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# Population dynamics of *Campylobacter jejuni* in healthy dairy calves reared with and without exposure to wild birds

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## Abstract

**Introduction:** In order to understand the emergence, persistence, and transmission of *Campylobacter jejuni* in livestock, this longitudinal study characterized the *C. jejuni* population in young calves and assessed the impact of exposure to wild birds during the pre-weaning period.

**Methods and results:** Faecal samples were collected on eight occasions from 48 calves housed in three pens between birth and 10 weeks of age, two of the pens being covered with orchard netting. From the 250 *C. jejuni* isolates obtained from the positive faeces, seven distinct sequence types (7-gene legacy MLST) were identified, with high genetic similarity of circulating strains. On each pen, sequential dominance of ST was observed, with ST-508 and ST-50 prevalent in calves under 4 weeks old, and ST-520 or ST-422 prevalent in the 8- to 10-week-old calves. Exposure of calves to wild birds did not influence genotypes distribution, $\alpha$-diversity, and community dissimilarity. However, a difference in *C. jejuni* populations between the two groups of calves that were not exposed to birds suggests that calving rank may shape the type and succession of ST detected over time.

**Conclusion:** This study provided no evidence for exposure to wild birds being a driver for *C. jejuni* population changes in healthy pre-weaned calves.

## Impact Statement

Cattle are an important source of human campylobacteriosis via food and environmental exposures. Understanding the factors shaping *Campylobacter jejuni* populations in cattle, including the role of herd management practices around calving, will help with developing interventions to reduce carriage in this reservoir and protect public health.

**Keywords:** *Campylobacter jejuni*; One Health; livestock; wild bird; early life infection; on-farm mitigation

## Introduction

*Campylobacter jejuni* is a leading cause of bacterial foodborne diarrhoeal illness worldwide. Human infections with *C. jejuni* are typically associated with mild clinical symptoms and are self-limited; however, late-onset complications include reactive arthritis or Guillain-Barré syndrome, a type of autoimmune-mediated paralysis (WHO 2020). In recent years, the hospital admission rate due to severe campylobacteriosis has risen significantly, reaching 10%–18% of the reported cases in several countries (Tack et al. 2020, Horn et al. 2023, ECDC 2024).

*Campylobacter jejuni* is carried in the digestive tract of animals, including poultry, cattle, pigs, and wild birds (Ogden et al. 2009). Poultry and ruminants have been identified as the most important sources of human infections, with cattle estimated to be responsible for 14%–28% of human cases in developed countries (Mughini Gras et al. 2012, Lake et al. 2021, Pascoe et al. 2024). Routes for infection from cattle include consumption of raw or unpasteurized milk and dairy products, drinking water from private wells, swimming in natural waters, or exposure to contaminated faeces or animals (Gilpin et al. 2008, Gilpin et al. 2020, Lake et al. 2021, Sorgentone et al. 2021). Campylobacter notification rates increase with decreasing urbanization, as rates in rural areas are found to be three-fold greater than those in urban areas (Lake et al. 2021, Horn et al. 2023). In rural areas, children <10 yrs old were found to be particularly at risk of being infected from a ruminant source (Mullner et al. 2010). From a One Health perspective (Mettenleiter et al. 2023), risk mitigation in cattle could thus have direct beneficial impact on the public health sector.

Several studies have examined the potential sources and pathways for cattle infection, and *C. jejuni* has been repeatedly detected in several wild bird species in bovine production environments (Busato et al. 1999, Ellis-Iversen et al. 2009, Hald et al. 2016). Birds' ecological habitats or feeding habits affect *Campylobacter* prevalence, infection risk, and transmission potential (Waldenström et al. 2002, Olvera-Ramírez et al. 2023). For example, wild birds that eat animal-origin food or forage on the ground near animal farms were found to have a greater *Campylobacter* prevalence than those foraging far away from farmed animals or hunt in the air, and a

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positive correlation between *C. jejuni* prevalence in wild birds and cattle manure has been established (Hald et al. 2016). Another study showed that accessibility of cattle feed to birds was a possible risk factor for *C. jejuni* presence in dairy cows (Wesley et al. 2000). Molecular characterization of *Campylobacter* strains emphasized that wild birds carry strains of *Campylobacter* indistinguishable from those in domestic animals, but also carry a more diverse *Campylobacter* population compared to cattle and may introduce novel or rare genotypes into the herds (Sippy et al. 2012, Hald et al. 2016, Rapp et al. 2020). These epidemiological studies highlighted the possible role of wild birds in sustaining the presence of *C. jejuni* on farms. However, the likelihood and direction of transmission from wild birds to cattle have not yet been clearly determined.

One of the additional complicating factors in *Campylobacter* transmission on cattle farms is the considerable variation in shedding duration and levels among animals, with a small proportion of animals appearing to be more susceptible to *C. jejuni* and excreting the bacteria frequently or in high concentration (Inglis et al. 2004, Minihan et al. 2004, Hakkinen and Hänninen 2009, Rapp et al. 2012). Field studies of naturally infected cattle showed that young animals can have a higher prevalence and higher faecal concentration than older animals (Nielsen 2002). It is unclear whether the different shedding patterns of *C. jejuni* by dairy animals are due to an initial infection and subsequent colonization with a single population of bacteria, or if they are the result of multiple repeated infections from the same source—including wild birds living on cattle farms.

In this study, we performed whole-genome sequencing analysis on *C. jejuni* isolates from dairy calves exposed or not to wild birds during the pre-weaning period. The aim was to compare genotypic profiles recovered from calves over time, to better characterize the impact of exposure to wild birds on *C. jejuni* infection in calves and to trace strain transmission among animals housed in the same pen. We hypothesized that calves exposed to wild birds would excrete a more diverse population and would excrete *C. jejuni* more frequently. A second aim was to examine the genetic variation of *C. jejuni* isolates within an individual animal or group of animals, to explore how these pathogens adapt and survive within their host. Understanding how certain strains may be introduced and survive within an individual or a group of bovine animals may advance the identification of novel management practices for better control of this important zoonotic bacteria on cattle farms.

## Materials and methods

### Animals and experimental study design

The study was conducted on a research dairy farm (Massey University, Palmerston North, New Zealand). It was a descriptive observational study involving three pens of calves. Each pen represented a different combination of exposure to birds (as part of improved biosecurity measures) and calving period (described below). All procedures involving animals were approved by the AgResearch Animal Ethics Committee (AE application number 15 024) under the New Zealand Animal Welfare Act 1999.

