Prevalence and types of *Campylobacter* on poultry farms and in their direct environment

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ABSTRACT

To study whether broiler and layer farms contribute to the environmental *Campylobacter* load, environmental matrices at or close to farms, and caecal material from chickens, were examined. Similarity between *Campylobacter* from poultry and environment was tested based on species identification and Multilocus Sequence Typing. *Campylobacter* prevalence in caecal samples was 97% at layer farms ($n = 5$), and 93% at broiler farms with *Campylobacter*-positive flocks ($n = 2/3$). *Campylobacter* prevalence in environmental samples was 24% at layer farms, and 29% at broiler farms with *Campylobacter*-positive flocks. *Campylobacter* was detected in soil and surface water, not in dust and flies. *Campylobacter* prevalence in adjacent and remote surface waters was not significantly ($P > 0.1$) different. Detected species were *C. coli* (52%), *C. jejuni* (40%) and *C. lari* (7%) in layers, and *C. jejuni* (100%) in broilers. Identical sequence types (STs) were detected in caecal material and soil. A deviating species distribution in surface water adjacent to farms indicated a high background level of environmental *Campylobacter*. STs from layer farms were completely deviant from surface water STs. The occasional detection of identical STs in broilers, wastewater at broiler farms and surface water in the farm environment suggested a possible contribution of broiler farms to the aquatic environmental *Campylobacter* load.

Key words | *Campylobacter*, environment, poultry farm, sequence type, surface water

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INTRODUCTION

Campylobacter infections are a leading cause of bacterial gastroenteritis worldwide. In the European Union, *Campylobacter* is the most commonly reported gastrointestinal bacterial pathogen in humans (EFSA 2014). The notification rate in the European Union was 55.49 per 100,000 people in 2012. In the United States, the FoodNet network identified 6,621 cases of *Campylobacter* infections in 2013, yielding an incidence of 13.82 per 100,000 people (Crim *et al.* 2014). Poultry, comprising broilers, laying hens, turkeys, ducks, and ostriches, is estimated to account for 50–70% of human *Campylobacter* infections (Epps *et al.* 2013).

Commonly reported symptoms of campylobacteriosis include diarrhoea, abdominal pain, fever, malaise and headaches (Epps *et al.* 2013). In humans, *C. jejuni* causes most infections, followed by *C. coli*, although *C. lari*, *C. fetus* and *C. upsaliensis* and other species have also been associated with human disease (Patrick *et al.* 2013; EFSA 2014). Infections are generally self-limiting, but approximately 10% of laboratory confirmed cases require hospitalization. Complications associated with *Campylobacter* infections are rare and include Guillain-Barré syndrome, a disease of the nervous system, and Reiter's syndrome, reactive arthritis (Whiley *et al.* 2013).

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Most human cases of campylobacteriosis are considered foodborne. A systematic review of case-control studies of human cases of campylobacteriosis and a meta-analysis of the retrieved data indicated that travelling abroad, eating undercooked chicken, exposure through environmental routes, direct contact with farm animals, and having a pre-existing chronic disease were significant risk factors. Consumption of chicken and poultry products was identified as the most important foodborne transmission route (Domingues *et al.* 2012). This was confirmed by the demonstration of a strong reduction in the number of reported campylobacteriosis cases in Belgium following the withdrawal of poultry products from retail outlets because of high dioxin levels in feed (Vellinga & Van Loock 2002). Source attribution analysis of campylobacteriosis cases in the Netherlands determined that the majority of human infections originated from chicken (66%), followed by cattle (21%), environment (10%), sheep (2.5%), and pigs (0.3%) (Mughini-Gras *et al.* 2012). A survey of 1,174 slaughter batches of broilers in the United Kingdom, processed at 37 abattoirs, demonstrated the colonization of the broilers with *Campylobacter* in 79.2% of the batches, emphasizing the importance of the handling or consumption of undercooked chicken as a major risk factor for human infection (Lawes *et al.* 2012).

The decline in the number of human campylobacteriosis cases in the Netherlands occurring after extensive culling of laying hens during an outbreak of avian influenza in poultry could, however, not be explained by reduced consumption of chicken meat (Friesema *et al.* 2012). Culling was mainly among laying hens, of which the meat is not consumed unprocessed in the Netherlands. Sales of poultry meat were nevertheless reduced, particularly in the culling area, but recovered rapidly, whereas the reduction in the number of cases in this area remained prominent for at least half a year. Reduced environmental contamination from the affected poultry farms, that were disinfected and remained empty for extended periods, is a possible explanatory factor for the reduced number of campylobacteriosis cases, which is supported by other studies (Domingues *et al.* 2012; Mughini-Gras *et al.* 2012) that similarly indicated the environment as a risk factor.

Environmental reservoirs of *Campylobacter* include the faeces of domestic animals such as dogs and cats, the faeces of wild birds and other wild animals, land application of farmyard manure, compost, and sewage (Whiley *et al.* 2013). *Campylobacter* spp. have also been isolated from various environmental water sources including rivers, lakes, streams, and coastal waters, which can become contaminated through direct fecal droppings from (agricultural or wild) mammals and birds, runoff from agricultural land, and (partially) treated and untreated wastewater. Domingues *et al.* (2012) identified contact with wild bird droppings (direct or on contaminated objects), drinking untreated water, and swimming in recreational waters as the most important environmental transmission routes. A case-case study in Canada identified consumption of water from private wells in rural areas as another risk factor for human campylobacteriosis (Lévesque *et al.* 2013).

This study investigated whether broiler and laying-hen farms contribute to the environmental load of *Campylobacter* spp. by examining various environmental matrices at the farms or close to the farms, such as soil, surface water, wastewater, air, dust and flies, as well as the caecal content of the chickens. Similarity between strains from poultry and from the environment was determined by using Multilocus Sequence Typing (MLST), that measures the variations in seven housekeeping genes yielding sequence types (STs) that can be grouped in clonal complexes with a common ancestor (Dingle *et al.* 2002).

