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# Seasonality of *Clostridium difficile* in the natural environment

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## Abstract

*Clostridium difficile* is considered the leading cause of antibiotic-associated disease worldwide. In the past decade, a large number of studies have focused on identifying the main sources of contamination in order to elucidate the complete life cycle of the infection. Hospitals, animals and retail foods have been considered as potential vectors. However, the prevalence of *C. difficile* in these types of samples was found to be rather low, suggesting that other contamination routes must exist. This study explores the presence of *C. difficile* in the natural environment and the seasonal dynamics of the bacterium. *C. difficile* was isolated from a total of 45 samples out of 112 collected (40.2%) on 56 sampling points. A total of 17 points were positive only during the winter sampling (30.4%), 10 were positive only during the summer sampling (17.9%) and 9 sampling points (16.1%) were positive in both summer sampling and winter sampling. Spore counts in soil samples ranged between 50 and 250 cfu/g for 24.4% of the positive samples, with the highest concentrations detected in samples collected in the forest during winter campaign (200–250 cfu/g). A total of 17 different PCR ribotypes were identified, and 15 of them had the genes coding for toxins A and B. Most of those ribotypes had not previously been found or had been isolated only sporadically (<1% of samples) from hospitals in Belgium. Regarding antimicrobial susceptibility, most of the resistant strains were found during the summer campaign. These findings bear out that *C. difficile* is present in the natural environment, where the bacterium undergoes seasonal variations.

**KEYWORDS**
animals, *Clostridium difficile*, crops, forest, walking areas

## 1 | INTRODUCTION

*Clostridium difficile* (*Clostridioides difficile*) is a well-known pathogen of humans and animals, responsible for several outbreaks in hospitals (Aldeyab et al., 2011; Martinez, Leffler, & Kelly, 2012; Pawar et al., 2012), as well as losses and treatment costs in production (Moono et al., 2016; Nagy & Bilkei, 2003) and companion animals (Weber, Kroth, & Heil, 1989). This opportunistic bacterium can proliferate and produce toxins and disease when there is an event that causes a disruption of the normal microbiota (Crobach et al., 2018), mainly antibiotic therapy; the clinical manifestations of *C. difficile* infection (CDI) range from mild or moderate diarrhoea to fulminant and fatal pseudomembranous colitis (Cohen et al., 2010).

In the past decade, a large number of studies have focused on identifying the main reservoirs of *C. difficile* and sources of contamination in order to elucidate the complete life cycle of the infection. Direct or indirect contact with an infected individual is a potential route of infection (Dionne et al., 2013; Crobach et al., 2018), and healthcare settings have been classically identified as the main source of contamination (Enoch & Aliyun, 2012). However,

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there has been a recently observed increase in community-acquired CDI (Leffler & Lamont, 2012), which can be even more severe than healthcare-acquired infections (Ogielska et al., 2015). Recently, it has been suggested that *C. difficile*-colonized patients may be the most important unexplained reservoir for *C. difficile* (Crobach et al., 2018).

Animals and retail foods have been repeatedly considered as potential vectors for *C. difficile* transmission (Rodriguez, Seyboldt, & Rupnik, 2018; Durham, Olsen, Dubberke, Galvani, & Townsend, 2016; Gould & Limbago, 2010; Rupnik, 2007). However, the prevalence of the bacterium in these types of samples has been found to be rather low (Cho et al., 2015; Hoffer, Haechler, Frei, & Stephan, 2015; Rodriguez et al., 2015; Shaughnessy et al., 2016), suggesting that other contamination routes in the environment must exist (Rodriguez et al., 2018).