Forty-eight (Friesian x Jersey breed) female dairy calves were used in the study. A detailed description of selection, housing, and rearing conditions of the calves can be found in Rapp et al. (2023). Briefly, calves born on straw bedding in a covered calving area were transferred to the farm calf-rearing facility within a maximum of 12 h after birth. This facility consisted of a barn with a solid roof and three side walls. There were three adjacent pens (identified by Pen 1, Pen 2, and Pen 3) separated by 1.8 m high clear plastic sheeting to prevent physical interaction between calves in the different pens. Pens 1 and 3 were fully enclosed with orchard bird netting (1 $cm^2$ square holes) to exclude wild birds; birds could access Pen 2 from one side. Pen 2 was located between Pen 1 and Pen 3 (Fig. 1). The water troughs in Pens 1 and 3 were connected to the town drinking water supply, while the water trough in Pen 2 was connected to roof-collecting rainwater tanks and was potentially contaminated by wild birds. To reduce pathogen spread between pens, all personnel used a boot wash before entering the rearing facility. They also wore waterproof gear that was cleaned with hot soapy water and surface disinfecting wipes (Mediwipes, Sulco, Auckland, New Zealand) before entering any pen. Furthermore, single-use cover shoes and gloves were worn and changed between pens. Calves were fed 4 L of colostrum within 12 h of birth, then 5.2 L in two daily feeds for 8 d, and 6 L of milk in two feeds per day after day 10. Both colostrum and milk were sourced from the farm milking herd and collected aseptically from the bulk tank. Feeding equipment (e.g. teats and bottles) was allocated to a given pen for the duration of the trial and washed daily with warm soapy water. All calves were offered hay and calf starter (20% CP, 40%–42% starch, 15%–16% NDF; Calf Max 20% pellets; SealesWinslow Ltd, Morrinsville, New Zealand) in open feed troughs before they were 1 and 3 weeks old, respectively. Calf starter allocated to Pen 1 and Pen 3 was stored in a 2000 L Smart Stakka silo (Advantageplastics, Waimakariri, New Zealand) to prevent contamination by bird droppings, while calf starter allocated to Pen 2 was stored in open plastic containers. Visual inspections of the feed troughs, water troughs, and pen walls for moist bird droppings confirmed the exposure status of calves to wild birds throughout the study period, with the presence of fresh bird droppings in the feed troughs recorded for 37% (22/60) of the daily inspections in Pen 2; but for none (0/59) of the inspections in both Pen 1 and Pen 3.

New Zealand has a seasonal calving system where the majority of dairy cows calve within a short timeframe (~6 weeks). The first calves born in the study's season were allocated to Pen 1, when the number of calves born per day was low (7 d to fill the pen to capacity; 23 calves). When more calves were born per day, Pen 2 and Pen 3 were filled simultaneously until they reached the pen capacity (25 calves/pen; 8 d to fill both pens). This was done to minimize the age difference between calves in Pens 2 and 3. Space allowance on each pen was 2.4 $m^2$/calf. The first 16 calves introduced in each pen were enrolled in the study so that most studied calves entered an environment (pen) with as few pathogens as possible. Each calf was identified by a unique farm identification ear tag.

Faecal samples were collected from each enrolled calf within 2 d after arrival in the pen ('week 0'), then calves were sampled once weekly for 4 consecutive weeks ('week 1' to 'week 4'). Another three-week sampling period with weekly samplings was undertaken when the calves were 2 months old ('week 8' to 'week 10'). Calf ages at sampling were 1.5 ± 0.5 d, 6.5 ± 2 d, 13.5 ± 2 d, 20.5 ± 2 d, 28 ± 2 d, 58 ± 3 d, 65 ± 3 d, and 72 ± 3 d for weeks 0, 1, 2, 3, 4, 8, 9, and 10, respec-

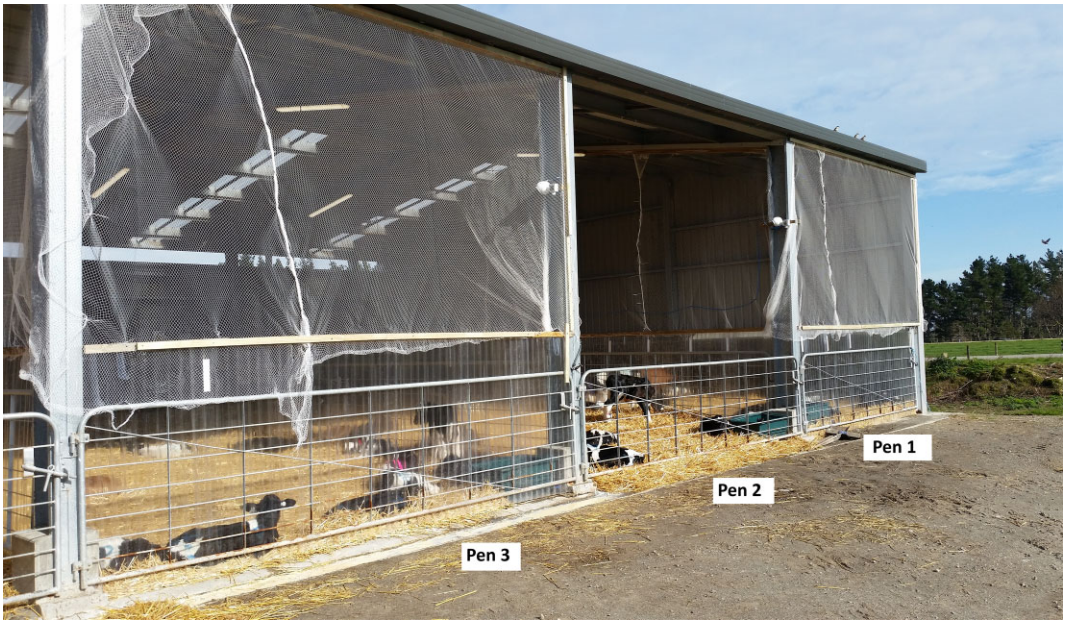


**Figure 1.** Experimental setup of calf pens. Three adjacent pens (Pen 1, Pen 2, and Pen 3) were separated by clear solid plastic sheeting to prevent physical interaction between calves. Two of the pens (Pen 1 and Pen 3) were fully enclosed with orchard bird netting, while Pen 2 (located between Pen 1 and Pen 3) was accessible to wild birds. Water troughs in Pens 1 and 3 were connected to the town supply, while Pen 2 used roof-collected rainwater. The supplementary feed given to the calves in Pens 1 and 3 was stored in closed containers silos, while it was left in open bags for Pen 2. Visual inspections (presence of bird droppings in the feed and water troughs) confirmed the exposure status to wild birds in Pen 2 but not in Pens 1 and 3. Personnel used strict hygiene protocols to minimize pathogen spread among pens. All calves remained in their allocated pen for the entire duration of the trial (day 1 to week 10).

tively. Faecal sample collection was done by direct retrieval after digital rectal stimulation. All faecal samples were transported to the laboratory in insulated containers and analysed for *C. jejuni* within 6 h of collection.

## Detection and isolation of *C. jejuni*

The presence of *C. jejuni* in the collected samples was tested by inoculation of 0.1 g aliquots of each calf's homogenized faecal sample in Exeter Broth (Fort Richard Laboratories Ltd, Auckland, New Zealand) and on mCCDA agar, as previously described (Rapp et al. 2023). Confirmation of the microbial cells as *C. jejuni* was done using TaqMan *Campylobacter* Multiplex Assay Beads (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA) in an ABI 7500 Fast qPCR instrument (Applied Biosystems™). Bacterial cells were stored in charcoal Amies transport medium (Fort Richard Laboratory Ltd) at −80°C until purification of single-colony isolates for genotyping.

