, BD–D, BD–H, and BD–G) were in NSW and 3 (BD–A, BD–E, and BD–F) were in Queensland (**QLD**). All farms were part of an integrated poultry production company based in NSW, Australia. The 3 free-range broiler farms were within the same vicinity (approximately 800 m apart), and they were 60 km away from Sydney.

After being reared in commercial closed barns in the first 21 d of age, the broiler chickens were free to roam in a fenced outdoor environment through barn flaps during daytime until reaching market weight (Free Range Egg and Poultry Australia, 2012), with the maximum stock density of 28 to 34 kg/m^2 (Australian Chicken Meat Federation, 2018). In this study, a flock was defined as the entire population of chickens housed in the same barn.

This study was conducted over 2 free-range broiler farm production cycles (designated experiment 1 and experiment 2; Figure 1). For both experiments, one barn from each broiler farm was selected as the target barn (designated T), focusing on *Campylobacter* transmission. The adjacent barns (designated A1 and A2) were used to assess potential transmission between flocks.

The codes for free-range broiler barns used in this study were composed of 3 components and presented as "the farm–the barn–the experiment". Thus, the target barn (T) on free-range broiler farm 1 (FB1) in experiment 1 (Exp.1) was coded as FB1–T–Exp.1; and the adjacent barns (A1 and A2) were separately coded as FB1–A1–Exp.1 and FB1–A2–Exp.1. The farm codes used in the study are listed in Table 1.

Determination of Sample Size

The sample size was determined using Epitools (AusVet Animal Health Services) with the population size of 12,000, test sensitivity of 0.9, the desired herd sensitivity of 0.95, and the designed prevalence of 0.1 via http://epitools.ausvet.com.au/content.php?page=FreedomFinitePop&Population (accessed on April 2nd, 2016). The designed prevalence of *Campylobacter* used in this study was justified at 0.1 (10%) to collect 34 fecal samples from chickens of each barn. Owing to time and logistical limitations, a total of 35 fecal samples from the target barns and 10 fecal samples from each of the adjacent barn were collected (Supplementary Table 1). For the breeder farms, 5 fecal samples per barn were collected (Supplementary Table 1). Therefore, a total of 20 or 30 fecal samples per farm were obtained.

Sample Collection

Fresh fecal and cecal excretions were collected and defined as fecal samples. The environment within and surrounding the barn was also selected for sampling and referred to as the environmental samples for *Campylobacter*.

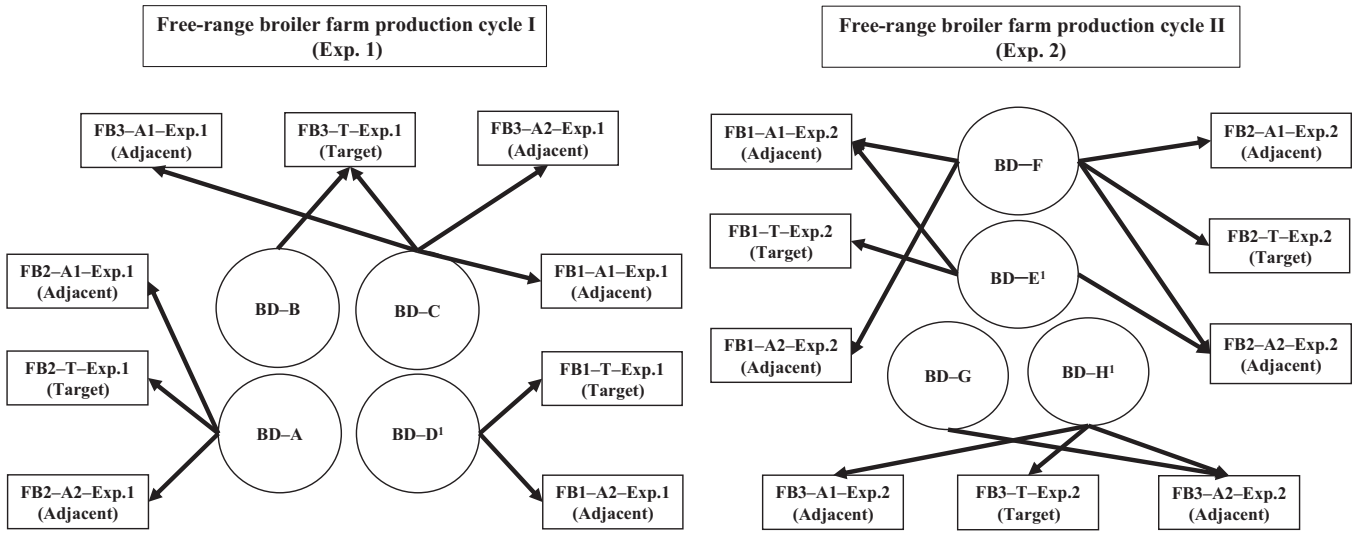

Figure 1. Diagram of free-range broiler and their parent breeder farms in experiments 1 and 2 of this study.[1] Indicates all depopulated breeder farms.

Table 1. Summary of the supplied free-range broiler barns and their parent breeder farms for experiments 1 and 2.

	Broiler farm				
Experiment	Farm	Barn	Chickens (n)	Barn code	Breeder farm
1	1	Adjacent1	14,670	FB1–A1–Exp.1	BD–C
		Target	14,670	FB1–T–Exp.1	BD–D[1]
		Adjacent2	15,390	FB1–A2–Exp.1	BD–D[1]
	2	Adjacent1	15,030	FB2–A1–Exp.1	BD–A
		Target	15,030	FB2–T–Exp.1	BD–A
		Adjacent2	14,850	FB2–A2–Exp.1	BD–A
	3	Adjacent1	11,980	FB3–A1–Exp.1	BD–C
		Target	11,980	FB3–T–Exp.1	BD–B and BD–C
		Adjacent2	15,030	FB3–A2–Exp.1	BD–C
2	1	Adjacent1	15, 480	FB1–A1–Exp.2	BD–F and BD–E[1]
		Target	14,760	FB1–T–Exp.2	BD–E[1]
		Adjacent2	14,760	FB1–A2–Exp.2	BD–F
	2	Adjacent1	14,670	FB2–A1–Exp.2	BD–F
		Target	14,670	FB2–T–Exp.2	BD–F
		Adjacent2	15,390	FB2–A2–Exp.2	BD–F and BD–E[1]
	3	Adjacent1	11,880	FB3–A1–Exp.2	BD–H[1]
		Target	11,880	FB3–T–Exp.2	BD–H[1]
		Adjacent2	14,850	FB3–A2–Exp.2	BD–G and BD–H[1]

[1]Indicates the depopulated breeder farms.

Fecal samples from the breeder farms were obtained on day 7 after the placement of broiler chicks for logistic reasons. Of further note, farms BD–D, BD–E, and BD–H were completely depopulated and consequently, samples from the 3 farms were not available.

All free-range broiler farms were sampled before chick placement (day 0) and then weekly, starting from the day of chick placement (day 1 or 3) until all fecal samples of the target barns tested were positive for *Campylobacter* spp. During each visit, fecal and environmental samples were collected from each broiler barn (Supplementary Table 1). All samples were kept in insulated boxes containing ice packs and transported to the laboratory for processing within 24 h.

Fresh fecal samples were randomly collected from each barn using Amies swabs containing charcoal transport medium (Copan Diagnostics Inc., Murrieta, CA) on the day of chick placement (day 1 or 3) and a sterile fecal container with a spoon (Techno Plas, St Marys, SA, Australia) on week 1 (day 8 or 10), 2 (day 15 or 17), and 3 (day 22 or 24). Additional samples using Amies swabs were obtained from the barn wall (swabbing a 100-cm^2 area on each side), water and feed pans, and footwear. In addition, drinking water samples (250 mL each) were collected from drinkers in 3 to 6 areas of each barn and kept in separate sterile plastic containers (Techno Plas). Water samples (250 mL each) from the main tank and puddles (outside the barn) were also collected and kept in separate sterile plastic containers. The presence of other potential hosts of *Campylobacter* (i.e., mammals, insects, and undomesticated birds) on the broiler farms was considered in this study. However, only fresh rodent feces, darkling beetles, and flies were identified on the farms during the sample collection period. Fresh rodent feces (dark in color, soft and moist textures, and spindle-shaped) and insects (darkling beetles and flies) were collected from the anteroom of each barn and placed in separate sterile plastic bags. Floor drag swabs (inside the barn and anteroom) were also collected using sterile tampons (Libra regular; Svenska Cellulosa Aktiebolaget, Springvale, VIC, Australia) moistened with sterile buffered peptone water (Acumedia; Neogen Corporation, Lansing, MI) by swabbing the floor in a zigzag pattern, including the perimeter and center of the room. Soil samples at free-range areas were obtained by drag swabbing a moist sterile tampon along the barn's outside perimeter. The swabs were placed in separate sterile plastic bags. A total of 1,865 samples were collected in this study (Supplementary Table 2).