*Clostridium difficile* is classically associated with the intestinal tract of humans and animals. However, it is possible that the primary habitat of the bacterium is soil, and some types have adapted themselves to inhabit the gut niche after passage into the host by various forms of oral contamination, as in the case of other spore-forming anaerobes (Haagsma, 1991). In this context, some studies have previously investigated the presence of *C. difficile* in the environment, including soils and water samples (Janezic, Potocnik, Zidaric, & Rupnik, 2016). The bacterium has been recovered from soil samples taken from sand playgrounds and public gardens (Al-Saif & Brazier, 1996; Moono, Lim, & Riley, 2017; Orden et al., 2017), from environmental soil samples and puddle water (Janezic et al., 2016), from rivers (Zidaric, Beigot, Lapajne, & Rupnik, 2010) and from rural environments in close contact with farm animals (Bäverud, Gustafsson, Franklin, Aspan, & Gunnarsson, 2003; Simango & Mwakurudza, 2008).

This study was designed as an in-depth exploration of the presence of *C. difficile* in the natural environment, specifically away from large urban cores and from animal farms, to discover new important reservoirs of the bacterium. Importantly, differences in the prevalence, spore counts, PCR-ribotype distribution and antibiotic resistance of the bacterium in nature during warm and cold months were also investigated.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design and sample collection

The study was conducted over a total of four months in two phases, from November to December 2016 (winter sampling) and from May to June 2017 (summer sampling). Samples were collected in the Walloon municipality of Esneux, located in the Belgian province of Liège. This region has an average of 181 days of rainfall per year, with a mean total annual precipitation of 827 mm (data obtained from the Meteoblue database, developed at the University of Basel, Switzerland; www.meteoblue.com). During the winter sampling (WS), daytime temperatures reached a minimum of 2°C and a maximum of 8°C. During the summer sampling (SS), the highest daytime temperature reached 27°C, while the minimum recorded daytime temperature was 17°C.

Four different areas were sampled. Further information about each sampling area and the approximate distances between them are detailed in Figure 1 and Table S1. These areas support a rich wild fauna (Figure S1), in addition to being privileged zones for the recreation of pets, notably dogs, cats and horses. For each sampling point, soil samples were collected on two different sampling days (winter and summer sampling). Each sample was collected from the top of the soil (the uppermost 2 cm) in sterile 100 ml containers. Between 40 and 60 g of soil was sampled, potentially including mud, grass, moss, roots and stones depending on the sample point. In order to ensure that the sampling point was exactly the same in winter and summer sample collection, a straight metal shaft was firmly fixed in the soil (25 cm deep), in the coordinates indicated in Table S1. These rods were periodically checked (weekly or biweekly) through the entire study to ensure that they have not been removed before the second sampling day. Samples were stored at room temperature for a maximum of 3 days before processing.

### 2.2 | *C. difficile* detection

Culture of soil samples was performed following a protocol for *C. difficile* enumeration and detection based on the work of Delmée, Broeck, Simon, Janssen, and Avesani (2005). Briefly, 10 g of soil sample was inoculated into 40 ml of the medium cycloserine cefotaxime fructose taurocholate broth (CCFT) (Delmée, Vandercam, Avesani, & Michaux, 1987), which was freshly prepared in the laboratory. Subsequently, 100 µl of the broth was spread on cycloserine cefotaxime fructose agar with taurocholate (CCFAT) (Delmée et al., 1987), and the plates were incubated for 48 hr at 37°C in an anaerobic workstation Concept Plus (Led Techno, BE). Three parallel plates were used for each single sample. The rest of the broth was also incubated for 72 hr at 37°C under anaerobic conditions for *C. difficile* detection after enrichment. After incubation, a 10 µl aliquot of the enriched broth was spread onto CCFAT, and these plates were incubated anaerobically at 37°C for 2 days. With this method, our limit of detection is 50 ufc/g for the direct culture and ≤10 ufc/g for the enrichment culture. Colonies of *C. difficile* were identified from culture plates by morphological criteria (irregular yellowish colonies with an appearance of ground glass and characteristic horse manure odour), subcultured onto blood agar (5% sheep blood; Biorad, BE) and checked using a rapid *C. difficile* latex agglutination test kit (DR 1107A, Oxoid, FR). Multiple colonies were taken when morphologies suggested more than one type of PCR ribotype or when the presumptive colonies were too small to ensure isolation on the blood agar. Confirmation of *C. difficile* was performed by detection of a species-specific internal fragment of the *tpi* gene and detection of genes for toxin A, toxin B and binary toxin (*cdtA*) by classical PCR (Rodriguez et al., 2013). For the enumeration in direct culture, if there was more than one colony in the same plate, we tested at least three of them by PCR to confirm *C. difficile* identity. For the rest, if they presented the same morphology as the others next and they were

