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Published in:
Microbial Risk Analysis

Link to article, DOI:
10.1016/j.mran.2022.100214

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

Microbial Risk Analysis 21 (2022) 100214

Contents lists available at ScienceDirect

Microbial Risk Analysis

journal homepage: www.elsevier.com/locate/mran

Assessing *Campylobacter* cross-contamination of Danish broiler flocks at slaughterhouses considering true flock prevalence estimates and ad-hoc sampling

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ARTICLE INFO

Keywords:
Campylobacter spp.
Data integration
Prevalence
Cross-contamination
Field investigation

ABSTRACT

Campylobacter cross-contamination of Danish broiler flocks at slaughterhouses was investigated using data from two national surveillance components and from ad-hoc sampling. The animal level (AL) and food safety (FS) components from 2018 were compared. The AL component contained results of PCR on pools of cloacal swabs from 3,012 flocks processed at two Danish slaughterhouses (S1-S2), while the FS component regarded culture testing of leg skins from 999/3,012 flocks. The monthly "apparent" (AP) and "true" flock prevalence (TP) were estimated. Agreement between components was measured in percentage and in weighted-Kappa values. The relationship between the occurrence of cross-contamination (flock positive only in the FS component = cross-contaminated or *CC*, vs. flock negative in both components or *NegBoth*), slaughterhouse and surveillance period (quarter: Q1 to Q4) was evaluated by a generalized linear mixed effects (GLM) model. Thereafter, a linear mixed effects (LME) model was used to investigate the relationship between the level of meat contamination of carcass positive flocks (y = log10 colony forming units per gram, cfu/g), slaughterhouse, surveillance period, and flock type (*CC* vs. positive in both components or *PosBoth*). For both models, the farm was the random effect. Finally, in autumn 2019, ad-hoc field investigations were carried out testing caecal and neck skin samples, from two consecutive flocks at S1 and S2. Whole genome sequencing (WGS) was performed on isolates, for multilocus sequence typing (MLST) and single nucleotide polymorphisms (SNP) analysis. The monthly TP was always higher for the FS than for the AL component. Agreement between the components was substantial, but 8.1–8.6% of the flocks were *CC*. Those had median cfu/g 21–28 times lower than that of *PosBoth* flocks. In the GLM model, the explanatory variables were both significant (P-value <0.05). For example, the odds ratios (ORs) were 8.4 (95% CI: 4.0; 17.6) for Q3 vs. Q1, and 3.1 (1.8; 5.2) for S2 vs. S1. In the LME model, the flock type and the interaction between the other two variables, were significant. In the field study, a caecal positive flock was succeeded by an initially negative flock, in one out of five sampling sessions at S2. The cecal negative flock was positive in 58.3% of the neck skins with the isolate genetically similar to that from the caecal positive flock. Those results show that cross-contamination can be affected by surveillance periods and slaughterhouses, and it can contribute significantly to the TP of carcass positive flocks.

1. Introduction

Campylobacter spp. is considered the most common bacterial cause of human gastroenteritis in the world (EFSA and ECDC, 2016; WHO, 2020). Human cases are usually linked to consumption of contaminated food or drink. Poultry meat is considered one of the main sources of human infections (Robyn et al., 2015; EFSA and ECDC, 2016; Gantzhorn et al., 2018).

In Denmark, a National Action Plan is in place to control *Campylobacter* spp. (Gantzhorn et al., 2018; Anonymous, 2019). Information is continuously collected through different surveillance data streams along the food chain (Anonymous, 2019; Houe et al., 2019). Within this rich data environment, integrating information from different sectors, databases and surveillance components has a significant potential, when

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https://doi.org/10.1016/j.mran.2022.100214
Received 26 October 2021; Received in revised form 22 March 2022; Accepted 22 March 2022
Available online 24 March 2022

the epidemiology of *Campylobacter* "from farm to fork" must be investigated. For example, surveillance information can be used to estimate prevalence of surveillance units (animals, food packages etc.) infected or contaminated with foodborne pathogens, at different steps of the food chain (e.g. farm, slaughterhouse, retail levels). This allows: timely detection of changes in burden of disease, evaluating efficacy of disease control plans, and targeting control actions when and where needed.

Since multiplication of *Campylobacter* takes place in the chicken gut, it is logical to prioritize control efforts in the primary broiler production, to reduce the proportion of infected flocks at the animal/farm level, and the concentration of *Campylobacter* on broiler carcasses. A number of on-farm control options such as feed/water additives have been studied and assessed, but none of them is deemed as sufficiently effective (Robyn et al., 2015; Rasschaert et al., 2020) and strict biosecurity/control, to limit introduction of the pathogen to the farms is still considered highly important (Nauta and Ellis-Iversen, 2020). At the same time, contamination of broiler carcasses can occur during slaughter and processing, especially during the evisceration process (if the viscera ruptures) (Seliwiorstow et al., 2016), although optimizations of Good Manufacturing Practice (GMP)/General Hygiene Practice (GHP) principles and process hygiene measures at slaughterhouses, are expected to reduce the carcass contamination (EFSA BIOHAZ Panel, 2011).

Cross-contamination can occur during processing if a *Campylobacter*-free flock is slaughtered after an infected flock, because equipment located along the processing line can become contaminated (Berndtson et al., 1996; Rasschaert et al., 2020). Carcasses of cross-contaminated flocks are expected having lower contamination levels than carcasses of flocks that arrive at the abattoir as positive. Although dose-response models exist (e.g. see review by Nauta et al., 2009) and can be used to assess differences in the probability of human infection given low doses; the use of detailed surveillance data, to evaluate the occurrence of flocks cross-contamination in a systematic way, is still uncommon.

The overall aim of this study was to investigate the occurrence of *Campylobacter* cross-contamination between Danish conventional broiler flocks at slaughterhouses and the related meat contamination levels. Furthermore, a small field investigation was conducted, to determine the extent of the broiler carcass cross-contamination on (originally) *Campylobacter* negative flocks, which were found contaminated following a *Campylobacter* positive flock.

2. Materials and methods

This study was carried out using data obtained from two national surveillance components (2018) and from a small (ad-hoc) field investigation (2019). The surveillance data regarded flocks processed at the two major Danish slaughterhouse companies (S1 and S2), which slaughtered the majority (> 90%) of the Danish broilers (Gantzhorn et al., 2018). A third slaughterhouse company (S3) opened recently, but it was not yet opened when the baseline (national) risk level was defined in 2013 for the Danish Action Plan, which is mainly based on data from S1 and S2. Therefore, data from S3 was not considered for this study.