Campylobacter spp. Isolation

All samples were processed following the standard ISO 10272:2006 method for *Campylobacter* isolation (ISO, 2006), with slight modifications. Briefly, all fecal samples were directly streaked onto a *Campylobacter*-selective agar (*Campylobacter* agar, Skirrow's agar, and Campy Food Agar; BioMérieux, Marcy l'Etoile, France). Water samples were filtered using a membrane that was 47 mm in diameter with a pore size of 0.45 μm (Merck Millipore, Burlington, MA). The membranes were then enriched using 10 mL of Bolton broth (Oxoid, Cambridge, UK). All swab samples were enriched using 10 mL of selective enrichment Bolton broth (Oxoid).

All streaked plates and enriched samples were incubated at 42°C for 48 h under a microaerobic environment generated with a BD GasPak EZ container system (Becton Dickinson Microbiology, North Ryde, NSW, Australia). All enriched samples were screened with VIDAS *Campylobacter* assay (BioMérieux) for *Campylobacter* spp. detection before plating onto the selective agar plates and incubating under microaerobic conditions as described earlier.

After incubation, individual bacterial colonies (a maximum of 5 colonies per sample) showing morphological

characteristics typical of *Campylobacter* spp. were selected for species-level identification. Colonies morphologically identified as either *C. jejuni* or *C. coli* were plated onto sheep blood agar plates (BioMérieux) and incubated under microaerobic conditions to obtain pure colonies (isolates). Subsequently, all pure *C. jejuni* and *C. coli* isolates were stored in the FBP *Campylobacter* growth medium, as previously described (Gorman and Adley, 2004) at −80°C.

C. jejuni *and* C. coli *Identification*

A two-stage approach was used to confirm the identity and differentiate *C. jejuni* and *C. coli* colonies in this study. Initially (primary identification), species testing of colonies was performed in an industry laboratory using their established protocols with the VITEK MS system (BioMérieux). Briefly, the VITEK MS system is a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) which has an inbuilt capacity to identify *C. jejuni* or *C. coli* using the manufacturer's default analysis algorithms. The VITEK MS system was used to screen the individual colonies selected based on morphology to provide a putative identity as either *C. jejuni* or *C. coli*. All isolates identified as *C. jejuni* or *C. coli* were subsequently transported to an academic laboratory, where PCR assays (tertiary identification) were used to designate each isolate as either *C. jejuni* or *C. coli* before genotyping.

Genomic DNA Extraction

DNA was extracted from pure colonies of *C. jejuni* and *C. coli* using the PREPMAN Ultra Sample Preparation (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's instructions. DNA samples were stored at −20°C until required.

C. jejuni *and* C. coli *Confirmation*

A conventional PCR assay (S1000 Thermal Cycler; Bio-Rad, Australia) was used to confirm *C. jejuni* and *C. coli*. A PCR protocol specific for the 16s rRNA (*Campylobacter* genus), *mapA* (*C. jejuni*), and *lpxA* (*C. coli*) genes was applied as previously described (Devi, 2019). Each PCR reaction volume was 25 µL, containing 2 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA), 1 × Green PCR Rxn Buffer/$MgCl_2$ (Invitrogen), 1.5 mmol $MgCl_2$ (Invitrogen), 0.2 mmol of dNTPs mixed (Invitrogen), 0.2 µmol of each primer set (Integrated DNA Technologies, Singapore) as described in Table 2, RNase-free water (to a final volume of 24 µL), and 1 µL of DNA template (10–30 ng) as previously described (Devi, 2019).

The PCR cycling conditions consisted of 94°C for 2 min, followed by 40 cycles of 94°C for 10 s, 60°C for 20 s, 72°C for 30 s, and finally 72°C for 5 min. The PCR products were stained with the Midori Green Stain (Nippon Genetics, Duren, Germany) and analyzed using 1.5% gel electrophoresis at 80 V for 40 min. The amplicon sizes were compared with a molecular weight marker (1-kb ladder, New England Biolabs, Ipswich, MA). *C. jejuni* ATCC 49943 and *C. coli* ATCC 33559 were used as positive controls for each PCR reaction. RNase water was used as the non-DNA template control.

Genotyping Process

Two processes, partial amplification of the *flaA* gene and genotyping analysis, were used to genotype study isolates. Based on results of these processes for *C. jejuni* and *C. coli* identification, 3 outcomes were used to classify samples: the samples containing *C. jejuni*, the sample containing *C. coli,* or the samples containing both *C. jejuni* and *C. coli* (different single purified colonies tested). For the purposes of genotyping, if a sample contained either *C. jejuni* or *C. coli,* then a single isolate was used for genotyping. If the sample contained *C. jejuni* and *C. coli*, then an isolate of each species was used for genotyping.

Amplification of the flaA Gene

C. jejuni and *C. coli* isolates were assessed for *flaA* amplification as described by Merchant-Patel et al. (2010) with minor modifications. Each *flaA* PCR reaction (20 µL) contained 1 × Type-it HRM-PCR kit (Qiagen, Hilden, Germany), 6.6 µL of MilliQ water, 0.7 µmol of *flaA* primers (Sigma-Aldrich, St. Louis, MO), and 2 µL of DNA template. The PCR assay was performed in a Rotor-Gene Q thermal cycler (Qiagen). The PCR conditions were applied at 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 15 s, followed by 72°C for 30 s.

Genotyping Analysis

The *flaA* amplicons were commercially sequenced using the Sanger sequencing method (Australian Genomic

Table 2. Oligonucleotide primers used for identification of *Campylobacter* spp., *Campylobacter jejuni,* and *Campylobacter coli* (Devi, 2019).

Group or species	Gene	Sequence 5′ to 3′	Amplicon size (bp)
Campylobacter	*16S rRNA*	Forward: CGTGCTACAATGGCATATACAATGA Reverse: CGATTCCGGCTTCATGCTC	113
C. jejuni	*mapA*	Forward: CACTTTAGACACTGGTATTGCTTTG Reverse: GATCGTTATTGTCAAGCACAACTATTC	191
C. coli	*lpxA*	Forward: GATGATGTTGTTATTGAGGCTTATG Reverse: GAAAGTATTCTCGCCCCTTG	92

Research Facility, Sydney, NSW, Australia). The nucleotide sequence alignment was performed using BioEdit Sequence Alignment Editor (version 7.2.5). The *flaA* allele and peptide numbers were identified by interrogation of each isolate in the *Campylobacter flaA* database, using the portal: http://pubmlst.org/campylobacter (accessed December 17th, 2019). The *C. jejuni* and *C. coli* genotypes were determined by analyzing the *flaA* allele numbers.

RESULTS

Campylobacter spp. were cultured from 526 (28.3%) of the 1,856 samples collected (Supplementary Table 2). Of these, 465 samples (88.4%) were fecal samples obtained from the breeder (n = 118) and free-range broiler farms (n = 347). The remaining 61 samples (11.6%) were from the environment of free-range broiler farms (Supplementary Table 2).

Campylobacter spp. Isolation From Breeder Farms

Campylobacter spp. were isolated from 118 (98.3%) of 120 fecal samples collected from 5 breeder farms, with the isolation rates ranging from 95 to 100% (Table 3). In this study, 7 isolates from 7 fecal samples initially identified as *C. jejuni* or *C. coli* by the VITEK MS system were reassigned by the PCR assays (Supplementary Table 3). The 12 additional isolates from the 7 fecal samples (as reculturable) were tested with the PCR assays. Of the 7 fecal samples retested, 5 and 2 samples were confirmed as *C. jejuni* and *C. coli*, respectively. Consequently, of the 118 positive fecal samples, 100 were identified as *C. jejuni* (n = 67) or *C. coli* (n = 33), and the remaining 18 contained both *C. jejuni* and *C. coli* (*C. jejuni* and *C. coli* were separately identified in different single colonies) as shown in Table 3. Hence, the genotypes of 85 *C. jejuni* (67 samples containing only *C. jejuni* and 18 samples containing both *C. jejuni* and *C. coli*) and 51 *C. coli* isolates (33 samples containing only *C. coli* and 18 samples containing both *C. jejuni* and *C. coli*) from breeder farms were further assessed with the *flaA* amplicon analysis. *C. jejuni* was the most frequently isolated species from the breeder farms in both experiments: Exp.1: BD–A and BD–C and Exp.2: BD–F and BD–G (Table 3).

