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# Analysis of reservoir sources of *Campylobacter* isolates to free-range broilers in Denmark

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**ABSTRACT** Campylobacter is a common cause of food poisoning in many countries, with broilers being the main source. Organic and free-range broilers are more frequently Campylobacter-positive than conventionally raised broilers and may constitute a higher risk for human infections. Organic and free-range broilers may get exposed to Campylobacter from environmental reservoirs and livestock farms, but the relative importance of these sources is unknown. The aim of the study was to describe similarities and differences between the genetic diversity of the Campylobacter isolates collected from free-range/ organic broilers with those isolated from conventional broilers and other animal hosts (cattle, pigs, and dogs) in Denmark to make inferences about the reservoir sources of Campylobacter to free-range broilers.

The applied aggregated surveillance data consisted of sequenced *Campylobacter* isolates sampled in 2015 to 2017 and 2018 to 2021. The data included 1,102 isolates from free-range (n = 209), conventional broilers (n = 577), cattle (n = 261), pigs (n = 30), and dogs (n = 25). The isolates were cultivated from either fecal material (n = 434), food matrices (n = 569), or of

nondisclosed origin (n = 99). Campylobacter jejuni (94.5%) dominated and subtyping analysis found 170 different sequence types (STs) grouped into 75 clonal complexes (CCs). The results suggest that CC-21 and CC-45 are the most frequent CCs found in broilers. The relationship between the CCs in the investigated sources showed that the different CCs were shared by most of the animals, but not pigs. The ST-profiles of free-range broilers were most similar to that of conventional broilers, dogs and cattle, in that order. The similarity was stronger between conventional broilers and cattle than between conventional and free-range broilers. The results suggest that cattle may be a plausible reservoir of C. *jejuni* for conventional and free-range broilers, and that conventional broilers are a possible source for freerange broilers or reflect a dominance of isolates adapted to the same host environment.

Aggregated data provided valuable insight into the epidemiology of *Campylobacter* sources for free-range broilers, but time-limited sampling of isolates from different sources within a targeted area would hold a higher predictive value.

Key words: one-health, source attribution, Campylobacter, broiler, epidemiology

#### INTRODUCTION

Food-borne illness and deaths due to disease caused by the zoonotic bacteria Campylobacter is estimated to be around 8.4% of the total burden of diarrheal diseases globally (Murray et al., 2012). A prior infection with Campylobacter may also increase the risk of

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inflammatory intestinal diseases such as inflammatory bowel disease (Gradel et al., 2009).

Campylobacter is currently considered the most common cause of food poisoning in many countries around the world, including in the European Union (**EU**) (Havelaar et al., 2015, EFSA and ECDC, 2019, Nastasijevic et al., 2020). Campylobacter jejuni and C. coli are the 2 species that are considered the main causes of human campylobacteriosis, but Campylobacter lari, Campylobacter upsaliensis, and Campylobacter concisus have also been reported to cause disease (Skarp et al., 2016).

Broilers are considered an important source of human cases of campylobacteriosis. It is estimated that 20 to 30% of campylobacteriosis cases can be attributed to

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handling, preparing, and consuming of broiler meat (EFSA BIOHAZ Panel, 2010). Probabilistic models of subtyping based on multilocus sequence typing (MLST) of *C. jejuni* isolates from USA and Australia suggest broilers may constitute as a reservoir, and that contributes to 50 to 80% of human cases (Mead et al., 1999; Hall et al., 2005; Hoffmann et al., 2007; Boysen et al., 2013). In addition to broilers, beef is considered the second most important source for human campylobacteriosis (Skarp et al., 2016; Wainaina et al., 2022). Controlling *Campylobacter* at the primary production level on farm is considered more effective than controlling the bacteria later in the food chain (EFSA BIOHAZ Panel, 2011).