## Genomic analysis of *C. jejuni*

A total of 250 *C. jejuni* isolates, comprising one isolate from each of the 239 *C. jejuni*-positive faeces and an additional second isolate from 11 of the positive faeces, were prepared for whole genome sequencing analysis. Resuscitation of the bacterial cells stored in charcoal Amies transport medium and isolation of single colonies was performed using the conditions described above. The confirmation that the single-colony isolates were *C. jejuni* was done by MALDI-ToF mass spectrometry using the Extended Direct Transfer Method in the Bruker MALDI Biotyper® (Bruker Corporation, Billerica, MA, USA) following the manufacturer's recommendations. High-quality genomic DNA was extracted from each confirmed isolate using the Qiagen PCR purification kit (QIAGEN, Hilden, Germany). Genomic DNA sequencing libraries were generated using the Illumina Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, USA). The libraries were sequenced by Massey Genome Service (Massey University, Palmerston North, New Zealand) on the HiSeq™ X platform (Illumina Inc.), producing 3.4 million 150 bp paired-end reads per sample. The quality of the raw sequence data was checked using the SolexaQA software (Cox et al. 2010). The raw reads were processed using the Nullarbor pipeline (version v.2.0.20191013) (Seemann et al. 2017). As part of this bioinformatics pipeline, the reads were assembled using SKESA (v.2.4.0) and annotated using Prokka (v.1.14.6). The assemblies were checked for completeness using BUSCO v.5.7.1 with the campylobacterales_odb10.2024–01-08 database (Manni et al. 2021). Clonal complexes (CC-) and sequence types (ST-) of the assembled genomes were determined using the multi-locus sequence typing (MLST) tool (v.2.19.0). Resistance genes were identified with ABRicate (v.1.0.1) using the NCBI AMRFinderPlus (v.2021–03-28). Core genome multi-locus sequence typing (cgMLST) was performed using the external platform Pathogenwatch (https://pathogen.watch, accessed 1 August 2023). The cgMLST profiles were assigned based on the combination of alleles detected at 1343 loci (cgMLST Oxford scheme) (Cody et al. 2017). The differences in cgMLST profiles were visualized using minimum spanning trees generated by Grape Tree (v.2.1) (Zhou et al. 2018) using the neighbour-joining (NJ) algorithm.

For each isolate, analysis of single-nucleotide polymorphisms (SNPs) was performed using the snippy pipeline v.4.6.0 (Seemann 2020) against the reference CC-21 genome *C. jejuni* subsp. *jejuni* NCTC 11168 (NCBI accession AL111168.1). As part of this pipeline, variants were called, and their potential effect on protein function was assessed.

Mutations in the DNA sequence were classified as synonymous (no change in the amino-acid sequence), non-synonymous (led to amino-acid substitution), and gene disruptive (frameshift or nonsense mutation that impaired or stopped a gene's function). The Illumina raw sequencing reads have been uploaded to NCBI database under BioProject (PRJNA1198386).

### Statistical analysis

All statistical analyses were performed using R v.4.4.1 (Team 2021). Differences in the number of disrupted gene functions among sequence types (ST) were assessed using analysis of variance (ANOVA) followed by post-hoc tests, using the predictmeans R package version 1.1.0. The relative levels of *C. jejuni* population diversity across the three different pens were investigated using the distribution of cgMLST genotypes. The $\alpha$-diversity was calculated using the R package iNEXT (Hsieh et al. 2016), which allows for the estimation of diversity across three parameter orders 'q' (q = 0, 1, 2), respectively representing cgMLST richness, Shannon diversity (the exponential of Shannon entropy), and Simpson diversity (the inverse of Simpson concentration). The relationship among *C. jejuni* populations in different pens was examined by generating dissimilarity matrices summarizing pairwise similarities of ST between each pair of pens. The degree of correlation between populations was tested using the Mantel test, where a value of 1 indicates indistinguishable populations. The significance of genetic differentiation was tested with 999 permutations. The Mantel tests were performed using the vegan R package v.2.6–8 (Dixon 2003). Correlations between the number of *C. jejuni*-positive samples, the number of ST detected, and the number of unique cgMLST profiles for each calf were calculated using the cor.test function in R using the Pearson's product moment as correlation coefficient.

## Results

### *Campylobacter jejuni* strains

A total of 250 *C. jejuni* isolates were obtained from the studied dairy calves over a 3-month period (84, 83, and 83 isolates from Pen 1, Pen 2, and Pen 3, respectively). Sequencing coverages ranged from 26-fold to 1208-fold, averaging 312-fold. The average nucleotide quality (Phred score) was over 30 for all samples. The number of contigs ranged from 12 to 56 per genome with a median of 25 (Supplementary material 1A). The N50 values ranged between 65 266 and 909 618 base pairs (bp), while the assembly size ranged from 1 610 443 to 1 735 324 bp with a GC content range of 30.2%–30.9%. The average size of the assembled *C. jejuni* genomes was 1.66 ± 0.012 million bp (range: 1.61–1.74 million bp). The genome completeness, assessed by BUSCO, ranged between 99.0% and 99.7%. A total of 1182 core genes were identified, while a set of additional 3253 genes was assigned to the accessory gene content, with each accessory gene identified in at least one of the genomes.

Characterization of the isolates by *in silico* 7-gene MLST revealed five different Clonal Complexes (CC-21, CC-508, CC-61, CC-45, and CC-42) and seven Sequence Types (ST-50, ST-520, ST-422, ST-508, ST-61, ST-45, and ST-42) (Table 1). CC-21 was dominant, accounting for 66% (166/250) of the isolates. Within this CC, the most frequently detected STs were ST-50 (30.8% isolates) and ST-520 (23.6% isolates). These

**Table 1.** Comparison of the genetic variations of the *C. jejuni* isolates ($n$ = 250) obtained from 48 dairy calves during their first 2 months of life.

| CC | ST (number isolates) | Genome size average ± SDT error | Number coding DNA Sequence (or CDS) average ± Sdt error | Number synonymous mutations average ± Sdt error | Number non-synonymous mutations average ± Sdt error | Number genes-disruptive mutations average ± Sdt error |
|---|---|---|---|---|---|---|
| CC-21 | ST-50 ($n$ = 77) | 1 660 547 ± 873 | 1711 ± 1 | 4825 ± 2 [a] | 2301 ± 2[a] | 114 ± 0[a] |
| | ST-422 ($n$ = 30) | 1 644 084 ± 1454 | 1698 ± 2 | 5554 ± 5 [b] | 2533 ± 4[b] | 122 ± 1[b] |
| | ST-520 ($n$ = 59) | 1 668 801 ± 2958 | 1709 ± 4 | 5731 ± 4 [c] | 2681 ± 3[c] | 143 ± 0[c] |
| CC-508 | ST-508 ($n$ = 62) | 1 656 627 ± 1253 | 1674 ± 2 | 16 521 ± 5 [g] | 6230 ± 5[g] | 227 ± 1[g] |
| CC-61 | ST-61 ($n$ = 20) | 1 640 047 ± 9507 | 1698 ± 12 | 10 035 ± 28 [d] | 4115 ± 15[d] | 174 ± 1[d] |
| CC-45 | ST-45 ($n$ = 1) | 1 610 443 | 1615 | 16 305 [f] | 5971[f] | 216[f] |
| CC-42 | ST-42 ($n$ = 1) | 1 636 871 | 1662 | 12 402 [e] | 4987 [e] | 194 [e] |
| | **Total = 250** | **1 657 612 ± 1261** | **1698 ± 2** | **8520 ± 307** | **3564 ± 103** | **155 ± 3** |
| | **ANOVA *P*-values** | | | **<0.001** | **<0.001** | **<0.001** |