Campylobacter spp. Isolation From Free-Range Broiler Farms

Campylobacter spp. were isolated from 17 of 18 barns, and one barn (FB3–A2–Exp.1) was negative (Table 4). Of the *Campylobacter*-positive barns, 9 had either *C. jejuni* or *C. coli* and 8 were positive for both. Based on the VITEK MS and PCR analyses, the same outcomes for the identification of *C. jejuni* and *C. coli* isolates from the broiler farms were identified (Supplementary Table 3). *Campylobacter* spp. were isolated from 408 (23.5%) samples (Table 4). *C. jejuni* and *C. coli* were isolated from 314 (77.0%) and 87 (21.3%), respectively, with 7 (1.7%) containing both species of interest (Table 4). Thus, the genotypes of 321 *C. jejuni* (314 samples containing only *C. jejuni* and 7 samples containing both *C. jejuni* and *C. coli*) and 94 *C. coli* isolates (87 samples containing only *C. coli* and 7 samples containing both *C. jejuni* and *C. coli*) from broiler farms were further assessed with the *flaA* amplicon analysis. *C. jejuni* was the most frequently isolated species in 14 positive barns and *C. coli* in the other 3 (Table 4).

Genetic Diversity of C. jejuni *and* C. coli

A total of 551 isolates (*C. jejuni*, n = 406 and *C. coli*, n = 145) identified in the breeder and broiler farms were genotyped by *flaA* amplicon analysis. The *flaA* nucleotide sequences were assigned into allele numbers. The 406 *C. jejuni* isolates were grouped into 29 genotypes: 24 recognized *flaA* alleles and 5 unassigned *flaA* alleles (Supplementary Table 4). The 145 *C. coli* isolates were grouped into 20 genotypes: 14 recognized *flaA* alleles and 6 unassigned *flaA* alleles (Supplementary Table 5).

Genetic Diversity of* C. jejuni *and* C. coli *in Breeder Farms The *C. jejuni* isolates (n = 85) from the breeder farms were grouped into 23 genotypes: 18 recognized *flaA* alleles and 5 unassigned *flaA* alleles (Supplementary Table 4). Of the 23 genotypes, 5 and 11 genotypes were isolated from Exp.1 and Exp.2, respectively, and the remaining 7 genotypes were isolated from both experiments. By contrast, the *C. coli* isolates (n = 51) were grouped into 18 genotypes: 12 recognized *flaA* alleles and 6 unassigned *flaA* alleles (Supplementary Table 5). Moreover, of these 18 genotypes, 8 and 7 were isolated from Exp.1 and Exp.2, respectively, and 3 were isolated from both experiments.

Table 3. Summary of *Campylobacter jejuni* and *Campylobacter coli* isolated from fecal samples from breeder farms based on polymerase chain reaction assays.

		Samples			*Campylobacter* species identified		
Farm	Barn (n)	Tested	Positive	%	*C. jejuni*	*C. coli*	*C. jejuni* and *C. coli*
BD–A	5	25	25	100.0	14	6	5
BD–B	4	20	19	95.0	8	9	2
BD–C	4	20	20	100.0	10	8	2
BD–F	6	30	30	100.0	13	9	8
BD–G	5	25	24	96.0	22	1	1
Total	24	120	118	98.3	67	33	18

Table 4. Summary of *Campylobacter jejuni* and *Campylobacter coli* isolated from samples collected from experiments 1 and 2 on 3 free-range broiler farms based on polymerase chain reaction assays.

	Samples			*Campylobacter* species identified		
Barn	Tested	Positive	%	*C. jejuni*	*C. coli*	*C. jejuni* and *C. coli*
FB1–A1–Exp.1	45	11	24.4	11	0	0
FB1–T–Exp.1	213	42	19.7	42	0	0
FB1–A2–Exp.1	45	20	44.4	20	0	0
FB2–A1–Exp.1	45	11	24.4	10	1	0
FB2–T–Exp.1	211	45	21.3	34	9	2
FB2–A2–Exp.1	45	12	26.7	0	12	0
FB3–A1–Exp.1	34	8	23.5	0	8	0
FB3–T–Exp.1	161	46	28.6	1	45	0
FB3–A2–Exp.1	34	0	0.0	0	0	0
FB1–A1–Exp.2	45	16	35.6	8	6	2
FB1–T–Exp.2	214	42	19.6	42	0	0
FB1–A2–Exp.2	45	21	46.7	20	1	0
FB2–A1–Exp.2	45	11	24.4	11	0	0
FB2–T–Exp.2	210	45	21.4	45	0	0
FB2–A2–Exp.2	45	11	24.4	11	0	0
FB3–A1–Exp.2	45	12	26.7	10	1	1
FB3–T–Exp.2	209	43	20.6	40	3	0
FB3–A2–Exp.2	45	12	26.7	9	1	2
Total	1,736	408	23.5	314	87	7

Genetic Diversity of* C. jejuni *and* C. coli *in Free-Range Broiler Farms The *C. jejuni* isolates (n = 231) were grouped into 9 genotypes, which belonged to 9 recognized *flaA* alleles (Supplementary Table 4). Among these, 3 (*flaA* alleles 14, 18, and 208) and 4 (*flaA* alleles 2, 18, 105, and 1033) were isolated from Exp.1 and Exp.2, respectively. The remaining 2 (*flaA* alleles 57 and 239) were isolated from both experiments. In comparison, the *C. coli* (n = 94) isolates were grouped into 5 genotypes, which were assigned to 5 recognized *flaA* alleles (Supplementary Table 5). Among these, one was identified exclusively in Exp.1 (*flaA* allele 769) and one exclusively in Exp.2 (*flaA* allele 16). The remaining 3 (*flaA* alleles 30, 36, and 256) were isolated from both experiments.

Dynamics of C. jejuni *and* C. coli *Colonization From Each F-Range Broiler Farm Between Experiments*

Free-range Broiler Farm 1 Seventy-three *C. jejuni* isolates from Exp.1 belonged to *flaA* alleles 14 (n = 47) and 57 (n = 26), respectively (Figure 2 and Supplementary Table 6). The *C. jejuni flaA* allele 14 was first isolated from 10 fecal samples of FB1–A2–Exp.1 on day 15. On day 22, this genotype was isolated from fecal samples from all barns (FB1–A1–Exp.1, n = 1; FB1–T–Exp.1, n = 23; and FB1–A2–Exp.1, n = 10), farm boots, and the environment of FB1–T–Exp.1 (drinking water and the free-range area). In addition, the *C. jejuni flaA* allele 57 was also isolated on the same time (day 22) from fecal samples of 2 barns (FB1–A1–Exp.1, n = 9; and FB1–T–Exp.1, n = 12) as well as the free-range area of FB1–A1–Exp.1 and the internal environment of FB1–T–Exp.1 (floors, walls, and barn boots).

In comparison, 72 *C. jejuni* isolates from Exp.2 belonged to *flaA* alleles 16 (n = 71) and 239 (n = 1) (Figure 2 and Supplementary Table 6). The *C. jejuni flaA* allele 16 was first isolated from 10 fecal samples of FB1–A2–Exp.2 on day 15. On day 22, this genotype was isolated from fecal samples (FB1–A1–Exp.2, n = 8; FB1–T–Exp.2, n = 35; and FB1–A2–Exp.2, n = 10), free-range areas (FB1–A1–Exp.2 and FB1–T–Exp.2), farm boots, and internal environment of FB1–T–Exp.2 (anteroom, floors, walls, barn boots). However, the *C. jejuni flaA* allele 239 was isolated only from a fecal sample of FB1–A1–Exp.2 on day 22, whereas the *C. coli* isolates (n = 9) isolated from FB1–A1–Exp.2 and FB1–A2–Exp.2 belonged to *flaA* alleles 16 (n = 1), 30 (n = 7), and 36 (n = 1) (Figure 2 and Supplementary Table 6). The *C. coli flaA* allele 30 was first isolated from 5 fecal samples of FB1–A1–Exp.2 on day 15. On day 22, fecal samples from the same barn were positive for *C. coli flaA* alleles 30 (n = 2) and 16 (n = 1), whereas the *C. coli flaA* allele 36 was isolated only from the free-range area of FB1–A2–Exp.2 on day 15.

