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Research Note

Fecal Shedding of Thermophilic *Campylobacter* in a Dairy Herd Producing Raw Milk for Direct Human Consumption

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ABSTRACT

Factors affecting the fecal shedding of thermophilic *Campylobacter* in Italian dairy farms were investigated in a 12-month longitudinal study performed on a dairy farm authorized to sell raw milk in Italy. Fifty animals were randomly selected from 140 adult and young animals, and fecal samples were collected six times at 2-month intervals. At each sampling time, three trough water samples and two trough feed samples also were collected for both adult and young animals. Samples were analyzed with real-time PCR assay and culture examination. Overall, 33 samples (9.7%) were positive for thermophilic *Campylobacter* by real-time PCR: 26 (9.2%) of 280 fecal samples, 6 (16.6%) of 36 water samples, and 1 (4.2%) of 24 feed samples. *Campylobacter jejuni* was isolated from 6 of 280 samples; no other *Campylobacter* species was isolated. A higher (but not significantly) number of positive fecal samples were found in younger animals (11.33 versus 6.92% of adult animals), and a significantly higher number of positive water samples were collected from the water troughs of young animals. A distinct temporal trend was observed during the study period for both cows and calves, with two prevalence peaks between November and December and between May and July. Several factors such as calving, housing practices, herd size, management practices forcing together a higher number of animals, and variations in feed or water sources (previously reported as a cause of temporal variation in different farming conditions) were excluded as the cause of the two seasonal peaks in this study. The factors affecting the seasonality of *Campylobacter* shedding in the dairy herds remain unclear and warrant further investigation. The results of the present study indicate that special attention should be paid to farm hygiene management on farms authorized to produce and sell raw milk, with increased surveillance by the authorities at certain times of the year.

Thermophilic *Campylobacter* strains are some of the most frequent etiological agents of bacterial gastroenteritis in humans in many developed countries. The *Campylobacter* species *C. jejuni, C. coli, C. lari,* and *C. upsaliensis* are considered thermophilic because of their ability to grow at 42°C. Most detection methods are designed for the human pathogens *C. jejuni* and *C. coli,* and most studies deal with *C. jejuni,* a common commensal in the gastrointestinal tracts of wild and farm animals that is ubiquitous in the natural environment *(19)*.

Ruminants constantly shed *Campylobacter* into the environment, where these pathogen populations are a reservoir for human infection via ingestion of contaminated food or direct or indirect contact with ruminants or their feces *(25)*. The most frequent food vehicle reported in outbreaks of campylobacteriosis is raw unpasteurized milk, whereas for sporadic cases, eating poultry is the greatest risk factor *(27)*. Cattle may contaminate the food chain, resulting in a hazard for the milk supply, which manifests itself as an increased risk associated with the consumption of raw, unpasteurized milk *(31)*. In Italy, contamination of milk by thermophilic campylobacters has assumed more importance since 2004 when the sale of raw milk for human consumption by self-service automatic vending machines became legal; two outbreaks of campylobacteriosis associated with the consumption of raw milk sold by automatic vending machines were reported, one in the Veneto region *(2)* and the other in the Marche region *(28)*. In Italy, there are no official data on the incidence of *Campylobacter* infection because the surveillance of acute gastrointestinal illness is part of the official surveillance program of infectious diseases and the laboratory-based surveillance network for enteric pathogens (Enter-net Italia). The acute gastrointestinal illness cases reported to the surveillance program of infectious diseases fall into two categories, those associated with *Salmonella* and those associated with other infectious agents, without distinguishing acute gastrointestinal illness caused by *Campylobacter* spp. from those due to other agents *(23)*. Therefore, it is not possible to establish a correlation between the legalization of vending machine raw milk sales and cases of campylobacteriosis. However, in Italy the presence of the main important foodborne pathogens, among which thermophilic campylobacters in

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raw milk sold at vending machines has been well documented, has been reported with prevalence rates that varied between 0.11 and 2% and that appeared to be static from 2008 to 2011 *(6, 10)*. A seasonal trend was observed in the number of human campylobacteriosis cases, with peaks reported in spring and autumn in the United States, Europe, and Italy; a similar trend was observed in *Campylobacter* dairy cattle fecal shedding *(25)*, suggesting a relationship between cattle fecal shedding and human illness.