METHODS

Poultry farms

During 2011 and 2012 three broiler (Br1–Br3) and five laying hen (Lh1–Lh5) farms in the Netherlands were visited multiple times. At each first orientation visit (t0), farms and their immediate surroundings were mapped, suitable sites for sampling were identified, and caecal material was sampled to establish presence or absence of *Campylobacter* in the flocks. During the second visit (t1), the farms were extensively sampled; samples included caecal samples and environmental samples such as soil, surface water, wastewater, dust, air and flies. At the laying-hen farms, the same flocks were present at t0 and t1, while at broiler farms the t0 flocks had been replaced with t1 flocks. All broiler farms were sampled a third time (t2), just after the t1

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flocks were removed and poultry-houses had been cleaned or were being cleaned; at these time-points caecal material was not present. Sampling strategies were different for the two farm types, due to the high turnover of flocks at broiler farms (every 6 to 7 weeks) and the lower turnover at laying-hen farms (approximately once in every 1.5 years). During the sampling, laying hens were 26–73 weeks old and broilers were 4–5 weeks old. Farm Br1 was extensively sampled during and after three different production rounds, at the paired time points t1/t2 (August–September 2011) and t3/t4 (November 2011) and t5/t6 (August–September 2012). At time points t1, t3 and t5, caecal material was sampled while the flocks were present, whilst at the subsequent time points t2, t4 and t6, wastewater was sampled after the same flocks had been removed for slaughter. All broiler farms used conventional barn farming, with capacities of 38,000 (Br3), 87,000 (Br1) and 150,000 (Br2) chickens. Two of the laying-hen farms were conventional barn farms with capacities of 78,000 (Lh2) and 80,000 (Lh4) chickens, and three of the laying-hen farms were free-range farms with capacities of 30,000 (Lh1, Lh5) and 43,000 (Lh3) chickens. All farms were located in rural areas (Figure 1) with

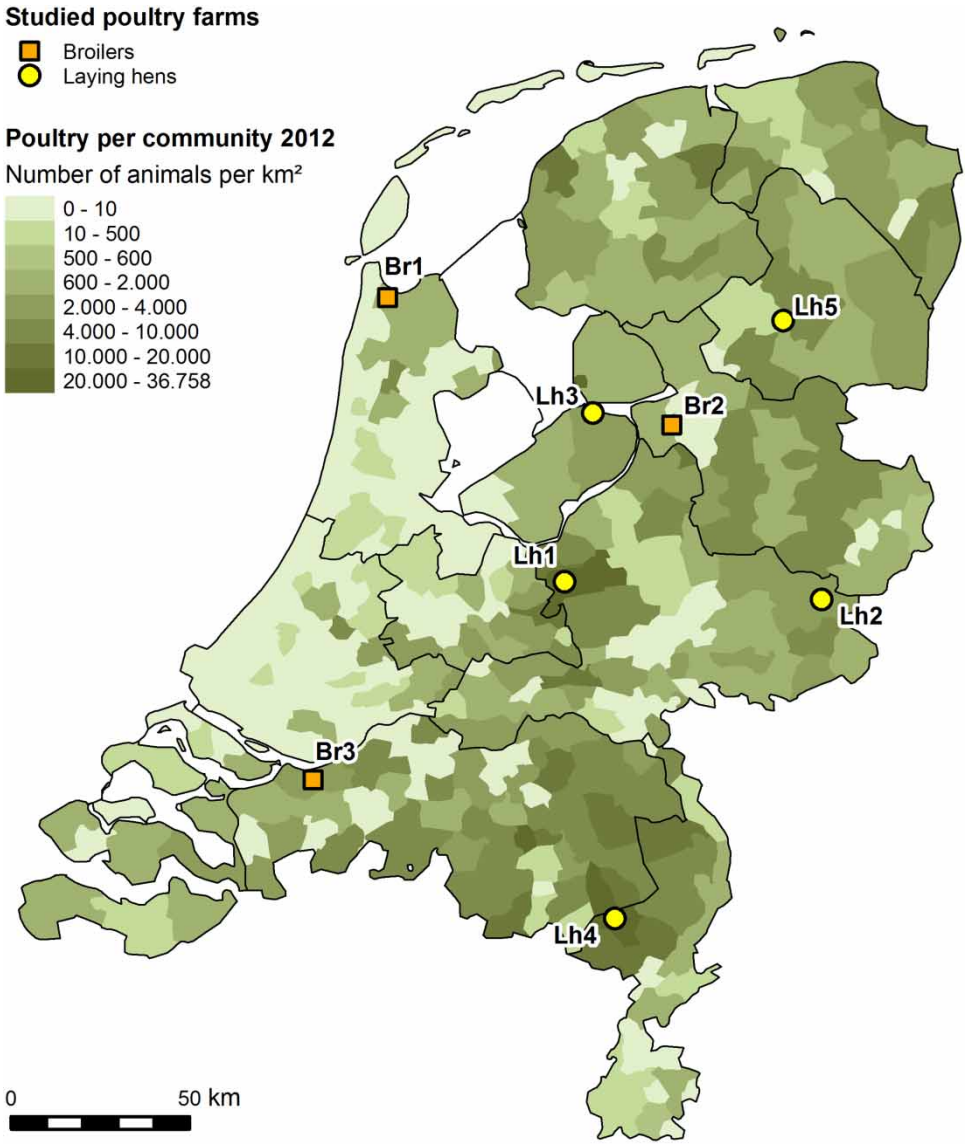

Figure 1 | Geographical location of the sampled broiler and laying-hen farms in a map of the Netherlands that displays the poultry density in the country.

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many small waterways (mainly ditches) nearby (<50 m) or further away (>50 m), except Lh5 which had no water nearby. An overview of farm details and sampling dates is available in Supplementary Table 1 (available with the online version of this paper).