Free-range Broiler Farm 2 Forty-six *C. jejuni* isolates from Exp.1 belonged to *flaA* alleles 14 (n = 15), 18 (n = 1), and 208 (n = 30) (Figure 3 and Supplementary Table 6). The *C. jejuni flaA* allele 18 was isolated only from rodent feces from FB2–T–Exp.1 on day 8. The *C. jejuni flaA* allele 14 was first isolated from fecal samples from FB2–A1–Exp.1 (n = 1) and FB2–T–Exp.1 (n = 11) and the environment of FB2–T–Exp.1 (walls and the free-range area) on day 22. At the same time, the *C. jejuni flaA* allele 208 was first isolated from fecal samples (FB2–A1–Exp.1, n = 9; and FB2–T–Exp.1, n = 20) and rodent feces from FB2–T–Exp.1. Moreover, the *C. coli* isolates (n = 24) belonged to *flaA* alleles 30 (n = 15), 256 (n = 8), and 769 (n = 1) (Figure 3 and Supplementary Table 6). The *C. coli flaA* allele 256 was first isolated from rodent feces from FB2–T–Exp.1 and the free-range

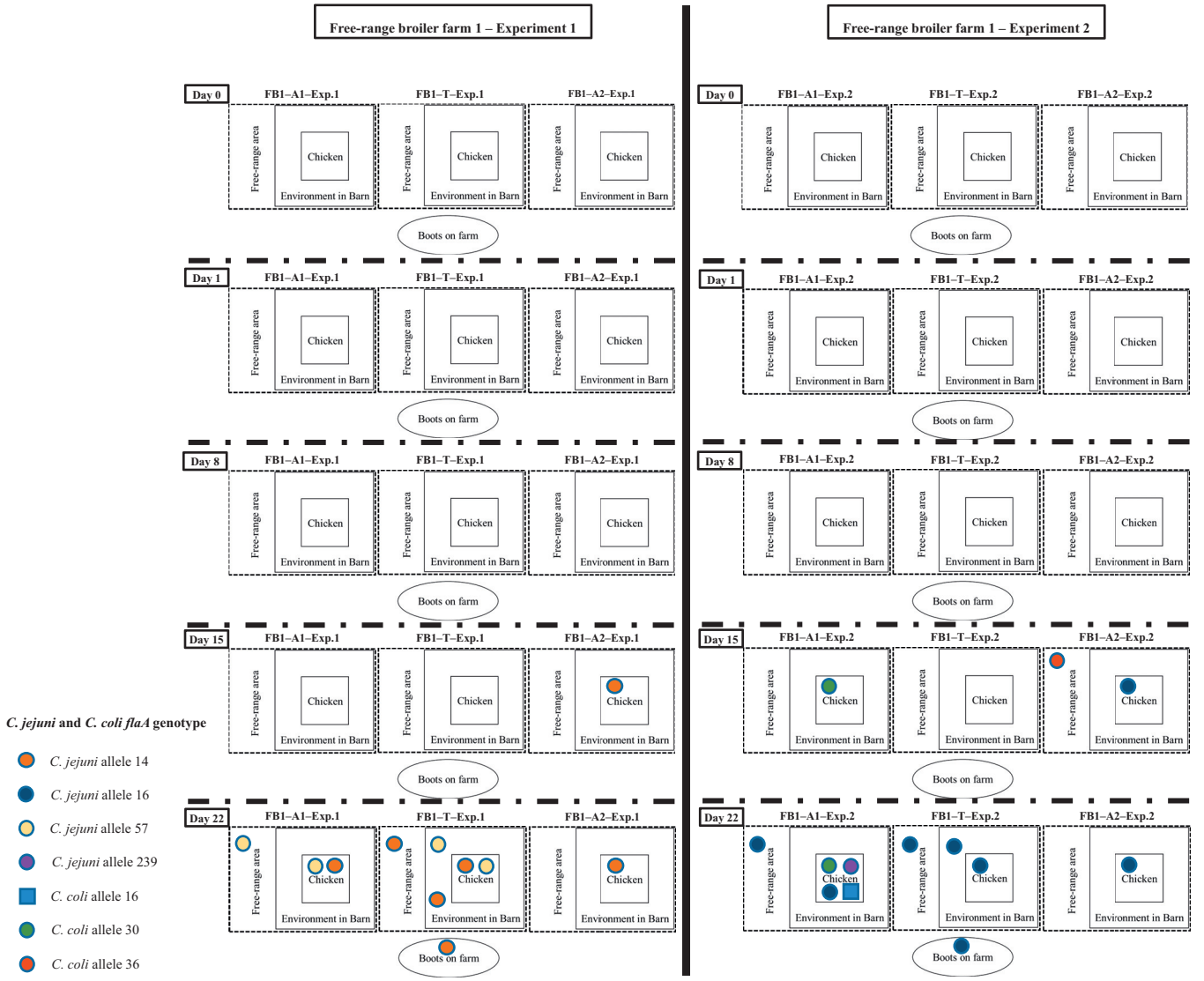

Figure 2. Schematic diagram of dynamics of *Campylobacter jejuni* and *Campylobacter coli* flaA types identified on free-range broiler farm 1 (FB1) from experiments 1 and 2.

area of FB2–A2–Exp.1 on day 1. This genotype was isolated from other samples from the same target barn (FB2-T-Exp.1) at different time points, such as barn boots (day 8) and rodent feces (day 8, 15, and 22) as well as 2 fecal samples of FB2–A2–Exp.1 (day 22). The *C. coli flaA* allele 769 was isolated only from the free-range area of FB2–A1–Exp.1 on day 8. The *C. coli flaA* allele 30 was first isolated from a fecal sample of FB2–A2–Exp.1 on day 15. One week later, this genotype coexisted between fecal samples of different barns (FB2–A2–Exp.1, n = 8; and FB2–T–Exp.1, n = 4) and the floors of FB2–T–Exp.1.

In comparison, the *C. jejuni* isolates (n = 67) from Exp.2 belonged to *flaA* alleles 2 (n = 2), 16 (n = 48), 105 (n = 1), 239 (n = 15), and 1033 (n = 1) (Figure 3 and Supplementary Table 6). The *C. jejuni flaA* alleles 1033, 105, and 239, isolated from rodent feces from FB2–T–Exp.2, were found on day 0, 1, and 8, respectively. One week later, on day 15, the *C. jejuni flaA* allele 2 was isolated only from rodent feces and the anteroom floor in FB2–T–Exp.2. Then, the *C. jejuni flaA* allele 16 was first isolated from the barns and the environment on day 22, including from fecal samples (FB2–A1–Exp.2, n = 6; and FB2–T–Exp.2, n = 35), free-range areas of all three barns (FB2–A1–Exp.2, FB2–T–Exp.2, and FB2–A2–Exp.2), the internal environment of FB2–T–Exp.2 (floors and barn boots), and farm boots. At the same time, the *C. jejuni flaA* allele 239 was isolated from fecal samples of FB2–A1–Exp.2 (n = 4) and FB2–A2–Exp.2 (n = 10).