The data was collected as part of the national action plan (Gantzhorn et al., 2018; Anonymous, 2019) and was used to assess occurrence and extent of *Campylobacter* spp. cross-contamination in quarterly (Q1, Q2, Q3 and Q4) and monthly (January to December) surveillance intervals, to account for within-year seasonality. Whereas the field investigation was carried out to provide more detailed information on the *Campylobacter* subtypes detected in cross-contaminated carcasses and on the frequency of cross-contamination at single slaughterhouse level. All data handling, integration and analysis were carried out in the free software R (R Core Team, 2013).

2.1. National surveillance data

2.1.1. Description of National surveillance data

The national data regarding surveillance of *Campylobacter* spp. in Danish broiler flocks raised on conventional farms, and slaughtered at S1 and S2 during 2018, were obtained from the Danish Veterinary and Food Administration (DVFA).

The datasets represented two different surveillance streams, which will be referred to as animal level (AL) and food safety (FS) surveillance components throughout the paper. The two components monitored presence of *Campylobacter* spp. at the farm (animal) and at post-slaughter (food) level, respectively. The AL component contained the results of polymerase chain reaction or PCR (Lund et al., 2004) on cloacal swabs collected by the slaughterhouse personnel just after stunning of the broilers. From every flock, 24 broilers were sampled randomly and the PCR was carried out on a unique pool of 12 swabs. Each swab represented a pair of broilers. Thus, a single PCR result (negative or positive) was recorded for all flocks slaughtered in 2018 at S1 and S2, together with meta-data. The variables used for this study were: Date of sampling, farm identification number from the Central Husbandry Register (CHR), flock ID, within-farm house-ID (where the flock was reared), and PCR results.

The FS component was a randomized survey stratified on slaughterhouses. *Campylobacter* contamination levels were obtained by culture testing (Nordic Committee on Food Analysis, 2007; Rosenquist et al., 2007) of one leg skin sample per flock, collected from chilled carcasses at the end of the slaughter line and ready for retail and human consumption. Laboratory testing results were recorded as colony-forming units/gram (cfu/g) and represented meat contamination levels. Samples with $\geq$ 10 cfu/g were considered as culture positive and otherwise negative. The meta-data variables used from this component were: Date of sampling, sample ID, CHR, flock ID, within-farm house-ID, name of slaughterhouse, project number, and culture results (cfu/g).

2.1.2. Surveillance data integration procedure

In both surveillance datasets, a flock was defined as the group of broilers reared in the same house on the farm and slaughtered on the same day. Where a CHR or the house-ID was missing, it was extracted from the flock-ID, which was a combined identifier, but was differently formatted between the two surveillance components and was sometimes incomplete. Therefore, to identify each flock tested in both components, the two datasets were matched using four different matching scenarios (I to IV) of: CHR, house-ID, and sampling dates. Differences in the matched flocks between scenarios, represented uncertainty of the matching process, because some flocks could have been entered with different formats, dates and IDs in the two datasets.

In scenario I, only flocks with the same combinations of CHR, house-ID, and sampling date were eligible and merged. In scenarios II, III and IV, discrepancies in sampling dates of +/- one, four or seven days were allowed, if the CHR and the house-ID matched between datasets. The discrepancy levels were acceptable, because the data providers informed us that sampling dates could be reported slightly differently across components (DVFA, personal communication).

2.1.3. Surveillance data analyses

The annual and monthly apparent prevalence (AP) of positive flocks were estimated for each surveillance component (AL and FS) and represented the probability of flock's positivity to *Campylobacter* spp. before and after slaughter (i.e. at pre and post-harvest levels).

In the veterinary field, the "true prevalence" (TP) is often estimated from surveillance data. This represents the proportion of "truly" infected surveillance units, which takes into account the performance (sensitivity or Se and specificity or Sp) of an imperfect test (Rogan and Gladen, 1978; Reiczigel et al., 2010), thereby making it possible comparing prevalence estimates obtained from different laboratory tests. The 95% confidence intervals (95% CI) could also be calculated (Blaker, 2000; Reiczigel et al., 2010), to get an idea of the uncertainty around the TP, by considering (besides the test performance) the sample size and the number of test-positives from which the AP is initially estimated. The TP was calculated as (Rogan and Gladen, 1978):

$$TP = \frac{(AP + Sp - 1)}{(Se + Sp - 1)}$$

The AP, the TP and the 95% CI were estimated using the "epiR" package and the "*epi*.prev" function in R. For more detailed information on the calculation of 95% CI we refer to (Blaker, 2000; Reiczigel et al., 2010; Stevenson and Sergeant, 2021).

For each component, the monthly number of tested flocks represented the sample size (N), while the number of positive flocks was the numerator (n). For the PCR test, Lund et al. (2004) estimated a diagnostic specificity of 96% and reported that, using *Campylobacter*--negative fecal samples spiked with various amounts of *Campylobacter*, at concentrations of 70 cfu of *C. jejuni*/ml, all PCR amplifications had a C_t value below 40; hence all reactions were positive. Accordingly, for the PCR test, we used Se and Sp equal to 100% and 96%, respectively. For the culture test, the Se and Sp were 82.8% and 98.6% (Rosenquist et al., 2007).

Two by two tables were created for descriptive statistics, to explore the number and the percentage of tested flocks, for which there was agreement (or discrepancy) between results recorded in the two datasets. Moreover, the weighted-K values (Cohen, 1960; Cohen, 1968) were estimated with the respective 95% CI, to measure agreement behind chance, and were interpreted using the scale by Landis and Koch (1977). Accordingly, agreement could be interpreted as: "poor" (<0), "slight" (0–20%), "fair" (21–40%), "moderate" (41–60%), "substantial" (61–80%) or "almost perfect" (81–100%).

2.1.4. Statistical modeling

For the statistical modeling, the flocks positive in both components (*PosBoth*) were considered as infected and carcass-contaminated. Whereas the flocks negative in both components (*NegBoth*) were classified as not-infected from the farm level and as *Campylobacter*-free on carcasses. Then, flocks positive only in the FS component, were counted as not infected from the farm level, but cross-contaminated (*CC*) at slaughterhouse, while flocks that were positive only in the AL component (*ALposOnly*) were considered as infected from the farm level and "*Campylobacter*-free" (or eventually contaminated at undetectable levels) on carcasses.

Thereafter, a generalized linear mixed effects (GLM) model was used to investigate cross-contamination occurrence = "yes" or "not". The flock type was set as binary response variable, including all *CC* flocks and all flocks negative in both components (i.e. *y* = flock type = yes = *CC* vs. flock type = no = *NegBoth*). The explanatory variables included in the GLM model were: the surveillance period of the year when flocks were sampled ("quarter" = Q1, Q2, Q3 or Q4), and the slaughterhouse where flocks were processed (S1 or S2). These with the lowest frequency of *CC* flocks (Q1 for the "quarter" variable and S1 for "slaughterhouse") were used as the reference levels to calculate the odds ratios (ORs) of cross-contamination. Thereafter, the post-hoc Bonferroni and Tukey's methods were used, to investigate the model's outputs more in detail and to see in which pairwise comparisons of explanatory variable's levels the statistical significance occurred.

