Identification of risk factors influencing *Clostridium difficile* prevalence in middle-size dairy farms

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**Abstract**

Farm animals have been suggested to play an important role in the epidemiology of *Clostridium difficile* infection (CDI) in the community. The purpose of this study was to evaluate risk factors associated with *C. difficile* dissemination in family dairy farms, which are the most common farming model in the European Union. Environmental samples and fecal samples from cows and calves were collected repeatedly over a 1 year period on 20 mid-size family dairy farms. *Clostridium difficile* was detected in cattle feces on all farms using qPCR. The average prevalence between farms was 10% (0–44.4%) and 35.7% (3.7–66.7%) in cows and calves, respectively. Bacterial culture yielded 103 *C. difficile* isolates from cattle and 61 from the environment. Most *C. difficile* isolates were PCR-ribotype 033. A univariate mixed effect model analysis of risk factors associated dietary changes with increasing *C. difficile* prevalence in cows (*P* = 0.0004); and dietary changes (*P* = 0.004), breeding Simmental cattle (*P* = 0.001), mastitis (*P* = 0.003) and antibiotic treatment (*P* = 0.003) in calves. Multivariate analysis of risk factors found that dietary changes in cows (*P* = 0.0001) and calves (*P* = 0.002) increase *C. difficile* prevalence; mastitis was identified as a risk factor in calves (*P* = 0.001). This study shows that *C. difficile* is common on dairy farms and that shedding is more influenced by farm management than environmental factors. Based on molecular typing of *C. difficile* isolates, it could also be concluded that family dairy farms are currently not contributing to increased CDI incidence.

**Introduction**

*Clostridium difficile* is a spore forming Gram positive anaerobe, which causes hospital and antimicrobial-associated intestinal disease in humans and some animal species. The incidence, severity, and recurrence rates of *C. difficile* infections in humans are increasing [1–6]. Recent prevalence studies suggested that farm animals can be the source for human infection [7–10], which has not been scientifically confirmed [11].

Antibiotic treatment, hospitalization, change of diet and neonatal period were suggested risk factors for *C. difficile* perpetuation in farm and companion animals [12–21]. Most studies investigating the epidemiology of *C. difficile* in bovines were performed on large scale intensive dairy and/or beef operations [8, 11, 22, 23], which is not reflective of the European agriculture. These studies mostly investigated the risk of age [11, 22, 23] or age and antibiotic use [8] for *C. difficile* shedding with feces, many times excluding several possible farm management and environment related risk factors [24].

Family farming is the most common operating farming model in the European Union (EU) [25]. It is strongly supported by the European commission and the majority of member EU states, because of its positive contribution to the socio-economic and environmental sustainability of rural areas [25]. They directly supply the local community and the market in general with products of animal and non-animal origin, within a rich epidemiological environment comprised of people, pet animals, farm animals, wild animals and vermin. To date, there are no studies investigating the epidemiology of *C. difficile* in...
such an environment. The purpose of this study was, therefore, to determine the prevalence of *Clostridium difficile*, to characterize *C. difficile* isolates and to determine risk factors for *C. difficile* perpetuation within the most common operational farming model in Europe.

**Materials and methods**

This study underwent ethical review and was given approval by the National Animal Care Committee at the Ministry of Agriculture, Forestry and Food–Veterinary administration.

**Animal samples**

Twenty family dairy farms (Table 1) located in the Slovenian Prealps were included in this study. The average milk yield was 6605.2 L/milk/cow/year (3727.32–8876.64 L milk/cow). Diseases recorded and treated on the farms were mostly mastitis, pneumonia, diarrhea, displaced abomasum or other gastrointestinal diseases, ketosis and endometritis/metritis. During the study every herd was checked for infectious diseases, such as paratuberculosis, listeriosis, bovine viral diarrhea and infectious bovine rhinotracheitis (Table 1).

Products from farms included in this study are mostly sold within the local community, whereas surplus milk and meat are sold to different dairy and meat processing plants in Slovenia, Austria and north-east Italy.

Calves were categorized into three age groups at the time of each sampling (age group one: 0–21 days; age group two: 22–56 days; age group three: 57–180 days), based on their nutritional and digestive physiology [26]. Feces were sampled individually from cows and calves under the age of 6 months in exactly 2 week intervals for a period of 1 year (27 sampling days from November, 2011–November 2012). For all the farms, the same sampling protocol was followed. Samples were taken from the rectum using clean latex gloves (Shield, UK). Cow and calf fecal samples from each farm were pooled separately in the laboratory within 24 h after collection: One gram of fecal sample from each individual was used. Pooled samples were then diluted with sterile saline solution in a 1:3 ratio. The aliquot of 2 mL of every pooled sample, and individual samples from all calves were stored in 2 mL sterile vials (Eppendorf Tubes<sup>®</sup>, Germany) at −70 °C for future analysis.

Heifers and bulls over 6 months of age were excluded from the study because of their limited contact with humans. They are not subjected to significant stress factors of production animals and are usually not used to human handling.