Free-Range Broiler Farm 3 The *C. jejuni flaA* allele 239 was isolated only from a sample of rodent feces from FB3–T–Exp.1 on Day 3 (Figure 4 and Supplementary Table 6). Although the *C. coli* isolates (n = 53) from Exp.1 belonged to *flaA* alleles 30 (n = 7) and 36 (n = 46) (Figure 4 and Supplementary Table 6), the *C. coli flaA* allele 36 was first isolated from the free-range area of FB3–A1–Exp.1, farm boots, and samples of FB3–T–Exp.1 (fecal samples; n = 3 and barn boots) on day 10. A week later (day 17), this genotype persisted, being isolated from the farm boots and other samples from FB3–T–Exp.1, such as fecal samples (n = 35), free-range

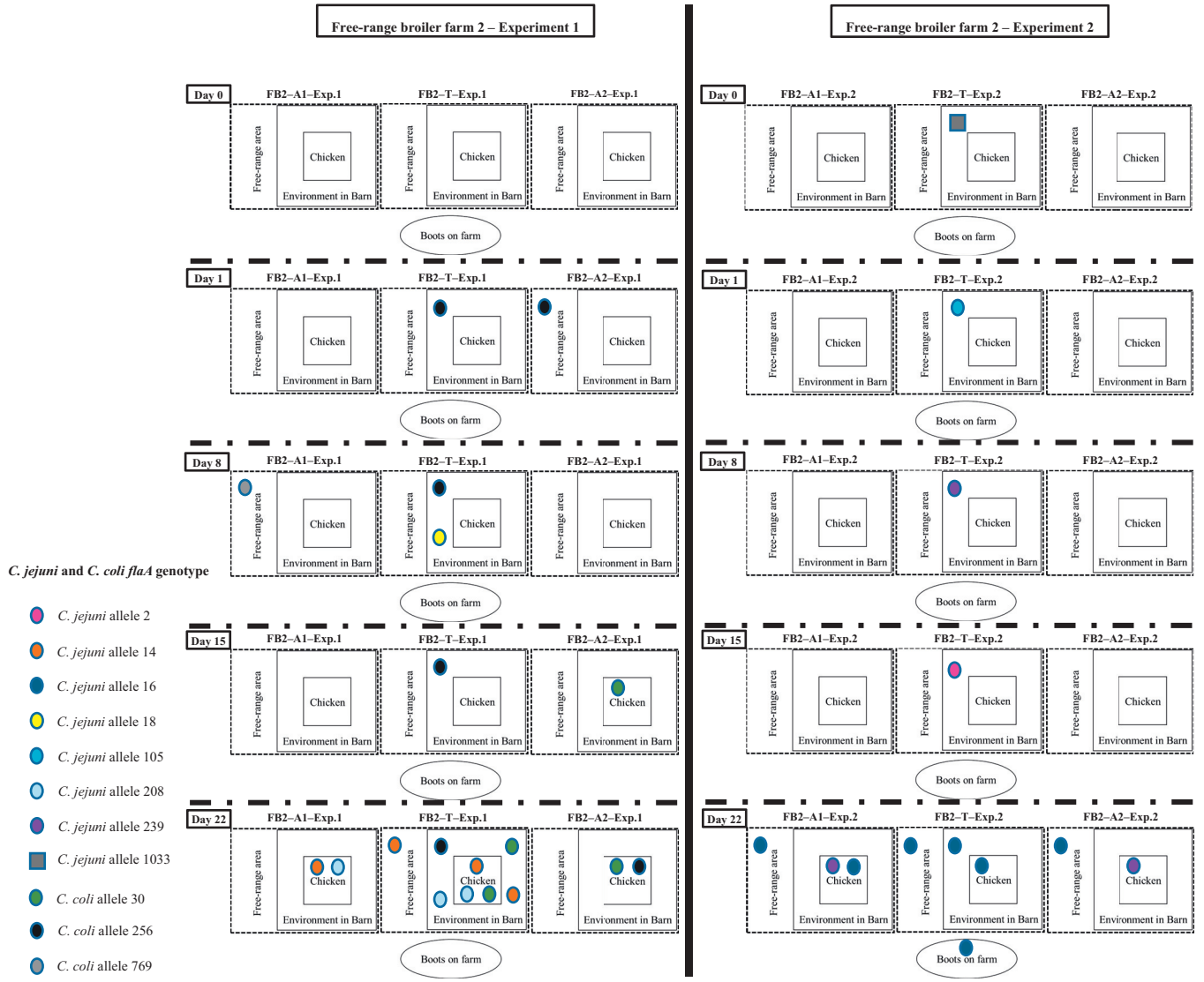

Figure 3. Schematic diagram of dynamics of *Campylobacter jejuni* and *Campylobacter coli* flaA types identified on free-range broiler farm 2 (FB2) from experiments 1 and 2.

area, and internal environment (floor, walls, water pans, and barn boots). The *C. coli flaA* allele 30 was isolated from only 7 fecal samples of FB3–A1–Exp.1 (day 17).

In comparison, the *C. jejuni* isolates (n = 62) from Exp.2 were isolated only from day 24 and belonged to *flaA* alleles 16 (n = 1), 57 (n = 36), 239 (n = 22), and 105 (n = 3) (Figure 4 and Supplementary Table 6). The *C. jejuni flaA* allele 57 was isolated only from FB3–T–Exp.2, including the free-range area, fecal samples (n = 32), and the internal environment (floors and barn boots). The *C. jejuni flaA* allele 239, previously isolated from samples in Exp.1, was also isolated from farm boots, free-range areas of FB3–A1–Exp.2 and FB3–A2–Exp.2, as well as fecal samples of FB3–A1–Exp.2 (n = 10) and FB3–A2–Exp.2 (n = 9). The *C. jejuni flaA* allele 105 was found in 2 fecal samples of FB3–T–Exp.2 and a fecal sample of FB3–A2–Exp.2. Furthermore, the *C. jejuni flaA* allele 16 was found only in a fecal sample of FB3–T–Exp.2. By contrast, the *C. coli* isolates (n = 8) from Exp.2 were assigned to *flaA* alleles 36 (n = 7) and 256 (n = 1) (Figure 4 and Supplementary Table 6). The *C. coli flaA* allele 36, previously isolated in Exp.1, was detected in Exp.2. This genotype was first isolated from the free-range area of FB3–A1–Exp.2 before chick placement (Figure 4). Two weeks later (day 17), this genotype was isolated from rodent feces from FB3–T–Exp.2, a fecal sample of FB3–A2–Exp.2, and farm boots. At a later time point (day 24), this genotype was subsequently isolated from a fecal sample of FB3–A1–Exp.2 and 2 fecal samples of FB3–A2–Exp.2. Moreover, the *C. coli flaA* allele 256 was only isolated from rodent feces from FB3–T–Exp.2 on day 24 (Figure 4 and Supplementary Table 6).

Similarity of C. jejuni *and* C. coli *Isolates From Breeders and Their Broiler Progeny*

Three *C. jejuni* genotypes (Supplementary Table 4) and 3 *C. coli* genotypes (Supplementary Table 5) were isolated from samples from both breeder farms and free-range broiler flocks.