Furthermore, a linear mixed effects (LME) model was used to investigate the level of contamination across carcass positive flocks. All flocks positive at the FS component were considered for the analysis. In this case, the log10 transformed cfu/g were set as response variable (y), while the explanatory variables were: the "surveillance period", the "slaughterhouse" and the "flock type" (but now set = *CC* or *PosBoth*).

For both models, the farm from where the flock originated (the CHR) was set as the random effect. A backward selection process was applied to select the final model, starting with the most complex version including all possible interactions between the explanatory variables; because any interaction, if significant (with P-value < 0.05), would have been considered of epidemiological interest. Both models were also re-run using monthly surveillance periods, i.e. substituting the variable "quarter" with the variable "month".

2.2. Field investigation

2.2.1. Sampling method

The samples of the field investigation were taken on five different days at S1 and S2 between August and October 2020. On each sampling day, in each slaughterhouse, caeca and neck skin samples were taken from two broiler flocks slaughtered consecutively.

To minimize the possibility of contamination from environmental sources contaminated in previous slaughtering days, each sampling occasion was on the first working day of the week of the slaughterhouses (Monday or Sunday), and the sampled flocks were the first and the second flock of the day (flock 1 and flock 2).

Ten caecal samples were planned to be taken from both flock 1 and flock 2, while 20 and 60 neck skin samples had to be taken from flock 1 and flock 2, respectively.

Samples were stored at 2–5 °C immediately after collection, before they were sent to the DVFA laboratory for microbiological analyses.

2.2.2. Isolation and enumeration of Campylobacter jejuni

Caecal samples were analyzed quantitatively for *Campylobacter* with a modified method of NMKL 119, 2007, where the amount of sample was reduced from 10 g to 1 g. Neck skin samples were analyzed quantitatively for *Campylobacter* according to NMKL 119, 2007 (Nordic Committee on Food Analysis, 2007; Rosenquist et al., 2007).

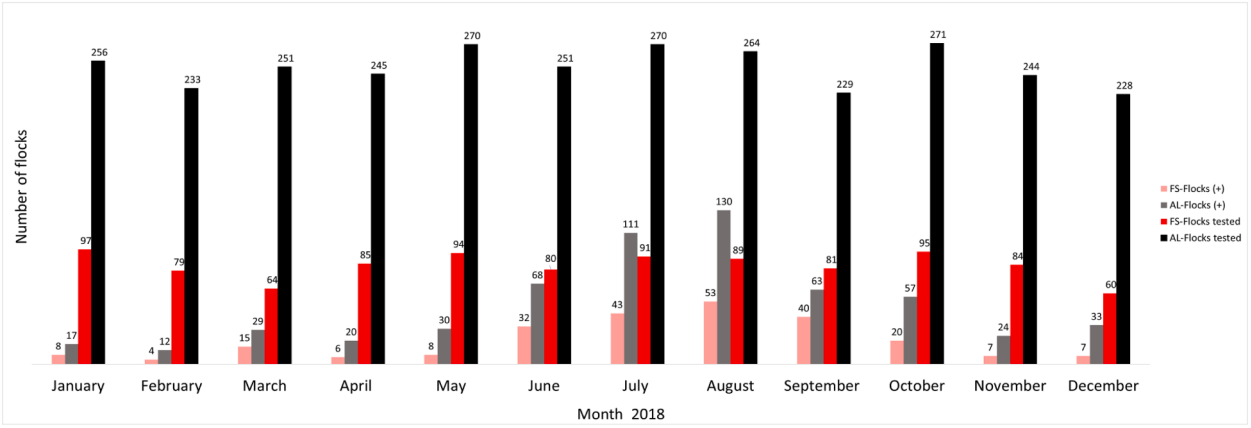

Fig. 1. Monthly number of conventional Danish broiler flocks tested for *Campylobacter* spp. during 2018, in the animal level surveillance component (AL) and in the food safety component (FS); with respective number of positive flocks (+). The data regards broiler flocks slaughtered at the two major Danish slaughterhouses (S1 and S2).

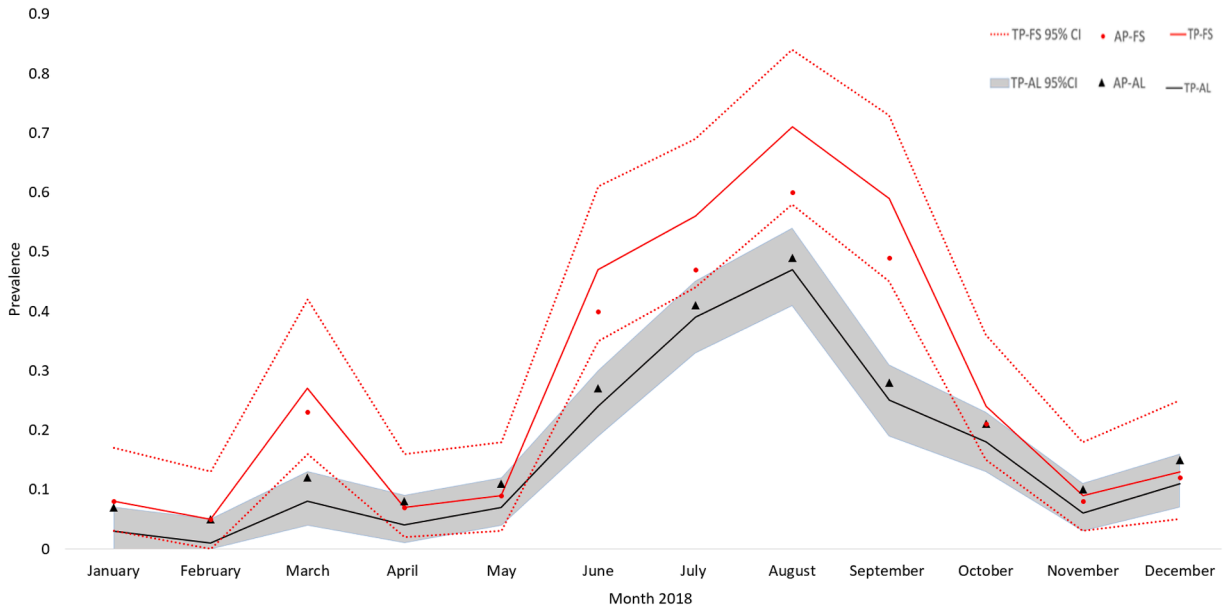

Fig. 2. Comparison of apparent (AP) and true (TP) *Campylobacter* flock prevalence estimated from the animal level (AL) and for the food safety (FS) surveillance components, with the respective 95% Confidence Intervals (TP 95% CI represented by the area between red dashed lines for the FS component, and by the grey area for the AL component). The data regards conventional broiler flocks slaughtered during 2018 at the two major Danish slaughterhouses (S1 and S2).