Sampled matrices

Overall, 354 samples were taken (Figure 2). Fresh caecal material was sampled from poultry houses and free-range areas. Soil was sampled at free-range areas where applicable, and from various other sites at the premises, i.e. in the vicinity (1–5 m) of poultry houses, manure storage sheds, or manure belts, as well as at sites not obviously prone to such faecal contamination sources. When present, surface water was sampled from ditches bordering on or within 50 m distance of the farm premises, as well as from more distant (50 m–1 km) water bodies. Wastewater (i.e. water present at the farm premises, largely originating from the cleaning of the poultry houses and farm premises) was sampled at broiler farms from basins, drains, storage pits, and run-off gullies. In some instances the water level in run-off gullies was low and sediment was sampled instead. Only one of the laying-hen farms had a wastewater pit that actually contained wastewater at the time of sampling, and this was sampled as well. When applicable, faeces of other farm animals was also collected: farmyard laying hens (i.e. a limited number of birds kept by the farmer to supply his immediate family with eggs) at Br1 and Br2, cattle at Br2 and Lh2, horses at Lh3, and swallows at Lh2. Flies were caught in indoor environments such as poultry houses, manure-storage sheds, egg-sorting areas, homes, the canteen or changing rooms, and stables of other animals, as well as outdoors. Dust was sampled from surfaces inside poultry houses or manure-storage sheds, from poultry-house ventilation fans, and, at Br1, outside one of the poultry houses from a fence positioned within 5 m range of the ventilation fan. Most air samples were collected inside poultry houses.

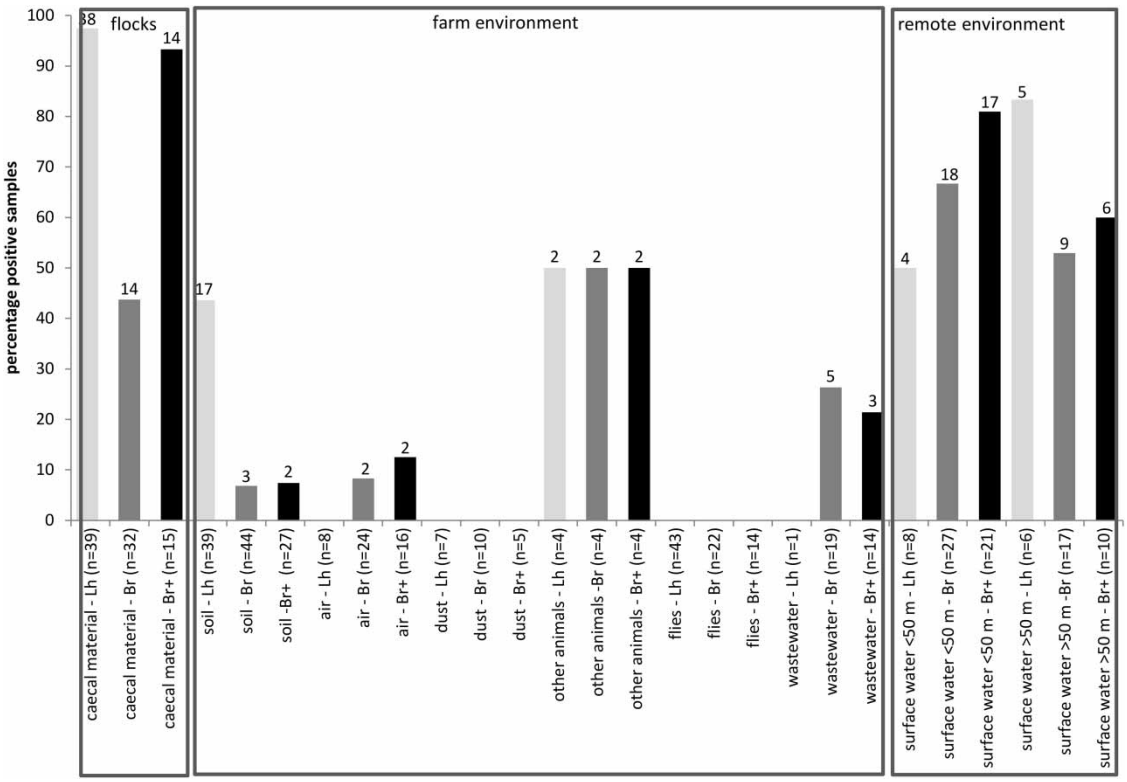

Figure 2 | Prevalence of *Campylobacter* at laying-hen farms and broiler farms and their environment. (Light grey bars: laying-hen farms (Lh); dark grey bars: all broiler farms (Br); black bars: broiler farms with *Campylobacter* positive flocks (Br+). Numbers between brackets indicate the total number of samples for each matrix, numbers above the bars display the number of positive samples for each matrix.)

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At farm Br1, outdoor air was also sampled (within 5 m of ventilator fans), as well as air from the canteen adjoining one of the poultry houses. All samples, except flies, were transported and stored at $5 \pm 3^{\circ}C$. Flies were transported and stored at room temperature. All samples were analysed within 24 hours of sampling.

Collection and preparation of samples

Caecal material

Caecal samples were collected from live birds by walking through the herds (with the appropriate protective clothing) and picking up freshly produced caecal material, either directly after it was seen to be produced, or from the stable floor when it was still soft, shiny and warm, indicating that it was freshly produced. Caecal material has a characteristic brown color.

Caecal samples consisted of material from three to five individual caecal droppings collected in a sterile stool sample container using the spoon attached to the lid. For analysis, samples were mixed using a cotton swab and, using the same swab, streaked onto CCDA agar (Oxoid B.V., Landsmeer, The Netherlands).

Soil, sediment and faeces

Soil was sampled from the surface to a depth of 5 to 8 cm, using a tubular soil sampler. At each site, nine to 12 grab samples were taken that were evenly distributed over a rectangular or square area with in-between distances of 0.5 to 1 m, and pooled in sterile filter bags (BagPage®, Interscience, St Nom la Bretêche, France). Sediments from (nearly) dried-up run-off gullies were sampled using a sterile spoon, and collected in sterile containers. Faeces from farmyard laying hens (picked up with gloved hand), cattle (scooped with sterile spoon), and swallows (picked up with gloved hand) were collected in sterile containers. Soil, sediment and faecal samples were diluted 1:1 in buffered peptone water (BPW), and homogenized using a Pulsifier® (Microgen Bioproducts, Camberley, UK). From these homogenates 20 g (i.e. equivalent of 10 g of soil, sediment or faeces) was put into 180 ml of Preston broth (Nutrient Broth No. 2, supplemented with Lysed Horse Blood, Preston Campylobacter Selective Supplement, and Campylobacter Growth Supplement; all components obtained from Oxoid B.V. and prepared according to the manufacturer's instructions).