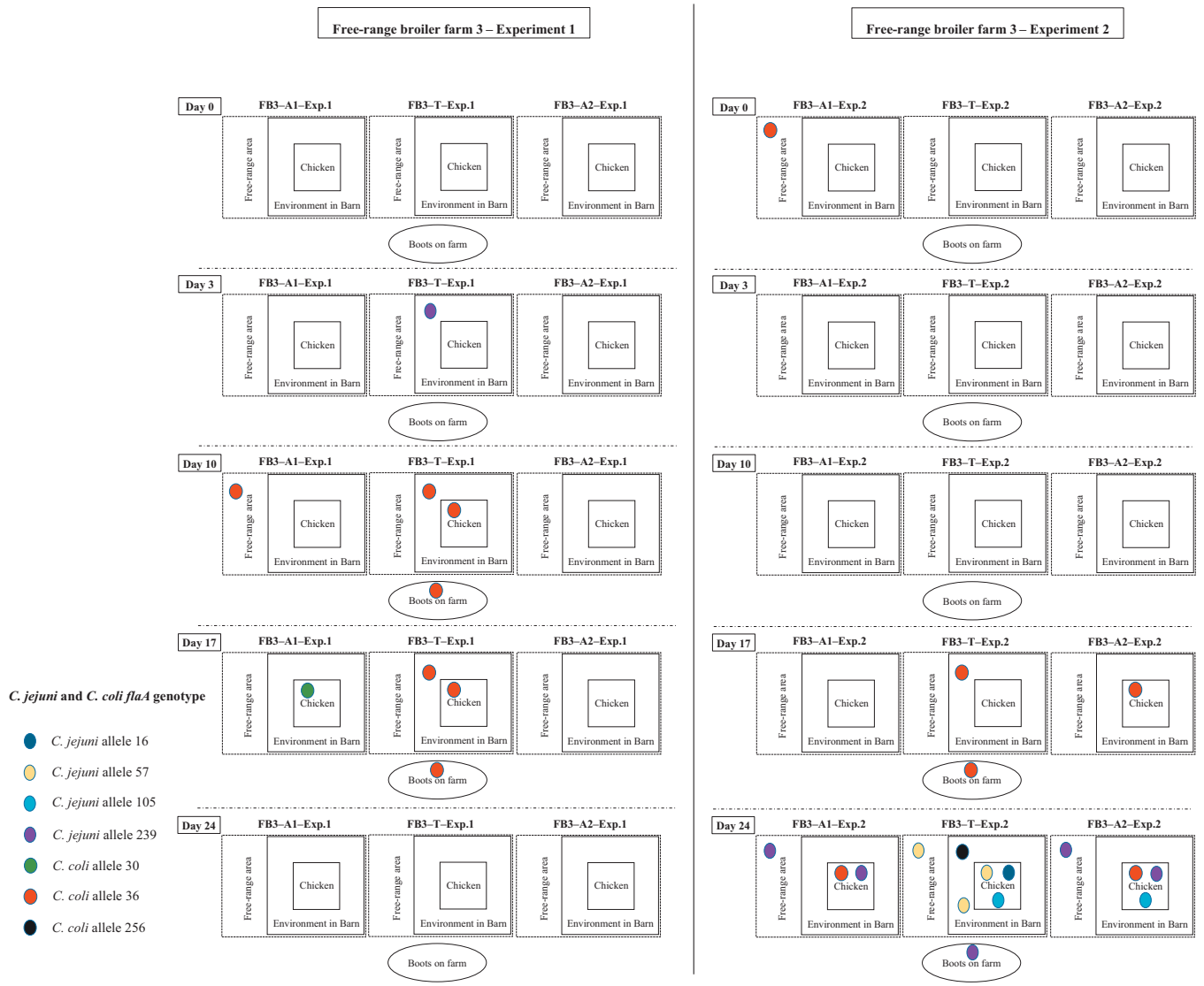

Figure 4. Schematic diagram of dynamics of *Campylobacter jejuni* and *Campylobacter coli* flaA types identified on free-range broiler farm 3 (FB3) from experiments 1 and 2.

Of the 3 *C. jejuni* genotypes (*flaA* alleles 18, 105, and 239), only *C. jejuni flaA* allele 239 was isolated from fecal samples of a breeder farm and in fecal samples from its broiler offspring, despite being located in geographically distant areas. The *C. jejuni flaA* allele 239 was isolated from 2 fecal samples of BD–F, located in QLD, and one fecal sample of FB1–A1–Exp.2, located in NSW, on day 22 (Figure 5B). However, this genotype was also identified in other linked broilers in FB2 located in NSW within the same experiment (Exp.2) (Figure 5B). However, it was also found in a sample of rodent feces (day 8) in FB2–T–Exp.2, and on day 22, it was identified in fecal samples of FB2–A1–Exp.2 (n = 4) and FB2–A2–Exp.2 (n = 10).

Notably, 2 of 3 *C. coli* genotypes (*flaA* alleles 16 and 30) isolated from fecal samples of 3 breeder farms and their progeny were genetically similar. The *C. coli flaA* allele 30 was isolated from both breeders and linked broilers, located in NSW and QLD. This genotype was isolated from 2 fecal samples of BD–A based in QLD and the samples from FB2 in Exp.1 based in NSW such as fecal samples of FB2–T–Exp.1 (n = 4, day 22) and FB2–A2–Exp.1 (n = 1, day 17; n = 8, day 22) and 2 floor samples of FB2–T–Exp.1 on day 22 (Figure 5A). This genotype was also isolated from a fecal sample from BD–C located in NSW and 7 fecal samples from FB3–A1–Exp.1 based in NSW (day 17) (Figure 5A). In addition, the *C. coli flaA* allele 16 was isolated from samples from Exp.2: from breeders and their progeny located in different states. This genotype was isolated from 2 fecal samples of BD–F, located in QLD, and one fecal sample of FB1–A1–Exp.2, located in NSW (day 22) (Figure 5B).

DISCUSSION

In this study, *C. jejuni* and *C. coli* were isolated from the breeder and free-range broiler farms, as has been previously reported (Vandeplas et al., 2010; O'Mahony et al., 2011; Prachantasena et al., 2016). Most studies

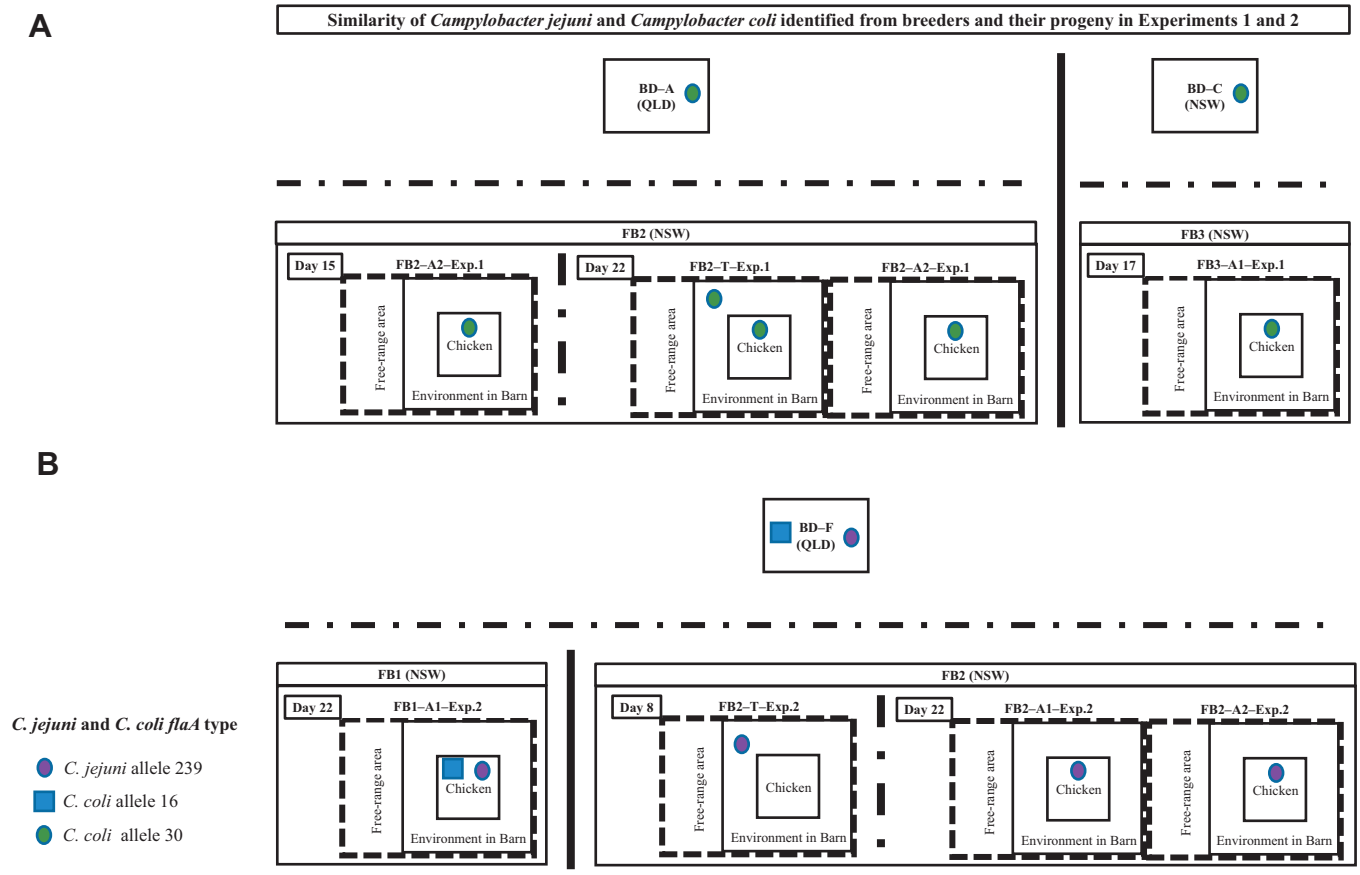

Figure 5. Schematic diagram of similarity of *Campylobacter jejuni* and *Campylobacter coli* flaA types between breeder farms and their progeny in experiments 1 (A) and 2 (B).

report *C. jejuni* and *C. coli* to be the first species isolated after 2 wk of rearing in commercial farms (Bull et al., 2006; Yano et al., 2013; Prachantasena et al., 2016), but these microorganisms have been detected earlier in free-range farms. For example, El-Shibiny et al. (2005) reported that a free-range broiler flock in the UK was colonized by *C. jejuni* within 1 wk of rearing (day 8). The present study, to the best of our knowledge, is the first to show the early detection of *C. coli* in fecal samples of chickens in a commercial free-range broiler flock approximately 1 wk (day 10) after chick placement.