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**FIGURE 1** Geographic locations of the different areas sampled and approximate distances between them

positive using *C. difficile* latex agglutination test kit, we assumed to be *C. difficile*. All PCR reactions were run with a positive and negative controls. Confirmation of these results was performed using the Genotype Cdiff system (Lifescience, GE) for *the tpi* gene as well as all toxin genes.

## 2.3 | PCR ribotyping

PCR ribotyping based on capillary gel electrophoresis was performed using the primers described by Bidet, Barbut, Lalande, Burghoffer, and Petit (1999) and the method proposed by Fawley et al. (2015). International numbers were used for *C. difficile* strains that presented a PCR-ribotype profile matching the Cardiff ribotypes (Anaerobic Reference Unit (ARU), UK) from the strain collection available in our laboratory. Otherwise, strains were identified with an internal system of nomenclature beginning with UCL (database at the Catholic University of Louvain, National Reference Laboratory for *C. difficile* in Belgium) or as rare profiles if the strains presented new PCR-ribotype profiles never detected in our laboratory before. As capillary gel electrophoresis-based PCR-ribotyping was performed, results were further interpreted using the web-based database WEBRIBO (http://webribo.ages.at). This web base application allows to obtain a ribotype identification for each typed isolate, and consequently it enables a rapid and easy inter-laboratory exchange of data (Indra et al., 2008).

## 2.4 | Antimicrobial susceptibility testing

The antimicrobial resistance of *C. difficile* isolates was studied using a panel of six antibiotics.

Resistance to erythromycin (15 μg), vancomycin (5 μg), clindamycin (2 μg), tetracycline (30 μg), metronidazole (5 μg) and moxifloxacin (5 μg) (Oxoid) was tested through a disc diffusion assay on Brucella Blood Agar with hemin and vitamin K1 (Oxoid) according to the French Society of Microbiology protocols (SFM, 2017). Zone diameters were measured after 24 hr of anaerobic incubation at 37°C and interpreted as previously described (Rodriguez et al., 2014).

## 2.5 | Statistical methods

Statistical analysis was performed to evaluate the influence of different parameters (season, direct and enrichment culture) using Fisher's test in GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

# 3 | RESULTS

## 3.1 | Prevalence of *C. difficile* in the environment

Overall, 112 soil samples were collected, including 56 samples during the WS and 56 samples during the SS. *C. difficile* was isolated from a

**TABLE 1** Detailed information about the detection of *C. difficile* in soil samples during both the summer and winter campaigns

| Sampling área | Subtype of area | Positive sample | Winter sampling (WS) | | | | Summer sampling (SS) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Direct detection (cfu/g) | Enrichment detection | PCR Ribotypes (WC) | Webribo results | Direct detection (cfu/g) | Enrichment detection | PCR Ribotypes (SC) | Webribo results |
| A | Farmlands | A1 | – | + | UCL86 | New Ribotype | – | – | – | – |
| | | A6 | + (150) | + | UCL16L | New Ribotype | – | – | – | – |
| | | A8 | + (50) | + | UCL15 | New Ribotype | – | + | UCL46 | New Ribotype |
| | | A9 | – | + | UCL46 | New Ribotype | – | – | – | |
| | Farmlands surroundings | A11 | – | + | UCL16 | New Ribotype | – | – | – | |
| | | A12 | – | – | – | – | – | + | Rare profile | New Ribotype |
| | | A13 | – | + | 003 | 003 | – | – | – | – |
| | Walking areas | A14 | + (100) | + | 003 | 003 | – | – | – | – |
| | | A15 | + (50) | + | 020 | 020 | – | – | – | – |
| | Residential area | A18 | – | + | 039 | 039 | – | – | – | – |
| | | A19 | + (100) | + | UCL16 | New Ribotype | + (50) | + | Rare profile | New Ribotype |
| | | A20 | – | – | – | – | – | + | 039 | 039 |
| | Forest | A21 | – | + | 014 | 014 | – | – | – | |
| | | A22 | – | – | – | – | + (100) | + | 039/020 | 039/020 |
| | | A23 | – | – | – | – | – | + | UCL20a | New Ribotype |
| | | A24 | – | – | – | – | – | + | UCL16 | New Ribotype |
| | | A27 | + (250) | + | UCL48b | New Ribotype | – | – | – | – |

