Clostridium difficile a new zoonotic agent?

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Mise en évidence du potentiel de transmission à l'homme de souches épidémiques de *Clostridium difficile* par la consommation de denrées alimentaires

Cristina Rodríguez Díaz

Thèse Présentée En Vue De L'Obtention Du Grade De Docteur En Sciences Vétérinaires

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My little, love, mi nena, te quiero Lhasa

Abbreviation list

A

A: Adenine

AFLP: Amplified fragment length polymorphism

AMOVA: Analysis of molecular variance

ANOVA: Analysis of variance

B

B. difficilis: *Bacillus difficilis*

B. heparinolyticus: *Bacteroides heparinolyticus*

Blast: Basic local alignment search tool

BMI: Body mass index

bp: Base pair

BSC: Bristol stool chart

C

°C: Degree Celsius

C: Cytosine

C. butyricum: Clostridium butyricum

C. difficile: Clostridium difficile

C. sordellii: Clostridium sordellii

C. sporogenes: Clostridium sporogenes

C. tertium: Clostridium tertium

CA-CDI: Community-acquired *C. difficile* infection

CC: Clindamycin

CCF: Cycloserine cefoxitin fructose

CCFA: Cycloserine cefoxitin fructose agar

CCFAT: Cycloserine cefoxitin fructose agar taurocholate medium

CCFBT: Cycloserine cefoxitin fructose taurocholate enrichment broth

CCFT: Cycloserine cefoxitin fructose taurocholate

CDA: *C. difficile* associated disease

CDI: *C. difficile* infection

CDIF: *C. difficile*

CDRN: *C. difficile* Network

CDT: *C. difficile* binary toxin

cdtA: *C. difficile* gene encoding for the enzymatic component of the binary toxin CDT

cdtB: *C. difficile* gene encoding for the binding component of the binary toxin CDT

cdtLoc: Full binary toxin locus

CE: Cytotoxicity assay

CEF: Cefquinone

CI: Confidence interval

CLSI: Clinical and laboratory standard institute

cm: Centimeter

cm^2: Square centimeter

D

D: Dependant resident

DNA: Deoxyribonucleic acid

DNTP: Deoxyribonucleotide triphosphate

E

€: Euros

E: Erythromycin

E. lenta: Eggerthella lenta

E. faecalis: Enterococcus faecalis

E. gallinarum: Enterococcus gallinarum

E. faecium: Enterococcus faecium

ECOFF: Epidemiological cut-off value

EFSA: European food safety authority

EIA: Enzyme immunoassay

ELISA: Enzyme-Linked immunoSorbent assay

ENR: Enrofloxacin

ESBL: Extended spectrum B-lactamasa

F

F: Female

F. magna: Finelgoldia magna

FAM: Carboxyfluorescein

F+M: Flunixin meglumine

FSM: French society of microbiology

G

g: Gram

G: Guanine

G+C: Guanine + cytosine

GDH: Glutamate dehydrogenase

GEN: Gentamicin

GM: Gentamicin

Gram +: Gram positive

gyrA: DNA gyrase coding gene from *C. difficile*

H

h: Hours

H2 blockers: (or H2 antagonists) Blockers of histamine at the histamine H2 receptors

HIV/AIDS: Immunodeficiency virus

I

I: Intermediate antimicrobial resistance

ICU: Intensive care unit

ID: Index of diversity

i.e.: That is

IgA: Immunoglobulin A

IgG: Immunoglobulin G

K

kb: Kilo base

kDa: Kilo Dalton

Kg: Kilogram

L

L. graminis: *Lactobacillus graminis*

L. rhamnosus: *Lactobacillus rhamnosus*

LCT: Large clostridial toxins

LSR: Lipolysis-stimulated lipoprotein receptor

LZ: (or Met) Metronidazole

M

M: Male

MAR: Marbloxacin

Met: (or LZ) Metronidazole

min: Minutes

ml: Millilitres

MLST: Multi-locus sequence typing

MLVA: Multi-locus variable number tandem repeat analysis

mm: Millimeter

mol: Mole unit

MRC-5: Medical Research Council cell strain 5

MUT: Mutation

M(o)X(F): Moxifloxacin

N

n: Number

NAP1: North America pulsotype 1

ng: Nanograms

NSAIDS: Non-steroidal anti-inflammatory drugs

O

OTU: Operational taxonomic unit

P

P: Penicillin

PaLoc: Pathogenicity locus

PCoA: Principal coordinate analysis

PCR-ribotyping: Polymerase chain reaction-ribotyping

PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism

PEN: Penicillin

PFGE: Pulsed-field gel electrophoresis

PMC: Pseudomembranous colitis

PYG: Peptone yeast glucose

R

R: Resistance

RA: Rifampicin

RAPD: Random amplified polymorphic DNA

rDNA: Ribosomal deoxyribonucleic acid

REA: Restriction endonuclease analysis

rep-PCR: Repetitive sequence-based PCR-typing

rpm: Revolutions per minute

rRNA: Ribosomal ribonucleic acid

RT-PCR: Real-time PCR

S

S: Susceptibility

s: Second

S. capitis: *Staphylococcus capitis*

S. haemolyticus: *Staphylococcus haemolyticus*

SD: Semi-dependant resident

SFM: French society of microbiology

SIDS: Sudden infant death syndrome

slpAST: Surface-layer protein A gene sequence typing

S-layer: Surface immuno-protein layer

spp.: Species

ST: Sequencing type

STRD: Summed tandem repeat difference

SXT: Trimethoprim sulfamethoxazole

T

TcdA: *C. difficile* toxin A

tcdA: *C. difficile* gene encoding toxin A

TcdB: *C. difficile* toxin B

tcdB: *C. difficile* gene encoding toxin B

tcdC: *C. difficile* gene (putative negative regulator gene of toxin production)

tcdC 39bp: *C. difficile tcdC* gene with a 39 base pair deletion

TE: Oxytetracycline

TET: Tetracycline

T: Thymine

tpi: Triose phosphate isomerase gene

U

UCL: Université Catholique de Louvain

UK: United Kingdom

USA: United States of America

USD: U.S. Dollars

UV: Ultraviolet light

V

V: Volt

VA: Vancomycin

Vit: Vitamin

VNTR: Variable number of tandem repeats

VPI: Virginia Polytechnic Institute

W

WGS: Whole genome sequencing

X

XNL: Ceftiofur

Other symbols

µg: Microgram

µl: Microliter

µM: Micromolar

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CHAPTER 1: Introduction

1.1 *C. difficile* Era: understanding the bacterium and associated infections

Clostridium difficile is a strict anaerobe, Gram-positive spore-forming bacterium that was first identified by Hall and O'Toole in 1935, in a study of the daily microbial changes in the feces of ten normal breast-fed infants. Since then, *C. difficile* emerged as a major human enteropathogen and nowadays the bacterium is considered as the leading cause of antibiotic-associated nosocomial diarrhea, responsible for outbreaks in hospitals and other health-care settings worldwide, capable of causing mild or serious diarrhea, fulminant colitis and death.