The number of organic and free-range broilers is increasing in the EU (European Commission, 2016), and has tripled in Denmark in the last decade (Statistics Denmark, 2023). Organic broilers are 1.5 to 3 times more likely to be *Campylobacter*-positive than conventionally raised broilers at slaughter (Anonymous, 2023), and the risk per serving of a meal from organic broilers present a higher risk of infection in humans (Rosenquist et al., 2013). In Denmark, organic and free-range broilers cover different requirements to space, limitations to feed and hygiene, but both systems require access to the outdoors for the broilers. Production systems that allow the broilers outdoor access make it impossible to guarantee the same level of biosecurity as for conventional indoorraised broilers. The magnitude of freedom within outdoors may also be important. Broilers that are unrestricted in their range or ability to roam have been correlated with a higher risk of flocks being Campylobacter-positive, compared to fenced-in broilers (Huneau-Salaün et al., 2007). In our present paper, broilers with any access to outdoors, including organically produced broilers, will be referred to as "free-range broilers."

Despite efforts have been made to trace different sources of *Campylobacter* that may result in broilers hosting the bacteria, it is still unclear how the broilers get exposed to *Campylobacter*, especially in free-range production systems. Some genetic variants of C. jejuni found in water and soil seem to be better at surviving in the environment than other variants (Schaffter et al., 2004; Epps et al., 2013; Bronowski et al., 2014; Reichelt et al., 2022) and could pose a risk to free-range broilers. From the environmental reservoirs C. jejuni may transfer to new hosts, such as broilers, via the reservoir itself, or potentially via rodents, flies, beetles, and wild birds (Jorgensen et al., 2011; Hald et al., 2016; Royden et al., 2016; Rossler et al., 2020). Livestock farms in general may also contribute to the spread, as C. jejuni DNA can be detected in air samples taken >1,000 m from the farms (de Rooij et al., 2019).

Attributing the source of human infections to specific foods and production systems, such as broilers with outdoor access, has been instrumental in focusing efforts to reduce the risk to humans. Source attribution is traditionally used to trace sources for human infections, but the study of potential sources of contamination to the production animals is important epidemiological knowledge that can guide interventions strategies. The comparison of the distribution of phenotypic and genotypic variants of *Campylobacter* isolates from different food and animal sources allows us to make inferences about the epidemiology of the zoonosis. Provided that a sufficient number of subtyped isolates is available, it may be possible to identify the most likely sources of *Campylobacter* found in food production (Sommer et al., 2013). MLST has successfully been used to explore links between sources of *Campylobacter* isolates and clinical human cases (Cody et al., 2019) and animals (Litrup et al., 2007). The MLSTs can be further grouped in clonal complexes (CCs) that can have common special features that indicate whether the bacteria have adapted to become a host generalist or specialist (Püning et al., 2021).

To limit zoonotic *Campylobacter* entering the food chain at the primary production level, it is crucial to understand the epidemiology of *Campylobacter* in freerange broiler farms. Therefore, the study aim was to investigate whether the whole-genome-sequenced (**WGS**) *Campylobacter* isolates from free-range broilers in Denmark were similar to those that were isolated from conventional indoor-raised chicken as well as from cattle, pigs, and dogs. Distribution and diversity of *Campylobacter* isolates from different sources (animal hosts) were characterized and compared based on their subtypes.

### MATERIALS AND METHODS

#### National Surveillance Data

In Denmark, zoonotic pathogens in livestock and foodstuffs are monitored and controlled through centrally coordinated projects initiated by the Danish Veterinary and Food Administration (**DVFA**) as described in the Annual Report on Zoonoses in Denmark (Anonymous, 2021). The present study utilized and aggregated such historical surveillance data for *Campylobacter*, which were collected between 2015 to 2017 and 2019 to 2021 (no surveillance was carried out in 2018). The original dataset contained isolates from broilers (organic, free-range, and conventional), cattle, pigs, dogs, ducks, turkeys, bathing water, and vegetables (N = 2,665).

The sample materials consisted of intestinal contents (fecal swab samples, or cecal content), meat and other food products (sampled either at a slaughterhouse or in the retail trade originating from Danish or foreign production), and bathing seawater. The samples were examined for the presence of *Campylobacter* at the Laboratory of the DVFA according to NMKL 119 (2007) (Rosenquist et al., 2007) and subsequently analyzed by WGS (Foddai et al., 2022; Wainaina et al., 2022). The sequenced genomes were transferred and stored at the Denmark's National Life Science Supercomputing Center (Computerome, https://www.computerome.dk) for further genomic analyses. Metadata were provided by the DVFA and included: Sample ID, isolate ID, The Central Husbandry Register (CHR, or farm registration

ID), source or host where the samples were taken from, *Campylobacter* species identified, sampling date, and country of origin. Broilers samples were further categorized into free-range (covering both organic and free-range broilers) or conventional broilers based on their status in the CHR.