Surface water and wastewater

All water samples were taken by submerging sterile glass bottles, according to ISO 19458 (ISO 2007). Water was filtered through 0.45 μm pore size membrane filters (Millipore, Amsterdam, The Netherlands) in total volumes ranging from 140 to 700 ml for surface water (depending on the turbidity) and 2–25 ml for wastewater. Filters were placed in 20–25 ml Preston broth.

Air

Air was sampled through 8 μm pore size membrane filters using a MD8 Airport portable air sampler (Sartorius Netherlands B.V., Rotterdam, The Netherlands), or alternatively, by placing CCDA agar-containing plates without lids on top of heat convectors that were suspended approximately 1 m above the poultry-house floors, for 50 to 75 minutes. Using the Airport air sampler, 250 or 500 L of air was sampled at 40 or 50 $l\,min^{-1}$. Upon sampling, membrane filters were directly placed in 180 ml Preston broth.

Dust

Dust was sampled from surfaces with sterile sponges that were pre-soaked with BPW using the Meat and Turkey Carcass Sampling Kit (Antonides BV, Oosterzee, The Netherlands). After sampling, the sponges were placed in sterile bags for transport to the laboratory, where they were placed in 90 ml Preston broth.

Flies

Flies were caught by using non-toxic, sticky flypaper, and collected within 24 hours after placement of the flypaper or alternatively, by using a fly swatter (gently, to avoid completely crushing the flies) and stored in sterile containers. Flies ($n = 297$) were analysed in 65 pools, each consisting of one to eight flies that were identical with respect to collection site and fly species/genus/family

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(Blaak *et al.* 2015). Flies were transferred aseptically from the flypaper and containers to sterile filter bags (Bag-Page®, Interscience, St Nom La Bretêche, France) containing 24 to 33 ml phosphate-buffered saline (Biotrading, Mijdrecht, The Netherlands) with 0.5% Tween-20 (VWR International B.V., Amsterdam, The Netherlands). Flies were thoroughly crushed by squeezing the filter bag between thumb and forefinger and then homogenized, using a Stomacher® 400 (Seward, Worthing, UK) at 230 rpm. Of these homogenates, 10 ml was added to 90 ml Preston broth.

Isolation and confirmation of *Campylobacter*

CCDA plates and Preston broth cultures were incubated in a microaerobic environment using CampyGen sachets (Oxoid B.V., Landsmeer, The Netherlands), for 48 ± 4 hours at 41.5 ± 1°C. Enriched samples where then streaked (10 µl) on CCDA agar and incubated under the same conditions. From each sample, a maximum of five suspected colonies was inspected for the typical 'corkscrew' morphology of *Campylobacter* by light microscopy. A maximum of three microscopically confirmed *Campylobacter* isolates per sample was subsequently subjected to polymerase chain reaction (PCR) analysis for genus confirmation and species identification.

Genus confirmation was performed by amplification of partial 16S rDNA using a real-time PCR based on the PCR described by Keramas *et al.* (2003). Species identification was achieved by using species-specific *glyA* PCRs for the detection of *C. coli*, *C. jejuni*, *C. lari* and *C. upsaliensis*, with a slight adaptation of the real-time PCR described by Jensen *et al.* (2005) (Table 1). For PCR analyses, material from one single colony was suspended in 50 µl Tris EDTA buffer (pH 8.0, Sigma-Aldrich, Zwijndrecht, The Netherlands) and cells were lysed at 95°C for 5 min. DNA extracts were stored at −20°C. For amplification 2 µl of 10× diluted DNA extract was mixed with 600 nM of each primer, 200 nM of the probe (Table 1) and 12.5 µl iQ Supermix (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) in a final volume of 25 µl. PCRs were run on a iQ5 real-time PCR detection system (Bio-Rad) using the following conditions: 2 min 50°C, 10 min 95°C, 40 cycles of 15 s 95°C, 60 s 60°C.

Table 1 | Primers and probes used for genus confirmation and species identification

Primer/probe	Sequence (5′-3′)	Reference
Genus confirmation		
UniversalCampy-F	CAACGAGCGCAACCCACG[a]	Keramas *et al.* (2003)
UniversalCampy-R	GCATAAGGGCCATGATGACTTG	This study
UniversalCampy-P	FAM-CGTCGTCCACACCTTCCTCCTCCT-TAMRA	This study
Species identification		Jensen *et al.* (2005)
C. coli-F	GGAGCTTATCTTTTTGCAGAC[a]	
C. coli-R	TGAGGAAATGGACTTGGATGC[a]	
C. coli-P	FAM-TGCTACAACAAGTCCAGCAATGTGTGCA-TAMRA	
C. jejuni-F	GTTCAGCCTAATTCAGGTTCTC[a]	
C. jejuni-R	GAACTTACTTTTGCACCATGAG[a]	
C. jejuni-P	FAM-AATCAAAGCCGCATAAACACCTTGATTAGC-TAMRA	
C. lari-F	CAGGCTTGGTTGTAGCAGGTG	
C. lari-R	ACCCCTTGGACCTCTTAAAG[a]	
C. lari-P	FAM-CATCCTAGTCCATTCCCTTATGCTCATGTT-TAMRA	
C. upsaliensis-F	CGTAGCTGGTGAGCATCCTAG[a]	
C. upsaliensis-R	GGTTTTGTGTGTGGTTGAGC[a]	
C. upsaliensis-P	FAM-CCTTTCCCTCACGCACACATCG-TAMRA	

[a]Primer and probes were slightly modified relative to the reference study.