The major research studies of the last years have been focused on rapid diagnosis of *C. difficile* infection (CDI) and its epidemiology. In the past two decades CDI has increased in severity, with the emergence of epidemic strains such as PCR-ribotype 027. This strain has substantially contributed to the rise in CDI incidence in North America and Europe. In addition, many different factors, like increased antibiotic therapy prescription or prolonged hospitalisation, have contributed to the spread of the bacterium. Recently, severe manifestations of CDI as well as recurrent episodes of infection, especially among elderly people over 65 years in whom a worse outcome is more probable, have alarmed clinicians. Therefore, because the life expectancy in humans now exceeds 81 years in several countries worldwide and the proportion of the elderly population is increasing, more serious CDI will probably occur in the near future. Furthermore, in the last decade, the increasing number of CDI acquired in the community suggests possible transmission outside healthcare settings. In view of this situation, many efforts have been made based on a list of educational measures that could reduce the exposure to the bacterium, as well as several clinical guidelines to better diagnose and treat the infection.

The introduction of this dissertation is intended to describe the history of *C. difficile*, starting from the first descriptions up to the present, including the current knowledge regarding detection, typing methods and laboratory diagnosis of CDI.

C. difficile infection: early history, diagnosis and molecular strain typing methods

Cristina   Rodriguez,   Johan   Van   Broeck,   Bernard   Taminiau,   Michel   Delmée,   Georges   Daube

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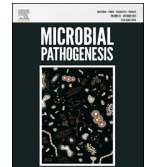
Clostridium difficile infection: Early history, diagnosis and molecular strain typing methods

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ABSTRACT

Recognised as the leading cause of nosocomial antibiotic-associated diarrhoea, the incidence of *Clostridium difficile* infection (CDI) remains high despite efforts to improve prevention and reduce the spread of the bacterium in healthcare settings. In the last decade, many studies have focused on the epidemiology and rapid diagnosis of CDI. In addition, different typing methods have been developed for epidemiological studies. This review explores the history of *C. difficile* and the current scope of the infection. The variety of available laboratory tests for CDI diagnosis and strain typing methods are also examined.

Contents

1. Introduction

Clostridium difficile is one of the most important nosocomial pathogens in humans. It is responsible for outbreaks of hospital-acquired infection, with symptoms including serious diarrhoea

and, in several cases, pseudomembranous colitis and even death. Although the principal risk factors in patients are a history of antibiotic treatment, an age of over 65 years, and prolonged hospitalisation [1,2], in recent years, studies have described the bacterium spreading further into the community [3] and an increase in the incidence and severity of nosocomial *C. difficile* infection (CDI) in North America and Europe [4]. This rise has been attributed to the emergence of new hypervirulent strains, including PCR-ribotype 027 [5] and PCR-ribotype 078 [6], which has been associated with antimicrobial exposure. Furthermore, a significant

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correlation between the lack of PCR-ribotype diversity in health-care settings and greater antimicrobial resistance has been observed [7].

In the past years, several studies and guidelines have been published to compare CDI incidence among different clinical settings, to increase the awareness of *C. difficile* and to improve the diagnosis and management of the infection [8]. This review is intended to describe the history of *C. difficile*, starting from the first descriptions up to the present, including the current knowledge regarding the detection, typing methods, and laboratory diagnosis of CDI.

2. *Clostridium difficile* discovery and its early history in humans

C. difficile was first identified by Hall and O'Toole in 1935, in a study of the daily microbial changes in the faeces of ten normal breast-fed infants up to the tenth day, when they left the hospital. The bacterium was described as a strict anaerobe with sub-terminal, non-bulging, elongate spores. In recognition of the difficulty of its isolation and study, it was originally named *Bacillus difficilis* [9]. Another remarkable property was its pathogenicity. Some strains were capable of producing toxins and caused respiratory death, with marked edema in the subcutaneous tissues of guinea pigs, rabbits, cats, dogs, rats and pigeons and convulsions in guinea pigs similar to those of tetanus. Its toxin was thermolabile, being inactivated in 5 min at 60 °C, but was not absorbed from the intestinal tract of the guinea pig, rat and dog: it acted only upon injection into the tissues [10]. In 1938, the bacterium *B. difficilis* was reclassified into the genus *Clostridium* [11] and *C. difficile* nomenclature was adopted by the Approved List of Bacterial Names [12].

Between 1940 and 1962, only two studies in the literature refer to *C. difficile* in humans [13,14]. However, there was no evidence in these cases that *C. difficile* was pathogenic. In the 1970s, a number of reports focused on the isolation of *C. difficile* from different hospitalised cases [15–21], but there did not seem to be an obvious pathogenic role in these cases, and *C. difficile* was still considered to be part of the normal faecal flora of humans. During this period, the first studies in animal models were published [22,23]. One of these studies [23] reported a cytopathic toxin in tissue-cultured cells and suggested the activation of an uncultivated virus. However, in retrospect, these findings could represent a description of the cytopathic effect of *C. difficile* induced by its toxins [24].

Pseudomembranous colitis (PMC) was first described in 1893 [25], prior to the availability of antibiotics, as a post-operative complication of gastrojejunostomy for an obstructive peptic ulcer in a young woman. Ten days after surgery, the patient developed haemorrhagic diarrhoea and died. After autopsy, the disease was identified as diphtheric colitis [26]. In subsequent years, many other early cases of PMC were recorded after surgical operations, in particular for patients with obstructive colorectal carcinoma [27] or under antimicrobial therapy [28–30]; however, while many studies showed important clues, its association with *C. difficile* would not occur until 1978 [31–36]. The finding was reported by three studies that were published in the literature almost simultaneously. In March 1978, one study [37] suggested that *C. difficile* was the causative agent of PMC. The authors found high titres of toxin in the faeces of all patients with PMC studied and hypothesised that the bacterium might be present in small quantities in the intestines of healthy adults and that under the appropriate conditions, it was able to multiply and cause postoperative diarrhoea or PMC due to its potential for toxin production. In April 1978, a second study [38] reported the isolation of *C. difficile* from the faeces of a patient with clindamycin-associated PMC and demonstrated both the presence of a faecal toxin and the toxigenicity of the isolate using a tissue-culture assay. In May 1978, a third study [39] reported that *C. difficile* was responsible for PMC and that previous antibiotic therapy produces susceptibility to infection, presumably as a result of a change in the bacterial flora. Finally, in late 1978, it was demonstrated that vancomycin eliminates toxin-producing *C. difficile* from the colon and is associated with rapid clinical improvement in patients with pseudomembranous colitis [40]. Fig. 1 summarises the early history of *C. difficile* in humans.