The epidemiology of thermophilic *Campylobacter* in dairy cow populations and the raw milk products that enter the food chain remains poorly understood, and a multitiered approach that begins as close as possible to the primary source (cattle) is needed and has been recommended to control campylobacteriosis *(31)*. Variables such as herd size and type, season, climate, animal age, geography, diet and husbandry practices, and the presence of wild birds have been implicated as the factors affecting *Campylobacter* fecal shedding on bovine dairy farms.

The prevalence of thermophilic *Campylobacter* in dairy cows on a herd basis has been widely studied, but few studies have been conducted on the longitudinal shedding of *Campylobacter* species in individual cattle. Most of the studies in which temporal variation and the impact of several factors (e.g., housing, season, climate, and age of animal) on thermophilic *Campylobacter* fecal shedding have been found were performed in countries applying different housing systems in different seasons. These housing practices are not common in Italy, and therefore the consequence of these variables on the shedding of thermophilic *Campylobacter* has yet to be determined in the typical Italian housing system in which cattle are usually housed inside the barn all year.

Research on the prevalence of fecal shedding of thermophilic *Campylobacter* on bovine dairy farms authorized to produce and sell raw milk and the factors affecting their shedding will be important for assessing risk and determining the design and implementation of management strategies. A 12-month longitudinal study was conducted on fecal shedding of thermophilic campylobacters on a bovine dairy farm in northern Italy that was authorized to produce and sell raw milk.

MATERIALS AND METHODS

The study was performed between October 2012 and September 2013 on a dairy farm of average size. This dairy herd was chosen on the basis of the results of a previous investigation *(11, 12)* as representative of a typical Italian dairy farm directly selling raw milk to consumers and meeting the following requirements: size, type of housing, type of feeding system, high hygiene standards, and known positive thermophilic *Campylobacter* status of in-line milk filters (14.2% as determined by real-time PCR) *(12)*.

The farm housed about 140 animals during the study: 70 cows (>24 months old, i.e., adults) and 70 calves and heifers (2 to 24 months old, i.e., young animals). Adults and young animals were housed in different parts of the barn; adult animals were kept in cubicles and divided into three groups depending on their milk production, whereas young animals were housed on straw in a yard with an external paddock and grouped in five pens depending on age from a minimum of 3 months to a maximum of 24 months.

Feeding for all animal groups and the total number of animals housed in the herd remained the same throughout the study. Water was provided by a municipal supply system. During the study period, the farmer intermittently split, mixed, or separated and moved the herd of dairy cattle between different fields for husbandry reasons.

A total of 50 animals were randomly selected from the dairy herd (25 adult and 25 young animals), and individual fecal samples were collected rectally from each animal six times at 2-month intervals throughout the duration of the study. Seven of the 25 selected adult animals were culled or sold during the study; thus, a total of 280 individual fecal samples were collected. In addition, three water trough samples and two feed trough samples for both young and adult animals were collected at each sampling time, for a total of 36 water samples and 24 feed samples. Water and feed sampling sessions were planned to collect at least one water trough sample for each group of animals and at least one sample for each type of feed given to animals (unifeed and concentrated feed in pellets fed to cows, hay fed to calves and heifers, and concentrated feed in pellets fed to heifers). Overall, 340 samples were collected and analyzed for the presence of thermophilic *Campylobacter* spp.