The clonal complexes (CC) and sequence type (ST) of the assembled genomes were determined using the multi-locus sequence typing (MLST) tool (v.2.19.0) of the Nullarbor pipeline (v.2.0.20191013). Variants were called using the snippy pipeline v.4.6.0. Differences in the number of gene mutations among ST were assessed using analysis of variance (ANOVA) followed by post-hoc tests, utilizing using the predictmeans R package version 1.1.0. Superscript letters indicate significant difference at $P < 0.05$.

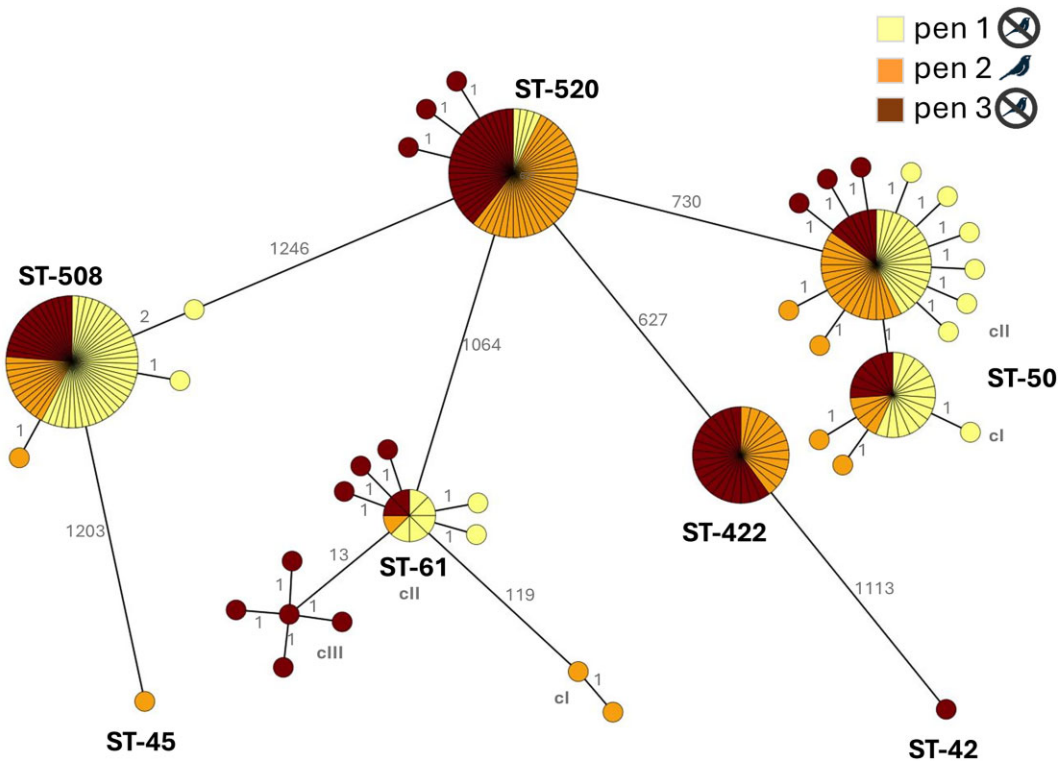


**Figure 2.** Minimum spanning tree (MST) of *Campylobacter jejuni* core genome sequence types (cgMLST) from 48 calves that were group-reared with (Pen 2) or without (Pen 1 and Pen 3) exposure to wild birds during the first 2 months of life. The 250 isolates were obtained from repeat collection of faeces from 16 of the calves housed on Pen 1, 16 of the calves housed on Pen 2 and 16 of the calves housed on Pen 3. One colony was obtained for each *C. jejuni*-positive faecal sample, except for 11 faecal samples (2 colonies). MST was calculated by pairwise comparison of 1343 target genes. Nodes correspond to a unique clonal group, the circle diameter is proportional to the number of isolates within it and the colour corresponds to a pen. cI, cII, and cIII indicate genetic clusters within each ST. The number of allelic differences between nodes is shown on branches. The MST analysis was carried out by GrapeTree version 1.5.0 using the neighbour-joining (NJ) algorithm.

two STs were widespread, detected in 28 and 26 calves out of the 48 studied calves, respectively. ST-422 (also belonging to CC-21) accounted for 12% of the isolates (14 calves). CC-508 (ST-508) was the second most dominant clonal complex, accounting for 25% isolates (27 calves). CC-61 (ST-61; 8% isolates) was present in fewer (11) calves. CC-42 (ST-42) and CC-45 (ST-45) were infrequently detected (<1% isolates), found in one calf each. Intra-sample diversity was low: in 9 out of the 11 samples from which more than one isolate was tested, the first and second isolate belonged to identical sequence types. The exceptions were for isolates from calf 44 at 9 weeks old (ST-61 and ST-508) and isolates from calf 26 at 2 d old (ST-61 and ST-42).

There were significant ($P < 0.05$) differences in the number of non-synonymous mutations and gene disruptive mutations among the ST (Table 1), with ST-508 isolates yielding the largest number of mutations affecting protein expression compared to the reference genome. All isolates except the ST-508 isolates and the ST-45 isolate carried a $bla_{\text{OXA-605}}$ gene. No other predicted resistance to antimicrobial compounds was identified via the Nullarbor pipeline.

The visualization of the genomic relationships among the *C. jejuni* isolates based on cgMLST allelic profiles revealed the presence of two closely related genetic clusters for ST-50 (identified by cluster I and cluster II, and represented by 51 and 26 isolates, respectively) and three different clusters for ST-61 (represented by 2, 13, and 5 isolates, respectively) (Fig. 2). For ST-50, most isolates belonged to two cgMLST profiles (accounting for 40 and 23 isolates), separated by a single allele variant (in CAMP1213). The two ST-50 cgMLST clusters were detected in all three pens regardless of exposure to birds or calving period. For ST-61, the population was more diverse. There were 211 consistent allele differences between cluster I and cluster II (including 91 alleles only present in either I or II), 223 allele difference between cluster I and cluster III (including 94 only present in either I or III), and 16 allele difference between cluster II and cluster III (including 5 only present in either II or III). This clustering was confirmed by core SNP analysis for both ST-50 (<6 SNP difference among strains attributed to ST-50) and ST-61 (557 SNP difference between cI and cII; 578 SNPs difference between cI and cIII; 21 SNP difference between cII and cIII) (Supplementary material 1B). Of the three ST-61 cgMLST clusters, ST-61 cI was detected in the group of calves exposed to birds (Pen 2), ST-61 cIII was detected in one group of calves not exposed to birds (Pen 3), while ST-61 cII was detected in all three pens. ST-508, ST-520, and ST-422 clusters had low genetic variability according to both the cgMLST analyses and core genome SNP, with similar cgMLST allelic profiles, and low number (<5, <4, and < 2) of core SNPs. Both ST-508 and ST-520 were found irrespectively of the exposure of the calves to wild birds, as they were detected in each three pens. ST-422 was detected in one group of calves that were not exposed to birds (Pen 3) but not in the other (Pen 1); it was also detected in calves that were exposed to birds (Pen 2).