| Farm characteristics and health status |
|---------------------------------------|
| **Farm characteristics** | **Health status** |
| Farm | Housing type | Cattle type | Farm location | No. dairy cows | Paratuberculosis | Listeriosis | BVD | IBR |
| Farm 1 | Free + Grazing | Holstein–Friesian | Rural | 17–21 | – | – | – | – |
| Farm 2 | Tie | Holstein–Friesian | Rural | 11–13 | – | – | – | + |
| Farm 3 | Tie | Holstein–Friesian | Village | 10–13 | – | – | – | + |
| Farm 4 | Tie | Holstein–Friesian | Village | 23–27 | – | + | – | – |
| Farm 5 | Free | Holstein–Friesian | Village | 21–24 | – | + | – | – |
| Farm 6 | Tie | Holstein–Friesian | Village | 14–18 | – | – | – | – |
| Farm 7 | Free | Holstein–Friesian | Village | 23–26 | – | – | – | – |
| Farm 8 | Tie | Holstein–Friesian | Village | 29–33 | – | + | – | + |
| Farm 9 | Tie | Holstein–Friesian | Village | 16–19 | – | – | – | – |
| Farm 10 | Tie | Holstein–Friesian | Village | 16–20 | – | + | – | + |
| Farm 11 | Tie + Grazing | Simmental | Rural | 14–16 | – | – | – | – |
| Farm 12 | Tie | Holstein–Friesian | Rural | 27–33 | – | + | – | – |
| Farm 13 | Tie | Mixed | Rural | 16–18 | – | – | – | – |
| Farm 14 | Tie | Simmental | Town | 11–18 | – | + | – | – |
| Farm 15 | Tie | Simmental | Town | 13–15 | – | – | – | – |
| Farm 16 | Tie | Simmental | Town | 13–15 | – | – | – | – |
| Farm 17 | Free | Simmental | Village | 31–40 | – | + | – | – |
| Farm 18 | Tie | Simmental | Rural | 11–14 | – | + | – | – |
| Farm 19 | Free | Holstein–Friesian | Village | 32–37 | – | + | – | – |
| Farm 20 | Tie | Simmental | Rural | 9–11 | – | + | – | – |
| Total (%) | 9–40 | 0/20 (0%) | 10/20 (50%) | 1/20 (5%) | 4/20 (20%) |

BVD: Bovine viral diarrhea, IBR: Infectious bovine rhinotracheitis, Free: Free range.
Environmental samples
Environmental samples were collected from every farm during the meteorological autumn, winter, spring and summer. Manure, silage/hay, water from drinking bowls and soil samples from around the barn were collected in sterile 10–50 mL tubes (Sarstedt, Germany). Samples from other domestic animals present on the farm were collected with sterile swabs (Deltalab, Spain). Barn flies (Stomoxys calcitrans) and Barn swallow droppings (Hirundo rustica) were sampled only once during the summer. Barn flies were captured alive with hands, using clean latex gloves (Shield, UK) and stored in 10 mL sterile tubes (Deltalab, Spain). Barn swallow droppings were collected from surfaces under the nests within the barn using sterile swabs (Deltalab, Spain).

Detection of *Clostridium difficile*
All pooled fecal samples were used for molecular detection of *C. difficile* 16S rDNA gene. Samples were processed within 2 days after collection. For total DNA isolation, SmartHelix™ First DNAid kit (IFB, Slovenia) was used as described previously [27]. *Clostridium difficile* 16S rDNA gene was detected using an improved quantitative PCR (qPCR) that has the lowest detection (7.72 CD cells/g feces) and quantification limit (77.2 CD cells/g feces) published to date [27]. Calf fecal samples were analyzed individually when pooled fecal samples tested positive on qPCR.

Pooled fecal samples from cows and individual calf fecal samples, which were positive for *C. difficile* 16S rDNA gene, were then cultured for *C. difficile* [7]. Samples were inoculated into cyloserine-cefoxitin fructose enrichment broth (Oxoid, UK) supplemented with 0.1% sodium taurocholate (Sigma, Aldrich) and cultured for 1 week in anaerobic conditions. Thereafter, 1 mL of inoculated broth from each sample and 1 mL of ethanol were mixed and left for 0.5 h at 20–25 °C. Samples were later inoculated onto standard selective medium enriched with cycloserine and cefoxitin (*C. difficile* agar base and *C. difficile* selective supplement; Oxoid, UK) and left to incubate for 48 h anaerobically at 37 °C. Preliminary identification of isolates was based on typical odor and morphologic criteria. One gram per sample, a swab or one mL of water sediment was used for culture. Environmental samples were cultured as described above.

Molecular characterization of *Clostridium difficile*
*Clostridium difficile* isolates recovered from fecal and environmental samples were characterized by PCR-ribotyping and toxinotyping. PCR-ribotyping was performed with primers for intergenomic region 16S-23S [28]. Amplification with PCR and electrophoresis of the PCR products on 3% agarose gel were done according to Janezic et al. [29]. PCR ribotypes were named using standard Cardiff/Leeds nomenclature (3-digit code). If reference strains were unavailable, the PCR ribotype was named using keys designated by internal nomenclature. Toxinotyping was performed using subsequent restriction PCR fragments for A3 (part of *C. difficile* toxin gene A, tcdA) and B1 (part of *C. difficile* toxin gene B, tcdB) [30], while the gene for the binary toxin was detected using the protocol described by Stubbs et al. [31].

Parasite burden on farms
Parasitological evaluations of pooled fecal samples from cows and calves were performed every month during the sampling period using standard flotation and sedimentation techniques [32].

Data collection and statistical analysis
Information regarding feeding regimens, diseases, and treatments were obtained from farmers, farm veterinary services, and the Central Husbandry Register. Heat index [33] was obtained from the nearest National Meteorological Service weather station (Ljubljana, Slovenia—14°5’E, 46°1’N). A mean value for heat index was calculated over 7 days prior to each sampling day.

The outcome in this study was the presence of *C. difficile* (present, not present) in four subgroups: (1) cows, (2) calves aged 0–21 days (first group), (3) calves aged 22–56 days (second group) and (4) calves aged 57–180 days (third group). The following risk factors were considered for each subgroup: Intestinal parasites, dietary change (a change from conserved to fresh feed), heat index, breed (Holstein–Friesian and Simmental), antibiotic treatment, other treatment (non-antibiotic treatment prescribed by the veterinarian), gastrointestinal disease, mastitis, other diseases, and meteorological season (Tables 4 and 5). The absence of a risk factor was considered as a reference category for odds ratio; a reference category for the outcome “Breed” was Holstein–Friesian.

The analyses were performed at the farm level. First, the univariate assessment of the association between each risk factor and different outcomes was performed by means of logistic regression where farm was included as the random effect. The week of sampling was included in each model as a fixed effect to adjust for the possible confounding effect of time. The variable was treated as continuous and a possible non-linear association was modelled using restricted cubic splines, however none of the models showed a significant effect of the non-linear term as judged by the likelihood ratio test (p > 0.05); therefore, only results for the linear association are reported. P-values were adjusted with the Benjamini-Hochberg method (P(bh)) to control the false discovery rate. Significance level was set to 0.05 for the adjusted
Statistical computing (R version 3.0.1)[34].