Thermophilic *Campylobacter* detection. Five grams of feces was homogenized in 5 ml of Bolton broth (Oxoid, Basingstoke, UK) and decanted at room temperature for 10 min; 100 μl of this suspension was then subjected to DNA extraction and real-time PCR by iQ-Check *Campylobacter* (Bio-Rad, Hercules, CA) according to the manufacturer's instructions *(4, 8)*. Reactions were carried out on a CFX-96 instrument (Bio-Rad) equipped with CFX Manager Ide software. The reference strain *C. jejuni* ATCC 49943 was included as a positive extraction control. *Campylobacter* isolation and identification was attempted from PCR-positive Bolton broth cultures according to the *Manual of Diagnostic Tests and Vaccine for Terrestrial Animals (21)*. PCR-positive Bolton broth cultures were plated onto selective modified charcoal cefoperazone deoxycholate agar (Microbiologics, St. Cloud, MN), and 100 μl was filtered with a 0.45-μm-pore-size cellulose acetate filter onto blood agar (Oxoid). After incubation at 41.5 ± 1°C under microaerophilic conditions for 44 ± 4 h, the plates were examined for colonies. Isolates were then confirmed by microscopic examination of morphology and motility, oxidase reaction, biochemical testing (glucose, lactose, and sucrose fermentation and gas and hydrogen sulfide production), and growth both in a microaerobic atmosphere at 25°C and in an aerobic atmosphere at 41.5°C. *C. jejuni, C. coli,* and *C. lari* were identified based on hippurate and indoxyl acetate hydrolysis.

For water and feed samples, 25 g of feed or 25 ml of water was homogenized in 225 ml of Bolton broth, incubated for 4 h at 37 ± 1°C, and then transferred to 41.5 ± 1°C for 20 h; 100 μl of the enriched cultures was subjected to DNA extraction and real-time PCR amplification by iQ-Check *Campylobacter* as described above. PCR-positive Bolton broth cultures were incubated for an additional 24 h at 41.5 ± 1°C, and then *Campylobacter* isolation and identification was attempted according to ISO 10272-1:2006 *(15)* by plating onto selective modified charcoal cefoperazone deoxycholate agar and *Campylobacter* selective medium–Skirrow agar (Biogenetics, Padova, Italy), and 100 μl was filtered with a 0.45-μm-pore-size cellulose acetate filter onto Columbia blood agar (Oxoid). After incubation at 41.5 ± 1°C under microaerophilic conditions for 44 ± 4 h, plates were examined and isolates were identified as described above.

Statistical analysis. Data analysis was performed using Stata 11.2 (2009, Stata Statistical Software: release 11, StataCorp.,

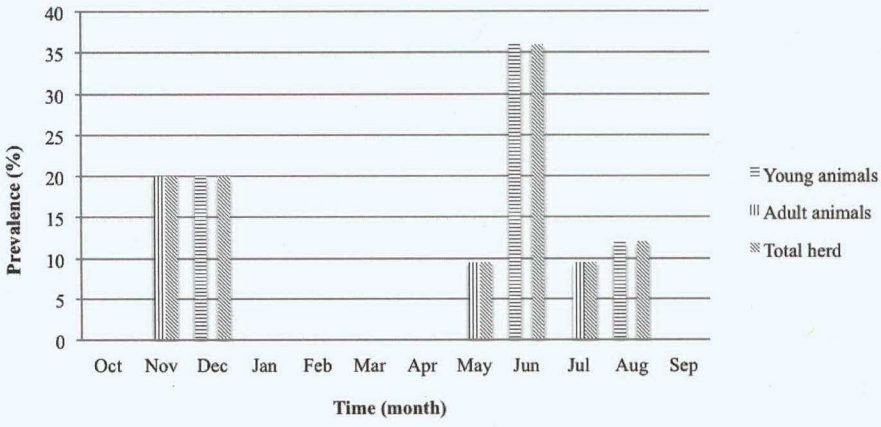

FIGURE 1. *Temporal variation of intra-herd prevalence of thermophilic* Campylobacter *during 1 year of sampling on the investigated dairy farm.*

College Station, TX). A logistic regression random effects model for longitudinal or panel data was built to evaluate the influence of animal age (young versus adult), average monthly temperature (°C), and rainfall (millimeters), on *Campylobacter* fecal shedding. Temperature was coded as low (<15°C), medium (15 to 20°C), or high (>20°C), and rainfall was coded as low (≤50 mm), medium (50 to 100 mm), or high (≥100 mm). The interaction between rainfall and temperature was included in the model. The average monthly temperature and the rainfall data were retrospectively collected from a meteorological archive *(3)*. Individual animal identifiers were used as the panel variable; sampling month was used as the time variable. After the model estimation, predicted prevalence was calculated on the basis of the observed age, temperature, and rainfall.