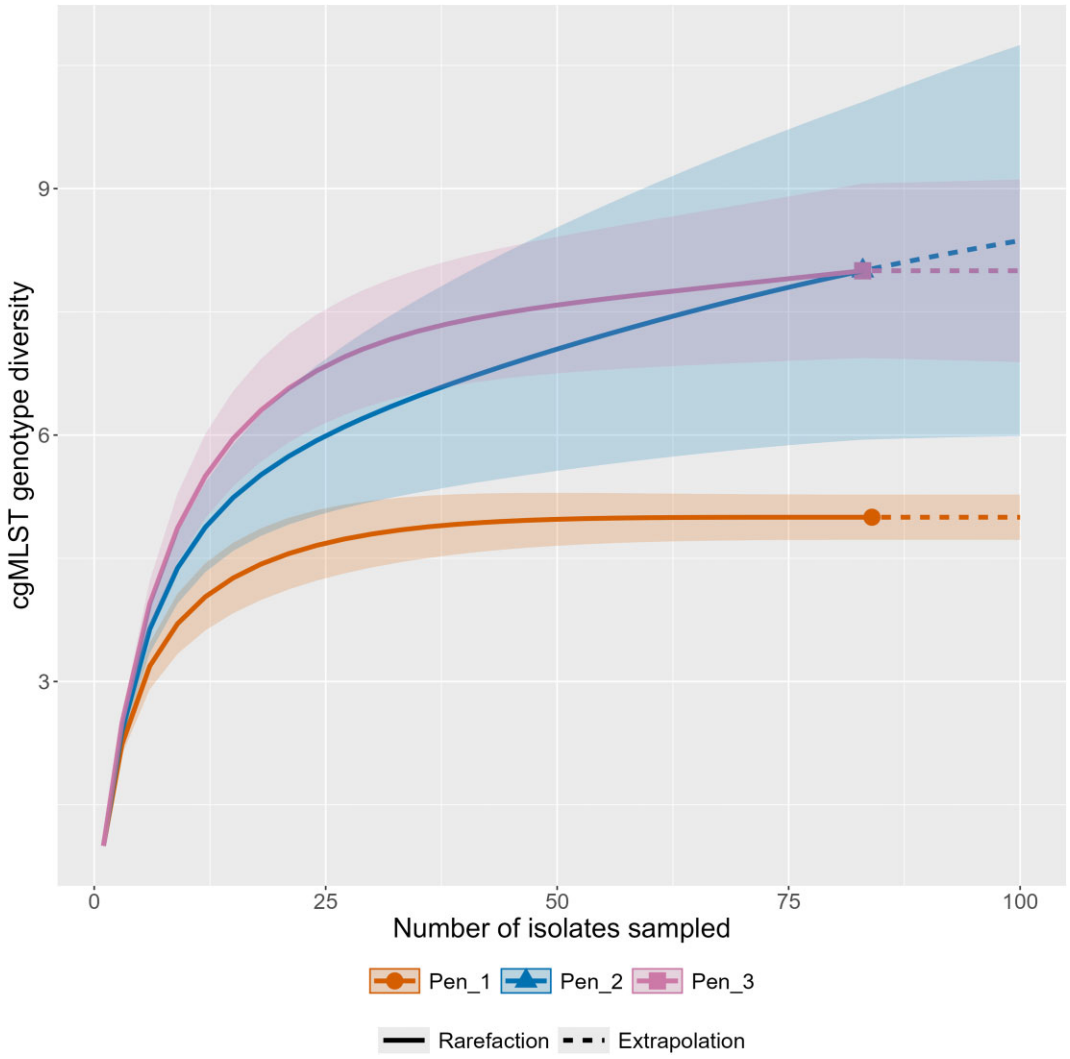


**Figure 3.** Sample size-based rarefaction curves of *C. jejuni* cgMLST isolated from calves housed in three pens (16 studied calves/pen) during the preweaning period. Birds were excluded from Pen 1 and Pen 3 through the use of orchard netting, town water supply, and closed storage conditions for the supplementary feed. The horizontal axis of the plot represents the number of samples used for analysis and the vertical axis represents the diversity or the number of cgMLST ST identified in the specified number of samples. Bootstrapped 95% confidence intervals were indicated by shaded areas.

## Number of unique cgMLST profiles

Several single locus variants of the cgMLST profiles were observed for the ST-50 clusters (14 variants in 77 isolates) and the ST-61 clusters (10 variants in 20 isolates). Single locus variants were less frequently detected within ST-520 (3 variants in 59 isolates) and ST-508 (3 variants in 62 isolates). Across the 1343 loci defined by the cgMLST *C. jejuni* scheme, the variants constantly exhibited allelic differences—excluding missing alleles—in eight loci (CAMP 0194, CAMP 0254, CAMP 0368, CAMP 0410, CAMP 0811, CAMP 1109, CAMP 1213, or CAMP 1413) for ST-50, in one locus only (CAMP 1214) for ST-61, in two loci (CAMP0805 and CAMP 1130) for ST-520, and in three loci (CAMP0410, CAMP 1458, CAMP 1461) for ST-508.

## *Campylobacter jejuni* population structure and differentiation over time

Rarefaction curves (Fig. 3) indicated a greater ST richness of the *C. jejuni* population in calves born during the peak of the calving season, independent of exposure to birds (Pen 2 and Pen 3; eight distinct cgMLST profiles), compared to calves born earlier and reared without exposure to birds (Pen 1; five distinct cgMLST profiles). $\alpha$-diversity of the *C. jejuni* community was not associated with the exposure of calves to wild birds (Supplementary material 2). However, there was a difference in $\alpha$-diversity indexes between the two groups of calves that were not exposed to birds, with lower diversity observed for calves born early (Pen 1: asymptotic estimate of Shannon diversity: 4.03, 95% CI: 3.52–4.53; asymptotic estimate of Simpson diversity: 3.48, CI: 2.85–4.12) compared to calves born at the peak of the calving season (Pen 3; asymptotic estimate of Shannon diversity: 6.42, CI: 5.51–7.33; asymptotic estimate of Simpson diversity: 5.53, CI: 4.40–6.67).