Models were built for each outcome. Backward selection for C. difficile; only one isolate was then successfully cultured. No C. difficile was isolated from fecal droppings from Barn swallows sampled during their peak breeding season.

Molecular characterization

Overall from 103 C. difficile strains 16 PCR-ribotypes and 4 toxinotypes were cultured from cows and calves. In cows, only a toxin negative PCR-ribotype 071 was cultured, which was also identified in calves from the same farm. The most predominant C. difficile strain in calves was PCR-ribotype 033 (toxinotype X1a; 75.5%). PCR-ribotypes 071, SLO 084 and SLO 116 were toxin negative, whereas ribotype 023 was toxinotype IV. All other ribotypes (001/072, 002, 003, 005, 012, 014/020, 018, 077, SLO 029, SLO 036, SLO 195 new) were toxinotype 0 (Table 2).

Sixty-two C. difficile strains grouped into 19 different PCR-ribotypes (one new) and 6 different toxinotypes (toxin negative, 0, IV, V, X1a and X1c-new) were identified in the environment (Table 3). The most predominant C. difficile types were SLO 060 and 033 (toxinotype X1a,c). Toxin negative PCR-ribotypes were SLO 057, SLO 116 and SLO 196, whereas PCR-ribotype 023 was toxinotype IV and PCR-ribotype 045 was toxinotype V. All other ribotypes (001/072, 002, 003, 012, 014/020, 018, 077, SLO 025, SLO 036, SLO 053, SLO 063) were toxinotype 0.

PCR-ribotype 001/072 was found in manure, soil and silage, while PCR-ribotype 014/020 was recovered from manure, soil and water samples.

Two strains of C. difficile were recovered from an adult rooster (PCR-ribotype/toxinotype; 045/V and SLO 060/X1a) and one new strain was isolated from a two-week-old rooster (SLO 196/toxin negative). Stable flies were infected with C. difficile PCR-ribotype/toxinotype 033/X1c (new toxinotype), which was also present in manure and soil samples.

Parasite burden between farms

Parasites identified in pooled fecal samples were: Stron-gylida (65%), Paramphistomum cervi (30%), Nematodirus sp. (55%), Strongyloides (15%), Eimeria sp. (100%), Mon-ezia sp. (40%) and Fasciola hepatica (5%).

Univariate analysis of risk factors

In cows (Table 4), the only risk factor associated with C. difficile prevalence after adjusting for time of sampling were dietary changes (OR 5.0; 95% CI 2.0–12.1; P = 0.004; Pbh = 0.007).

In the first age group of calves (Table 5) risk factors increasing C. difficile prevalence were dietary changes (OR 5.08; 95% CI 2.3–77.9; P = 0.004; Pbh = 0.04) and breeding Simmental cattle (OR 5.3; 95% CI 1.9–14.7; P = 0.001; Pbh = 0.03).
Table 2  Results for individual fecal samples from calves and molecular characterization of isolated *C. difficile* strains

| Samples | Farm | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | Overall | % |
|---------|------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|------|----|
| No. calves | | 171 | 48 | 97 | 160 | 121 | 69 | 263 | 125 | 48 | 153 | 73 | 98 | 110 | 313 | 83 | 59 | 157 | 70 | 184 | 40 | 2442 | 100 |
| Analysis DNA isol. | | 44 | 24 | 6 | 92 | 83 | 26 | 11 | 34 | 6 | 6 | 185 | 62 | 26 | 81 | 41 | 52 | 12 | 918 | 37.6 |
| qPCR + | | 11 | 8 | 1 | 17 | 32 | 15 | 2 | 15 | 2 | 10 | 9 | 1 | 1 | 30 | 29 | 12 | 24 | 14 | 6 | 4 | 243 | 10 |
| Culture + | | 2 | 5 | 1 | 8 | 7 | 6 | 2 | 4 | 1 | 5 | 2 | 1 | 0 | 11 | 17 | 7 | 11 | 10 (9) | 2 | 0 | 102 (101) | 42 (41) |
| Ribotype | Toksinotype | | | | | | | | | | | | | | | | | | | | | |
| 001/072 | 0 | 1 | | | | | | | | | | | | | | | | | | | | |
| 002 | 0 | | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| 003 | 0 | | | 1 | | | | | | | | | | | | | | | | | | | | |
| 005 | 0 | | | | 1 | | | | | | | | | | | | | | | | | | | |
| 012 | 0 | | | | | 3 | 1 | | | | | | | | | | | | | | | | |
| 014/020 | 0 | | | | | 1 | | | | | | | | | | | | | | | | | | |
| 018 | 0 | | | | | | 1 | | | | | | | | | | | | | | | | | |
| 023 | IV | | | | | | | | | | | | | | | | | | | | | |
| 033 | XIX | | | | | | | | | | | | | | | | | | | | | |
| 071 | Tox- | | | | | | | | | | | | | | | | | | | | | |
| 077 | 0 | | | | | | | | | | | | | | | | | | | | | |
| SLO 029 | 0 | | | | | | | | | | | | | | | | | | | | | |
| SLO 036 | 0 | | | | | 1 | | | | | | | | | | | | | | | | | |
| SLO 084 | Tox- | | | | | | | | | | | | | | | | | | | | | |
| SLO 116 | Tox- | | | | | | | | | | | | | | | | | | | | | |
| SLO 195 | 0 | | | | | | | | | | | | | | | | | | | | | |