Since then, the number of reports documenting *C. difficile* infection in hospitals increased, and it became the pathogen of the 90s [41]. In the early 2000s, a rise in the incidence, severity and mortality rate of CDI was reported in Europe and North America, associated with the emergence of a new hypervirulent strain, PCR-ribotype 027 [5]. *C. difficile* is now a worldwide public health concern, as it is considered the major cause of antibiotic-associated infections in healthcare settings. Three previous reviews have addressed the recent epidemiology of CDI in hospitals, nursing homes and in the community as well as the principal outbreaks reported [2,42,43].

In recent years, with the availability of next-generation sequencing technologies, it has been demonstrated that *C. difficile* is closely related to the *Peptostreptococcaceae* family. It has therefore been suggested that *C. difficile* should be attributed to a new *Peptoclostridium* genus, renaming *C. difficile* to *Peptoclostridium difficile*. The newly proposed genus, *Peptoclostridium*, are Gram-positive, motile, spore-forming obligate anaerobes. All strains are mesophilic or thermophilic, grow in a neutral to alkaline pH and are oxidase- and catalase-negative. The G + C content of the genomic DNA ranged from 25 to 32 mol % [44].

3. The scope of CDI

C. difficile intestinal colonisation can be asymptomatic or produce disease. The clinical manifestations of CDI range from mild or moderate diarrhoea to fulminant pseudomembranous colitis [8]. Other symptoms described are malaise, fever, nausea, anorexia, the presence of mucus or blood in the stool, cramping, abdominal discomfort and peripheral leucocytosis. Extraintestinal manifestations (arthritis or bacteraemia) have been described but are rare. Severe disease can present colonic ileus or toxic dilatation and distension with little or no diarrhoea. The worst outcome of CDI is sepsis and death [8], which is estimated to occur in 17% of cases; however, this percentage is higher among older people [45].

Antibiotic treatment [1] and advanced age have classically been associated with *C. difficile* infection and related to an increased mortality rate [46]. A recent review regarding CDI cost-of-illness describes a mean cost ranging from 8911 to 30,049 USD for hospitalised patients (per patient/admission/episode/infection) in the USA [47]. In Europe, the annual economic burden is estimated to be approximately 3000 million euro [48]. However, it is necessary to note that the diagnostic strategy remains suboptimal in a large number of healthcare facilities, and a significant proportion of infections may remain undiagnosed [49].

Colonisation by non-toxigenic *C. difficile* has also been described, with a prevalence ranging between 0.4% and 6.9% [50], although this prevalence is lower than the estimated asymptomatic colonisation by toxigenic strains, which is between 7% and 51% [51,52]. Furthermore, it has been hypothesised that asymptomatic carriers can be colonised by both types of strains (toxigenic and non-toxigenic) for long periods of time without developing the disease [53]. However, these asymptomatic carriers could play an important role in transmission as a source for many unexplained cases [54]. It has been suggested that the presence of non-toxigenic *C. difficile* in the intestinal tract protects against CDI, although there is no clear evidence to explain how these avirulent strains reduce
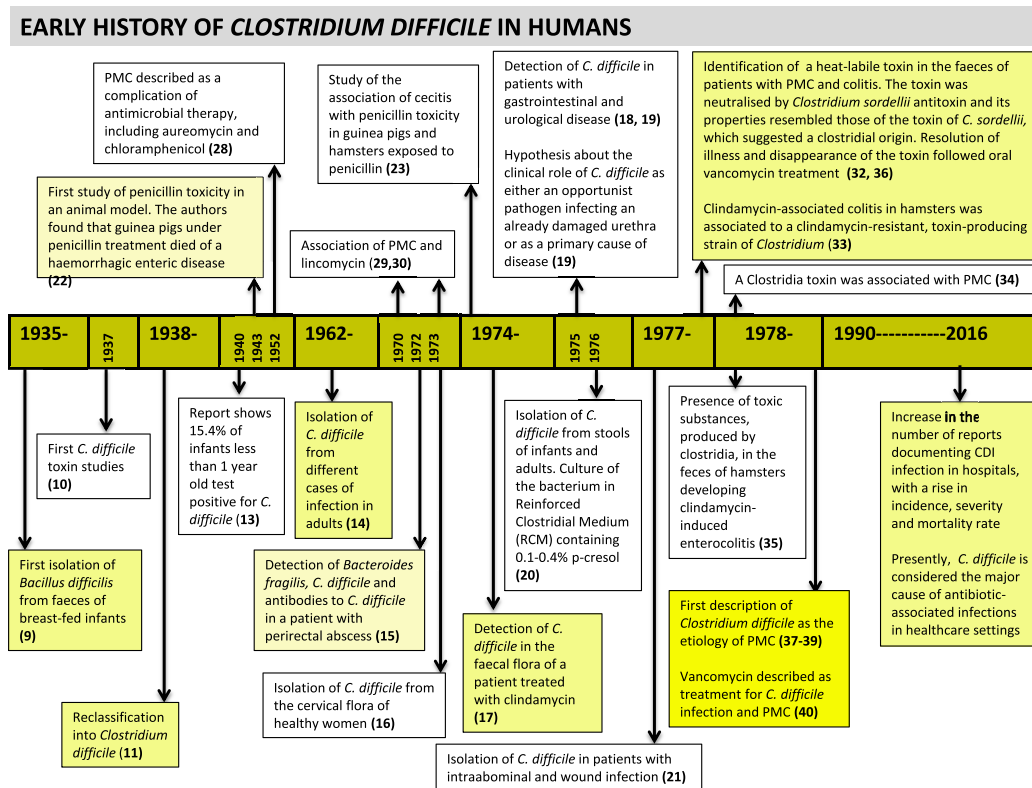
Fig. 1. *Clostridium difficile* history in humans PMC: pseudomembranous colitis.

the risk of developing an infection [50]. Simple competition for a niche in the gastrointestinal tract or other complex effects on mucosal immunity and nutrient acquisition have been hypothesised [50]. A variation in *C. difficile* non-toxigenic colonisation with age has been described, ranging from 6.9% for patients aged 60 years or more [55] to 22.8% for patients younger than 20 years of age [56], and up to 53—96% in neonatal units [57,58], supporting the hypothesis that these strains are more prevalent in younger patients and infants [50].