The result of the laboratory analyses were stored in Excel. Data handling and analyses were carried out in the free software R (R Core Team, 2013). Samples with >10,000 cfu/g were converted as 10,000 and samples <10 cfu/g as 0 (considered negative). The number of cfu/g found on positive samples was transformed to log10 cfu/g.

2.2.3. Whole genome sequencing data analyses

All *Campylobacter* isolates were stored at −80 °C and analyzed by whole genome sequencing (WGS). This was performed using MiSeq or NextSeq sequencing machines (Illumina Inc., San Diego, USA) with the Nextera XT Library preparation protocol for paired-end reads of 150 bp or 250 bp.

WGS data were processed using an internal QC Pipeline, performing trimming of low quality and adaptor sequences, and de novo assembly using SPAdes.

The species identification was done by KmerFinder v.3.2 (Clausen et al., 2018; Hasman et al., 2014; Larsen et al., 2014). Multilocus sequence typing (MLST) and single nucleotide polymorphism (SNP) calling, were performed to assess the genotypic associations among the isolates. MLST typing was done by MLSTFinder v.2.0 (Larsen et al., 2012), which uses the PubMLST database (WHO, 2020). SNP calling and construction of the SNP matrix were performed using assembled genome by CSIPhylogeny v.1.4 (Kaas et al., 2014). One of the isolates was selected as a reference. KmFinder, MLSTFinder and CSIPhylogeny are all publically available web tools from the CGE website (WHO, 2020).

3. Results

3.1. National surveillance data

3.1.1. Flock prevalence per surveillance component

In 2018, the total number of flocks tested in the AL and in the FS component, was 3,012 and 999 respectively. The annual apparent flock prevalence was 19.7% and 24.3%, respectively (Table, 1). For the FS component, this is the prevalence of flocks slaughtered at S1-S2 and positive in the leg skin samples, and could differ from the prevalence reported in the national surveillance report where more data (250 additional flocks) from the S3 slaughterhouse (disregarded in this study) was considered (Anonymous, 2019).

In Fig. 1, for each component, are reported the monthly number of tested flocks together with the respective number of positive flocks. The monthly median number of tested flocks was 251 (min. = 228; max= 271) in the AL and 85 (min. = 60; max. = 97) in the FS component, while the monthly median number of positive flocks was 32 (min = 12; max= 130) and 12 (min = 4; max= 53), respectively.

Comparing the AP and TP estimated within the same component (Fig. 2, dots or triangles vs lines of the same color), it can be noted that for the AL component the AP was consistently higher than the TP, while for the FS component, the AP was lower or almost similar to the TP. This difference between AP and TP obtained from the same sample size and component, showed the effect of estimating the flock prevalence considering not only the observed percentage of test-positives (AP), but also the error of the test used (Rogan and Gladen, 1978; Reiczigel et al., 2010)

When comparing the prevalence estimates between components, the AP of the FS component (red dots) was higher than that of the AL component (black triangles) in: January (+2%), March (+12%), June (+13%), July (+6%), August (+10%) and September (+22%). While it was equal in February and October, and lower in April (−1%), May (−3%), November (−2%) and December (−3%). The TP of the FS component was always higher than that of the AL component (Fig. 2, red line vs. black line). In that case the median difference between the two TP estimates was 6% (min = +1% in May; max = +34% in September).

The width of the 95% CI represented the uncertainty around each TP estimate (Fig. 2). The median width of the 95% CI was 9% (min. = 5%; max. = 13%) for the TP of the AL component (Fig. 2, grey interval), and 21% (min. = 13%; max. = 28%) for the TP of the FS component (Fig. 2, red interval). Thus, there was less uncertainty around the former estimate. The 95% CIs around the two TP values overlapped in all months apart from March, June, August and September, suggesting that (potentially) only for those four months, differences of TP could be considered statistically significant. When the two intervals overlapped, the minimum distance (1%) between the lower limit of the FS component and the upper limit of the AL component was observed in July (Fig. 2).

3.1.2. Agreement on binary testing results between components

Approximately 33% (999/3012) of the PCR tested flocks were also

tested by culture. Among those, 500 were slaughtered at S1 and 499 at S2. The maximum matching of flocks across the two components (949/999 = 95.0%) occurred in scenarios III-IV, which allowed for more than one day of registered sampling date discrepancy between datasets. In scenarios I and II, the percentage of matched flocks was lower (28.3% and 92.3%, respectively). Scenarios III-IV yielded the most inclusive and representative matched dataset, but did include some uncertainty around sampling dates and matching. Scenarios I and II were less comprehensive, but also less likely to be influenced by missing or faulty values, because flocks were matched on perfect combination of CHR, house unit and sampling date in scenario I, or differed only by one day in the sampling dates reported in the two datasets in scenario II.

Despite these differences in the matching precision, the four scenarios showed similar percentages of flocks (8.1–8.6%) arriving negative at the slaughterhouse (AL component), but resulting positive when leaving the slaughterhouse (FS component) (Table, 2). Those *CC* flocks were of main interest to the study, because they should have been cross-contaminated at the slaughterhouse.

In scenario I, the weighted Kappa was 63.0% (95% CI: 50%; 75%), whereas it was 70% (95% CI: 65%; 76%) in scenario II and 70% (95% CI: 64%; 75%) in scenarios III-IV. Thus, agreement between components was classified as "substantial" in all matching scenarios.

3.1.3. Descriptive comparison of contamination levels between flocks positive in both components and flocks positive only in the FS component

According to the scenarios with the maximum matching frequency across components (III-IV), the annual number of *PosBoth* flocks was 55 at S1 and 97 at S2; while the number of *CC* flocks was 26 and 56, respectively. The number of *NegBoth* flocks was 377 and 324, while the number of *ALonlyPos* flocks was 4 and 10, respectively.

In Table 3, the distributions of cfu/g on leg skins from the *PosBoth* and *CC* flock types were compared for each matching scenario. In scenario I, the median count of cfu/g was approximately 28 times higher in the *PosBoth* flocks, than in the *CC* flocks. Whereas in scenarios II to IV the median cfu/g was ≈ 21 times higher. The differences in contamination levels between the two groups suggested that contamination probably occurred differently, supporting the hypothesis that flocks which arrived as negative at the slaughterhouse, but resulted positive in carcasses (i.e. the *CC* flocks), were likely to be originally "*Campylobacter*-free" at the farm level, but became cross-contaminated during the slaughtering.