### Table 3  *Clostridium difficile* isolates from environmental samples and their molecular characterization

| Environmental samples | C. difficile culture results | C. difficile strain characterization |
|-----------------------|-----------------------------|-----------------------------------|
|                       | Winter          | Spring       | Summer      | Autumn       | All year     | Ribotypes       | Toxinotypes       |
| Manure                | 4/20 (20%)  | 7/20 (35%)  | 5/20 (25%)  | 7/20 (35%)  | 23/80 (28.7%) | 001/072, 002, 014/020, 023, 033, 077, SLO 036, SLO 053, SLO 060 |
| Soil                  | 5/20 (25%)  | 8/20 (40%)  | 7/20 (35%)  | 8/20 (40%)  | 28/80 (35%)  | 001/072, 012, 014/020, 018, 023, 033, 081, SLO 025, SLO 057, SLO 060, SLO 063 |
| Silage/hay            | 0/20 (0%)   | 1/20 (5%)   | 0/20 (0%)   | 2/20 (10%)  | 3/80 (3.75%) | 001/072, 003, SLO 045, SLO 060, SLO 196 (new) |
| Water                 | 1/20 (5%)   | 0/20 (0%)   | 1/20 (5%)   | 1/20 (5%)   | 3/80 (3.75%) | 0, tox- |
| Other animals on farms| 1 (2 strains)/32 (3.1%)--adult rooster | 0/33 (0%) | 1/24 (4.2%)--rooster 0/26 (0%) | 2/115 (1.7%) | 0, tox- |
| Barn swallows (Hirundo rustica) | / | / | 0/20 (0%) | / | 0/20 (0%) |
| Stable flies (Stomoxys calcitrans) | / | / | 2/20 (10%) | / | 2/20 (10%) 033 Xlc (new) |
| Total                 | 11 (12)/112 (9.8%) | 16/113 (14.2%) | 16/144 (11.1%) | 18/106 (17%) | 61/475 (12.8%) |

### Table 4  Risk factors associated with the prevalence of *C. difficile* in cows and third age group of calves (57–180 days)

| Age group | Cows | Calves 57–180 days |
|-----------|------|---------------------|
| Risk factors | Odds ratio | CI, low | CI, up | P value | Pbh | Odds ratio | CI, low | CI, up | P value | Pbh |
| Intestinal parasites cows | 0.67 | 0.27 | 1.67 | 0.397 | 0.685 | 1.11 | 0.45 | 2.73 | 0.805 | 0.899 |
| Intestinal parasites calves | 0.97 | 0.47 | 1.98 | 0.937 | 0.976 | 1.12 | 0.53 | 2.37 | 0.760 | 0.899 |
| Dietary change | 5.00 | 2.06 | 12.11 | 0.001 | 0.007 | 2.84 | 1.10 | 7.35 | 0.030 | 0.289 |
| Heat index | 0.95 | 0.91 | 0.99 | 0.046 | 0.323 | 0.98 | 0.93 | 1.02 | 0.423 | 0.899 |
| Breed | 1.80 | 0.58 | 5.57 | 0.304 | 0.685 | 2.82 | 1.23 | 6.47 | 0.014 | 0.268 |
| Antibiotic treatment: cows | 1.72 | 0.86 | 3.43 | 0.118 | 0.323 | 1.26 | 0.59 | 2.69 | 0.545 | 0.899 |
| Other treatment: cows | 1.85 | 0.91 | 3.74 | 0.087 | 0.323 | 1.16 | 0.51 | 2.60 | 0.715 | 0.899 |
| GI diseases: cows | 1.60 | 0.54 | 4.68 | 0.390 | 0.685 | 0.86 | 0.18 | 4.18 | 0.862 | 0.909 |
| Mastitis | 1.30 | 0.58 | 2.88 | 0.513 | 0.750 | 1.16 | 0.48 | 2.78 | 0.736 | 0.899 |
| Other diseases: cow | 3.38 | 0.72 | 15.73 | 0.119 | 0.323 | 0.00 | 0.00 | ∞ | 1.000 | 1.000 |
| Antibiotic treatment: calves | 0.64 | 0.19 | 2.11 | 0.468 | 0.741 | 0.56 | 0.12 | 2.64 | 0.472 | 0.899 |
| Other treatment: calves | 1.18 | 0.46 | 3.02 | 0.723 | 0.858 | 1.26 | 0.44 | 3.61 | 0.664 | 0.899 |
| GI diseases: calves | 0.00 | 0.00 | ∞ | 0.976 | 0.976 | 1.55 | 0.16 | 14.32 | 0.696 | 0.899 |
| Other diseases: calves | 1.40 | 0.42 | 4.66 | 0.5764 | 0.782 | 1.33 | 0.35 | 5.06 | 0.669 | 0.899 |
| Meteorological season-winter vs | 0.60 | 0.323 | 0.230 | 0.899 |
| Spring | 1.20 | 0.48 | 3.00 | 0.693 | 0.858 | 1.52 | 0.57 | 4.01 | 0.395 | 0.899 |
| Autumn | 0.55 | 0.14 | 2.10 | 0.391 | 0.685 | 0.58 | 0.14 | 2.36 | 0.451 | 0.899 |
| Summer | 0.32 | 0.09 | 1.14 | 0.079 | 0.323 | 0.64 | 0.17 | 2.34 | 0.503 | 0.899 |

CI: 95% confidential intervals.
Pbh: P values adjusted with Benjamini and Hochberg method.
Antibiotic treatment (OR 3.1; 95% CI 1.4–6.6; \( P = 0.003 \); Pbh = 0.03) and mastitis (OR 3.4; 95% CI 1.5–7.9; \( P = 0.003 \); Pbh = 0.03) increased \( C. \) difficile prevalence in the second age group of calves (Table 5).

No risk factors associated with the \( C. \) difficile prevalence in the third age group of calves (Table 4) were identified after adjusting for time of sampling.

Multivariate analysis of risk factors
In cows, dietary changes were associated with the prevalence of \( C. \) difficile (OR 5.8; 95% CI 2.4–14.4; \( P = 0.0001 \)). Similarly, in the first age group of calves, dietary changes were associated with the prevalence of \( C. \) difficile (OR 17.2; 95% CI 2.8–106.0; \( P = 0.002 \)). Mastitis was identified as a risk factor in the second group of calves (OR 1.6; 95% CI 0.7–3.4; \( P = 0.001 \)). Dietary changes also increased the prevalence of \( C. \) difficile in the third group of calves (OR 2.8; 95% CI 1.0–7.4; \( P = 0.03 \)) (Table 6).