(Continues)

**TABLE 1** (Continued)

| Sampling área | Subtype of area | Positive sample | Winter sampling (WS) Direct detection (cfu/g) | Winter sampling (WS) Enrichment detection | PCR Ribotypes (WC) | Webribo results | Summer sampling (SS) Direct detection (cfu/g) | Summer sampling (SS) Enrichment detection | PCR Ribotypes (SC) | Webribo results |
|---|---|---|---|---|---|---|---|---|---|---|
| B | Walking areas | B1 | + (50) | + | 039 | 039 | – | – | – | – |
| | | B2 | – | + | Rare profile | New Ribotype | – | – | – | – |
| | | B3 | – | + | Rare profile | New Ribotype | – | – | – | – |
| | | B5 | – | + | UCL48d | New Ribotype | – | + | UCL36 | New Ribotype |
| | Forest | B6 | + (200) | + | 039 | 039 | – | – | – | |
| | | B7 | – | + | UCL46 | New Ribotype | – | + | UCL16b* | 449 |
| | | B10 | – | + | UCL9 | 039 | – | – | – | |
| C | Forest | C2 | – | – | – | – | – | + | 039 | 039 |
| | | C4 | – | – | – | – | – | + | UCL540 | New Ribotype |
| | Walking areas | C8 | – | + | UCL55a | New Ribotype | – | + | UCL9 | 039 |
| | | C9 | – | + | 039 | 039 | – | + | 020 | 020 |
| D | Parking and walking areas | D1 | – | – | – | – | – | + | UCL16 | New Ribotype |
| | | D2 | + (50) | + | 020 | 020 | – | – | – | – |
| | | D3 | – | – | – | – | – | + | UCL122 | New Ribotype |
| | | D4 | – | + | 020 | 020 | – | + | UCL16b | New Ribotype |
| | School and walking areas | D5 | – | + | UCL16b | New Ribotype | – | – | – | |
| | | D6 | – | + | 039 | 039 | – | + | 039 | 039 |
| | | D8 | – | + | 020 | 020 | – | + | 020 | 020 |
| | | D9 | – | – | – | | – | + | UCL16a | New Ribotype |

*Note:* WINTER SAMPLING [colonies/plate/0,1 μl in triplicate] **A6**: [(4) (3) (2)]; **A8** [(0) (2) (1)]; **A14**[(2) (3) (1)]; **A15** [(1) (1) (1)]; **A19** [(3) (3) (0)]; **A27** [(7) (5) (3)]; **B1** [(2) (1) (0)]; **B6** [(4) (5) (3)]; **D2** [(1) (1) (1)] SUMMER SAMPLING [colonies/plate/0,1 μl in triplicate] **A19** [(2) (1) (0)]; **A22** [(2) (2) (2)]