Fisher's exact test was used to compare the proportion of positive samples collected from water troughs of young and adult animals. Significance was set at $P < 0.05$.

RESULTS

A total of 33 samples (9.7%) were positive for thermophilic *Campylobacter* by real-time PCR examination: 26 (9.2%) of 280 fecal samples, 6 (16.6%) of 36 water samples, and 1 (4.2%) of 24 feed samples. Only six samples (1.7%) tested positive for *C. jejuni* by culture examination: 3 (1.1%) of 280 fecal samples and 3 (8.3%) of 36 water samples. No thermophilic *Campylobacter* spp. were isolated from feed samples. No *Campylobacter* spp. other than *C. jejuni* were isolated from any sample.

Only water troughs located in the young animal areas were *Campylobacter* positive (33.3% of the water samples by real-time PCR) at the first four sampling times (from October to May), and no *Campylobacter* was detected in any of the water samples from adult animal areas. The proportion of positive samples from young animal water troughs differed significantly from that from adult water troughs.

A higher *Campylobacter* prevalence was observed by real-time PCR examination in fecal samples of younger animals (11.33 versus 6.92% of adult animals), but the logistic regression model showed no significant difference in prevalence between young and adult animals.

Compared with *Campylobacter* prevalence at medium temperature and medium rainfall, the logistic regression highlighted a significant increase in prevalence when both temperature and rainfall were low and when the temperature was high and the rainfall was medium.

The significant relationship with weather variables can explain the peaks observed between November and December and between May and July, as can be seen by comparing the observed prevalences (Fig. 1) with the predictions of the logistic regression model (Fig. 2).

DISCUSSION

This report is the first to document temporal variation in fecal shedding of thermophilic campylobacters in a typical Italian dairy farm housing cattle inside the barn all the year and authorized to produce and sell raw milk to consumers.

The reported prevalence of thermophilic campylobacters in cattle worldwide varies widely. The individual *Campylobacter* prevalence in beef and dairy cattle, i.e., the percentage of positive animals within a single herd, ranges from 1.6 to 89%. In our study, only *C. jejuni* was isolated; the isolation of only *C. jejuni* rather than other thermophilic *Campylobacter* species is in agreement with previous studies in which *C. jejuni* accounted for 55.4 to 100% of the *Campylobacter* species isolated *(20)*. This result may also be affected by the fact that most detection methods are designed for the human pathogens *C. jejuni* and *C. coli*.

The *Campylobacter* prevalence observed in our study is relatively low, similar to other studies performed in dairy herds *(10, 16)* but significantly different from that reported by Bianchini et al. *(7)* in an area close to that of our study but involving farms producing milk for different dairy products (i.e., raw or pasteurized milk and soft or hard cheeses). The lower prevalence found in our study could be related to several factors. Our focus was on a dairy farm authorized to produce and sell raw milk for direct human consumption, which according to specific regulations *(16)* must implement higher hygiene standards than other dairy farms, must have a self-control plan, must use specific procedures for cleaning and disinfection of facilities and equipment, and must comply with more restrictive parameters for the total bacterial counts in milk (50,000 versus 100,000 CFU/ml for other farms) and specific requirements for pathogenic bacteria in milk (i.e., *Salmonella, Listeria monocytogenes, Escherichia coli* O157:H7, *C. jejuni,* and *Staphylococcus aureus*). The type of sampling performed, based on the main objective of the study, allowed us to follow the same young and adult animals throughout the study, and the analytical procedures we used were different