3.1.4. Output of statistical modeling

The GLM model did not show any significant interaction between the two explanatory variables, but both the surveillance period set as "quarter" (P-value = 1.91e-08) as well as the "slaughterhouse" (P-value = 2.93e-05) were significantly associated with cross-contamination occurrence. For instance, for Q3 the OR was 8.4 (95%CI = 4.0; 17.6) compared to the reference category Q1. Using monthly surveillance periods: June, July, August and September showed high statistical significance (P-values = 0.01; 0.002; 4.65e-05, and 0.0005, respectively) and the highest ORs, which were equal to 7.5 (1.5; 37.5); 11.8 (2.5; 55.8); 24.5 (5.3; 114.0); and 15.8 (3.3; 74.9); respectively. March showed borderline significance (P-value = 0.049) and OR 5.2 (1.0; 27.3). For the variable "slaughterhouse" = S2, the OR was = 3.1 (1.8; 5.2) compared to the reference category S1.

At the Bonferoni and Tukey's post-hoc tests, Q3 was the quarter of the year when cross-contamination was significantly more likely to occur. Moreover, independently of the surveillance period, S2 was significantly (P-value = 2.93e-05) more likely to have *CC* flocks than S1. In both post-hoc methods, August had significantly higher estimates than all other months, apart when compared to March, June, July and September. Whereas the latter showed significantly higher estimates than January, February and April.

The LME model used to investigate the meat contamination levels, showed a significant interaction between the surveillance period set as "quarter" (P-value = 0.004) or "month" (P-value = 0.03) and the variable "slaughterhouse". Moreover, the *PosBoth* flock type, carried significantly (P-value < 2.2e-16) higher contaminations (mean 2.7 log10 cfu/g with 95%CI = 2.6; 2.9) than the *CC* type (mean 1.7 log10 cfu/g; 95%CI = 1.6; 1.9). Therefore, flocks classified as originally infected from the farm, had significantly higher contaminations than flocks which were classified as originally "*Campylobacter*-free" and became cross-contaminated with detectable cfu/g on carcasses.

3.2. Field investigation

A total of 100 caecal and 390 neck samples were taken during the five sampling sessions of the field investigation (Table 4). All caecal samples were negative, except for flock 1 on the 19th of October 2020 at S2. In the positive flock, all 10 caecal samples were positive and identified as carrying *Campylobacter jejuni* with >4 log10 cfu/g. All caecal samples from the subsequent flock (flock 2), on the same sampling occasion, were negative.

Neck skin samples were negative on four out of five sampling days. On the 19th of October 2020, all neck skin samples from flock 1 were positive (20/20) with a mean of 2.8 log10 cfu/g (min = 2.2; max = 3.8) and 58.3% (35/60) of the samples from flock 2 (on the same day) was positive for *Campylobacter jejuni* with a mean of 1.7 log10 cfu/g (min = 1.0; max = 3.8).

All of the isolates (10 cloacal and 55 neck skin samples) were identified as sequence type (ST) 21. The maximum number of SNPs among the 65 isolates were 6.

4. Discussion

This study provides evidence by different approaches that *Campylobacter* cross-contamination of broiler flocks can occur at considerable levels in slaughterhouses, especially during summer. Firstly, national surveillance data were utilized to investigate the presence of *Campylobacter* at the beginning and at the end of the slaughter line. Thereafter, the contamination levels on carcasses were explored more in detail by sampling of two consecutive flocks, at two slaughterhouses, on five different days. We found that cross-contamination could be a considerable contributing factor to the prevalence of carcass positive flocks and this is relevant for authorities and industries working on national action plans aiming to improve *Campylobacter* control along the poultry meat chain, to reduce the risk of infection for consumers.

4.1. Epidemiological information obtained from national surveillance data analysis

The national surveillance data showed that overall, 19.7% and 24.3% of flocks slaughtered in 2018 at the two major Danish slaughterhouses, and tested for *Campylobacter* in the AL and FS component (respectively), were positive. Monthly TP was also higher in the FS than in the AL component (Fig. 2), and the two 95% CI around the TP did not overlap with each other in March, June, August, and September. Those findings suggested that some cross-contamination could have occurred at slaughterhouses, especially during summer months, and could have caused the difference between the TP estimates obtained from the two components.

It is well-known that there is seasonality of *Campylobacter* occurrence in birds and in poultry meat ready for human consumption. In the Northern European countries like Denmark, broiler flock prevalence and incidence of human cases peak during summer months (Wedderkopp et al., 2000; Wedderkopp et al., 2001; Patrick et al., 2004; Kuhn et al., 2020). Different reasons have been suggested for differences in summer peaks of flock prevalence, such as: the abundance of flies, higher water run-off, increased ventilation needs related to high environmental temperatures, etc. (EFSA BIOHAZ Panel, 2011).

The agreement between the two components was "substantial" in all

Table 1
Summary of two national surveillance datasets from 2018, which were used for *Campylobacter* status classification of the broiler flocks slaughtered at the two major Danish slaughterhouses (S1 and S2).

General information	Animal level (AL) component	Food safety (FS) component
Samples	Cloacal swabs	Leg skin
Sampling unit	24 animals per flock (12 swabs of 2 animals each)	1 leg skin per flock
Diagnostic method	PCR	Culture
Tested farms	160	160
Total number of flocks	3012	999
Campylobacter positive flocks	19.7% (594/3012)	24.3% (243/999)

PCR = polymerase chain reaction.

matching scenarios (I to IV), and thus, for most (≈ 91%) of the matched flocks, the two components agreed on the binary status classification (Table 1). On the other hand, for approximately 8.1% (scenario I) - 8.6% (scenarios II to IV) of the matched flocks, the results were positive in the FS but not in the AL component (Table 2). It was expected that all flocks where the two components disagreed, would be positive in the AL but negative in the FS component, due to higher flock (group) level sensitivity in the former (see next Section).

It is very likely that, the percentage of flocks found positive in the FS component but negative in the AL component (i.e. the *CC* flocks), were actually due to cross-contamination, rather than being caused by low sensitivity issues related to cloacal sampling. In fact, the contamination levels (cfu/g) observed in flocks positive in both components (namely the *PosBoth* flocks) were remarkably higher than in those that arrived at the slaughterhouse as AL negative, but became FS positive after processing (Table 3). Those flocks could have been cross-contaminated with "few" cfu/g persisting on the slaughter line from highly cloacal positive flocks.

4.2. Impact of testing strategy and uncertainty on the interpretation of surveillance outputs

Estimating the TP with the related 95% CI, helped understanding the pathogen's occurrence at both farm (AL component) and slaughterhouse levels (FS component) and the association between them, while taking into account for different sample types, sample sizes and tests used. This facilitates distinguishing changes in *Campylobacter* prevalence, which are due to the actual epidemiology of the pathogen (e.g. due to seasonality), from other variations of prevalence that could otherwise be caused, by the sampling and testing strategy applied.