4. *C. difficile* outside Europe and North America

As previously cited, *C. difficile* is the most frequent bacteria associated with nosocomial diarrhoea in Europe and North America. However, little information is available regarding the extent of the infection in other regions or developing countries. In Zimbabwe, a study conducted in a healthcare centre reported a prevalence of 8.6% in a total of 268 diarrhoeal stool samples. Further characterisation of the isolates showed that all were susceptible to metronidazole and vancomycin, but approximately 70% were resistant to co-trimoxazole, which is an antibiotic widely used in this region as prophylaxis against infections in HIV/AIDS patients [59]. In a study of the gut microbiota of 6-month-old Kenyan infants consuming home-fortified maize porridge daily for 4 months and receiving micronutrient powder containing 2.5 ng of iron, *C. difficile* was detected with a high prevalence (56.5%). The results obtained showed that iron fortification in infants adversely affected the gut microbiota, with an increase in the proportion of some pathogenic bacteria, including *Escherichia coli*, *Salmonella*, *Clostridium*

perfringens and *C. difficile* [60]. A review [61] on the epidemiology of *C. difficile* in Asia shows that infection occurred at similar rates to other areas but with a predominance of variant toxin A and toxin B positive strains, including PCR-ribotypes 017 and 018. In contrast with the situation in America and Europe, PCR-ribotypes 027 and 078 have rarely been reported in Asia. The unregulated use of antibiotics in some Asian regions and the lack of surveillance raise concerns over the risk of bacterial mutation and infection [61]. An additional review describes the situation in Thailand in detail. A lack of data regarding *C. difficile* epidemiology is reported along with a high level of indiscriminate use of antimicrobials. *C. difficile* strains isolated from Thai patients showed a high degree of resistance for a wide range of antibiotics, including clindamycin, cefoxitin and erythromycin. Nevertheless, the strains were fully susceptible to metronidazole and vancomycin. In the same review, the authors concluded with the recommendation for a monitoring plan for *C. difficile* infections in hospital and community settings in Thailand and other Asian countries [62]. The same observation has been made for Latin America, where little data are available regarding the epidemiology of *C. difficile* in hospitals, and increased awareness and vigilance among healthcare professionals and the general public seem essential [63]. In an epidemiological study of *C. difficile*-associated diarrhoea in a Peruvian hospital, the reported overall incidence per 1000 admissions was 12.9. As the presence of another patient with CDI in the same room was significantly associated with the development of diarrhoea, the authors concluded that *C. difficile* transmission commonly occurred in this healthcare setting and highlighted the need for implementing adequate hygiene programmes [64] (Table 1).

Table 1
C. difficile infection in Asia, Africa and South America.

Continent	Country	Patients enrolled in the study/type of samples	CDI cases	Main PCR-ribotypes	Date of study[a]	Reference
Africa	Zimbabwe	Diarrhoeal stools of outpatients over 2 years of age presenting at healthcare centres	8.6% (23/268)	–	2014	[59]
	Kenya	6 month-old Kenyan infants consuming home fortified maize porridge and 2.5 ng of iron daily for 4 months	56.5% (65/115)[b]	–	2015	[60]
	Nigeria	HIV-positive inpatients of a University Teaching Hospital	43.5% (10/23)	–	2008–2009	[67]
		HIV positive outpatients a University Teaching Hospital	14% (10/71)	–		
Asia	Japan	–	–	018/014/002/001	2013	[61]
	Korea	Adult patients from 17 tertiary hospitals with a diagnosis of CDI	2.7/1000[c]	–	2004–2008	
	Malaysia	Stool samples from hospitalised inpatients with antibiotic associated disease	13.7% (24/175)	–	2008	
	India	Stool samples from hospitalised patients with antibiotic associated disease	22.6% (21/93)	–	1983–1984	
		Hospitalised patients with diarrhoea over 1 year	11.1% (38/341)	–	1991	
		Diarrheal hospitalised patients	16.7% (26/156)	–	1999	
		Hospitalised patients suspected of suffering CDI	17.2% (17/99)	–	2006–2008	
		Children with acute diarrhoea in hospitals	7–11%	–	1991/2001/2005	
		HIV seropositive adult subjects with diarrhoea	18.08%	–	2008–2011	
	Bangladesh	Children admitted to hospital with diarrhoea	1.6% (13/814)	–	1999	[66]
	China	Patients from a 1216 bed hospital in Shanghai	17.1/10,000[d]	017/012/046	2007–2008	[61]
	Taiwan	Stools samples from patients with CDI at all high-risk units	0.45/1000[d] 7.9/1000[e]	–	2010	
	Hong-Kong	Stools samples from patients suspected of CDI collected at a university-affiliated teaching hospital	5.1% (37/723)	027[f]	2008	[137]
	Singapore	Samples from patients of tertiary and secondary general hospitals	5.16/10,000[h] 2.99/10,000[h]	–	2006 2008	[138]
	Indonesia	Patients with diarrhoea in community and hospital settings	1.3% (2/154)	–	1997–1999	[140]
	Thailand	Hospitalised patients of all ages	52.2% (106/203)	–	1990	[62]
		Diarrheal stools of patients between 0 and 3 years	84.8%	–		
		Patients over 15 years	10% (20/140)	–	1991_1994	
		Antimicrobial treated group	1.4% (2/140)	–		
		Control group	4.8%–52.2%	–		
		Immunocompromised patients	36.7% (11/30)	–	1998	
		Febrile neutropenia paediatric oncology	58.8% (20/34)	–		
		Human immunodeficiency virus HIV positive cohort	36.5% (99/271)	–		
		Diarrheal patients Non diarrheal patients	15; 6% (16/102)	–		
		Acquired immunodeficiency syndrome in HIV positive patients				
		Patients of all ages	41.7% (20/48)	–	2001	
		Treated with antimicrobials Non-treated with antimicrobials	15.5% (13/84)	–		
		Hospitalised patients	18.6% (107/574)	–	2000–2001	
		Hospitalised patients over 15 years	26.9% (47/175)	–	2012	
South America	Chile	Hospitalised patients suspected of having CDI	20.6% (81/392)	012 (14.8%) 027 (12.3%) 046 (12.3%) 012/020 (9.9%)	2011–2012	[139]
	Argentina	Faecal specimens from hospitalised and ambulatory patients	6.5% (16/245)	–	1998–1999	[63]
		Diarrheal stool samples from hospitalised patients	36.8% (32/87)	–	2000–2001	
		Hospitalised patients	37/10,000[d] 84/10,000[d] 67/10,000[d] 43/10,000[d] 48/10,000[d] 42/10,000[d]	017 (90.8%)/001 (5.3%)/014 (3.1%)/031 (0.7%)	2000 2001 2002 2003 2004 2005	
	Brazil	Faeces of children over 1 year with acute diarrhoea	5.5% (10/181)	–	2000–2001	
		Faeces of children aged between 3 months and 7 years	6.7% (14/210)	–	2003	