**TABLE 2** PCR ribotypes, toxin gene profiles and antimicrobial susceptibility of *C. difficile* PCR ribotypes isolated from soil samples

| PCR ribotype (Webribo) | Number of isolates | | | Toxin gene profile | | | Antibiotic susceptibility profile (number of isolates) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WS | SS | Total | *tcdA* | *tcdB* | *cdtA* | Va-R | Met-R | Mox-R | Clin-R | Te-R | E-R |
| 003 (003) | 2 | 0 | 2 | + | + | – | S | S | S | S | S | S |
| 014 (014) | 1 | 0 | 1 | + | + | – | S | S | S | S | S | S |
| 020 (020) | 4 | 3 | 7 | + | + | – | S | S | S | R (*n* = 2 SC) | S | S |
| 039 (039) | 6 | 5 | 11 | – | – | – | S | S | S | R (*n* = 1 WC; *n* = 3 SC) | S | R (*n* = 1 WC) |
| UCL15 (NR) | 1 | 0 | 1 | + | + | – | S | S | S | S | S | S |
| UCL16 (NR) | 2 | 2 | 4 | + | + | – | S | S | S | R | S | S |
| UCL16a (NR) | 0 | 1 | 1 | + | + | – | S | S | S | R | S | S |
| UCL16b (NR) | 1 | 1 | 2 | + | + | – | S | S | R | R | S | S |
| UCL16b*(449) | 0 | 1 | 1 | + | + | – | S | S | R | R | S | S |
| UCL16L (NR) | 1 | 0 | 1 | + | + | – | S | S | S | S | S | S |
| UCL20a (NR) | 0 | 1 | 1 | + | + | – | S | S | S | R | S | S |
| UCL36 (NR) | 0 | 1 | 1 | – | – | – | S | S | S | S | S | S |
| UCL46 (NR) | 2 | 1 | 3 | + | + | – | R (*n* = 1 SC) | S | S | R (*n* = 1 SC) | S | R (*n* = 1 SC) |
| UCL48b (NR) | 1 | 0 | 1 | + | + | – | S | S | S | S | S | S |
| UCL48d (NR) | 1 | 0 | 1 | + | + | – | S | S | S | R (*n* = 1 SC) | S | S |
| UCL55a (NR) | 1 | 0 | 1 | + | + | – | S | S | S | S | S | S |
| UCL86 (NR) | 1 | 0 | 1 | + | + | – | S | S | S | S | S | S |
| UCL122 (NR) | 0 | 1 | 1 | + | + | – | S | S | S | S | S | S |
| UCL540 (NR) | 0 | 1 | 1 | + | + | – | S | S | S | R | S | S |
| Rare profile (NR) | 2 | 2 | 4 | + | + | – | S | S | S | S | S | S |

Abbreviations: Clin-R, clindamycin resistance; E-R, erythromycin resistance; Met-R, metronidazole resistance; Mox-R, moxifloxacin resistance; NR, new ribotype; R, resistant; S, susceptible; Te-R, tetracycline resistance; Va-R, vancomycin resistance; WC, summer sampling; WS, winter sampling.

total of 45 samples (40.2%). Positive samples were detected as follows: 17 positive samples were from collection points that tested positive only during the WS (30.4%), 10 positive samples were from points that tested positive only during the SS (17.9%) and 9 samples (16.1%) were from points that tested positive during both the SS (*n* = 9) and the WS (*n* = 9). Therefore, the overall proportion of positive samples was highest in the WS. Eleven out of 45 samples (24.4%) tested positive on direct culture; 9 of them (81.8%) were detected during the WS, while only 2 (18.2%) tested positive on direct culture during the SS. Soil samples had detectable levels of *C. difficile* ranging from 50 to 250 cfu/g. All of the positive samples detected by direct culture were also positive after enrichment. By contrast, 34 out of 45 positive samples (75.6%) were detected only after an enrichment step (Table 1). Statistical analysis showed a significant effect of seasonality in *C. difficile* detection by direct culture ($p = 0.0089$) with higher spore levels in soil during winter months.

The highest percentage of positive *C. difficile* soil samples was detected in the busiest areas, including residential and walking areas, parking lots and schools, with more than half of positive samples collected from such areas (56.5%; 26 positive samples out of 46 samples studied from those areas). The next highest prevalence was found in farmlands and their surroundings (30.8%; 8 positive samples out of 26 samples studied from those areas). Forested areas had a very similar prevalence (27.5%; 11 positive samples out of 40 samples studied from those areas). Samples from busy areas were more frequent positive for *C. difficile* by direct culture than the two other areas investigated. Nevertheless, the highest concentrations of *C. difficile* spores were detected in samples collected in the forest (200–250 cfu/g) (Table 1).