#### Data Management

To elucidate the potential sources of *Campylobacter* in the free-range broiler farms, samples were removed from the dataset if: 1) duplicates were found; 2) incomplete metadata; 3) country of origin was other than Denmark; 4) sources with fewer than 20 isolates; and 5) ST or CC could not be identified. The final dataset contained only isolates from broilers, cattle, pigs, and dogs (N = 1,102).

De novo assembly was carried out using the Food QC & Assembly pipeline that includes assembler SPAdes 3.15.4 (Bankevich et al., 2012). The quality of the assembly was assessed using the number of contigs (N50) and the total size of the assembly. N50 is the median of the lengths of contigs and the longer, the better is the assembly. Assemblies were scaffold assemblies, and genome assemblies with less than 500 contigs were kept in the dataset. Finally, the total size of the assembly was checked to match the expected size for a *C. jejuni* genome ( $\sim 1.6-1.7$  million base pairs (**Mbp**)).

#### Subtyping by MLST

We performed 7 loci MLST (or classical MLST) using the MLST pipeline available from the Center for Genomic Epidemiology (www.genomicepidemiology.org) (Larsen et al., 2012). It uses the MLST scheme developed by Dingle et al., and is updated weekly from PubMLST (https://pubmlst.org/organisms/campylo bacter-jejunicoli) (Jolley et al., 2018). In order to batch upload all the genomes the pipeline was run in Computerome 2.0 (https://www.computerome.dk). An inhouse script in python was used to gather results in a matrix with the 7 loci as columns, STs, CCs, and genomes as rows.

Because both *C. jejuni* and *C. coli* are characterized using the same MLST method, they were analyzed together (Dingle et al., 2001; Miller et al., 2005).

To assess the relationship among the isolates Entero-Base GrapeTree (MSTreeV2) was used to create the minimum spanning tree of CC stratified according to the animal source of each isolate (Zhou et al., 2018) (Supplementary Materials S1 and S2).

#### Statistics

The genetic diversity within each source was evaluated using the Gini-coefficient. The Gini-coefficient was calculated using the *ineq* package in Rstudio (Version 1.2.5033, Rstudio, Inc.) (Zeileis et al., 2014). The Ginicoefficient is a quantification of the statistical dispersion within a population, in this case the CCs in each source. A Gini-coefficient of 0 equals to a completely equal distribution and a nonspecific genetic profile of the CCs found. A Gini-coefficient of 1 represents completely unequal distribution, and a specific genetic profile of the CCs is found. The results were classified as described by Henri et al., 2016: <0.4 indicating an equal distribution of CCs (a nondistinct genetic profile), 0.4 to 0.6 indicating a moderate association of CCs (moderately distinct genetic profile), and >0.6 indicating an unequal distribution of CCs for the source (a distinct genetic profile).

The Czekanowski proportional similarity index (**PSI**) was used to estimate the similarity of 2 nonparametric frequency distributions, in this case the proportional distribution of STs of the *Campylobacter* isolates in different sources (Rosef et al., 1985; Ramonaite et al., 2017). A Czekanowski PSI of 0 indicates no similarity of the distribution of STs frequencies in 2 sources, while 1 indicates a complete overlap.

The distribution of CCs of broiler production type (free-range vs. conventional), material (fecal material vs. food matrices) and time periods (broiler isolates collected May-October vs. November-April, isolates collected 2015-2017 vs. 2019-2021) were examined using slope charts (Rstudio). Dominating STs were defined as those isolates representing  $\geq 50\%$  of the isolates in the source. Dominating CCs were defined as being represented in the top 80% of the isolates.