Parasites were not shown to be a risk factor, which would directly influence the prevalence of \( C. \) difficile. However, they were identified to influence risk factors, which increased the prevalence of \( C. \) difficile in the multivariate analysis (Table 6).

Discussion
This study investigated the role of family farming on the ecology and epidemiology of \( C. \) difficile, which could be associated with the community-acquired CDI [7–10]. Dietary changes were the most prominent risk factor associated with the prevalence of \( C. \) difficile. The \( Clostridium \) difficile ribotypes identified in this study suggest that family dairy farming in Europe is an unlikely source for CDI.

Community-acquired CDI is a significant medical problem in human medicine. Animal contact is suggested as a potential risk factor for the development of community-acquired CDI [35, 36], because of the high prevalence of \( C. \) difficile in pigs, cattle and poultry on large scale intensive farms [8, 11, 20, 21, 35–38]. Intensive farming management subjects animals to a substantial stress, which increases the likelihood for pathogen transmission [39]. Human animal interaction in large intensive farms is reduced to a minimum and animals in intensive production have limited contact with other animal species that could harbor or transmit pathogenic organisms. Most likely transmission of a pathogen from large intensive farms, therefore, is through a food chain. Farming management on smaller family farms is less stressful for animals and has smaller negative impact on the environment [40]. Such farms are also more interlinked within the community, and a direct or indirect transmission of pathogens between animals, and animals and humans, is possible, including food chain transmission [41].
Several longitudinal studies investigated the prevalence of *C. difficile* in different domestic animal species during different ages or production stages, spanning over a period of 1 month to a year [11, 21–23, 38, 42]. The farm prevalence in this study varied from 3.7 to 74.1%; all farms were positive on at least one sampling day. Other studies also suggest transient shedding patterns of *C. difficile* [11, 42]. A prevalence of 10% in cows was found in this study. This is more than in large intensive dairy farms where prevalence of 0.95 [10], 1.5 [43], 2.4 [44] and 4.5% [45] were reported based on a single sampling interval. As expected, calves (35.7%) had much higher *C. difficile* prevalence than cows in this study. Studies reporting prevalence of *C. difficile* in calves reported prevalence from 6 to 22% [10, 22–24, 43, 46, 47], and even 56% in calves less than 7 days old [36]. The use of qPCR as a screening method made *C. difficile* detection more sensitive [27], which most likely accounted for the higher *C. difficile* prevalence in this study compared to other studies. Several sampling stages over a prolonged period are also more likely to identify bacteria in the investigated population.

*Clostridium difficile* bacterial culture results in this study, however, are more in line with previously published data. Considering the results of a bacterial culture, the prevalence in cows and calves would be 0.2% (1/540 pooled samples) and 4.1% (101/2442 individual samples), respectively. Results based on the bacterial culture indicated lower *C. difficile* prevalence than that reported in studies investigating animals on large intensive farms [8, 23, 48–50, 56], has not been detected in this study. It has been suggested that the exposure to less toxigenic strains of *C. difficile* such as PCR-ribotype 033 may help protect people or animals against more toxigenic strains and decrease the incidence of community-acquired CDI [57, 58]. The prevalence of *C. difficile* in cows was not associated with the presence of diseases, nor with antibiotic and non-antibiotic treatment, which is in agreement with previous studies [24, 48, 49].

| Outcome | Regression coefficient | Odds ratio | CI, low | CI, up | P value |
|---------|------------------------|------------|---------|--------|---------|
| Cows    | Intercept              | −2.490     |         |        | <0.0001 |
|         | Dietary change         | 1.772      | 5.881   | 2.401  | 14.405  | 0.0001  |
|         | Intestinal parasites cows | −0.448   | 0.639   | 0.248  | 1.644   | 0.3531  |
| Calves  | Intercept              | −0.437     |         |        | 0.2357  |
|         | Dietary change         | 2.849      | 17.263  | 2.810  | 106.027 | 0.0021  |
|         | Intestinal parasites calves | −0.430   | 0.650   | 0.330  | 1.282   | 0.2145  |
| Calves  | Intercept              | −1.909     |         |        | <0.0001 |
|         | Mastitis                | 1.407      | 4.083   | 1.729  | 9.639   | 0.0013  |
|         | Intestinal parasites calves | 0.488   | 1.629   | 0.773  | 3.432   | 0.1991  |
| Calves  | Intercept              | −2.576     |         |        | <0.0001 |
|         | Dietary change         | 1.037      | 2.819   | 1.073  | 7.407   | 0.0354  |
|         | Intestinal parasites calves | 0.061   | 1.062   | 0.502  | 2.246   | 0.8739  |

Calves first age group: 0–21 days; Calves second age group: 22–56 days; Calves third age group: 57–180 days.

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Table 6 Multivariate analysis

196) and a new toxinoype (Xlc) were identified. PCR-ribotype 033 was the most frequently determined PCR-ribotype in this study, which has often been reported in calves and humans, but has not been linked to the community-acquired CDI [7, 10, 23, 43, 46, 50, 51]. Other PCR-ribotypes found were associated with CDI including ribotypes 001/072, 002, 012 and 014/020 [10, 29, 52, 53]. PCR ribotype 014/020 was previously isolated from meat products in Canada [54, 55]. PCR ribotype 078, which is closely associated with the rising incidence of community-acquired CDI [5, 10, 52] and has been identified on large cattle farms [8, 23, 48–50, 56], has not been detected in this study. It has been suggested that the exposure to less toxigenic strains of *C. difficile* such as PCR-ribotype 033 may help protect people or animals against more toxigenic strains and decrease the incidence of community-acquired CDI [57, 58].

The most important risk factor influencing the prevalence of *C. difficile* in this study were dietary changes. A similar result was found in horses [5, 13, 18] but not in other farm animals. Breeding/rearing Simmental cattle increased the risk for *C. difficile* shedding in the first age group of calves. They were at least five times more likely to shed *C. difficile* than Holstein–Friesian calves in the same age group. We were not able to identify the reason for this prevalence difference. Most *C. difficile* ribotypes identified in Simmental cattle were not regarded as dangerous for CDI, which could competitively reduce the presence of more dangerous strains of *C. difficile* in the environment and potentially make them a safer breed of cattle [57, 58].