The genotypes of *C. jejuni* and *C. coli* isolated in the present study were diverse, consistent with previous reports (Colles et al., 2011; Vidal et al., 2016). One reason for this could be that multiple *Campylobacter* genotypes from various sources can accumulate and persist simultaneously within broiler flocks (Ridley et al., 2008a). In addition, Ridley et al. (2008b) have suggested that *C. jejuni* could undergo genetic rearrangement by 4 wk after challenge with mixed strains in the birds due to the competitive environment in the chicken gut, thus leading to diverse genotypes. The data also showed more genetic diversity on the breeder farms compared with that on the free-range broiler farms. This has been reported previously and suggests that *Campylobacter* colonization of breeder chickens is a dynamic process, supported by the notion of repeat exposure in longer-lived breeders compared with broilers (Colles et al., 2011).

Some *C. jejuni* and *C. coli* genotypes isolated from the broiler farms were common among chicken feces from the different farms and environments isolated in this study. This suggests that free-range broiler flocks in the same area (although in different farms) are exposed to the same sources of *Campylobacter* and thus share similar genotypes. Some of these genotypes not only coexisted within a single free-range broiler barn and its environment but were also detected in the adjacent barns and farm environment; this suggests the spread of the microorganisms between the broilers and the surrounding environment. Similar findings have been described previously by Zweifel et al. (2008).

Our data indicated that the dynamics of *Campylobacter* spp. colonization and the dominant genotypes within a single barn depend on the time of sample collection. For example, the pre-existing dominant *C. coli* genotype was replaced with a new emerging *C. jejuni* in some free-range broiler barns. This implies that the newly acquired species could have been more successful in colonizing chickens. A new upcoming *C. coli* genotype isolated from the environment was unable to replace the pre-existing *C. coli* genotype, implying that it was less competitive than the pre-existing genotype. Competitive exclusion among *Campylobacter* species and genotypes in chickens during colonization may lead to one genotype replicating rapidly and becoming dominant (Hook et al., 2005; Colles et al., 2019).

The present study is the first to demonstrate that horizontal transmission (the environment to birds) played an essential role in the colonization of free-range broiler farms in Australia. Importantly, our data showed that the same *C. coli* genotype from the first production cycle in this study (Exp.1) remained in the environment before the chick placement and was subsequently detected in chicken feces in the associated flock during the following production cycle (Exp.2). This demonstrates the potential for carryover or reintroduction of *C. coli* between consecutive free-range broiler barns. Improved hygiene practices and appropriate biosecurity measures could reduce *Campylobacter* transmission in broiler farms (Smith et al., 2016).

As layer breeder chickens supply the eggs for multiple generations of broiler chickens, the possibility of vertical transmission of *Campylobacter* from layers to broilers is of interest. Carriage of *Campylobacter* spp. in the eggs, by the previous infection of the eggs within the breeder population, would provide a potential source for vertical transmission. Thus, if vertical transmission was an important source of broiler colonization, *Campylobacter* control in the layer birds could be an effective intervention point. Cox et al. (2012) suspected that *Campylobacter* could be transmitted from the breeder flock to the fertile eggs through the hatchery and to the broiler farms. However, a few studies have reported the same *C. jejuni* or *C. coli* strains in broiler breeder flocks and their progeny (Cox et al., 2002; Idris et al., 2006), thereby suggesting that the layer hens can be a potential source of *Campylobacter* spp. in broiler chickens, suggesting the possibility of vertical transmission.

The present study provides some evidence to support the possibility of vertical transmission as some isolates from breeder farms (n = 3) were the same genotypes as the isolates from their progeny in broiler flocks (n = 4) from the same region (approximately 500 km apart) and different regions (approximately 1,000 km apart). However, further studies are required to investigate this, as fecal samples from some breeder farms could only be collected after their corresponding chicks were placed at broiler farms or not at all in this study for commercial reasons. Consequently, it was not possible to determine the specific *Campylobacter* genotypes, if any, in the breeder farms at the time of egg laying.

Another possible mechanism of *Campylobacter* spp. transmission in hatching chicks could be the uptake of *Campylobacter* spp. from hatchery-related samples such as contaminated eggshells and tray liners in hatcheries (Byrd et al., 2007; Messelhausser et al., 2011). In the present study, sampling at the hatchery was not possible for commercial reasons. Because of these factors, directly tracing specific genotypes of *Campylobacter* spp. through the complete broiler production system was not possible. For future studies, sampling at the hatchery stage should be included to investigate the role of the hatchery in *Campylobacter* transmission. In addition, other molecular methods such as multilocus sequence typing and whole-genome analyses are required for greater understanding of *C. jejuni* and *C. coli* genotypes compared with global epidemiology.

CONCLUSIONS

Horizontal transmission was identified as the most frequent mode of colonization of free-range broiler chickens. Although dominant genotypes were identified, all free-range broiler flocks studied were exposed to or colonized by multiple *Campylobacter* genotypes earlier in the production cycle. Also of interest was the detection of diverse genotypes in the longer-lived layer birds, where it might be expected that the colonizing genotype may stabilize over time. Collectively, these data indicate that the colonization of chickens with *Campylobacter* is a complex and dynamic process and that effective ongoing control of this critical foodborne pathogen through the broiler production system will require a multifaceted approach.

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DISCLOSURES

The authors declare that they have no conflict of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.12.004.

REFERENCES

Australian Chicken Meat Federation. 2018. Chicken Meat Production. Accessed Dec. 2019. https://www.chicken.org.au/chicken-meat-production/.

Beery, J. T., M. B. Hugdahl, and M. P. Doyle. 1988. Colonization of Gastrointestinal Tracts of chicks by *Campylobacter jejuni*. Appl. Environ. Microbiol. 54:2365–2370.

Bull, S. A., V. M. Allen, G. Domingue, F. Jorgensen, J. A. Frost, R. Ure, R. Whyte, D. Tinker, J. E. Corry, J. Gillard-King, and T. J. Humphrey. 2006. Sources of *Campylobacter* spp. colonizing housed broiler flocks during rearing. Appl. Environ. Microbiol. 72:645–652.

Byrd, J., R. H. Bailey, R. Wills, and D. Nisbet. 2007. Recovery of *Campylobacter* from commercial broiler hatchery Trayliners. Poult. Sci. 86:26–29.

Colles, F. M., N. D. McCarthy, R. Layton, and M. C. Maiden. 2011. The prevalence of *Campylobacter* amongst a free-range broiler breeder flock was primarily affected by flock age. PLoS One 6:e22825.