#### RESULTS

#### Descriptive Analysis of the Dataset

The final dataset included 1,102 isolates from freerange (n = 209) and conventional broilers (n = 577). cattle (n = 261), pigs (n = 30), and dogs (n = 25) (Dataset: Supplementary Material 2, Table of samples collected: Supplementary Material 3). The isolates came from fecal material (n = 434), food matrices (meat collected at the slaughterhouse and packaged meat products at retail level, n = 569) or nondisclosed origin (n = 99). Campylobacter isolates were predominantly C. *jejuni* (94.5%) (Supplementary Material 4). A few isolates were C. coli (2.1%) in samples collected from broilers and these were predominantly found in freerange broilers. One isolate from free-range was nondistinguishable and was classified as C. coli or C. jejuni. Almost half of the isolates from pigs (n = 12, 40.0%) and all dogs (n = 25, 100.0%) were not classified to species level (*Campylobacter* spp.).

#### Subtyping Analysis

The 1,102 isolates had 170 different STs that were assigned to 75 different CCs. Half of the dataset consisted of 11 STs: ST-21 (n = 154, 14,0%), ST-45 (n = 72, 6.5%), ST-42 (n = 52, 4.7%), ST-48 (n = 46, 4.2%), ST-257 (n = 46, 4.2%), ST-61 (n = 42, 3.8%), ST-50 (n = 40, 3.6%), ST-19 (n = 35, 3.2%), ST-122 (n = 32, 2.9%), and ST-464/ST-441 (n = 31, 2.8% each) (Supplementary Material 5). Isolates identified as *C. jejuni* were

**Table 1.** Frequency and distribution of multilocus sequence type (ST) and clonal complexes (CC) of *Campylobacter* isolates from broiler, cattle, pigs, and dogs.

Source	ST ( <i>N</i> )	CC ( <i>N</i> )	CCs which were found only in that source $(N, \%)^1$	Dominating ( $\geq 80\%$ ) CCs of isolates from the source in declining order	Gini-coefficient
Free-range broilers $(n = 209)$	62	34	11, 32.4	21, 45, 828, 464, 353, 607, 48, 443, 1034, 6148, 1911, 2274	$0,\!590$
Conventional broilers $(n = 577)$	107	55	28, 50.9	21, 45, 206, 48, 257, 828, 22, 464, 353, 283, 42	0,754
Cattle $(n = 261)$	44	22	6,27.3	21, 61, 42, 48, 441, 257	0,688
Pigs $(n=30)$	12	2	0, 0.0	403	0,433
Dogs(n=25)	19	13	1, 7.7	45, 21, 283, 443, 48, 353, 692, 658, 206	0,375

Gini-coefficient: 0 = genetically equal distribution; 1 = genetically unequal distribution.

dominated by the same ST as the general sample, with the difference of ST-441 replacing ST-464. *Campylobacter coli* were only found in samples from 2019 and were dominated by ST-6148 (n = 9, 39.1%) and ST-854 (n = 4, 17.4%). Isolates not assigned to a specific *Campylobacter* species occurred between 2015 and 2017 and were dominated by ST-45 (n = 5, 13.5%) and ST-403 (n = 3, 8.1%), which are *C. jejuni* subtypes.

Isolates from free-range broilers consisted of 62 different STs assigned to 34 CCs of which 15 (44.1%) were singletons (Table 1 and Figure 1). The most common CC found in isolates from free-range broilers were CC-21 (n = 37, 17.7%), CC-45 (n = 25, 12.0%), CC-828 (n = 17, 8.1%), CC-464 (n = 14, 6.7%), and CC-353 (n = 13, 6.2%).

Conventional broiler isolates consisted of 107 STs assigned to 55 CCs of which 27 (49.1%) were singletons. The most common CC found in isolates from conventional broilers were dominated by CC-21 (n = 168, 29.1%), CC-45 (n = 91, 15.8%), and CC-206 (n = 37, 6.4%).

Cattle isolates consisted of 44 STs assigned to 22 CCs of which 10 (45.5%) were singletons. The most common

CCs found in isolates from cattle were CC-21 (n = 69, 26.4%), CC-61 (n = 48, 18.4%), and CC-42 (n = 39, 14.9%).

Pig isolates consisted of 12 STs assigned to 2 CCs: CC-403 (n = 28, 93.3%) and CC-21 (n = 2, 6.7%).