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Country	Description	Rate/Percentage	Ribotypes	Period	Ref
	Patients with diarrhoea in the intensive care unit	44.9% (22/49)	–	2002	
	Faecal samples from children between 3 months and 7 years old	–	001/015/031/043/046/131/132/133/134/135/136/142/143	2007	
	Adult hospitalised patients	28.5% (6/21)[j]	014/106	2008–2009	
	Hospitalised patients suspected of having CDI	3.3/1000[d,j]	–	2002–2003	
	Patients of a medical surgical intensive care unit presenting nosocomial diarrhoea	19.7% (43/218)[k]	038/135	2006–2009	
	Immunosuppressed adult patients receiving antimicrobial treatment before an episode of nosocomial diarrhoea	21.7% (19/70)	010/020/133/233	2008–2009	
Chile	Hospitalised patients at a university tertiary hospital suspected of having CDI	28.2% (26/92)	–	2001	
	Hospitalised patients suspected of having CDI	0.53/100[l] 7/100[m]	–	2000–2001	
	Inpatients of a health unit	15.9% (112/706)	–	2003–2008	
Costa Rica	Patients presenting diarrhoea and receiving antimicrobial drugs	30% (31/104)	–	2008	
	Patients with CDI in a Costa Rica hospital	–	027 (54%)	2010	
Jamaica	Patients with and without immunosuppressive treatment and patients under radiotherapy	14.1% (16/113)	–	2009	
Mexico	Hospitalised patients at a tertiary hospital	5.04/1000[e]	–	2003–2007	
Puerto Rico	Hospitalised patients with diarrhoea	10.3%	–	2005	
Peru	Hospitalised patients at a tertiary hospital	156/4264 (35.5%)	–	2005–2006	[64]

[a] Study period if available or date of publication.
[b] Detection by 16S pyrosequencing and targeted real-time PCR.
[c] Incidence of CDI for adult admission.
[d] Incidence of CDI for admission.
[e] Incidence of CDI in medical care units.
[f] Only one strain identified as PCR-ribotype 027.
[h] Incidence of CDI cases per 100,000 inpatient-days.
[i] C. difficile was isolated from 4/6 of the patients with CDI.
[j] C. difficile was isolated from 16/138 stool samples of patients with CDI.
[k] Mean incidence of CDI 1.8/1000 patient days. Highest incidence between December 2007 and August 2008 (5.5/1000 patient days).
[l] Global incidence of CDI per year in the hospital.
[m] Incidence in the nephrology unit.

One of the most serious human health problems in developing regions is the microbial contamination of drinking water and foods, leading to severe gastrointestinal diseases that are exacerbated by under-nutrition and the lack of medical treatment in these regions. Water-, sanitation- and hygiene-related deaths occur almost exclusively in developing countries (99.8%), of which 90% are the deaths of children [65]. Indeed, children are the most at-risk group, especially in the first year of life. *C. difficile* was identified among a large number of bacteria associated with diarrhoea in this population. However, the source of contamination (water, food or environment) by the enteropathogens identified in diarrheic children was not elucidated [65].

Another issue of concern is CDI in immuno-compromised patients in developing countries. In a study conducted to assess the microbial aetiologies of diarrhoea in adults infected with human immunodeficiency virus (HIV) in India, *C. difficile* was the most common bacterial pathogen identified, with a reported prevalence of 18% [66]. Consistent with this study, HIV-positive inpatients and outpatients in Nigeria were shown to be *C. difficile*-positive in 43% and 14% of cases, respectively [67] (Table 1). Both studies show the importance of establishing controlled and regulated access to antibiotics in developing countries, as well as the importance of the early diagnosis of intestinal pathogens to reduce morbidity and mortality rates, especially among HIV-positive people. In a further study evaluating CDI in travellers, infection was reported to be more commonly acquired in low- and middle-income countries. Furthermore, CDI was often acquired in the community by young patients and associated with the empirical use of antimicrobials, frequently fluoroquinolones [68].

5. *C. difficile* is found everywhere

C. difficile is ubiquitous in the environment, and the bacterium has the capacity to persist on inanimate surfaces for as long as several months [69]. These contaminated areas can contribute towards *C. difficile* transmission in healthcare settings. Bed frames, floors or bedside tables have been described as the most commonly contaminated areas in rooms used to isolate patients with *C. difficile* diarrhoea [70], even after detergent-based cleaning [71]. Table 2 summarises the available studies in the literature regarding the dissemination of *C. difficile* spores in healthcare settings and related environments. However, the difference in prevalence among studies may be due to the sampling and culture methods used [70] and in the cleaning programmes used to control the spread of *C. difficile*. In this context, a previous study reported that unbuffered hypochlorite (500 ppm) was less effective than phosphate buffered hypochlorite (1600 ppm) for surface decontamination [72]. In addition to the patient room environment, the bacterium was isolated from the hands and stools of asymptomatic hospital staff and from the home of a patient suffering CDI. Furthermore, *C. difficile* inoculated onto a surface (floor) has been shown to persist there for five months [73]. In an intensive care unit, an outbreak of pseudomembranous colitis was attributed to the cross-contamination of inanimate environmental sources with persistence in the hospital for several weeks [77]. Regarding the medical equipment, two previous studies have reported that the replacement of electronic thermometers with single-use disposables significantly reduced the incidence of *C. difficile*-associated diarrhoea in both acute care and skilled nursing care facilities [78,79]. However, it has also been reported that with the use of disposable or electronic thermometers, there was no effect on either the overall rate of nosocomial diarrhoea or the rate of nosocomial infections [79]. A further study also describes how the use of tympanic thermometers reduces the risk of acquiring vancomycin-resistant *Enterococcus* and CDI by 60% and 40%, respectively [80].

Increased interest in the transmission of *C. difficile* has led to new studies in the literature reporting the presence of spores in other areas never studied before. Medical staff has increasingly used mobile technology devices in hospitals, such as iPads, to access electronic patient information. A recent study [82] evaluated the contamination of 20 iPads by *C. difficile* spores in a healthcare setting. Although with the number of samples tested, there was not sufficient data to estimate the prevalence, and in addition, there was no *C. difficile* recovery, the study also reported the effect of different agents on iPad disinfection. The results showed that bleach wipes were able to remove the inoculated spores completely from the screen surface, while a microfibre cloth was more effective than alcohol wipes. As there are no existing medical guidelines specific to electronic devices, and the manufacturer recommends avoiding the use of chemicals or abrasives to clean the device, the authors emphasised the importance of reducing the tablets' environmental contact in rooms housing patients suffering from CDI.