The prevalence of *C. difficile* in cows was not associated with the presence of diseases, nor with antibiotic and non-antibiotic treatment, which is in agreement with previous studies [24, 48, 49]. *Clostridium difficile*
prevalence in the second age group of calves, however, was sensitive to the antibiotic treatment in cows, as well as to the presence of mastitis on the farm. Possible reason for this finding is the shift in rumen microbiota in calves after the age of 3 weeks [26]. Another reason is a greater likelihood of the second age group of calves to be fed waste milk [59].

Gastrointestinal diseases were not linked to increased prevalence of *C. difficile* in cows or calves. Most animals included in the gastrointestinal disease group in this study had diarrhea. Diarrhea was [19, 37] or was not [24, 60–62] identified as a risk factor in other studies. Intestinal parasites have contributed to the potency of risk factors identified in the multivariate analysis. Interaction between intestinal pathogens is often the culprit for the development of gastrointestinal diseases in individuals and the population [63, 64] and warrants detailed investigation in the population.

Environmental temperatures and humidity are considered significant stress factors for production animals, which can influence the presence of *C. difficile* in feces [65–68]. Rodriguez-Palacios et al. [55] reported a positive association between *C. difficile* isolation in meat products in Canada with the months of January and February. In calves aged less than 1 month it was more likely to isolate *C. difficile* from their feces during the months of May, June and July when compared to August [24]. In the present study meteorological season and heat index did not influence the prevalence of *C. difficile*.

It is always important to be familiar with factors, which may influence the epidemiology of the disease and the biology of the etiological factor. This study provides significant information with regards to the epidemiology of *C. difficile* on the most prominent farming model in Europe. The results of this study indicate that it is unlikely that mid-size family dairy farms in Europe harbor highly pathogenic *C. difficile* strains, which are found to cause disease in animals and humans. The predominant presence of the benign *C. difficile* PCR-ribotype 033 may even have a protective rather than pathologic role in the pathogenesis of the disease.

**Abbreviations**

BVD: bovine viral diarrhea; CDI: *Clostridium difficile* infection; IBR: infectious bovine rhinotracheitis; MAP: *Mycobacterium avium* subsp. *paratuberculosis*.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

The study was designed by MV, PB, MO and MR. The sampling protocol and population size were set by RB, MV and PB. The farms were selected by FB and PB. Sampling was carried out by PB, MV, FB and OF. Molecular detection and culture were performed by PB. Molecular characterization of isolates was performed by MR. Statistical analysis and interpretations were performed by RB and MV. Parasitological examination of samples was carried out by AVR. MV and PB wrote the manuscript. MQ, RB and MR critically revised the manuscript. All authors read and approved the final manuscript.

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**References**

1. CDCP (Center for Disease Control and Prevention) (2005) Severe *Clostridium difficile*-associated disease in populations previously at low risk-four states, 2005. MMWR Morb Mortal Wkly Rep 54:1201–1205
2. Paltansing S, van den Berg RJ, Guseinova RA, Visser CE, van der Vorm ER, Kuipjer EJ (2007) Characteristics and incidence of *Clostridium difficile*-associated disease in The Netherlands, 2005. Clin Microbiol Infect 13:1058–1064
3. Wilcox MH, Mooney L, Randall R, Settle CD, Favlen W (2008) A case-control study of community-associated *Clostridium difficile* infection. J Antimicrob Chemother 62:388–396
4. Limbago BM, Long CM, Thompson AD, Killgore GE, Hennett GE, Havill NL, McKie J, Lathrop S, Jones TF, Park MV, Harriman KO, Gould LH, McDonald LC, Angola FJ (2009) *Clostridium difficile* strains from community-associated infections. J Clin Microbiol 47:3004–3007
5. Keessen EC, van den Berk AJ, Haasjes NH, Hermanus C, Kuipjer EJ, Lipman LJA (2011) The relation between farm specific factors and prevalence of *Clostridium difficile* in slaughter pigs. Vet Microbiol 154:130–134
6. Juneau C, Mendias EN, Wagal N, Loefelholz M, Savidge T, Croisant S, Darrn S (2013) Community-acquired *Clostridium difficile* infection: awareness and clinical implications. J Nurse Pract 9:1–6
7. Avbersek J, Janezic S, Pate M, Rupnik M, Zidaric V, Logar K, Vengust M, Zemljic M, Pirš T, Ocepek M (2009) Diversity of *Clostridium difficile* in pigs and other animals in Slovenia. Anaerobe 15:252–255
8. Costa MC, Stämpfli HR, Arroyo LG, Pearl DL, Weese JS (2011) Epidemiology of *Clostridium difficile* on a veal farm: prevalence, molecular characterizations and tetacycline resistance. Vet Microbiol 152:379–384
9. Burt SA, Siemeling L, Kuipjer EJ, Lipman LJA (2012) Vermin on pig farms are vectors for *Clostridium difficile* PCR ribotype 078 and 045. Vet Microbiol 160:256–258
10. Koene MGJ, Mevius D, Wagenaar JA, Harmanus C, Hensgens MPM, Meetsma AM, Putrulan FF, Van Bergen MAP, Kuipjer EJ (2012) *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. Clin Microbiol Infect 18:778–784
11. Weese JS, Wakeford T, Reid-Smith R, Rousseau J, Friendship R (2010) Longitudinal investigation of *Clostridium difficile* shedding in piglets. Anaerobe 16:501–504
12. Frazier KS, Heron AJ, Hines ME II, Gaskin JM, Altman NH (1993) Diagnosis of enteritis and enterotoxemia due to *Clostridium difficile* in captive ostriches (*Struthio camelus*). J Vet Diagn Invest 5:623–625
13. Bäverud V, Gustafsson A, Franklin A, Lindholm A, Gunnarsson A (1997) *Clostridium difficile* associated with acute colitis in mature horses treated with antibiotics. Equine Vet J 29:279–284
14. Gustafsson A, Bléverud V, Gunnarsson A, Rantzien MH, Lindholm A, Franklin A (1997) The association of erythromycin ethylsuccinate with acute colitis in horses in Sweden. Equine Vet J 29:314–318

15. Bléverud V, Franklin A, Gunnarsson A, Gustafsson A, Hellander-Edman A (1998) Clostridium difficile associated with acute colitis in mares when their foals are treated with erythromycin and rifampicin for Rhodococcus equi pneumonia. Equine Vet J 30:482–488

16. Songer JG, Post KW, Larson DJ, Jost BH, Jayarao BM (2008) Veterinary microbiology. 7th ed. Blackwell Publishing, Ames, IA.