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Isolate selection

Overall, 299 PCR confirmed *Campylobacter* spp. isolates were obtained; 116 from caecal material and 183 from other matrices. To exclude bias introduced by inclusion of 'copy-isolates', i.e. descendants of the same original strain multiplied during enrichment, one isolate per species per positive sample was randomly selected for data analysis. This resulted in the inclusion of 135 isolates for data-analysis: 56 from caecal material (42 and 14 from laying hen and broiler farms, respectively) and 79 (33 and 46 from laying hen and broiler farms, respectively) from other matrices. Among these 135 isolates were 69 *C. jejuni*, 55 *C. coli*, eight *C. lari*, and three other unidentified *Campylobacter* strains. The *C. jejuni* and *C. coli* isolates were further characterized using MLST. From caecal material, at least one isolate per species per sampling site per farm was included, resulting in MLST analysis of 27 of 31 *C. jejuni* and 16 of 22 *C. coli* isolates. From environmental matrices, 37 of 38 *C. jejuni* and 19 of 33 *C. coli* were included for MLST analysis. The proportion of environmental *C. coli* isolates that was included for MLST analysis was relatively low compared with the proportion of environmental *C. jejuni*, because only isolates from farms where *C. coli* was also detected in caecal material were included.

Multilocus sequence typing

To establish the relation between *Campylobacter* isolates from caecal material and farm environment, *C. jejuni* and *C. coli* isolates were characterized using MLST. For MLST, seven housekeeping genes were amplified (*aspA*, *glnA*, *gltA*, *glyA*, *pgm, tkt* and *uncA*), as described by Dingle *et al.* (2002). Primer sequences for PCR and sequencing were obtained from the *Campylobacter* MLST webpage http://pubmlst.org/campylobacter/info/primers.shtml (for *C. jejuni* the primers marked as 'most successful' were used).

For amplification, 2 μl of 10× diluted DNA extract was mixed with 200 nM of each primer, 1× PCR buffer (Invitrogen, Bleiswijk, The Netherlands), 2.5 mM $MgCl_2$ (Invitrogen), 200 μM dNTP mix (Invitrogen), and 1.25 U Taq polymerase (Invitrogen) in a final volume of 50 μl. Amplification conditions were: 5 min 95°C, 35 cycles of 30 s 95°C, 30 s 50°C (*C. jejuni aspA*, *pgm*, *tkt*, *uncA; C. coli tkt*) or 30 s 58°C (all other targets), 45 s 72°C, and a final 10 min 72°C. PCR products were analysed on agarose gel and PCR products of the expected size were treated with ExoSAP-IT (GE Healthcare, Hoevelaken, The Netherlands) followed by sequencing using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Bleiswijk, The Netherlands). STs were identified by comparing with the online database available at the *Campylobacter* MLST webpage http://pubmlst.org/campylobacter/ using Bionumerics software (version 7.5; Applied Maths NV, Sint Martnes-Latem, Belgium). New STs (new allele sequences and/or new allele combinations) were submitted to the MLST webpage. Bionumerics software was used for the construction of maximal parsimony trees based on the concatenated sequences of the alleles.

Statistics

Differences in *Campylobacter* prevalence between farms and sampling sites were tested with the Pearson's Chi-Square Test using SPSS Statistics software, version 22 (IBM, Amsterdam, The Netherlands).

RESULTS

Prevalence of *Campylobacter* at laying hen and broiler farms

At all five laying-hen farms, *Campylobacter* spp. were detected in caecal material taken during the orientation visits (t0) and the subsequent visits (t1), and the flocks were therefore considered to be *Campylobacter* positive. In total, *Campylobacter* spp. were isolated from 52 caecal samples: 38 samples (97%) from the five laying-hen farms and 14 samples (93%) from the broiler farms Br2 (t0, t1) and Br1 (t3, t5) (Figure 2). *Campylobacter* spp. were detected in caecal material from t0 and t1 flocks at Br2 and in the wastewater sampled at this farm at t2, but not in the caecal material from flocks at Br1 and Br3 present at t0 and t1. Caecal material from Br1 was however *Campylobacter* positive when sampled 7 weeks (t3) and a year (t5) later. Even though at Br3 all caecal samples at t0 and t1 were negative, *Campylobacter* was detected in wastewater

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that was sampled at t2, which was 3 weeks later, after removal of the flock and cleaning of the poultry houses.

Campylobacter spp. were detected in 24% and 23% of the environmental samples obtained from laying-hen farms ($n = 28/116$) and broiler farms ($n = 39/167$), respectively. At broiler farms, the prevalence in environmental samples was 29% when *Campylobacter* was detected in caecal samples ($n = 32/111$) and 12% when *Campylobacter* was not detected in caecal samples ($n = 7/56$). *Campylobacter* was not detected in any of the dust and fly samples at any of the farms, irrespective of whether *Campylobacter* was present in caecal samples or not. The highest prevalence of *Campylobacter* in the environment was observed at the more remote (>50 m) surface water sampling sites in the farm environment of laying-hen farms with *Campylobacter*-positive flocks (83%); the prevalence in surface water adjacent (<50 m) to these laying-hen farms was lower (50%) (Figure 2). These differences between adjacent and remote surface waters were however not significant ($P > 0.1$). The opposite was observed for broiler farms with *Campylobacter*-positive flocks, with the prevalence in surface water adjacent to the farms being higher (81%) than that in more remote surface water in the farm area (60%). Again, the observed differences between adjacent and remote surface waters were not statistically significant ($P > 0.1$). In soil, *Campylobacter* was more frequently detected at laying-hen farms: in 44% of samples compared to 7.4% of samples at broiler farms with positive flocks ($P = 0.002$) (Figure 2). This difference was mainly due to a high prevalence in free-range areas at laying-hen farms (14 of 18 samples positive (78%)). At broiler farms, *Campylobacter* was detected in soil close to poultry houses with a prevalence similar to that observed near manure storage sheds at laying-hen farms (10%). *Campylobacter* was also detected in other farm animals: cattle at Lh2 and farmyard laying hens at Br1. *Campylobacter*-negative animals included cattle and a farmyard laying hen at Br2, horses at Lh3 and swallows at Lh2.