For instance, the increase of AP and TP during the summer months in both components (Fig. 2), were likely to be caused by the actual epidemiology of *Campylobacter* infections/contaminations as reported in literature (Wedderkopp et al., 2000; Wedderkopp et al., 2001; Patrick et al., 2004). Whereas regarding the high spike of both estimates observed mainly from the FS component in March (Fig. 2), we speculate it could have been caused by: i) the actual epidemiology of the pathogen (i.e. actual increase of prevalence), and/or ii) by the sample size used.

On one hand, an actual change of prevalence due to unknown reasons, could be postulated because, in the previous four years (2014–2017) a similar pattern was observed in March 2014 and 2017, when a bump of flock prevalence was evident in the FS component, but not in the AL component (Gantzhorn et al., 2018). On the other hand, in March 2018, a "lower than usual" number of flocks were tested in leg skins (Fig. 1). In that period, Easter vacations could have affected (at least in part) the slaughtering and/or the sampling patterns. The number of culture tested flocks in the FS component (64 in Fig. 1) was lower than: i) the monthly median number of flocks tested in the same FS component (85) and ii) the number of PCR tested flocks in the AL component (251). Moreover, while the overall monthly median percentage of flocks tested in cloacal swabs and followed up in carcasses was around 34%, it was only 64/251 = 25% in March (the second lowest follow up observed after December, when Christmas and New Year holidays could have also affected the slaughtering/sampling patterns) (Fig. 1). Hence, it seems that in March 2018, compared to other months of the same year, a relatively higher numerator of FS positive flocks was divided by a relatively lower denominator. This observation is also supported by the fact that, the 95% CI (i.e. the uncertainty) was wider in March (8%) than in the previous (5%) and in the following month (7%) (Fig. 2), when similar environmental conditions should have applied (see Section 4.3 for discussion on statistical significance).

Regarding the comparison of the AP and TP obtained from the same surveillance stream, it was found that within the AL component, the AP was usually higher than the TP, while for the FS component, it was lower (March, June-September) or similar (other months) (Fig. 2). This difference between AP and TP estimated through the same sample size and component, showed the effect of correcting the AP estimate for the error of the test used. In the AL component, flocks were tested with the PCR, which had higher diagnostic sensitivity (Se 100% vs. 82.8%) and lower specificity (Sp 96.0% vs. 98.6%) than the culture test (Lund et al., 2004; Rosenquist et al., 2007). Thus, the AP estimated with the PCR was an overestimation of the TP investigated through the AL component, due to the low test's specificity (false positive results could have occurred). Whereas the AP estimated with the culture test was an underestimation of the TP investigated through the FS component due to the low test's sensitivity (false negative results could have occurred).

The uncertainty around the two TP estimates, was larger (wider 95% CIs) around the TP estimated with the FS component than around the TP estimated through the AL component (Fig. 2), due to the combination of smaller sample sizes tested in the former (Fig. 1) with the used: sensitivity, specificity, and prevalence (Reiczigel et al., 2010; Stevenson and Sergeant, 2021).

Therefore, using TP and its respective 95% CI, enhances the comparability and interpretability of surveillance information collected across sectors, which apply different sampling strategies and tests. For these reasons, the TP can be considered as a more objective, transparent and repeatable surveillance output-based standard than the AP. It also allows for historic comparisons, even if sampling strategies or test methodologies change during National Action Plans, and for comparisons between surveillance components, programs, countries, and populations.

4.3. Considerations from statistical modeling

At S2 a higher frequency of (direct) *Campylobacter* introduction through AL positive flocks (*PosBoth* plus *ALonlyPos* flocks), as well as a higher frequency of cross-contamination (in number of *CC* flocks) were observed compared to S1 (Section, 3.1.3); despite the number of flocks tested in both components was similar between the two slaughterhouses.

According to the GLM model, cross-contamination was significantly more likely to occur during summer and at S2. In March and from June to September, the chances of cross-contamination were significantly higher compared to January. The statistical significance of those months appeared to be in line with the interpretation of 95% CIs reported in Fig. 2, although in the figure, the comparison was made between components within the same month. Whereas in the GLM model, the comparison was made for all months against January. In other words, the AP measured through the FS component, represented the probability that a flock was carcass-contaminated with cfu/g detectable by the culture test during a specific month, while the TP estimated from the same component (Fig. 2), represented the probability that a flock was carcass-contaminated in reality (detected or not, by the culture test) during the specific month. Instead, the ORs estimated from the GLM model, suggested how much more likely was for an AL-negative flock, to become cross-contaminated (with cfu/g detectable by culture test) during month "x", compared to an AL-negative flock slaughtered in January. The

borderline level of significance observed for March (P-value = 0.049), seems in line with the discussion carried out in Section 4.2, about the potential impact of sample sizes (Fig. 1) for that month.

When the Bonferroni and Tukey's post hoc methods were applied to the pairwise comparisons of quarterly surveillance periods, it was confirmed that Q3 was the only quarter of the year with a significant effect for cross-contamination occurrence. This was evident by comparing Q3 vs Q1 (level of reference in the GLM model), but also comparing the former vs. Q2 or Q4 (through the Bonferroni and Tukey's methods). None of the other pairwise comparisons between Q-periods appeared significant.

When considering monthly surveillance periods, at both post-hoc methods, the statistical significance was shown only for August (against all months, apart for March, June, July and September), and for September (against January, February and April). It is known that the Bonferroni and Tukey's methods are conservative on showing significance and using "month" instead of "quarter" caused having less observations per surveillance period.

In the LME model, it was found that, the meat contamination was significantly higher on the flocks arrived as AL-positive to the slaughterhouse, than on the *CC* flocks. This finding was in line with those of previous studies (Allen et al., 2007; Johannessen et al., 2007; Elvers et al., 2011; Seliwiorstow et al., 2016). Moreover, there was a significant interaction between surveillance period and slaughterhouse; meaning that the effect of one variable (on meat contamination), changed across the levels of the other.

The results from the two statistical models are of high importance because, if needed, they could be used to prioritize risk-based control actions in a cost effective manner, i.e. in particular surveillance periods and/or slaughterhouses. For this purpose, the availability of consistent surveillance data from the AL and FS components, can allow repeated investigations through systematic data integrations, as shown in this study.

4.4. Information obtained from field investigations

The field study showed that 58.3% of the neck skin samples from the (caecal) negative flock following a positive flock were positive, while all other negative flocks slaughtered after negative flocks remained uncontaminated.