### 3.2 | Characterization of *C. difficile* strains isolated from soil samples

A total of 20 different PCR ribotypes were identified among the 45 positive samples. Following our internal nomenclature, only 4 of these PCR ribotypes had profiles that corresponded to the ARU Cardiff collection (PCR ribotypes 003, 014, 020 and 039), while using WEBRIBO we have been able to identify with the international nomenclature one additional type (449). The remaining PCR ribotypes (*n* = 15) were identified with an internal system of nomenclature (beginning with UCL). The most prevalent PCR ribotype was identified as 039 (UCL9) (non-toxigenic strains), isolated in a total of 11 samples, followed by PCR ribotype 020, isolated from 7 samples (Table 2). PCR ribotype 039 was detected more frequently in busy

areas (*n* = 7) and forest areas (*n* = 4) than in other areas. Almost all the strains identified as PCR ribotype 020 (*n* = 7) were detected in busy areas, with the exception of one isolate that was collected in the forest. The same PCR ribotypes were detected by direct culture and enrichment in most of the samples that were positive after both analyses. However, in a single sample (from a forest (A22)), different PCR ribotypes were detected by direct culture (PCR ribotype 039) and after enrichment (PCR ribotype 020). Only two subtypes of areas (sample points D6 and D8) yielded the same PCR ribotypes (039 and 020) during both the WS and the SS (Table 1).

Thirty-three isolates (18 different PCR ribotypes) had the genes encoding for toxins A and B; therefore, they were identified as toxigenic. None of the strains presented the *cdtA* gene coding for binary toxin CDT. No association was observed between the WS or SS and the isolation of toxigenic versus non-toxigenic strains.

### 3.3 | *C. difficile* antimicrobial susceptibility

All of *C. difficile* isolates collected during summer sampling and winter sampling were fully susceptible to metronidazole and tetracycline. Regarding vancomycin, only one isolate, recovered during summer sampling and identified as PCR-ribotype UCL46, was resistant to this antibiotic. Moxifloxacin resistances were found in only two isolates (PCR-ribotypes UCL16b and 449), which were obtained from samples collected in summer and winter sampling. Similarly, erythromycin resistances were found in two additional isolates (039 and UCL46) in winter sampling and summer sampling, respectively. Finally, a great number of isolates presented resistances to clindamycin, and they were found in both sampling periods (Table 2). Multidrug resistances were identified for clindamycin and erythromycin; moxifloxacin and clindamycin; and vancomycin, clindamycin and erythromycin. Isolates with antimicrobial resistances were obtained mainly from busy areas (parking and walking areas) and from farmlands.

## 4 | DISCUSSION

This study refers to the presence of *C. difficile* in environmental soil samples in different locations and across winter and summer. Forty-two per cent of soil samples tested positive for the bacterium, and among them, 73% harboured toxigenic strains. All the samples tested corresponded to the top layer of the soil (the uppermost 2 cm). These findings confirm the results of previous studies reporting that *C. difficile* is very prevalent in different soils (Rodriguez et al., 2018; Janezic et al., 2016; Moono et al., 2017). Furthermore, some of the PCR- ribotypes found are the same as those isolated from humans and animals.