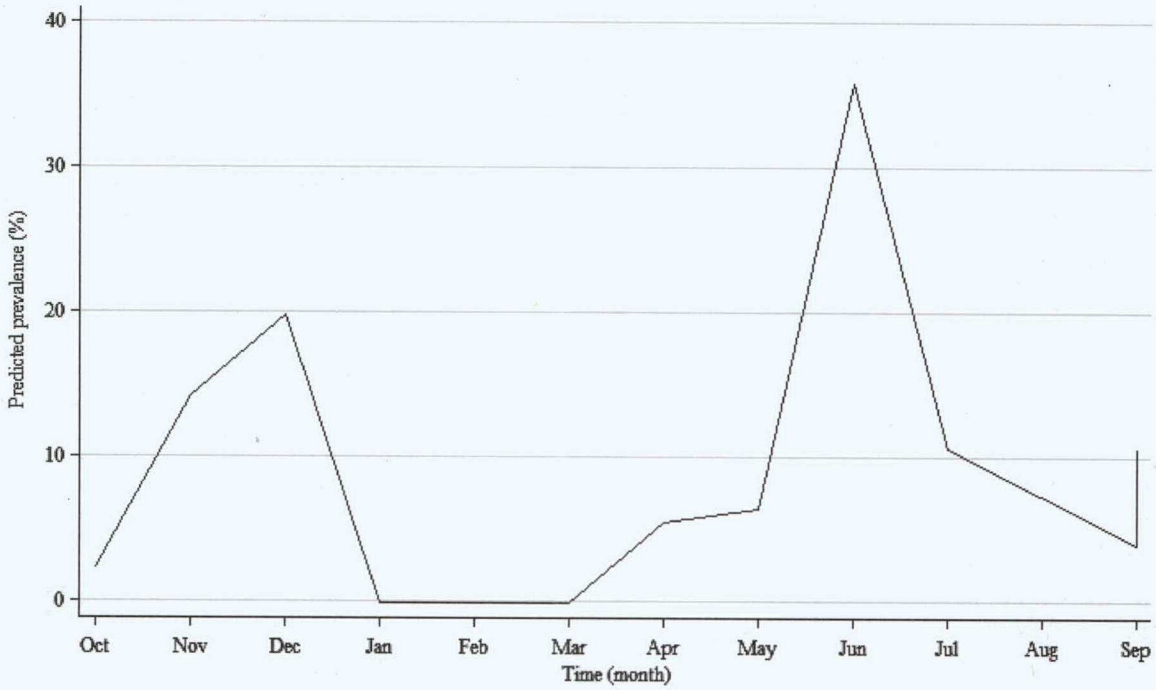

FIGURE 2. *Intraherd prevalence of thermophilic campylobacters predicted by the logistic regression model.*

from those used in the previous study. The real-time PCR detected 6.7-fold more positive samples than did the culture methods, in line with previously reported data *(10)*. The higher sensitivity of the PCR-based methods for foodborne pathogens in different types of samples has been well documented *(1, 5, 11, 12, 17, 29, 32)*.

The results of this study did not reveal a significant difference in *Campylobacter* prevalence between young and adult animals, although a higher number of positive fecal samples was detected in young animals. This finding is in contrast with those of other reports of a significantly higher prevalence of *C. jejuni* shedding in calves *(20)*. Several aspects of the present study may have influenced the result: the type of sampling (longitudinal) in animals of different ages (calves, heifers, and cows), the aging of animals during the study as previously observed *(9)*, the limited number of samples, and the use of animals from a single herd.

Water samples collected from water troughs were frequently contaminated (16.6%), and all *Campylobacter*-positive water samples were collected from troughs of only young animals. Contaminated water has been implicated as an important source of *Campylobacter* infection for cattle *(7, 14, 22, 25, 30)*. Ellis-Iversen et al. *(9)* found that water trough hygiene was associated with *Campylobacter* excretion and that more frequent emptying and cleaning of water troughs reduced the risk of *Campylobacter* infection in cattle. In another study, the circulation of *Campylobacter* spp. in dairy herds was related to water trough and bedding cleanliness *(12)*. Several factors could explain the higher contamination of young animal water troughs on the investigated dairy farm: (i) the higher (although not significantly) number of young animals shedding *Campylobacter;* (ii) the different design of water troughs for young and adult animals, i.e., the water troughs of adult animals are more modern and can be completely emptied, whereas the troughs of young animals cannot be emptied and hence are difficult to clean; and (iii) the different type of housing (cubicles for adult animals and straw yard with external paddock for young animals), which makes bedding and animal cleanliness more difficult to maintain among younger animals mainly in wet periods. A temperate climate with abundant rainfall hinders the maintenance of general farm cleanliness and hygiene leading to an increase in contamination of the farm environment, which was proposed as a cause of higher fecal shedding of *C. jejuni* by cattle *(22)*. All these aspects could have influenced the reported higher *Campylobacter* prevalence in both fecal and water samples of the young compared with adult animals, highlighting the relationship between water trough contamination and *Campylobacter*-positive fecal samples and the mechanisms for maintenance and recycling of *C. jejuni* on the dairy farm. Thus, *Campylobacter* circulation in the dairy herds is correlated with factors affecting water trough cleanliness.