Several other studies have also shown cross-contamination at slaughterhouses, e.g. with varying degrees of 30% to 100% (Allen et al., 2007; Johannessen et al., 2007; Miwa et al., 2003; Sasaki et al., 2013), although it is difficult to directly compare the results of these studies due to different designs. The risk of carcass contamination as well as the counts of *Campylobacter,* can vary significantly between countries and slaughterhouses (EFSA, 2010). For example, the slaughterhouse-specific effect could relate to not only the different hygiene process standards, but also to the incoming flocks with varying *Campylobacter* infection status, as observed through the national surveillance data (Section, 3.1.3, Table, 3).

Similarly, the previous cross-contamination studies have consistently showed that the number of *Campylobacters* found on cross-contaminated broiler carcasses, is much lower than the number found on contaminated broiler carcasses of the originally infected flocks (Allen et al., 2007; Johannessen et al., 2007; Elvers et al., 2011; Seliwiorstow et al., 2016). However, it is worth noting that in the field investigation, some cross-contaminated carcasses contained as many *Campylobacters* as the carcasses originating from the caecal positive flock, and *Campylobacters* isolated from the (caecal) negative flock were of the same subtype as those isolated from the positive flock. While subtyping by MLST scheme is useful in characterizing bacterial isolates, it does not give enough discriminatory power to differentiate different strains, as the analysis is based only on seven house-keeping genes. On the other hand, SNP analysis compares isolates at the nucleotide level, giving high discriminatory power, and is often used in epidemiological investigations to assess the relatedness among the isolates (Schürch et al., 2018). There is no universal threshold for the number of SNPs to determine the relatedness (Schürch et al., 2018), but low number (tens) of SNPs, generally indicate a common source for outbreak investigations (Pightling et al., 2018). As an example, a recent Danish outbreak investigation has shown that *C. jejuni* outbreak strains had 0–6 SNPs, while there were 28–427 SNPs between the outbreak strains and non-outbreak strains of the same ST type (Joensen et al., 2021). In our field investigation, the SNP analysis showed up to 6 SNPs among the isolates from the two subsequent flocks, indicating that they were all closely related and likely to be originated from the preceding flock. This was as expected and further supports the occurrence of cross-contamination from the previous positive flock.

4.5. Considerations on the limitations of the study and potential improvements

While we tried to adjust for uncertainty and data quality, when analyzing the two national surveillance datasets, we acknowledge that it was still not perfect. The number of slaughtered birds per flock was not available in any of the two datasets, and the within-flock prevalence was not considered in the estimation of the flock level sensitivities. Normally, those parameters are important when sensitivities are calculated at (finite) group level (MacDiarmid, 1988), unless it is assumed that all units within the group are infected and that the individual diagnostic test sensitivity (Se) is similar to the group level sensitivity, e.g. when only one unit (n) is tested out of the total (N) present and infected. Usually, *Campylobacter* spreads quickly within infected flocks and in some way, the assumption of very high within-flock prevalence (e.g. ≈ 95–100%) is applicable (Berndtson et al., 1996; Miwa et al., 2003; Nauta et al., 2005; Van Gerwe et al., 2005; Koolman et al., 2014). The field study similarly showed that in the originally positive flock, all (10/10) caeca were contaminated. This is a common finding, especially if thinning is applied (Koolman et al., 2014).

Furthermore, an ideal estimation of flock prevalence, should be based on random sampling at all surveillance units levels (farm, flock and bird level). For this study, broilers to be tested from each flock were selected randomly in both surveillance components. The flocks to be tested in the FS component were also selected randomly at each slaughterhouse (S1 or S2) between all those slaughtered. Instead, the selection of flocks to be slaughtered and tested in the AL component, as well as their farms of origin, were more dependent on the age of the birds and on the farm's management, which could have introduced some level of bias on the estimates. Nevertheless, such a level of error could be considered relatively consistent across surveillance months, quarters and years, and it is not expected to affect the main results/conclusions of this study.

Regarding the unique sample identification number of the tested flocks (*Prøve.ID*), it was available only for the FS dataset, where each flock could be directly and unequivocally identified. In the AL component flocks could be identified by combinations of: CHR, house-ID, and sampling date. Due to this difference between datasets, it was decided to apply the same handling procedure for both of them. This procedure was validated using the FS dataset. It was checked that using the number of flocks counted by the unique ID gave the same number of flocks counted by the combination of the three variables mentioned above. The robustness of this approach was found acceptable. Furthermore, since scenarios III and IV led exactly the same matched flocks, it can be concluded that the same flock could be registered with a maximum discrepancy of four days between the two components. This discrepancy is very short and tolerable, despite the two surveillance components were designed for different purposes and were not originally planned to be continuously compared with each other (as made in this study). Nevertheless, the procedures of data handling and integration would become quicker and simpler to repeat, if data formatting was more similar between components. This could allow having a more detailed

Table 2
Comparison of binary testing results from two surveillance components (AL and FS) on conventional broiler flocks slaughtered during 2018 at the two major Danish slaughterhouses (S1 and S2).

Scenario	*Campylobacter* status	AL (+)	AL (-)	*Total*
I	FS (+)	30 (10.6%)	23 (8.1%)	*53 (18.7%)*
	FS (-)	5 (1.8%)	225 (79.5%)	*230 (81.3%)*
	Total	*35 (12.4%)*	*248 (87.6%)*	*283 (100.0%)*
Scenario	*Campylobacter* status	AL (+)	AL (-)	*Total*
II	FS (+)	151 (16.4%)	79 (8.6%)	*230 (25.0%)*
	FS (-)	14 (1.5%)	678 (73.5%)	*692 (75.0%)*
	Total	*165 (17.9%)*	*757 (82.1%)*	*922 (100.0%)*
Scenario	*Campylobacter* status	AL (+)	AL (-)	*Total*
III-IV	FS (+)	152 (16.0%)	82 (8.6%)	*234 (24.7%)*
	FS (-)	14 (1.5%)	701 (73.9%)	*715 (75.3%)*
	Total	*166 (17.5%)*	*783 (82.5%)*	*949 (100.0%)*

AL = Animal level component; FS = Food safety component. Matching scenarios: *I* = flocks matched across surveillance components on date, house-ID and CHR; II = allowing 1 day difference in dates reported across components; III-IV = allowing four to seven days difference if CHR and the house-ID matched between datasets; (+) = positive; (-) = negative.

knowledge of the pathogeńs occurrence along the poultry meat processing chain.

Considering the statistical modeling, it must be noted that the category of flocks positive only in the AL component (namely the *ALonlyPos* flocks) was disregarded from both models. In the GLM model, the *CC* and the *NegBoth* flocks were included to estimate, for the flocks that were negative from the farm level (i.e. AL-negative), the OR of becoming cross-contaminated on carcasses at the slaughterhouse, with detectable cfu/g (i.e. to become FS positive). Whereas in the LME model, the main aim was to compare the meat contamination levels between the *CC* and the *PosBoth* flocks. In reality some of the *ALonlyPos* flocks could have been "false positive" to the AL component, while others could have been "false negative" for the FS component, because the Sp of the PCR test and the Se of the culture test were <100%. Nevertheless, only 14 out of the 949 flocks tested and matched across components were classified as *ALonlyPos* (Table 2). Even considering all those flocks as "false positive" to the AL component (as worst case scenario) and including them into *NegBoth* category, would not have changed remarkably the main outputs of the GLM model. Furthermore, those flocks could not be included into the LME model, because they had no cfu/g.