Dog isolates consisted of 19 STs assigned to 13 CCs of which 8 (61.5%) were singletons. The most common CCs found in isolates from dogs were CC-45 (n = 8, 32.0%), CC-21 (n = 3, 12.0%), and C-48/283/443 (n = 2, 8.0% each). Between a quarter to half of the CCs of isolates from either free-range broilers, conventional broilers, or cattle were only found only in that specific source (Table 1).

#### Genetic Diversity in Each Source

The distribution analysis of CCs in each source showed an equal genetic distribution of CCs in dogs, and a moderately unequal genetic distribution of CCs in pigs and free-range broilers (Table 1). An unequal genetic distribution of CCs was observed for conventional broilers and cattle.



**Figure 1.** Minimum spanning tree of multilocus sequence type (ST) and clonal complexes (CC) of *Campylobacter* isolates from free-range broilers (red), conventional broilers (blue), cattle (green), pigs (orange), and dogs (yellow). The sizes of circles indicate the number of isolates, length of lines indicates the number of variations in loci between CCs. Circles with the same number indicate the CC belong to different STs.

Slope charts showed that CC-21 and CC-45 were the most frequent CC in samples from the broilers, regardless of comparing fecal material and food matrices, years of the sampling periods, or colder and hotter period of the year (Supplementary Material 6: Figures S2–S6). The genetic profile for conventional broilers was almost identical regardless of whether the isolate came from fecal material or a food matrix, while it varied for freerange broilers (Figures S2 and S3). Comparing broiler isolates collected in November to April with isolates collected in May to October showed that several isolates were evenly present in the genetic profile (e.g., CC-21, CC-45, CC-48, CC-464, CC-828, CC-607, CC-257, CC-1034, CC-353, CC-283), while others were more associated with a specific season (Supplementary Material 6: Figures S4 and S5). The dominant CCs were the same in broilers between 2015 to 2017 and 2019 to 2021, and most of the CCs were present but changed in frequencies, with the exception of CC-828 that appears to have emerged during 2019 to 2021 (Figure S6).

#### Source Analysis

The Czekanowski PSI indicated that the strongest overlap between the ST of isolates found in free-range boilers was with the ST of isolates found in conventional broilers (Table 2). This overlap consisted of 35 shared STs that represented 65.3% (n = 377/577) of the isolates found in conventional broilers. The second strongest overlap with the ST-profiles with free-range broilers was with isolates from dogs, sharing 10 STs that represented 64.4% (n = 16/25) of the isolates found in dogs. The Czekanowski PSI of the ST-profile of isolates from freerange broilers and cattle was less than half of that observed between the ST-profile of isolates from conventional broilers and cattle. Free-ranging broilers and cattle shared only 13 STs but these represented 64.8%(n = 169/261) of the isolates from cattle. The overlap between conventional broilers and cattle was almost as strong as that observed between the 2 broiler production types. The overlap between conventional broilers and cattle covered more (n = 19) of the common STs (ST-19, ST-21, ST-42, ST-257, ST-441) and represented a larger proportion of the isolates (83.5%, n = 218/261) from cattle. The overlap between broilers and pigs was poor, sharing only 2 STs (6.7%, n = 2/30).

The relationship between CCs in the investigated sources is illustrated in Figure 1 as a minimum spanning tree

**Table 2.** The Czekanowski proportional similarity index (PSI) of the distribution of multilocus sequence type (ST) of isolates in different sources.

Source	Free-range broilers	Conventional broilers
Free-range broilers	-	0.3833
Conventional broilers	0.3833	-
Cattle	0.1742	0.3628
Pigs	0.0572	0.0489
Dogs	0.2840	0.2514

PSI index: 0 =lowest similarity; 1 =largest similarity.

(same figure for STs are shown in Supplementary Material 7). The figure was set with CC-21 as the founding CC for the data and showed that the CCs of isolates from freerange generally can also be found in conventional broilers. The CCs from isolates from cattle and dogs showed a similar picture. Pigs was the only source that indicated a cluster of STs linked to CC-403 and were connected to the only other CC found in this source (CC-21).