Irrespective of exposure to wild birds or calving period, the *C. jejuni* population in each group of calves was characterized by early dominance of some STs, followed by subsequent rapid domination of other STs (Fig. 4). As observed for the *C. jejuni* population richness, the calves' exposure to birds was not associated with changes in ST detected over time: the

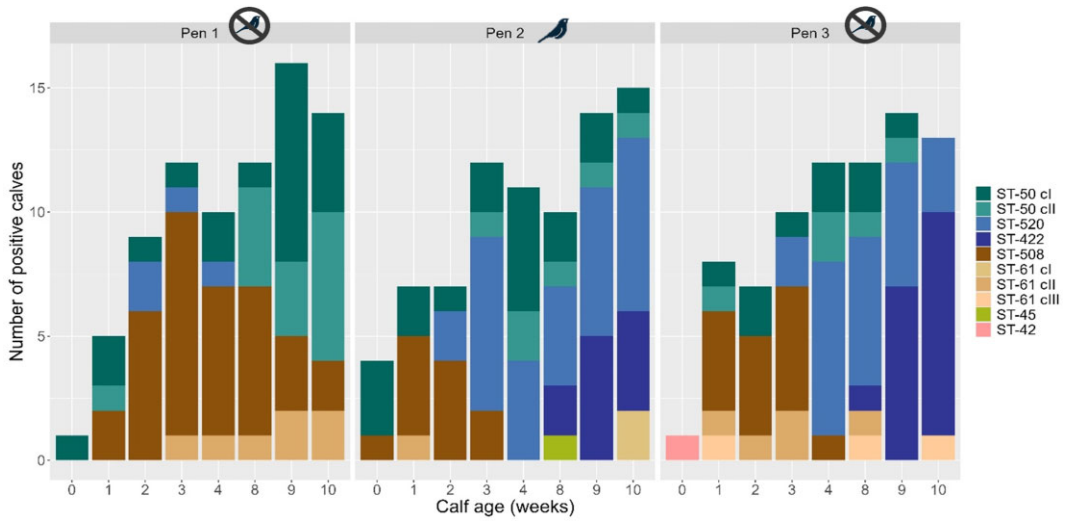


**Figure 4.** Genetic composition of *C. jejuni* population over time on each pen. *Campylobacter jejuni* isolates were obtained from 16 of the calves housed on Pen 1, 16 of the calves housed on Pen 2, and 16 of the calves housed on Pen 3, for a total of 84, 83, and 83 isolates from Pen 1, Pen 2, and Pen 3, respectively. Space allowance on each pen was 2.4 $m^2$/calf. Birds were excluded from Pen 1 and Pen 3 through the use of orchard netting, town water supply, and closed storage conditions for the supplementary feed. Each of the sequence type (ST) and cluster (cI, cII, or cIII) determined by the cgMLST Oxford scheme and the MST analysis is denoted using a specific colour.

distribution of STs was more similar between Pens 2 and 3 (Mantel test r = 0.4191, $P = 0.08$) when compared to Pen 1 (Mantel tests between Pen 1 and Pen 2 or between Pen 1 and Pen 3: r < 0.08, $P > 0.3$). In calves born early in the calving season and reared with restricted exposure to birds (Pen 1), ST-508 had a greater relative abundance in young (<4 weeks) calves, detected in nine calves, while ST-50 was the dominant ST detected in older (8- to 10-week-old) calves (11 calves). In calves born at the peak of the calving season and reared with or without restricted exposure to birds (Pens 2 and 3), ST-508 was also detected in the young calves (5 calves total) but was not detected in any of the older calves. Instead, the dominant STs in the 8- to 10-week-old calves were ST-520 (detected in 11 calves in Pen 2 and 8 calves in Pen 3) and ST-422 (5 calves in Pen 2 and 9 calves in Pen 3). ST-61 was detected in up to two calves per sampling occasion in Pen 1, and sporadically in Pen 2 and Pen 3.

### Calf-level variation

The studied calves excreted up to five different 7-gene MLST STs over the 10-week period (Fig. 5 and Supplementary material 1C). The large majority (77%; 37/48 calves) excreted between 2 and 3 different STs, and a minority (6%: calves C23, C25, and C26) excreted four or more different STs. As expected, the numbers of ST detected per calf correlated positively with the total number of *C. jejuni* isolates ($R^2$: 0.56, 95% CI: 0.32–0.73, $P < 0.001$). Similarly, the number of unique cgMLST profiles correlated positively to the total number of *C. jejuni* isolates ($R^2$: 0.66, 95% CI: 0.41–0.82, $P < 0.001$). Unique cgMLST profiles appeared to be more frequent in a small proportion (6/48) of calves, with four calves (C5, C8, C9, C26) found to each excrete two ST-50 unique profiles, one calf (C26) excreting four different ST-61 unique profiles, one calf (C34) excreting two of the three ST-520 unique profiles, and another calf (C13) excreting two of the three ST-508 unique profiles.

## Discussion

Establishing the genetic diversity and succession of the different strains of zoonotic organisms—such as *C. jejuni*—in livestock animals can provide information on transmission dynamics, persistence, or emergence of zoonosis. The results of our longitudinal monitoring of 48 calves from birth to 2 months of age demonstrated a time-dependent shift of the *C. jejuni* population in the gut of pre-weaned dairy calves. It provided evidence for a clear succession dynamic where the genotypes detected in early life were replaced by other genotypes as the calves grew. This finding was consistent across the three pens regardless of calf's exposure to wild birds. While not all calves on each pen were monitored, our findings also suggested that some genotypes might be consistently present in the animals, but not necessarily detected.

Overall, the *C. jejuni* isolates detected in the calves of our study were dominated by ST-21 and ST-508 complexes, followed by ST-61 and ST-42 and ST-45 complexes. Three of these complexes (CC21, CC42, CC61) have been previously detected in pre-weaned and weaned calves (Kwan et al. 2008, Klein-Jöbstl et al. 2016, Hansson et al. 2021) as well as in adult cattle (Kwan et al. 2008, Pascoe et al. 2024). Previously published studies reported STs prevalence according to cattle management class or broad age of animals (Kwan et al. 2008, Klein-Jöbstl et al. 2016, Hansson et al. 2021). In comparison, our monitoring revealed that a dominant ST-508 in the gut of young calves could be replaced within four to eight weeks post-partum by ST belonging to the ST-21 complex. This was unexpected, as ST-508, alongside ST-61 and ST-42 complexes, has been identified as a specialist lineage associated with cattle, while the ST-21 complex is a multi-host generalist lineage (Pascoe et al. 2024). However, our findings are in agreement with the study by Hansson et al. (2021), who observed that the CC21 was the CC most frequently detected in calves (mean age 113 d) on seven farms in Sweden, while the ruminant lineages CC61 and CC42 were detected more sporadically. Similarly, Kwan et al. (2008) reported a similar or greater prevalence of CC21 in pre-weaned and weaned calves compared to