There are few studies describing the presence of *C. difficile* in the natural environment and in the environment in the community (Table 3). The prevalence of *C. difficile* was recently studied in retail baskets, trolleys, conveyor belts and plastic bags in 17 different supermarkets from 2 cities in Saudi Arabia. The study reported a *C. difficile* prevalence of 0.75% on sampled surfaces, with the highest level of contamination in baskets and trolleys, which could suggest the need for the implementation of planned disinfection in supermarkets to control community-acquired CDI [83]. In the natural environment, the bacterium was detected in seawater, zooplankton [84], tropical soils [85] and rivers [86]. In the rural environment, *C. difficile* was recovered from homestead soils, household-stored water [87] and soils of stud farms with mature horses [88]. In this last study, *C. difficile* was inoculated in equine faeces and the bacterium was found to survive at least 4 years (no later time points were tested) when kept at room temperature and outdoors at an ambient temperature over the year.

While *C. difficile* is also known as an enteric pathogen in some food-producing and companion animal species, there are several reports describing the presence of the bacterium in the intestinal contents of apparently healthy animals (Table 3). Moreover, recently published data suggests that animals are an important source of human CDI that can spread disease through environmental contamination, direct or indirect contact, or food contamination, including carcass and meat contamination at slaughter or, in the case of crops, through the use of organic animal manure [129]. Table 3 summarises the prevalence of *C. difficile* reported in pets (dogs and cats), food animals (pigs and cattle), horses and wild animals. Despite the large number of studies describing the presence of human epidemic PCR-ribotypes in these animals, *C. difficile* has not been confirmed as a zoonotic agent, but it seems evident that there is a potential risk of transmission, especially in people with close contact with contaminated animals and their environment.

6. *C. difficile* characteristics and its toxins

Since its discovery in 1935, the characteristics of *C. difficile* growth, sporulation and virulence have been documented in detail. The fundamental aspects of the bacterium are summarised in Table 4. One of these characteristics is that *C. difficile* has no protease, phospholipase C or lipase, but it is among the few bacteria able to ferment tyrosine to *p*-cresol, which is a phenolic compound that inhibits the growth of other anaerobic bacteria [134,20]. Dawson et al. (2008) [134] found that *Clostridium sordellii* tolerated *p*-cresol but did not produce it. Therefore, the authors suggested that the mechanism of tolerance might not be linked to the production of this organic compound. Furthermore, the increased

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Table 2
C. difficile spores in the environment of healthcare settings.

Environment	Positive surfaces (number or percentage of positive surfaces/patient rooms)	Study conditions	Main PCR-ribotypes (% of toxigenic strains)	Reference
Hospital rooms previously accommodating CDI patients	Patient-helper trapeze (5) Call button (3) Bed table (4) Bedrail (6) Tap (3) Toilet (7) Inner door handle (1) Shackle of hand disinfectant (1) Door handle facing to outer sluice (1) Stethoscope (1) Rail at foot-end (2)	Surface sampling before being cleaned	PCR-ribotype 012 PCR-ribotype 020C PCR-ribotype SE121 PCR-ribotype 023	[70]
	Patient-helper trapeze (1) Bedrail (2) Toilet (3)	Surface sampling after being cleaned		
Hospital side rooms used for the isolation of patients with symptomatic CDI	Floor (45%) Light (35%) Bed (9%) Sink/table (8%) Window (3%)	Surface sampling after detergent-based cleaning	PCR-ribotype 1 (93% of toxigenic strains)	[71]
Hospital with an outbreak of antibiotic-associated colitis	Environmental cultures obtained on the ward (31.4%)	Surface sampling before ward disinfection	–	[72]
	Environmental cultures obtained on the ward (21%)	Surface sampling after ward disinfection with unbuffered hypochlorite[a]		
Environment and contacts of hospitalised patients carrying *C. difficile* in their stools	Floors and other surfaces (9.3%)	Areas where carriers had diarrhoea	(100% of toxigenic strains)	[73]
	Floors (2.6%)	Areas without *C. difficile* carriers		
Different areas of hospitals with and without positive patients for *C. difficile*	Environmental cultures (32.5%)	Case-related environments[b]	–	[74]
	Environmental cultures (1.3%)	Control sites[c]		
	Highest counts of *C. difficile* from toilet seats, toilet bowl rims, bathroom handrails and bathroom floors	Ambulatory patients		
	Highest counts of *C. difficile* from bed handrails and near the beds	Non ambulatory patients		
Two elderly medicine wards	Environmental cultures (35%)	Two different types of cleaning which included hypochlorite or neutral liquid detergent[d]	–	[75]
Samples of the inanimate ward environment on two elderly medicine hospital wards (A and B)	Environmental cultures (34%). Highest counts of *C. difficile* (sorted in descending order) from: sluice floor, commodes, toilet floors, ward floors, radiators and air vents	Environmental samples for ward A	–	[76]
	Environmental cultures (36%). Highest counts of *C. difficile* (sorted in descending order) from: commodes, toilet floors, ward floors/air vents/sluice floor and radiators	Environmental samples for ward A		
Samples from surfaces of a variety of areas in a nursing home	Environmental cultures (kitchen, kitchen-staff locker room and bathroom, resident's rooms, private bathrooms, residence hall, lifts and staircase railings (0%)	Sampling before and after cleaning routine	–	[81]

[a] If disinfection with phosphate buffered hypochlorite, 98% of reduction in surface contamination.
[b] Hospital bedrooms and bathrooms used by eight patients with faecal cultures positive for *C. difficile*.
[c] Hospital bedrooms and bathrooms where there were no known cases of diarrhoea.
[d] Decrease of CDI incidence on one ward when it was disinfected with hypochlorite.

production and *p*-cresol tolerance of some strains, including PCR-ribotype 027 strains, has led to hypotheses regarding its contribution towards *C. difficile* hypervirulence [134].

C. difficile is able to produce two major toxins, toxin A (308 kDa) and toxin B (270 kDa), as well as the binary cytolethal distending toxin (CDT). Toxins A and B belong to the group of Large Clostridial Toxins (LCT). Only strains that produce at least one of the three toxins cause disease. Toxin A is considered to be an enterotoxin because it causes fluid accumulation in the bowel. Toxin B does not cause fluid accumulation but is extremely cytopathic for tissue-cultured cells [130]. These toxins are encoded by two genes, *tcdA* and *tcdB*, mapping to a 19.6 kb pathogenicity locus (PaLoc) and containing 3 additional regulatory genes, *tcdC*, *tcdR*, and *tcdE*, that are responsible for the synthesis and regulation of toxins A and B

[135]. Deletions, insertions, or polymorphic restriction sites in one or more of the PaLoc genes can result in toxin variant strains that produce either toxin A or toxin B [135]. While the power of purified toxin A to produce pathology *in vitro* has been widely described, a study [136] reported that toxin B, but not toxin A, was essential for virulence. Finally, in 2011, it was shown that both mutant variants, toxin A+ toxin B− and toxin A− toxin B+, can cause disease [137]. It is worth noting that toxin A + B− isolates of *C. difficile* have not been described in nature. Toxin A− toxin B+ strains have been widely reported in human cases [138] but also in animals suffering from diarrhoea [139]. A previous study reported that toxin A− toxin B+ strains caused the same spectrum of disease that is associated with toxin A+ toxin B+ strains, ranging from asymptomatic colonisation to fulminant colitis, with outbreaks in hospitals and other

Table 3
C. difficile spores in the natural environment, in the environment in the community and in animals.