Distribution of *Campylobacter* species in poultry and the farm environment

In caecal samples from each of the investigated laying-hen farms, both *C. coli* and *C. jejuni* were detected (Figure 3). Caecal material from laying-hen farms Lh1 and Lh3 additionally contained *C. lari*. Caecal material samples from the two farmyard laying hens that were kept in hen houses at the farm premises of Br1 also contained *C. jejuni*, *C. coli*, and *C. lari*, in line with the observations at laying hen farms. All *Campylobacter*-positive caecal samples from broilers contained *C. jejuni* only. *C. jejuni* was also the only *Campylobacter* species detected in wastewater, soil and air at broiler farms (Figure 3). By contrast, in surface water, including water bodies near and further away from the broiler farms, approximately half of the *Campylobacter* isolates were *C. jejuni*, whereas *C. coli* was also abundantly present (Figure 3). This suggests that there were other *Campylobacter* sources than the broiler farms investigated. At laying-hen farms, both *C. jejuni* and *C. coli* were detected in farm soils, mirroring the mixed prevalence of these species in caecal material (Figure 3). In contrast to soil and caecal material, *C. coli* was not detected in surface water adjacent to the laying-hen farms. In surface water near Lh1 a fourth *Campylobacter* species was detected, which appeared to be a non-*jejuni*, non-*coli*, non-*lari* and non-*upsaliensis* *Campylobacter* species in the species PCR. This *Campylobacter* species was not detected in any of the other samples.

Molecular relationship between *Campylobacter* isolates from different matrices

In caecal material from laying-hen farms, nine different *C. coli* (17 isolates) and ten different *C. jejuni* (12 isolates) STs were detected (Figure 4). In caecal material from broiler farms, six different *C. jejuni* (14 isolates) STs were detected (Figure 4). Among these STs were three new *C. coli* STs (ST-7916, ST-7918, and ST-7937) and two new *C. jejuni* STs (ST-7913, ST-7914). The majority of STs were farm-specific: among the *C. coli* isolates, only ST-901 was detected at two different laying-hen farms (Lh2 and Lh3), and also among *C. jejuni* isolates only ST-2314 was detected at two different laying-hen farms (Lh1 and Lh4). None of the same STs were observed in broiler and laying hen flocks.

At laying-hen farms, 40% (6/15) of all *C. coli* isolates from soil and 67% (2/3) of the *C. jejuni* isolates from soil had a ST that was identical to at least one ST of the isolates from caecal material at the same farm. The vast majority of the *C. coli* from caecal material (15/17, 88%) and soil

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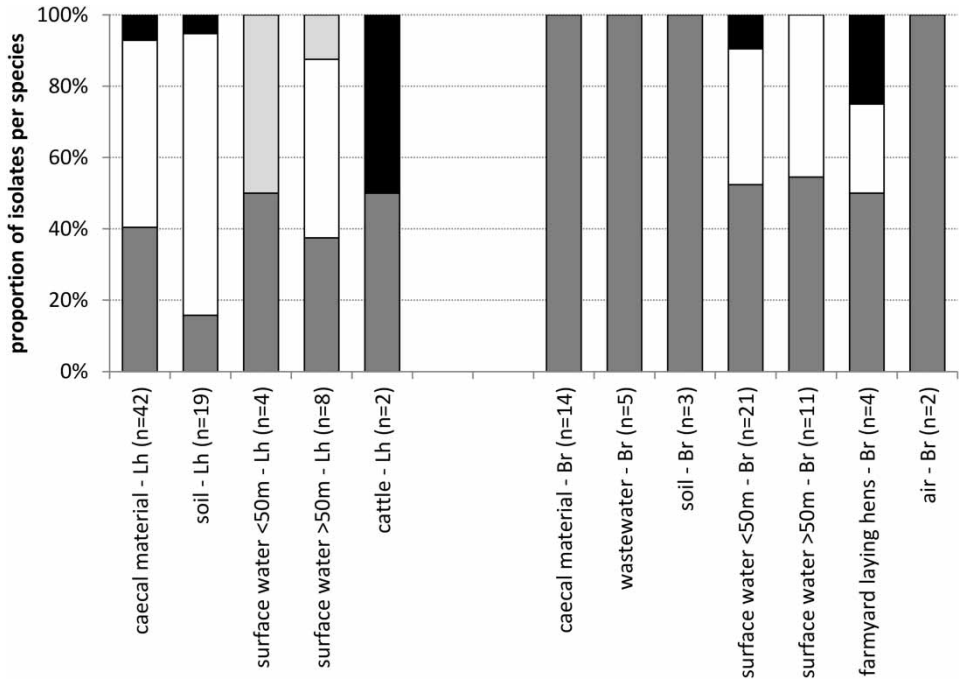

Figure 3 | Distribution of *Campylobacter* species among isolates from chickens and the environment at laying-hen farms (Lh) and broiler farms (Br). (White bars: *C. coli*; dark grey bars: *C. jejuni*; black bars: *C. lari*; light grey bars: *Campylobacter* species. Numbers between brackets indicate the total number of *Campylobacter* isolates for each matrix; percentages indicate the proportion of a species relative to the total number of *Campylobacter* isolates for each matrix.)

(13/15, 87%) belonged to the ST-828 clonal complex. The other four isolates (two from caecal material, two from soil) were more closely related to the ST-828 complex than the *C. coli* from surface water (Figure 3). All *C. coli* and *C. jejuni* isolates from surface water, both from adjacent and from remote water bodies, differed from 'caecal' STs. *C. jejuni* from cattle at Lh2 had a ST (ST-42) that was different from those found in the laying hen flock.

Fifty-three percent ($n = 9/17$) of the *C. jejuni* isolates from the farm environment at the broiler farms with *Campylobacter*-positive caecal material (Br1 and Br2) had a ST identical to those observed in caecal material at the same farms (Figure 4). Among these isolates were isolates from soil ($n = 2/2$,100%), poultry-house air ($n = 2/2$, 100%), wastewater ($n = 3/3$, 100%) and adjacent surface water ($n = 2/10$, 20%). Also, one of the more remote surface water sites ($n = 1/5$, 20%) in the area of farm Br1 contained a 'caecal' ST (ST-267). This water body was a ditch running alongside the farm premises, sampled at a distance of 100–150 m from the nearest poultry houses at the farmyard. *C. jejuni* ST-230 was detected in three different ditches in the vicinity of Br1, both during the presence of flocks and during cleaning. In two of these ditches, ST-230 was detected in samples taken adjacent to the farm as well as at a more remote site, in this case at 100–150 m distance.