The results of the present study confirmed the bimodal temporal distribution of fecal samples positive for thermotolerant campylobacters, which is the hallmark of *C. jejuni* epidemiology in agreement with several published surveys *(18, 22, 24–26)* performed under different farming conditions. However, it is not clear whether the periodicity in *Campylobacter* in fecal samples is due to recrudescence, i.e., fluctuations in population levels of indigenous campylobacters, or indicates seasonal reinfection *(25)*. Several explanations have been given for the observed seasonal trend. Stanley and colleagues *(25, 26)* speculated that the increased prevalence of thermophilic campylobacters in spring and autumn in four dairy herds in the United Kingdom could be due to the increase in calving or changes in diet or water source associated with the spring transition from winter housing to summer grazing and the autumn

return to winter housing. In a longitudinal study on 15 dairy farms, Grove-White et al. *(13)* reported an increase in the fecal shedding of *C. jejuni* in summer, attributing it to grazing more than to true seasonality. Other explanations include the presence of migratory birds, rodents or insects *(26),* housing practices that enhance the contact time between young and adult animals *(18),* management practices forcing a higher number of animals together in certain periods of the year, and hormonal changes due to calving *(22).*

The cause of the two peaks in fecal shedding in the dairy herd in the present study could not be identified unequivocally, but grazing pasture and changes in diet and water source can be ruled out because the cows, heifers, and calves were housed in the barn all year and no changes in feed or water source were made during the study. The concentration of animals in the barn and seasonal management of calving practices also cannot explain the enhanced fecal shedding because the number of animals remained almost the same during the study and seasonal calving practices were not used in this dairy farm. Higher environmental contamination due to migration of birds could explain the first peak (November and December) but not the second (May through July); instead, the May through July peak may be related to an increase in the number of flies, which are usually present in lower numbers in November and December.

Other environmental factors such as changes in rainfall, temperature, and day length have been implicated as probable causes for the seasonality of thermophilic *Campylobacter* excretion by cattle. In the present study, the relationship between *Campylobacter*-positive fecal samples and the average monthly temperature and rainfall was inconsistent but merits further investigation in a specifically designed study.

The findings of our study indicate that factors responsible for the seasonality of *Campylobacter* shedding reported previously in dairy herds in other countries with different housing systems are not directly applicable to Italian conditions; consequently, these factors could be differently relevant to the housing and climate scenarios under which the Italian dairy cattle were managed. Thus, the factors responsible for the seasonality of *Campylobacter* shedding in these dairy cattle have not been completely defined and warrant further investigation.

Although this study included only a single farm authorized to produce and sell raw milk and the low prevalence of *Campylobacter* precluded any epidemiological investigation, the findings allow some conclusions concerning the potential consequences for milk contamination. Because of the well-documented presence of *Campylobacter* in raw milk for human consumption that is sold at vending machines *(6, 10, 11)* and the fact that fecal contamination during milking is responsible for *Campylobacter* contamination of milk *(19),* the seasonality of thermophilic *Campylobacter* fecal excretion by cattle should be considered from a public health perspective. Therefore, the results of the present study suggest that special attention be paid to hygiene management on farms authorized to produce and sell raw milk, with increased surveillance based on specific risk-based plans by the authorities at certain times of the year.

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