Environment/animals	Prevalence (%)	Samples	Main PCR-ribotypes	Reference
17 supermarkets from two cities in Saudi Arabia	20/1600 (0.75%)	Retail baskets and trolleys (4/400) Conveyor belts (1/400) Plastic bags (3/400)	PCR-ribotype 027 4 different PCR-ribotypes (with NIN)	[83]
Five sampling stations along the coastline of the Gulf of Naples	9/21 (42.9%)	Sea water (2/5) Sediment (0/5) Zooplankton (3/5) Shellfish (4/6)	PCR-ribotype 003 PCR-ribotype 005 PCR-ribotype 009 PCR-ribotype 010 PCR-ribotype 056 PCR-ribotype 060	[84]
Samples from grassland, non-agricultural soils from five different regions of Costa Rica	3/117 (3%)	Central Plateau/Dry Pacific/North	–	[85]
Water samples (n = 69) from 25 different rivers in Slovenia	42/69 (60.9%) 17/25 (68%)	Water samples Rivers	34 different PCR-ribotypes PCR-ribotype 014 predominant (16.2% of all isolates)	[86]
Samples from a rural community in Zimbabwe	54/146 (37%) 14/234 (6%) 20/115 (17.4%) 7/161 (4.3%)	Soil samples Water samples Chicken faeces samples Faecal samples of other animals	–	[87]
Samples from studfarms and farms with horses and samples from faecal samples of horses	14/132 (11%) 2/220 (1%) 4/72 (6%) 18/43 (42%) (5%–63%) 5/134 (3.7%)	Outdoor soil samples Soil samples from farms with mature horses Diarrhoeic mature horses Faecal samples of horses with colitis Foals and adults horses with gastrointestinal disease Faecal samples of horses at hospital admission	– – PCR-ribotype 014 4 additional PCR-ribotypes with NIN	[87] [88] [111] [112]
	10/73 (13.7%)	Faecal samples of hospitalised horses	PCR-ribotype 014 6 additional PCR-ribotypes with NIN	[113]
	4/82 (4.8%) 14/62 (23%)	Faecal samples of horses at hospital admission Faecal samples from diarrheic horses	17 different PCR-ribotypes 6 different PCR-ribotypes	[114] [115]
Wild animals	2 fatal cases of elephants with enterocolitis	Samples of intestinal contents of two Asian Elephants	PCR-ribotype I	[89]
	1 clinical case of an ocelot with diarrhoea	Stool sample of a male Ocelot	–	[90]
	11/30 (36.7%) 2/34 (5.9%) 7/175 (4%)	Pooled faecal samples of captive white-tailed deer Non-diarrheic maned wolf and a diarrheic ocelot Droppings from barn Swallows	PCR-ribotype 078 – PCR-ribotype SB3, PCR-ribotype SB159 PCR-ribotype S166	[91] [92] [93]
	0/465 50% 7/200 (3.5%)	Cloacal samples of migrating passerine birds Faecal samples from zoo animals (chimpanzee, dwarf goat, Iberian ibex and plains zebra)	– PCR-ribotype 078 PCR-ribotype 039 PCR-ribotype 110	[94] [95]
	15 cases of enterocolitis in harbor seals	Faecal samples of juvenile harbor seals (Phoca vitulina)	–	[96]
	3/46 (6.5%)	Stool samples from free-living South America coati	PCR-ribotype 014/020 PCR-ribotype 106 PCR-ribotype 013	[97]
	5/109 (4.6%) 95/724(13.1%) 7/161 (4.4%) 41/60 (25%)	Faecal samples of free-living South America coatis Faecal samples of black and Noway rats Faecal samples of feral swine Faecal samples of Iberian pigs (free range system)	35 different PCR-ribotypes – PCR-ribotype 078	[98] [99] [100] [101]
Pets (dogs and cats)	14/139 (10%)	Faecal samples of dogs	PCR-ribotype 001	[102]
	62/204 (30%) 16/117 (13.6%)	Faecal samples of dogs Faecal samples of dogs	3 other PCR-ribotypes (with NIN) 29 PCR-ribotypes	[103] [104]

Animal	Sample	Prevalence	PCR-ribotype	Ref.
	Faecal samples from 18 puppies aged between 7 and 55 days	(33.3%–100%)	PCR-ribotype 012, PCR-ribotype 014, PCR-ribotype 046, PCR-ribotype 056, PCR-ribotype 010, PCR-ribotype 078, PCR-ribotype 213, PCR-ribotype 009, PCR-ribotype 020	[105]
	Faeces from healthy dogs	2/50 (4%)	PCR-ribotype 009, PCR-ribotype 010	[106]
	Faeces from dogs with diarrhoea	2/20 (10%)	PCR-ribotype 014	[107]
	Faeces from dogs	48/93 (52%°)	—	[108]
	Faeces from dogs	9/165 (5.5%)	PCR-ribotype 010, PCR-ribotype 014/020, PCR-ribotype 039, PCR-ribotype 045	
	Faeces from cats	5/135 (3.7%)	PCR-ribotype with NIN	
	Faecal samples of two adult cats	Two cats with acute diarrhoea	—	[109]
Pigs (piglets and adult pigs at slaughter)	Faecal samples of cats in a veterinary hospital	23/245 (9.4%)	—	[110]
	Faecal samples from pigs at slaughter (5–6 months)	1/100 (1%)	PCR-ribotype 078	[116]
	Faecal samples from neonatal piglets	45/67 (67.1%)	PCR-ribotype 046	[117]
	Faecal samples from pigs at slaughter	2/61 (3.3)	—	[118]
	Faecal samples from pigs at slaughter	58/677 (8.6%)	PCR-ribotype 078, PCR-ribotype 014, PCR-ribotype 013	[119]
	Faecal samples from pigs at slaughter	0/165 (0%)	—	[120]
	Faecal samples from neonatal piglets	241/513 (47%)	—	[121]
	Faecal samples from pigs at slaughter	30/436 (6.9%)	PCR-ribotype 078	[122]
	Faecal samples from neonatal diarrhoeic piglets	103/174 (59.2%)	PCR-ribotype 273	[123]
	Faecal samples from neonatal non diarrhoeic piglets	11/11 (100%)		
	Faecal samples of finishing pigs at farm (13–27 weeks)	2/250 (0.8)	—	[124]
Cattle (calves and adult cattle)	Faecal samples of calves (<12 weeks)	4/42 (9.5%)	PCR-ribotype 077, PCR-ribotype 038, PCR-ribotype 002	[125]
	Faecal samples of cattle at slaughter (15—56 months)	10/101 (9.9%)	PCR-ribotype 078, PCR-ribotype 029	[116]
	Faecal samples of cattle at slaughter	3/67 (4.5%)	—	[118]
	Faecal samples of calves	176/999 (17.6%)	PCR-ribotype 078, PCR-ribotype 033, PCR-ribotype 045	[126]
	Faecal samples of calves aged between 3 and 25 days	90/150 (60%)	—	[127]
	Faecal samples of dairy and beef cow	2/330 (0.61)	PCR-ribotype 027	[128]

With NIN: with non international nomenclature.