Figure 4 shows maximum parsimony trees constructed based on the concatenated sequences of the seven MLST alleles. Node sizes reflect the number of isolates per ST and node colors represent different matrices; STs are indicated next to the nodes. Branch lengths are on a logarithmic scale, the numbers indicate number of nucleotide distances between nodes. In Figure 4(a), underlined STs belong to clonal complex ST-828. Note that per sample maximally one isolate of each variant was included, and that multiple isolates of a specific variant reflects detection in multiple samples.

DISCUSSION

As expected (Jore *et al.* 2010; Jones *et al.* 2012), *Campylobacter* was highly prevalent in caecal material from

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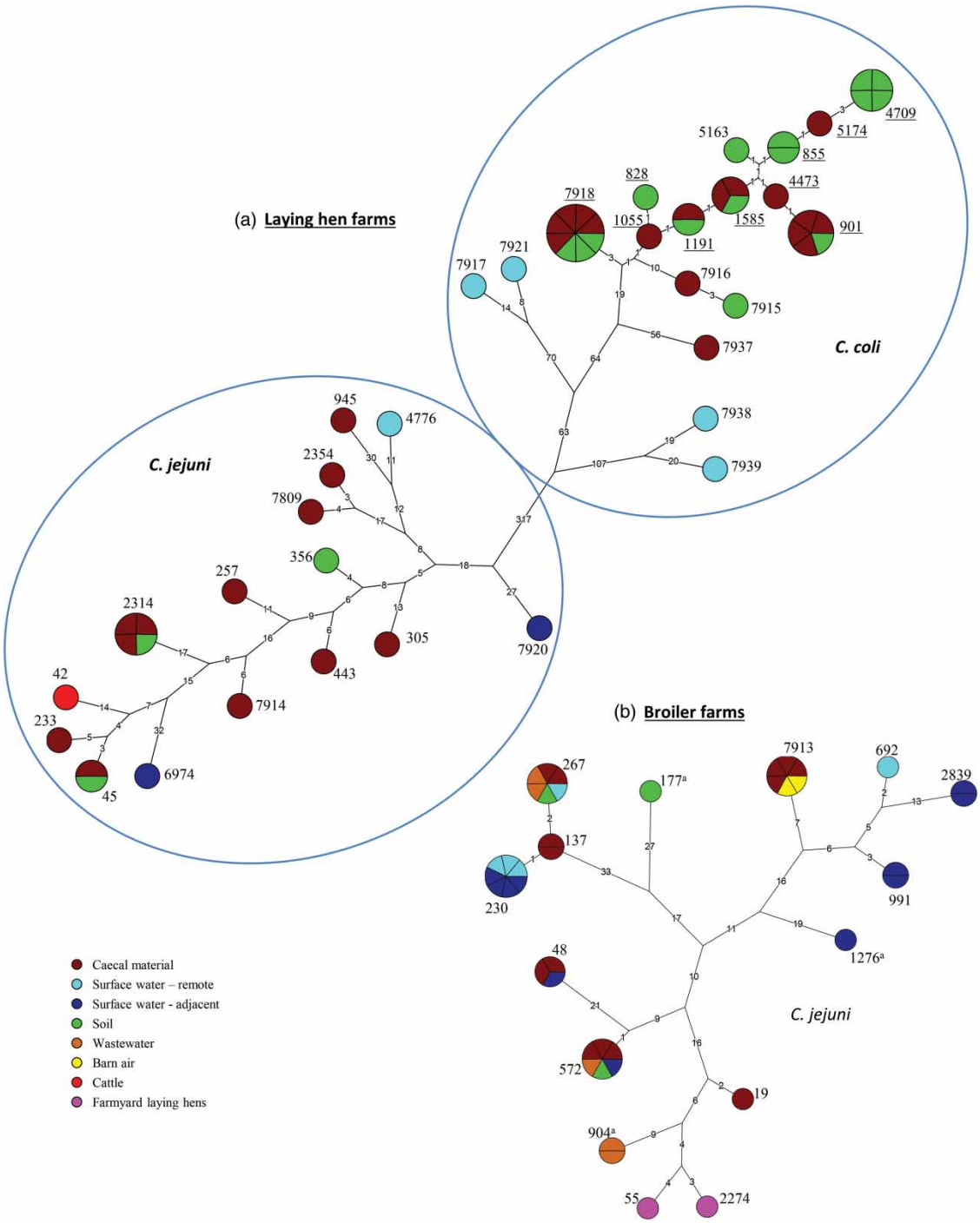

Figure 4 | Molecular relationship between isolates from laying-hen farms (a) and broiler farms (b).

Campylobacter-positive flocks, both at broiler farms and at laying-hen farms. *Campylobacter* was also detected in various environmental matrices at or close to the farms, predominantly in soil and surface water, the latter also containing *Campylobacter* when sampled 50 to 150 m away from the farms. *Campylobacter* was not detected in dust samples or on flies caught at any of the farms, suggesting that these matrices are of limited relevance for

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the dissemination of *Campylobacter* from poultry farms. However, the negative results do not necessarily reflect the absence of *Campylobacter* in these matrices, as numbers may have been very low. In other studies, *Campylobacter* has been detected on flies caught at poultry farms (Hald *et al.* 2004; Hansson *et al.* 2007), and calculations based on a worst-case scenario using data from literature, suggest that human exposure to contaminated flies from poultry farms cannot be ruled out (Evers *et al.* 2016).

Soil samples were found to contain *Campylobacter* when soil was likely to be contaminated by poultry faeces, e.g. when sampled from free-range areas at laying-hen farms, close to poultry-houses or manure storage sheds, and under manure transport belts. Sequence typing suggested that *Campylobacter* in soil originated from poultry since identical (or highly related) STs were found in caecal material and soil at all four laying-hen farms where *Campylobacter* was detected in soil, and at both broiler farms with *Campylobacter*-positive flocks. Similar results were obtained in Ireland, where identical STs in broilers and in soil were detected (O'Mahony *et al.* 2011). In that study, the *Campylobacter* prevalence in soil was 12%, which is in the same order of magnitude as the prevalence in soil samples at broiler farms with *Campylobacter*-positive flocks in the current study (7.4%). Since the soil that was found to be *Campylobacter*-positive does not leave the farm premises, soil as such is not considered a particularly relevant vehicle of spread of *Campylobacter* from poultry farms to the outside-farm environment where humans may be exposed, unless indirectly through run-off to adjacent surface water. However, contaminated soil on poultry farms may contribute to horizontal transmission of *Campylobacter* between flocks (Whiley *et al.* 2013).