17. Bléverud V, Gustafsson A, Franklin A, Aspán A, Gunnarsson A (2003) Clostridium difficile: prevalence in horses and environment, and antimicrobial susceptibility. Equine Vet J 35:465–471

18. Gustafsson A, Bléverud V, Gunnarsson A, Bringle J, Franklin A (2004) Study of faecal shedding of Clostridium difficile in horses treated with penicillin. Equine Vet J 36:180–182

19. Clooten J, Kruth S, Aroyo L, Weese JS (2008) Prevalence and risk factors for Clostridium difficile colonization in dogs and cats hospitalized in an intensive care unit. Vet Microbiol 129:209–214

20. Hopman NEM, Keessen EC, Harmanus C, Sanders IM, van Leengoed LAMG, Kuiper EJ, Lipman LJA (2011) Acquisition of Clostridium difficile by piglets. Vet Microbiol 149:186–192

21. Hawken P, Weese JS, Friendship R, Warner K (2013) Longitudinal study of Clostridium difficile and Methicillin-resistant Staphylococcus aureus associated with pigs from weaning through to the end of processing. J Food Prot 76:624–630

22. Houser BA, Soehnlen MK, Wolfgang DR, Lysczek HR, Burns CM, Jayarao BM (2010) Development of a qPCR protocol for rapid detection and quantification of Clostridium difficile. Lett Appl Microbiol 51:367–372

23. Janezic S, Ocepek M, Zidaric V, Janezic S, Kocuvan A, Rupnik M (2008) Distribution of Clostridium difficile PCR ribotypes in healthy calves and pigs at slaughter and in minced meat in Switzerland. J Food Prot 71:1302–1306

24. Rodriguez-Palacios A, Staempfli HR, Duffield T, Peregrine AS, Troitz-Williams LA, Arroyo LG, Brazier JS, Weese JS (2006) Development of a qPCR for Clostridium difficile in animal products. J Food Prot 69:925–931

25. European Commission. Agriculture and rural development – Family farming. 2015. http://ec.europa.eu/agriculture/family-farming/index_en.htm.

26. Drackley JK (2008) Calf nutrition from birth to breeding. Vet Clin North Am Food Anim Pract 24:55–86

27. Hensgens MP, Goorhuis A, Notermans DW, van Benthem BH, Kuijper EJ (2009) Decrease of hypervirulent Clostridium difficile in retail meat, Canada. Emerg Infect Dis 15:802–805

28. Bandelj P, Logar K, Usenik AM, Vengust M, Ocepek M, Rodriguez-Palacios A, Staempfli HR, Duffield T, Peregrine AS, Troitz-Williams LA, Arroyo LG, Brazier JS, Weese JS (2006) Development of a qPCR protocol for rapid detection and quantification of Clostridium difficile in cattle feces. FEMS Immunol Med Microbiol 48:151–155

29. Janezic S, Ocepek M, Zidaric V, Rupnik M (2012) Detection of toxigenic Clostridium difficile in animals and environmental samples. J Food Prot 75:333–337

30. Rupnik M, Avesani V, Rupnik M (2008) Isolation of Clostridium difficile from healthy food animals: optimized isolation and prevalence. J Food Prot 71:1302–1306

31. Schmitzberger I, Wrbka Th, Steurer B, Aschenbrenner G, Petersell J, Zechmeister HG (2005) How farming styles influence biodiversity maintenance in Austrian agricultural landscapes. Agric Ecosyst Environ 108:274–290

32. Hensgens MP, Goorhuis A, Notermans DW, van Benthem BH, Kuijper EJ (2009) Decrease of hypervirulent Clostridium difficile in retail meat, Canada. Emerg Infect Dis 15:802–805

33. Steadman RG (1979) The assessment of sultriness. Part I: a temperature-humidity index based on human physiology and clothing science. J Appl Meteor 18:861–873

34. Williams LA, Arroyo LG, Brazier JS, Weese JS (2006) Detection and characterization of Clostridium difficile isolated from feedlot cattle. J Food Prot 71:1302–1306

35. Simango C, Mwakurudza S (2008) Clostridium difficile in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int J Food Microbiol 124:268–270

36. Knight DR, Thean S, Putsatih P, Fenwick S, Riley TV (2013) Cross-sectional study reveals high prevalence of Clostridium difficile non-PCR ribotype 078 strains in Australian veal calves at slaughter. Appl Environ Microbiol 79:2630–2635

37. Hammitt MC, Bueschel DM, Keel MK, Glock RD, Cuneo P, DeYoung DW, Reggiardo C, Tinţă HT, Songer JG (2008) A possible role for Clostridium difficile in the etiology of calf enteritis. Vet Microbiol 127:343–352

38. Zidaric V, Zemljic M, Janezic S, Kocuvan A, Rupnik M (2008) High diversity of Clostridium difficile genotypes isolated from a single poultry farm producing replacement laying hens. Anaerobe 14:325–327

39. CMIW (Compassion in world farming) (2013) Zoonotic diseases, human health and farm animal welfare. http://www.cmiw.org/index.php/documents/cmi_docs/2013/2/zoonesis_4page_report.pdf. Accessed 20 May 2015

40. Schmitzberger I, Wrbka Th, Steurer B, Aschenbrenner G, Petersell J, Zechmeister HG (2005) How farming styles influence biodiversity maintenance in Austrian agricultural landscapes. Agric Ecosyst Environ 108:274–290