#### DISCUSSION

Free-range and conventional broilers shared STs and CCs that made up most isolates in both. The CCs were heavily dominated by CC-21 and CC-45. Isolates covered by these 2 CCs are known to be host generalists isolates that infect both chicken, cattle, and humans (Dearlove et al., 2016). Without a representative sample of isolates collected from the environment around the broiler farms, it is difficult to establish if the dominant CCs found in the broilers living in different production systems are present due to adaptation to this host, or if it is due to an exchange of genetic material between the production systems. Many unique CCs were found in free-range broilers but more so in conventional broilers. This is counterintuitive, if we assume that free-range broilers have an increased chance of being infected with a greater variety of *Campylobacter* isolates than conventional broilers, due to exposure to the outdoors environment (Rivoal et al., 2005). The larger sample size from conventional broilers in the study has likely contributed to the finding of rarer CCs. This is reflected in the Ginicoefficient that is correlated with the sample size—the more samples, the more genetic diversity is found. Though it is difficult to deduct anything from unequal sample sizes, an alternative explanation may be that the larger and denser populations in conventional farms than in free-range farms may facilitate a higher exchange of genetic material once *Campylobacter* is present.

The second most similar overlap of ST-profile of freerange broilers was to that of dogs. This was based on a relatively small number of isolates from dogs, and mainly included more generalist STs belonging to CC-21, CC-45 and CC-48. The isolates from dogs were all collected from the Copenhagen area between 2016 and 2017, and almost all of the broiler farms are located in the opposite end of Denmark in Jutland. The geographical distance makes a direct transmission from the sampled dogs to broilers unlikely. Alternatively, it could be that dogs are fed with undercooked meat from conventional and free-range chicken and can acquire an infection this way. However, it is possible that dogs and broilers generally are infected from a shared environmental source. Human outbreak data from around this period (2015-2016), showed that almost half (48.3%) of the C. jejuni STs (ST-21, ST-45, ST-48) identified, were shared with those found in dogs in this study (Joensen et al., 2018). Some of the human isolates with ST-48 were identified in the Copenhagen area. An explanation

for the overlap could thus be that isolates from dogs due to their closely shared environment with humans may act as a proxy for human clinical cases, some of which may have been food-borne campylobacteriosis from broiler meat.

Considering that conventional broilers can be a source of *Campylobacter* to free-range broiler flocks or vice versa, it was necessary to assess the source of *Campylo*bacter to the conventional broilers. The Czekanowski PSI of the ST-profile of conventional broilers and cattle was almost identical to the PSI observed between the 2 broiler production forms. The similarity between the ST-profile of free-range broilers and cattle was less than half of that observed between conventional broilers and cattle. This difference could possibly be explained by a diversity of *Campylobacter* in free-range broilers that is reflecting their contact with environmental isolates, which may alter the composition of their ST-profile. In Denmark, a higher density of cattle farms around the location of the broiler houses, and the number of chimneys on the broiler house, have been found to be associated with the occurrence of *Campylobacter* in the broiler flocks (Sommer et al., 2013). Similar findings have been observed in other countries in similar climates such as Sweden (Hansson et al., 2010; Frosth et al., 2020) and United Kingdom (Ellis-Iversen et al., 2009). Flies on cattle farms have been found to carry C. *jejuni* and may act as vectors of *Campylobacter* from cattle farms to broiler farms (Adhikari et al., 2004; Jonsson et al., 2012). Attaching fly screens on broiler houses during the summer season has been demonstrated to reduce the number of flocks testing positive for *Campylobacter* in Denmark (Hald et al., 2007; Bahrndorff et al., 2013). The similarities between ST-profiles presented here thus offer further evidence to the role of cattle as a potential reservoir of C. *jejuni* colonizing broilers.

According to ST type similarity, free-range and conventional broilers share a considerable number of strains, which seem to support the theory of some species-preference of some *Campylobacter*. However, the strong association between cattle and conventional broilers is not found between cattle and free-range broilers, which may be explained by the larger sample size from conventional broilers and associated diversity of isolates. Alternatively, different environmental sources may exist for free-range broilers, or that different subtypes of *Campylobacter* are circulating in free-range farms. Longitudinal studies with repeated sampling on the same free-range farms would likely capture the epidemiology more successfully.