The detection of *Campylobacter* in environmental samples, including wastewater and soil, at farm Br3 after removal of a *Campylobacter*-negative flock (time point t2), indicates that *Campylobacter* may persist in the farm environment, as has been previously demonstrated (Jones 2001; Nicholson *et al.* 2005). It is assumed that *Campylobacter* in environmental samples from Br3 originated from a previous flock. However, the flock present during sampling at time point, t1, may have contained *Campylobacter* at a low or undetectable level, or may have been colonized with *Campylobacter* in the time between taking caecal samples and the later visit to sample the environment after the flock had been slaughtered.

In contrast to soil, surface water may transport *Campylobacter* over longer distances with water flow or current, to water bodies that the general human population may be exposed to, e.g. during recreational activities or when the water is used for irrigating crops. Surface water adjacent to poultry farms may become contaminated with *Campylobacter* from poultry farms through run-off of water containing poultry faeces from the farm premises during (heavy) rainfall, but also during poultry-house cleaning activities.

The high prevalence of various *Campylobacter* species in surface water in the broiler farm areas, both close by and distant from the farms, in contrast to the sole detection of *C. jejuni* at the farms, suggests that there is a high background level of environmental *Campylobacter*. These *Campylobacter* probably originate from sources other than the investigated poultry farms, such as wild birds (Ogden *et al.* 2009), other (poultry) farms (Figure 1), or wastewater of urban origin (Jones 2001; Rodríguez & Araujo 2010). However, the detection of poultry STs in surface water close to Br1 and Br2 (ST-572, ST-48) and at 100–150 m distance from poultry houses at Br2 (ST-267), as well as the detection of one ST (ST-230) in three different ditches surrounding Br1, suggests that broiler farms could contribute to the *Campylobacter* load in surface water. This assumption is supported by the slightly (although statistically not significant) elevated prevalence in surface water adjacent to broiler farms compared with the prevalence at more remote sampling sites.

In laying-hen flocks, *C. jejuni*, *C. coli* and *C. lari* were detected. Even though *C. coli* was the most prevalent species in caecal material and farm soil, *C. coli* was not detected in surface water close to the farms. *C. coli* was, however, detected in surface water further away from the farms. The two *C. jejuni* isolates that were detected in surface waters close to laying-hen farms both had another ST than those that were detected in caecal material. Moreover, while all *C. coli* isolates from caecal material and farm environment either belonged to the same clonal complex (ST-828), or differed only slightly from the most related ST-828 clonal complex variant, the surface water isolates were completely deviant. This underscores the unrelatedness of these isolates

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to those present at the laying-hen farms. These findings suggest a minor contribution of laying-hen farms to the aquatic environmental *Campylobacter* load, if at all.

Our study results suggest that broiler farms contribute more to the *Campylobacter* load in the aquatic environment than laying-hen farms. This may be related to the more frequent cleaning of the poultry-houses at broiler farms as a result of the shorter life span of broilers (6–7 weeks) as compared to that of laying hens (approximately 1.5 years). During cleaning of poultry houses, large amounts of wastewater are generated which are partly stored in wastewater pits or enter the sewage system, but which may also reach adjacent surface water by running off the farm premises. The possible contribution of broiler farms to the environmental load of *Campylobacter* is supported by the observation of more positive environmental samples from farms with *Campylobacter*-positive flocks, in contrast to farms without positive flocks.

Although a limited contribution of poultry farms, and particularly broiler farms, to the *Campylobacter* load in surface water is suggested by our findings, this result should be considered as an indication that requires further study, rather than a conclusion. The relatively low number of water isolates, and the observed large variety of *Campylobacter* STs in the aquatic environment do not allow firm conclusions to be drawn. The study was designed to determine the contribution of poultry farms to the environmental load of extended-spectrum β-lactamase producing *Escherichia coli* (Blaak *et al.* 2015) and *Campylobacter* through various environmental routes, and did not specifically focus on water. Different environmental matrices were equally sampled and screened, which resulted in a limited subset of water samples and therefore a limited number of *Campylobacter* isolates from water.

The contribution of one individual farm, as in our study, may be limited, while the presence of multiple farms in a watershed may account for a higher proportion of the environmental load. In a study of a rural mixed watershed in the United States, it was observed that the prevalence of *Campylobacter* in surface water samples from the watershed increased with an increased number of poultry houses in the watershed, but also with increased influence of wastewater treatment plants (Vereen *et al.* 2013).

CONCLUSIONS

The study results indicate that poultry farms contribute to the environmental load of *Campylobacter* by contaminating soil at farm premises, and suggest that broiler farms could, more than laying-hen farms, contribute to the *Campylobacter* load in the aquatic environment. Recent findings from a study that inferred the origin of *Campylobacter* isolates from surface water in the Netherlands and Luxembourg demonstrated that isolates from the Netherlands were mainly attributed to poultry (Mughini Gras *et al.* 2016). Therefore, further study is necessary, using a study protocol that specifically aims at determining the influence of poultry farms on the aquatic environmental load of *Campylobacter*. This requires the inclusion of several poultry farms that are surrounded by water bodies, the testing of a larger number of water samples, including longitudinal sampling, and the isolation of more *Campylobacter* isolates, particularly because of the high level of background contamination and the large variation of *Campylobacter* STs in the environment. Such an approach additionally allows the study of co-circulating STs, because of larger numbers of samples and isolates. For development of intervention strategies to prevent contamination of the aquatic environment, the study of the contribution of risk factors for contamination, such as cleaning practices, the influence of rainfall, and the combined influence of multiple (poultry) farms, should be included.

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