Studies addressing the presence of *C. difficile* in the natural environment are less numerous than those investigating the bacterium in foods and animals (Rodriguez et al., 2018). There are recent studies describing its presence in wastewater treatment plants or rivers (Moradigaravand et al., 2018; Zidaric et al., 2010), with some of them performed in soil farms (Bäverud et al., 2003) and in other rural areas (Janezic et al., 2016; Simango, 2006) or public environments (Al-Saif & Brazier, 1996; Moono et al., 2017; Orden et al., 2017). Wastewater treatment plants are highly contaminated, with up to 96% of samples being positive (Romano et al., 2012; Xu, Weese, Flemming, Odumeru, & Warriner, 2014; Nikaeen, Aghili-Dehnavi, Hssanzadeh, & Jalali, 2015). Common ribotypes associated with CDI were found in these types of samples, suggesting that humans are the main source of this contamination and that effluent can contribute to the dissemination of the spores in the environment (Moradigaravand et al., 2018; Xu et al., 2014). In this context, *C. difficile* was reported to be present in 68% of positive samples from rivers near densely populated areas, with the same predominant ribotypes found in hospitals (Zidaric et al., 2010). Regarding public environments, such as parks and other playgrounds, the available data are not yet conclusive, since the reported prevalence varies from zero detection to 52% (Al-Saif & Brazier, 1996; Orden et al., 2017). In this study, the overall prevalence found (40.2%) matched with the data reported previously from environmental samples. Considering each type of area separately, walking and residential areas were the environments with the highest positive rates of *C. difficile* (56.5% of total samples in this category). These areas are frequented every day not only by domestic animals (dogs and cats) but also by various aquatic birds (Figure S1). Aquatic birds could be contaminated by *C. difficile* spores from water sources (like nearby rivers) and spreading them to the forest. Regarding dogs, *C. difficile* has been previously reported with a prevalence varying between 4.8% and 11.9% (Alvarez-Perez, Blanco, Harmanus, Kuijper, & Garcia, 2017; Diniz et al., 2017). Sandboxes used by dogs have been also reported to be contaminated with a *C. difficile* rate of 60% (12 positive sandboxes out of 20 tested) (Orden et al., 2017). Regarding the results of our study, it is possible that dogs and are colonized by *C. difficile* after contact with soil, which is contaminated with spore levels of 50–100 cfu/g in busy areas. Most of the available studies reported low levels of *C. difficile* spores in foods, animals or in the environment, as in most of them the bacterium was only detected after anaerobic enrichment. The infectious dose for *C. difficile* in human disease is still unknown. In a previous study using animal models, infection occurred after a very small dose of *C. difficile* (less than 10 ufc) in hamsters under antibiotic therapy (Larson & Borriello, 1990). Similarly, a further study estimated that the environmental spore load required to infect 50% of those naïve mice exposed for 1 hr in a closed cage was 5–10 spores/cm$^2$ (Lawley et al., 2010). Regarding this data, it seems that a small number of *C. difficile* spores to cause CDI in susceptible individuals. Although the risk factors for the infection in the community are not fully understood, it has been suggested that continuous exposure to these contamination sources over days, weeks or even years can finally trigger the infection (Rupnik, 2010; Weese, Avery, Rousseau, & Reid-Smith, 2009).

*Clostridium difficile* was detected in farmlands and forest with similar prevalence (30.8% and 27.5%, respectively). A previous study reported the survival of *C. difficile* in manure compost derived from pigs, which can potentially contaminate the land (Usui et al., 2017). Bäverud et al., (2003) reported the presence of *C. difficile* spores in outdoor soil samples from farms with mature horses and from stud

farms, with a prevalence ranging between 1% and 11%, respectively. Furthermore, in the present study, the sampling points in farmlands and surroundings were located close to forests, which means that there is a constant circulation of native fauna (Figure S1). Although data regarding the intestinal carriage of *C. difficile* in wild animals are very limited, there is some evidence in the literature that indicates a prevalence similar to that in companion species. The presence of *C. difficile* has been previously reported in rodents and birds living near farms (Andres-Lasheras et al., 2017) and in a few other wild animals with a prevalence of up to 6.5% (Silva, Ribeiro de Almeida, et al., 2014 and Silva, D'Elia, et al., 2014; Jardine, Reid-Smith, Rousseau, & Weese, 2013). However, a further study reported zero prevalence of *C. difficile* in wild passerine birds (Bandelj et al., 2011). Therefore, it is not clear to what extent wild animals (and eventually dogs, horses, and human pedestrians), contribute to this soil contamination in forests. Surprisingly, the highest spore count (250 cfu/g) was found in this area.