Colles, F. M., S. G. Preston, K. K. Barfod, P. G. Flammer, M. C. J. Maiden, and A. L. Smith. 2019. Parallel sequencing of *porA* reveals a complex pattern of *Campylobacter* genotypes that

differs between broiler and broiler breeder chickens. Sci. Rep. 9:6204.
Cox, N. A., L. J. Richardson, J. J. Maurer, M. E. Berrang, P. J. Fedorka-Cray, R. J. Buhr, M. D. Lee, C. L. Hofacre, P. M. O'Kane, A. M. Lammerding, A. G. Clark, S. G. Thayer, and M. P. Doyle. 2012. Evidence for horizontal and vertical transmission in *Campylobacter* passage from hen to her progeny. J. Food Prot. 75:1896–1902.
Cox, N. A., N. J. Stern, K. L. Hiett, and M. E. Berrang. 2002. Identification of a new source of *Campylobacter* contamination in poultry: transmission from breeder hens to broiler chickens. Avian Dis. 46:535–541.
Devi, A. 2019. Prevalence of *Campylobacter* spp. in human clinical samples, PhD Thesis. Charles Sturt University, Wagga Wagga, Australia.
El-Shibiny, A., P. L. Connerton, and I. F. Connerton. 2005. Enumeration and diversity of Campylobacters and bacteriophages isolated during the rearing cycles of free-range and organic chickens. Appl. Environ. Microbiol. 71:1259–1266.
Ellis-Iversen, J., A. Ridley, V. Morris, A. Sowa, J. Harris, R. Atterbury, N. Sparks, and V. Allen. 2012. Persistent environmental reservoirs on farms as risk factors for *Campylobacter* in commercial poultry. Epidemiol. Infect. 140:916–924.
Free Range Egg and Poultry Australia. 2012. FREPA Range Care – Chicken Meat Bird Standards. Accessed Dec. 2019. http://www.frepa.com.au/standards/meat-standards/.
Gomes, C. N., R. A. Souza, J. Passaglia, S. S. Duque, M. I. Medeiros, and J. P. Falcao. 2016. Genotyping of *Campylobacter coli* strains isolated in Brazil suggests possible contamination amongst environmental, human, animal and food sources. J. Med. Microbiol. 65:80–90.
Gorman, R., and C. C. Adley. 2004. An evaluation of five preservation techniques and conventional freezing temperatures of -20°C and -85°C for long-term preservation of *Campylobacter jejuni*. Lett. Appl. Microbiol. 38:306–310.
Hermans, D., F. Pasmans, M. Heyndrickx, F. Van Immerseel, A. Martel, K. Van Deun, and F. Haesebrouck. 2012. A tolerogenic mucosal immune response leads to persistent *Campylobacter jejuni* colonization in the chicken gut. Crit. Rev. Microbiol. 38:17–29.
Hiett, K. L., N. J. Stern, P. Fedorka-Cray, N. A. Cox, and B. S. Seal. 2007. Molecular phylogeny of the *flaA* short variable region among *Campylobacter jejuni* isolates collected during an annual evaluation of poultry flocks in the Southeastern United States. Foodborne Pathog. Dis. 4:339–347.
Hook, H., M. A. Fattah, H. Ericsson, I. Vagsholm, and M. L. Danielsson-Tham. 2005. Genotype dynamics of *Campylobacter jejuni* in a broiler flock. Vet. Microbiol. 106:109–117.
Idris, U., J. Lu, M. Maier, S. Sanchez, C. L. Hofacre, B. G. Harmon, J. J. Maurer, and M. D. Lee. 2006. Dissemination of fluoroquinolone-resistant *Campylobacter* spp. within an integrated commercial poultry production system. Appl. Environ. Microbiol. 72:3441–3447.
Ingresa-Capaccioni, S., S. Gonzalez-Bodi, E. Jimenez-Trigos, F. Marco-Jimenez, P. Catala, S. Vega, and C. Marin. 2015. Comparison of different sampling types across the rearing period in broiler flocks for isolation of *Campylobacter* spp. Poult. Sci. 94:766–771.
ISO (International Organization for Standardization). 2006. Microbiology of food and animal feeding stuffs - Horizontal method for detection and enumeration of *Campylobacter* spp. Part 1: detection method. ISO 10272-1:2006. ISO, Geneva, Switzerland.
Meinersmann, R. J., L. O. Helsel, P. I. Fields, and K. L. Hiett. 1997. Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. J. Clin. Microbiol. 35:2810–2814.
Merchant-Patel, S., P. J. Blackall, J. Templeton, E. P. Price, S. Y. Tong, F. Huygens, and P. M. Giffard. 2010. *Campylobacter jejuni* and *Campylobacter coli* genotyping by high-resolution melting analysis of a *flaA* fragment. Appl. Environ. Microbiol. 76:493–499.
Messelhausser, U., D. Tharigen, D. Elmer-Englhard, H. Bauer, H. Schreiner, and C. Holler. 2011. Occurrence of thermotolerant *Campylobacter* spp. on eggshells: a missing link for food-borne infections? Appl. Environ. Microbiol. 77:3896–3897.
Messens, W., L. Herman, L. De Zutter, and M. Heyndrickx. 2009. Multiple typing for the epidemiological study of contamination of broilers with thermotolerant *Campylobacter*. Vet. Microbiol. 138:120–131.
Miele, M. 2011. The taste of happiness: free-range chicken. Environ. Plan A. 43:2076–2090.
Naald, B. V., and T. A. Cameron. 2011. Willingness to pay for other species' well-being. Ecol. Econ. 70:1325–1335.
O'Mahony, E., J. F. Buckley, D. Bolton, P. Whyte, and S. Fanning. 2011. Molecular epidemiology of *Campylobacter* isolates from poultry production units in southern Ireland. PLoS One 6:e28490.
Petersen, L., and S. L. W. On. 2000. Efficacy of flagellin gene typing for epidemiological studies of *Campylobacter jejuni* in poultry estimated by comparison with macrorestriction profiling. Lett. Appl. Microbiol. 31:14–19.
Prachantasena, S., P. Charununtakorn, S. Muangnoicharoen, L. Hankla, N. Techawal, P. Chaveerach, P. Tuitemwong, N. Chokesajjawatee, N. Williams, T. Humphrey, and T. Luangtongkum. 2016. Distribution and genetic Profiles of *Campylobacter* in commercial broiler production from breeder to slaughter in Thailand. PLoS One 11:e0149585.
Ridley, A. M., V. M. Allen, M. Sharma, M. Harris, and D. G. Newell. 2008a. Real-time PCR approach for detection of environmental sources of *Campylobacter* strains colonizing broiler flocks. Appl. Environ. Microbiol. 74:2492–2504.
Ridley, A. M., M. Toszeghy, S. A. Cawthraw, T. M. Wassenaar, and D. G. Newell. 2008b. Genetic instability is associated with changes in the colonization potential of *Campylobacter jejuni* in the avian intestine. J. Appl. Microbiol. 105:95–104.
Singh, M., and A. J. Cowieson. 2013. Range use and pasture consumption in free-range poultry production. Anim. Prod. Sci. 53:1202.
Singh, P., and Y. M. Kwon. 2013. Comparative analysis of *Campylobacter* populations within individual market-age broilers using *fla* gene typing method. Poult. Sci. 92:2135–2144.
Smith, S., L. L. Messam, J. Meade, J. Gibbons, K. McGill, D. Bolton, and P. Whyte. 2016. The impact of biosecurity and partial depopulation on *Campylobacter* prevalence in Irish broiler flocks with differing levels of hygiene and economic performance. Infect Ecol. Epidemiol. 6:31454.
Templeton, J. 2014. *Campylobacter* genotypes in chickens– national and regional influences from RIRDC report. Accessed Dec. 2019. https://rirdc.infoservices.com.au/items/14-032.
Vandeplas, S., R. Dubois-Dauphin, P. Palm, Y. Beckers, P. Thonart, and A. Théwis. 2010. Prevalence and sources of *Campylobacter* spp. contamination in free-range broiler production in the southern part of Belgium. Biotechnol. Agron. Soc. Environ. 14:279–288.
Vidal, A. B., F. M. Colles, J. D. Rodgers, N. D. McCarthy, R. H. Davies, M. C. Maiden, and F. A. Clifton-Hadley. 2016. Genetic diversity of *Campylobacter jejuni* and *Campylobacter coli* isolates from conventional broiler flocks and the Impacts of sampling Strategy and laboratory method. Appl. Environ. Microbiol. 82:2347–2355.
Walley, K., P. Parrott, P. Custance, P. Meledo-Abraham, and A. Bourdin. 2015. A review of French consumers purchasing patterns, perceptions and decision factors for poultry meat. Worlds Poult. Sci. J. 71:5–14.
WHO. 2012. The Global View of Campylobacteriosis: Report of an Expert Consultation. Accessed Dec. 2019. https://apps.who.int/iris/bitstream/handle/10665/80751/9789241564601_eng.pdf;jsessionid=B297E0E7F6F9707F4F55E20FC43740B2?sequence=1.
Yano, S., T. Kira, Y. Morishita, K. Ishihara, T. Asai, T. Iwata, M. Akiba, and T. Murase. 2013. Colonization of chicken flocks by *Campylobacter jejuni* in multiple farms in Japan. Poult. Sci. 92:375–381.
Zweifel, C., K. D. Scheu, M. Keel, F. Renggli, and R. Stephan. 2008. Occurrence and genotypes of *Campylobacter* in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms. Int. J. Food Microbiol. 125:182–1.