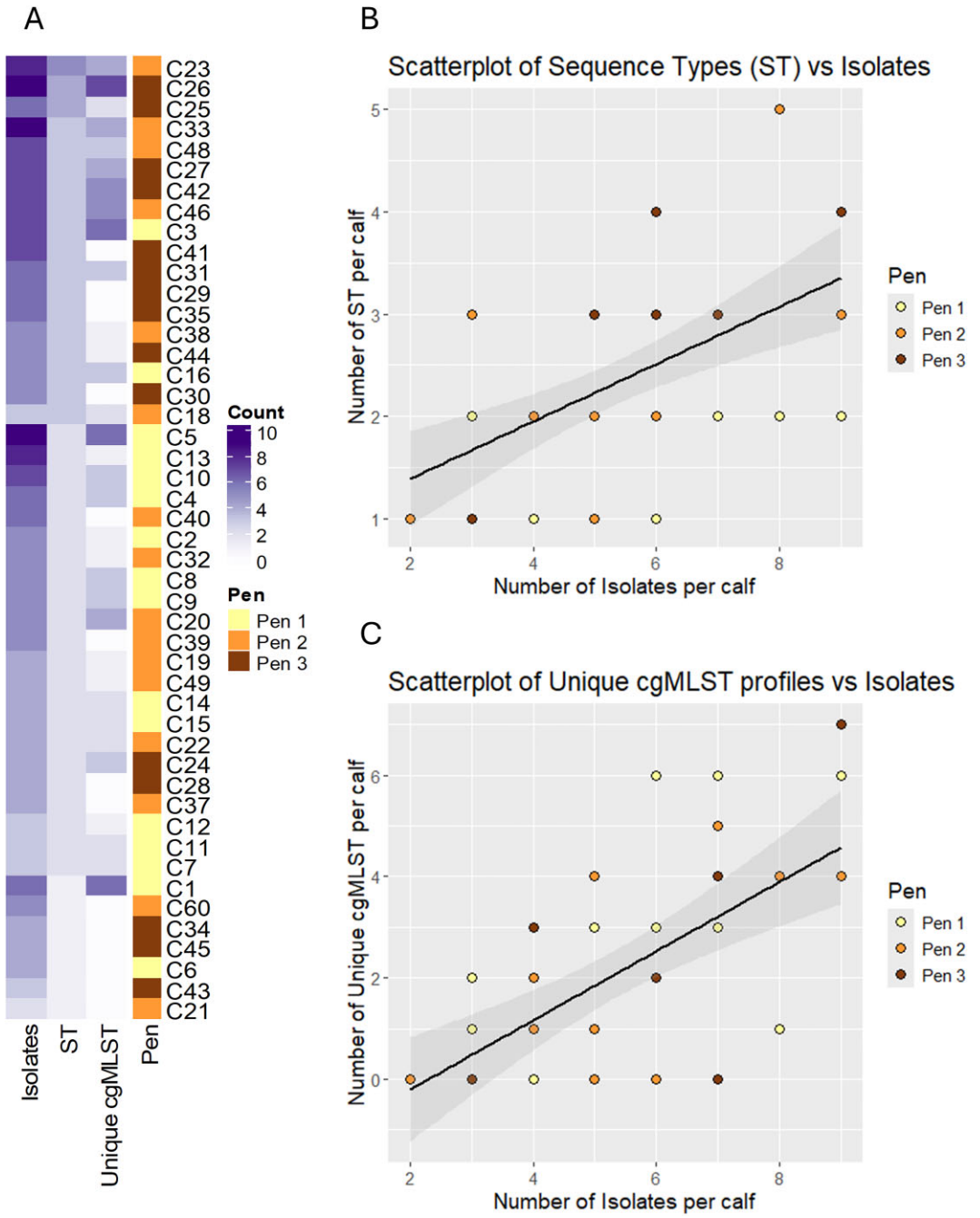


**Figure 5.** (A) Heat map showing the number of *C. jejuni* isolates, the number of ST (7-gene legacy MLST) identified from these isolates, and the number of unique cgMLST profiles obtained per calf. Calves were housed either in pens where exposure to wild birds was limited through increased biosecurity measures (Pen 1 and Pen 3) or in a pen the wild birds had access to (Pen 2) (16 studied calves/pen). Colour intensity of the counts represents the number of isolates, from 0 (clear) to 9 (dark). The alphanumeric labels for each calf (rows) denote the farm-allocated calf ID number. One calf (C36 in Pen 3) of the 48 studied calves is not included in the heat map, as it excreted detectable levels of *C. jejuni* on only one sampling occasion, and no assembled *C. jejuni* genome was obtained from this faecal sample. (B) Correlations between the number of isolates and the number of 7-gene MLST ST. (C) Correlations between the number of isolates and the number of unique cgMLST profiles. The grey area indicates the 95% confidence interval. Each dot represents one calf, jitter was added to help with dot separation. The Pearson's product moment was used as correlation coefficient.

the ruminant lineages ST-61 and ST-42, although a farm effect was noticed. ST-422 has also been detected in cattle and sheep in New Zealand (Mullner et al. 2010), further confirming its presence in the bovine host.

Complex ecological relationships among ST have been demonstrated in poultry using mathematical modelling approaches (Rawson et al. 2022). Specific dynamics among ruminant-associated clonal complexes have also been noted in cattle, in particular between the ST-61 and ST-42 complexes, which were found to be almost mutually exclusive (Kwan et al. 2008). Our work provided further evidence of a competitive hierarchy in *C. jejuni* population in a group of calves, where a generalist ST was capable of outcompeting ruminant ST, and hence replacing them as the dominant strain within the host cattle. Our finding that ST-61 was detected sporadically and/or in low occurrence within each pen suggests moreover

that at least one ruminant-associated ST might have co-existed with the generalist ST and persisted in the group of calves for a long period of time.

Calves are born with an immature rumen, and the pre-weaning period is associated with profound physiological changes in the morphology and role of intestinal cells and in the diversity and richness of the associated intestinal microflora (Yang et al. 2015, Meale et al. 2017, Malmuthuge et al. 2019). As a host generalist lineage, ST-21 CC has been shown to exhibit an enhanced capability to respond to environmental challenges (Yahara et al. 2017, Mouftah et al. 2021). The abilities of this lineage to adapt to environmental changes could have contributed to its dominance in the late pre-weaning period. The molecular mechanisms modulating the colonization of the bovine host by *C. jejuni* are yet to be fully understood. A wide range of *C. jejuni* genes associated with motility and chemotaxis, formation of the protective polysaccharide capsule at the cell surface, adhesion to host cells, and cellular invasion, have been identified as being essential for colonization of human and avian hosts (Lopes et al. 2021). Additionally, gene expression in *C. jejuni* is known to be finely regulated, with phase variation processes crucial for reversible phenotypic changes and growth of the bacteria in response to environmental changes (Burnham and Hendrixson 2018). While in-depth genomic analysis of *C. jejuni* strains was out of the study scope, our results suggest that the observed population succession in the gut of young calves could be attributed to possible genetic differences among *C. jejuni* strains. Further studies are required to investigate the regulation and expression of the key genes promoting the adaptation of different strains of *C. jejuni* to the bovine host, as well as to investigate the interactions between *C. jejuni* strains, commensal microbiota in the gut, and gut epithelial cells in young calves.