*Campylobacter* isolates collected from pigs did not show similarities with other sources and made up a distinct group in the minimum spanning tree. Our data did not indicate that isolates found in pigs shared genetic material with other sources. It was previously found that *C. coli* is predominantly found in pigs (Thakur et al., 2006), but was not identified in samples from pigs in our data. The isolates from pigs were collected during 2015 to 2017 and belonged to CC-403 and CC-21. However, *C. coli* clade I isolates belonging to CC-828 emerged in the samples in 2019 as one of the third most common CC found in free-range broilers, but our data did not include any samples from pigs collected after 2019. Therefore, further investigation is warranted to assess whether pigs can act as a potential source for broilers by sampling isolates from both sources at the same time point.

The present study was based on aggregated data which present a number of limitations to the interpretation of the results. First, only the most dominant isolate in a sample is reflected, as sequencing is limited to 1 colony per Campylobacter-positive sample. Second, aggregated data present limitations to the representativeness of each source and their comparison. The data were largely collected as time-limited projects that focused on different sources, mainly of relevance to food-borne human campylobacteriosis. No sample collection was carried out in 2018, and the number and type of samples from each source varied from year to year. The lack of systematic sampling limits the ability to accurately capture potential links between sources. The samples were collected in different ways, which could be grouped into either intestinal contents (fecal swab samples, or cecal content) or from a meat food matrix (carcass or product). It is assumed that broiler meat is contaminated from feces during slaughter, and that the distribution of STs found in intestinal contents and on meat is similar. This was observed to be true for conventional broilers, and for CC-21 and CC-45 in general (Supplementary Material 6). However, for samples collected from freerange broilers there were differences in the frequency of different CCs in intestinal contents and meat, and more CCs that were not found in intestinal contents were found dominant on meat. This may be explained by no intestinal content samples collected from free-range broilers in the dataset after 2018, but meat samples were collected both before and after. Between 2018 and 2019, there was a change in which slaughterhouses accepted free-range broilers and from 2019 only 1 slaughterhouse specialized in this type of broiler. The changes in which isolates have become dominant in intestinal content and meat from free-range broilers may thus be a consequence of changes in the supply and processing.

Between May and November more broiler flocks tested positive for *Campylobacter* compared to December to April. Regardless of the production type, there seemed to be both a stable and a seasonal composition of CCs. These results indicate that the seasonality of *Campylobacter* includes variations in the occurrence of different CCs.

Our source analysis used MLST based on 7 loci, but other methods could be applied. A recent study used a weighted network-based approach to evaluate the accuracy of using WGS data for a higher-resolution source attribution for human clinical isolates (Wainaina et al., 2022). The study used the same data as the current study and concluded that whole genome MLST (wgMLST), may provide more accurate input than core genome MLST (cgMLST) and single-nucleotide polymorphism (SNP) detection. However, these methods require high computational resources, and the output seems largely unchanged compared to analyses done by 7-loci MLST (Pires and Christensen, 2017; Wainaina et al., 2022). Our choice of approach seems still appropriate to deduce an overall picture of subtypes of *Campylobacter* present in the Danish free-range broiler farms.

In conclusion, STs and CCs of the *Campylobacter* isolates collected from the free-range broilers showed the largest overlap with those collected from conventional broilers compared to other animal sources. The genetic subtypes of the *Campylobacter* isolates collected from conventional broilers showed some similarities to those collected from cattle. More epidemiological studies to link the routes Cam*pylobacter* enters different types of broiler farms as well as between broiler and cattle farms may be warranted, but at a sufficient sample size that it enables to capture the diversity of isolates present. However, the results should be interpreted with caution since the utilized data were not collected for the study purpose, varying in different sample collection periods, sample materials, and geographical coverage. A coordinated effort of sampling of a balanced dataset from several sources within a predefined geographical area during the same time period is necessary to corroborate the findings of our study.

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#### DISCLOSURES

B. L. and J. E.-I. report that financial support was provided by Danish Veterinary and Food Administration (DVFA) at the time of work. B. L., M. S., N. T. S., and T. H. periodically give scientific advisory work to DVFA. J. E.-I. is currently working at DVFA.

#### SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j. psj.2023.103025.

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