Regarding the field investigation, due to the limited number of positive flocks, it was not possible to determine if there was a correlation between the bacterial load in the preceding (caecal) *Campylobacter*-positive flock and the frequency of cross-contamination in the following negative flock. However, according to the slaughterhouse veterinarian, who took the samples the 19th of October 2020, sampling of the neck skins from the flock started approximately 20 min after the flock change, and it lasted for approximately one hour. With the line speed of 13,000 carcasses per hour at S2, this meant that samples were taken between 4000 and 18,000 carcasses within flock 2. This is different from outputs of a risk assessment model, which predicted that cross-contamination lasts only up to 23–24 animals in a negative flock slaughtered after a positive (Nauta et al., 2005). In UK, *Campylobacter* was hardly detected by position of about 5000 carcasses (Elvers et al., 2011). On the other hand, Miwa et al. (2003) detected *Campylobacter* in the originally negative flock following a positive flock, after 4000 carcasses had passed and Seliwiorstow et al. (2016) likewise showed the presence of *Campylobacter* in the negative flock after 20 min from the start of the flock, i.e. after 2000–4000 broilers had passed. Further study with a large sample size is warranted to investigate the extent of the cross-contamination.

5. Conclusion

This study provides evidence by different approaches that *Campylobacter* cross-contamination of flocks can occur at considerable levels in slaughterhouses. Using surveillance data from two different points in the food chain, the monthly true prevalence estimates were higher at the end of the slaughter line than at the beginning, especially during summer. The cross- contamination was further investigated through the disagreement observed between the flocks, which entered at the slaughterhouse as *Campylobacter* negative, but were *Campylobacter* positive at the end of the slaughter line. The surveillance period and the slaughterhouse variables, interacted on determining the meat contamination levels and were both significantly associated with the occurrence of cross-contamination. The cross-contaminated flocks had a significantly lower contamination level (log10 cfu/g) than the flocks, which were positive in both components. The cross-contamination effect was further investigated in a field study, by sampling consecutive flocks at two slaughterhouses. One flock arrived at the slaughterhouse caeca

Table 4
A summary of caecal and neck skins samples taken from broiler flocks at the two Danish slaughterhouses (S1 and S2).

Sampling Date	Slaughterhouse	Flock	% Positive caecal sample (positive/total)	% Positive neck skin sample (positive/total)
2020–08–30	S2	Flock 1	0% (0/10)	0% (0/20)
2020–08–30	S2	Flock 2	0% (0/10)	0% (0/60)
2020–09–13	S1	Flock 1	0% (0/10)	0% (0/20)
2020–09–13	S1	Flock 2	0% (0/10)	0% (0/60)
2020–09–21	S2	Flock 1	0% (0/10)	0% (0/10)
2020–09–21	S2	Flock 2	0% (0/10)	0% (0/60)
2020–10–04	S1	Flock 1	0% (0/20)	0% (0/20)
2020–10–04	S1	Flock 2	NA	0% (0/60)
2020–10–19	S2	Flock 1	100% (10/10)	100% (20/20)
2020–10–19	S2	Flock 2	0% (0/10)	58.3% (35/60)
Total			100	390

NA = not available.

Table 3
Distribution of *Campylobacter* cfu/g on Danish broiler flocks (2018) positive in both components (AL and FS) or only in leg skins (FS).

		The cfu/g *Campylobacter* on carcasses (log 10 transformed values)							
Matching scenario	Test result	Min	2.5th percentile	25th percentile	Median	Mean	75 percentile	97.5th percentile	Max
I	AL +; FS+	20 (1.3)	27 (1.4)	230 (1.4)	565(2.4)	1348 (2.8)	1150 (3.1)	7173 (3.8)	10,000 (4.0)
	AL -; FS +	10 (1.0)	10 (1.0)	10 (1.0)	20 (1.3)	152 (1.4)	25 (1.4)	1450 (3.1)	2000 (3.3)
II	AL +; FS+	10 (1.0)	28 (1.5)	250 (2.4)	620(2.8)	1349 (2.8)	1500 (3.2)	7825 (3.9)	10,000(4.0)
	AL -; FS +	10 (1.0)	10 (1.0)	10 (1.0)	30 (1.5)	423 (1.8)	200 (2.3)	3095 (3.5)	8200 (3.9)
III-IV	AL +; FS+	10 (1.0)	28 (1.5)	250 (2.4)	615(2.8)	1342 (2.8)	1500 (3.2)	7753 (3.9)	10,000(4.0)
	AL -; FS +	10 (1.0)	10 (1.0)	10 (1.0)	30 (1.5)	410 (1.8)	178 (2.3)	2975 (3.5)	8200 (3.9)

AL = Animal level component; FS = Food safety component. Matching scenarios: *I* = flocks matched across surveillance components on date, house-ID and CHR; II = allowing 1 day difference in dates reported across components; III-IV = allowing four to seven days difference if CHR and the house-ID matched between datasets.

negative, right after a positive flock. Nonetheless, 58.3% of the tested carcasses from the former was contaminated with similar genotypes as the previously slaughtered positive flock. All other negative flocks slaughtered after a negative flock remained uncontaminated. Those results may be useful for industry, decision makers and national *Campylobacter* reduction initiatives, to increase efforts along the food chain aimed at: i) lowering the prevalence of flocks entering the slaughterhouse as positive and ii) reducing cross-contaminations during processing. Consequently, the campylobacteriosis risk to consumers could be reduced.

CRediT authorship contribution statement

Alessandro Foddai: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nao Takeuchi-Storm:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Birgitte Borck Høg:** Data curation, Investigation, Validation, Writing – review & editing. **Jette Sejer Kjeldgaard:** Data curation, Investigation, Validation, Writing – review & editing. **Jens Kirk Andersen:** Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. **Johanne Ellis-Iversen:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

None.

Acknowledgements

This work was supported by funding from the European Union's Horizon 2020 Research and Innovation programme under grant agreement No 773830: One Health European Joint Programme. The field study was funded by the Danish Veterinary and Food Administration (No 5234). The authors are thankful to Mette Rørbæk Gantzhorn, Bettina Pyndt Jørgensen, and Hans Ebbensgaard Murillo, from the Danish Veterinary and Food Administration for providing the data, constructive discussions, and critical review of the manuscript during the project.

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