41. Swagemakers P, Wiskerke H, Van Der Ploeg JD (2009) Linking birds, fields and farmers. J Environ Manage 90(Suppl 2):S185–S192

42. Schuster A, Staempfli HR, Arroyo LG, Reiss-Smith RJ, Janecko N, Shewen PE, Weese JS (2012) Longitudinal study of Clostridium difficile and antimicrobial susceptibility of Escherichia coli in healthy horses in a community setting. Vet Microbiol 159:364–370

43. Romano V, Albanese F, Dumontet S, Krovec K, Petroni O, Pasquale V (2012) Prevalence and genotypic characterization of Clostridium difficile from ruminants in Switzerland. Zoonoses Public Health 59:545–548

44. Thistam SN, Frank JF, Lyon SA, Siragusa GR, Bailey JS, Lombard JE, Haley CA, Wagner BA, Dargatz DA, Fedorka-Cray PJ (2011) Clostridium difficile from healthy food animals: optimized isolation and prevalence. J Food Prot 74:130–133

45. Indra A, Lassnig H, Baliko N, Much P, Friedler A, Huhiulescu S, Allerberger F (2009) Clostridium difficile: a new zoonotic agent? Wien Klin Wochenschr 121:91–95

46. Pirs T, Ocepek M, Rupnik M (2008) Isolation of Clostridium difficile from food animals in Slovenia. J Med Microbiol 57:790–792

47. Rodriguez-Palacios A, Pickworth C, Loerch S, Leuene TT (2011) Transient fecal shedding and limited animal-to-animal transmission of Clostridium difficile by naturally infected feeding lot cattle. Appl Environ Microbiol 77:3391–3397

48. Costa MC, Reid-Smith R, Gow S, Hannon SJ, Booker C, Rousseau J, Benedict KM, Morley PS, Weese JS (2012) Prevalence and molecular characterization of Clostridium difficile isolated from feedlot beef cattle upon arrival and mid-feeding period. BMC Vet Res 8:38

49. Keel K, Brazier JS, Post KW, Weese JS, Songer JG (2007) Prevalence of PCR Ribotypes among Clostridium difficile isolates from pigs, calves, and other species. J Clin Microbiol 45:1963–1964

50. Hensgens MP, Goorhuis A, Notermans DW, van Benthem BH, Kuiper EJ (2009) Decrease of hypervirulent Clostridium difficile PCR ribotype 027 in the Netherlands. Euro Surveill 14:19402

51. Skablan J, Dzeroski S, Zenko B, Morgus D, Gangi S, Rupnik M (2013) Gut microbiota patterns associated with colonization of different Clostridium difficile ribotypes. PLoS One 8:e58005

52. Rodríguez-Palacios A, Staempfli HR, Duffield T, Weese JS (2007) Clostridium difficile in retail ground meat, Canada. Emerg Infect Dis 13:485–487

53. Rodríguez-Palacios A, Reiss-Smith RJ, Staempfli HR, Daigualt D, Janecko N, Avery BP, Martin H, Thompson AD, McDonald LD, Lembago B, Weese JS (2009) Possible seasonality of Clostridium difficile in retail meat, Canada. Emerg Infect Dis 15:802–805

54. Hofer E, Haeckler H, Frei R, Stephan R (2010) Low occurrence of Clostridium difficile in fecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. Appl Environ Microbiol 73:1973–1975

55. Sambol SP, Merrigan MM, Tang JK, Johnson S, Gerdling DN (2002) Colonization for the prevention of Clostridium difficile disease in hamsters. J Infect Dis 186:781–1789

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58. Songer JG, Jones R, Anderson MA, Barbara AJ, Post KW, Trinh HT (2007) Prevention of porcine Clostridium difficile-associated disease by competitive exclusion with nontoxigenic organisms. Vet Microbiol 124:358–361
59. Godden S, Feirtag J, Green L, Wells S, Fetrow J (2003) A review of issues surrounding the feeding of waste milk and pasteurization of waste milk and colostrum. Proceedings of the Minnesota Dairy Health Conference, 2003. College of Veterinary Medicine, University of Minnesota. http://purl.umn.edu/108982 Accessed 27 January 2016
60. Struble AL, Tang YJ, Kass PH, Gumerlock PH, Madewell BR, Silva J Jr (1994) Fecal shedding of Clostridium difficile in dogs: a period prevalence survey in a veterinary medical teaching hospital. J Vet Diagn Invest 6:342–347
61. Yaeger MJ, Kinyon JM, Songer JG (2007) A prospective, case control study evaluating the association between Clostridium difficile toxins in the colon of neonatal swine and gross and microscopic lesions. J Vet Diagn Invest 19:52–59
62. Alvarez-Perez S, Blanco JL, Bouza E, Alba P, Gibert X, Maldonado J, Garcia ME (2009) Prevalence of Clostridium difficile in diarrhoeic and non-diarrhoeic piglets. Vet Microbiol 137:302–305
63. Hammitt MC, Bueschel DM, Keel MK, Glock RD, Cuneo P, DeYoung DW, Reggiardo C, Trinh HT, Songer JG (2008) A possible role for Clostridium difficile in the etiology of calf enteritis. Vet Microbiol 127:343–352
64. Slovis NM, Elam J, Estrada M, Leutenegger CM (2014) Infectious agents associated with diarrhoea in neonatal foals in central Kentucky: a comprehensive molecular study. Equine Vet J 46:311–316
65. West JW (2003) Effect of heat-stress on production in dairy cattle. J Dairy Sci 86:2131–2144
66. Bohmanova J, Misztal I, Cole JB (2007) Temperature-humidity indices as indicators of milk production losses due to heat stress. J Dairy Sci 90:1947–1956
67. Norman KN, Harvey RB, Scott HM, Hume ME, Andrews K, Brawley AD (2009) Varied prevalence of Clostridium difficile in an integrated swine operation. Anaerobe 15:256–260
68. Smith DL, Smith T, Rude BJ, Ward SH (2013) Short communication: comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows. J Dairy Sci 96:3028–3033