We expected the *C. jejuni* population of calves in Pen 2 to be affected—at least partially—by the access of common wild birds (mainly *Passer domesticus*) to this pen. Wild birds carry a wide range of *C. jejuni* strains (Tawakol et al. 2023), and the exchange of strains between birds and bovine animals has been previously demonstrated (Adhikari et al. 2004, Sanad et al. 2013). However, our comparisons of the *C. jejuni* populations among the three groups of calves provided no evidence that exposure to bird droppings was a driver for the observed changes in *C. jejuni* population richness and temporal changes. They also highlighted that the *C. jejuni* populations in the two groups of calves born at a similar time in the calving season (Pen 2 and Pen 3) were more similar to each other than to the first group (Pen 1). Biosecurity/biocontainment efforts have been made throughout the study, as feeding teats and feed storage conditions were different among pens, but the possibility of cross-pen contamination through fomites (such as boots or farmer overalls) or aerosols (Villarroel et al. 2007, Heinemann et al. 2020) cannot be fully excluded. It is also possible that new common genotypes had been introduced by the animal bedding regularly added into the pens. Another hypothesis is that the calves had been colonized by multiple genotypes *before* being introduced into their respective rearing pens, with only a dominant genotype excreted or detected at later times. The observed changes in the *C. jejuni* population would thus have arisen from both the initial colonizing population and the subsequent acquisition or re-infection of *C. jejuni* strains through calf-to-calf transmission within each pen. The hypothesis of early colonization from a single source, followed by within-pen transmission, aligns with the high genetic similarity of the circulating strains. It is further supported by the fact that calves in Pen 1 were born ∼10 d before the calves in Pen 2 or Pen 3, hence were born in a different environment and received a different colostrum. Local environment, quality of colostrum, and physical interactions with the dam—even for a short period of time post-partum—can shape the succession and establishment of the calves' gut microbiota in the first 3 to 4 weeks of life, as well as the risk for being *Campylobacter* positive (Klein et al. 2013, Klein-Jöbstl et al. 2019, O'Hara et al. 2020, Barden et al. 2020). There is growing evidence for natural co-colonization of individual animals or humans by multiple genotypes, with longitudinal studies reporting persistence of some genotypes in the host gut for at least 12 months (Moore et al. 2001, Rapp et al. 2019, Djeghout et al. 2022). In chickens, Coward et al. (2008) failed to replicate population dynamics of *Campylobacter* within broilers and attributed this to a "founder effect" *e.g.* small variations in population level at the first inoculation having large consequences for the flock-wide population dynamics. The similarity in genotypes and succession pattern between pens reported in our study suggests that the succession of *C. jejuni* strains in pre-weaned calves was not entirely stochastic. Further assessments of the potential mechanisms associated with early colonization and how it might influence the *C. jejuni* population structure in the developed gut of bovine animals are warranted. For example, including factors such as dam's age or number of parturitions, environmental conditions the dam was exposed to before calving, or calving management practices, might need to be considered in future studies. If early colonization with multiple STs at birth is proven, its long-term effect would need to be investigated in order to control *C. jejuni* in the animal reservoir.

*Campylobacter jejuni* has developed resistance to multiple antimicrobial treatments, with increasing fluoroquinolone resistance reported in young calves (Châtre et al. 2010, EFSA and ECDC 2025). The origin of these resistant strains in young animals is unclear, in particular when they are found in animals with no prior exposure to fluoroquinolone (Goulart et al. 2022). Given the low levels of fluoroquinolone-resistant *C. jejuni* in cattle in New Zealand (Cornelius et al. 2024), our study did not include a detailed analysis of antimicrobial resistance. Future research targeting herds with naturally high levels of antibiotics resistant strains would be required to assess whether early-life exposure—potentially via contaminated faecal material during or immediately after calving—would facilitate acquisition of resistant strains. Understanding the implications of such early colonization would be essential for managing antimicrobial resistance in dairy systems.

Our monitoring of the natural evolution of the *C. jejuni* population within young healthy calves suggests that a small number of animals might be more likely to excrete different *C. jejuni* strains over time and/or excrete allelic variants. The impact of individual animals ('super shedders', that shed particular faecal bacteria at higher levels than other animals) on the maintenance and transmission of bacteria in a herd has been demonstrated (Matthews et al. 2006). In contrast, the role of individual animals to generate genetic diversity of the strains circulating within a group of animals has yet to be explored. In our study, the number of animals excreting allelic variants was too small for our observations to be considered significant, and no clonal expansion was observed within our study timeframe. As genetic variation is a pre-requisite to evolution-

ary change and host adaptation (Epping et al. 2021), further studies with larger sample sizes would be needed to confirm potential role of individual animals in influencing *C. jejuni* genomes and evolution within the bovine host.

Our observational study based on the monitoring of calves exposed or not to wild birds in the first 2 months of life provided insights into possible factors that can influence establishment and succession of *C. jejuni* populations in the bovine host. However, it has several limitations. Our small sample size and the lack of pen replicates restricted the generalizability of the findings, although our results could be used to inform power base sample size and design larger scale studies, including a pen-level randomized controlled trials. The recovery of *C. jejuni* isolates was based on culturing steps, possibly limiting the ability to detect all the ST types excreted at once or with enough sensitivity. Metabarcoding approaches have been successfully applied to obtain information about the presence of zoonotic bacterial pathogens—including *Campylobacter* sp.—in farm-dwelling rodents or urban wildlife (Jahan et al. 2021, Cabodevilla et al. 2024). They also demonstrated their potential to differentiate between *Campylobacter* ST in chicken (Kingsbury et al. 2021). A third limitation of our study was that the bird population may have been naturally contaminated with *C. jejuni*, and the presence and genotypes of *C. jejuni* were not confirmed in the wild bird droppings. Future works leveraging a metabarcoding approach alongside WGS-based approaches are warranted to improve characterizing the diversity and population structure of *C. jejuni* in livestock and wild birds.

In conclusion, our study emphasized the importance of a One Health approach to understand zoonotic risks. It provided valuable insights into the ecological dynamics of *C. jejuni* within the bovine host in contact with wild birds. It did not provide any evidence that exposure to wild birds and their droppings influenced *C. jejuni* population composition and changes in healthy young calves. Instead, the observed differences in *C. jejuni* populations related to the calf birth period, and pointed to the importance of herd management practices before or during calving. Future on-farm research to mitigate zoonotic transmission of *C. jejuni* from livestock to humans should investigate the impact of calving management practices on the establishment and evolution of *C. jejuni* population in the bovine reservoir.

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## Author contributions

Delphine Rapp (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing), Colleen M. Ross (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing), Halina E. Tegetmeyer (Data curation, Formal analysis, Visualization, Writing – review & editing), Paul Maclean (Formal analysis, Visualization, Writing – review & editing), Nigel P. French (Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing), and Gale Brightwell (Funding acquisition, Project administration, Writing – review & editing)

## Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

*Conflict of interest*: None declared.

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## Data availability

The datasets analysed during the study are available in the supplementary materials; the Illumina raw sequencing reads have been uploaded to NCBI database under BioProject (PRJNA1198386).

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