Other unexpected findings included the differences found in terms of prevalence, detection and spore counts during the WS and SS. The results indicate that *C. difficile* is more abundant in the soil during winter months than during summer months in this region. A few other studies in humans and food have reported possible *C. difficile* seasonality. In previous studies in hospitals in Taiwan and Australia, the incidence of CDI was found to be highest in March and lowest in the last quarter of the year (Lee, Hung, Lin, Tsai, & Ko, 2016; Worth, Spelman, Bull, Brett, & Richards, 2016). A further review on CDI seasonality reports a similar seasonal pattern in the Northern and Southern hemispheres, with a peak in spring and lower frequencies in summer and autumn (Furuya-Kanamory et al., 2015). Our study did not include a spring campaign, but we observed lower levels of *C. difficile* in the SS than in the WS. Similar situations matching our findings were reported in the incidence of CDI in a German hospital (Reil et al., 2012) and in the prevalence of *C. difficile* in retail meat in Canada (Rodriguez-Palacios et al., 2009). It is not clear why *C. difficile* counts in soils are lower in summer than in winter. People and their pets frequent the investigated areas more often in the summer months than in the freezing months. Therefore, natural soils and not pets may be the main reservoir and source of contamination for *C. difficile*, and animals and humans may contribute to the spread of the bacterium. On the contrary, it is possible that the decrease in the number of spores in soils during summer months could be related with the temperature. An increase in temperature increases protozoal activity. These protozoa could act as predators, attack indigenous soil bacteria and reduce their numbers. The outcome of this might be that some bacterial populations could be reduced (Casida, 1989), and therefore have an impact in *C. difficile* spore counts. In cattle, it has been reported that the bacterial composition of faunated and unfaunated animals are significantly different, with higher numbers of *Clostridia* in unfaunated cattle (protozoa elimination from the rumen) than in faunated cattle (Ozutsumi, Tajima, Takenaka, & Itabashi, 2005). Further studies are needed to investigate the impact of this hypothesis in the prevalence of *C. difficile* prevalence in soils.

Unexpectedly, a great variety of PCR ribotypes were found in soil. Four PCR ribotypes identified in this study (014, 020, UCL46 and UCL48d) were found in 3%–10% of human samples in 2016 in Belgium (Lambert, 2017). Surprisingly, most of the PCR ribotypes identified in the study, including UCL46, UCL58d, ULCL9, 039 among others, had not previously been found or had been isolated only sporadically (less than 1%) in hospitals in this country. Regarding antimicrobial susceptibility, no association between the areas studied and the isolation of drug-resistant strains could be established. However, a great number of the resistant strains were found during the summer sampling. Seasonal variations in drug resistance have not been previously described for *C. difficile* but has been suggested for other bacteria (Pathak, Bhattacherjee, & Ray, 1993). For some bacteria, such as *Streptococcus pneumoniae* and *Escherichia coli*, seasonality in drug resistance has been explained by the fact that there are also fluctuations in antibiotic use, which is especially high during the winter months (Sun, Klein, & Laxminarayan, 2012; Dagan et al., 2008). However, in the environment it has recently been reported that seasonal variations strongly influence specific transport patterns of tetracycline resistance genes (Keen, Knapp, Hall, & Graham, 2018). Therefore, the results of the present study highlight the need for further investigations in this area.

In conclusion, our results show that even in forest areas that are not heavily frequented by humans and their pets, *C. difficile* is present in the soil, with even higher spore levels than in busy areas. Therefore, contamination from soils probably occurs, but in addition to the degree of bacterial exposure (high in populated areas and low in wild areas), there is probable an element of bacterial adaptive character according to the type of animal, its body temperature, its diet and, therefore, its intestinal microbiota (Sonnenburg et al., 2016). In the human microbiota, according to a recent report, the total proportion of spore-forming bacteria shows high variability, with a high turnover of such bacteria over time (Browne et al., 2016). These findings combined with transmission dynamics and geographical areas could be the key to a better understanding of the epidemiology of *C. difficile* in both humans and animals.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## ETHICAL APPROVAL

Authors declare that Ethical Statement is not applicable.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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