Table 4
Fundamental aspects of the bacterium.

C. difficile characteristics		Data	Reference
Cells	Gram	Gram-positive	[130]
	Motility	Motile in broth cultures	
	Ciliature	Peritrichous	
	Size	0.5–1.9 μm wide	
		3.0–1.9 μm long	
	Chains	Some strains produce chains of 2–6 cells aligned end-to-end	
Spores	Shape and position	Oval, subterminal (rarely terminal) and swell the cell	
	Cogerminants for spores	Bile salts (cholate, taurocholate)	[131]
		Glycine, histidine	
Colonies	Morphology	Circular, occasionally rhizoid	[10,130]
	Size	2–5 mm	
	Colour	Opaque, greyish, whitish, with a matt-to-glossy surface[a]	[133]
		Yellow fluorescence[b]	
	Other characteristics	Non-haemolytic. They produce a characteristic odour, described as smelling like cow manure, a barnyard or horse stables	[132]
Growth temperature	Optimum	30–37 °C	[10,130]
	Range of growth	25–45 °C	
pH		5 (minimum)	
Water activity		0.969 (minimum)	
Atmosphere		Anaerobic conditions	
Cultures in PYG after 5 days of incubation[c]	pH	5.0–5.5	[130]
	Products in this medium	Acetic, isobutyric, butyric, isovaleric, valeric, isocaproic, formic and lactic acids[d]	
	Other characteristics	Cultures are turbid with smooth sediments	

[a] Colonies on blood agar.
[b] After 24 h of incubation on cycloserine cefoxitin fructose agar (CCFA) or after 48 h of incubation on blood agar under long-wavelength ultraviolet light.
[c] Peptone yeast glucose (PYG) broth is a liquid non-selective medium used to identify metabolic products of anaerobic bacteria.
[d] When lactate is not used, pyruvate is converted to acetate and butyrate, and threonine is converted to propionate.

healthcare settings worldwide [135]. It must be noted that toxin A– toxin B+ strains are sometimes reported solely on the basis of the lack of *tcdA* amplification; however, there are some A+ variant strains with a partially deleted *tcdA* fragment [140].

Both toxins A and B translocate to the cytosol of target cells and inactivate small GTP-binding proteins. By glycosylating small GTPases, the two toxins cause actin condensation and cell rounding, which is followed by cell death. Toxin A acts primarily within the intestinal epithelium, while toxin B has broader cell tropism [141]. Both toxins induce the production of tumour necrosis factor alpha and pro-inflammatory interleukins, which induce the inflammatory cascade and the pseudomembrane formation in the colon. The endoscopy of *C. difficile* colitis shows a colonic mucosa with multiple whitish plaques, usually raised and adherent, of a size varying from a few millimetres to 1–2 cm; the cells can even be confluent in severe disease. The intervening colonic mucosa can be oedematous, granular, hyperaemic or completely normal [142].

The genes *cdtA* and *cdtB*, encoding the CDT, which belongs to the group of clostridial binary toxins, are not found on the PaLoc. This toxin is encoded on a separate region of the chromosome (CdtLoc). It has been described that all strains with *cdtA* and *cdtB* genes are variant strains (with changes in the *tcdA* and *tcdB* genes) [143]. In contrast, most types not producing binary toxin have toxin genes very similar to the reference strain, VPI 10463.[1] These CDT + strains represent up to 6% of the toxigenic isolates from hospitalised patients [143]. The production of CDT is frequently associated with hypervirulent strains. CDT has been described as causing the collapse of the actin cytoskeleton and cell death. The lipolysis-stimulated lipoprotein receptor (LSR) is known to be the host receptor for the *C. difficile* CDT toxin [144]. Furthermore, the CDT toxin also induces the formation of microtubule-based protrusions and increases the adherence of the bacterium [145]. While CDT is still being investigated, some studies have already reported data regarding the clinical relevance of this toxin. Bacci et al. (2011) [146]

associated the presence of CDT in patients with higher case-facility (death) rates. Other authors also found that CDT was a marker for more virulent *C. difficile* strains or that it contributed directly to strain virulence. Tagashira et al. (2013) [147] described two cases of fulminant colitis due to CDT + strains in the same ward of a hospital in Japan occurring within ten weeks of each other. A further study suggested that CDT was a predictor of recurrent infection, and its presence may require longer antibiotic treatments [148]. CDT + *C. difficile* strains that do not produce toxins A and B have been described in independent cases of patients with diarrhoea suspected of having CDI [149].

7. Laboratory diagnosis of CDI

To aid in the surveillance of CDI and to increase comparability between clinical settings, standardised case definitions have been proposed (Fig. 2) [8]. A laboratory diagnosis of CDI must be based on the detection of *C. difficile* toxins or on the isolation of toxigenic *C. difficile* strains from stool samples [150]. However, these results should be combined with the clinical findings to diagnose the disease. The clinical manifestation includes diarrhoea with the passage of 3 or more unformed stools in 24 or fewer consecutive hours [8]. In this context, only unformed and fresh stools should be tested for diagnostic purposes (the specimen should be loose enough to take the shape of the container). The cytotoxic activity is lost very quickly, meaning if the analysis of fresh specimens is not possible, the samples should be stored at 4 °C or below. However, cultures are not affected by temporal conditions due to sporulation [150]. Formed stools only must be tested if they come from patients with ileus or potential toxic megacolon or in the case of epidemiological studies [150].

A recent guide to the utilisation of the microbiology laboratory for the diagnosis of infectious diseases [151] highlights the importance of the collection device, temperature and transport time because the interpretation of the results will depend on the quality of the specimens received for analysis. Specifically, regarding *C. difficile*, the recommendations are that the stool

[1] http://www.mf.uni-mb.si/mikro/tox/.
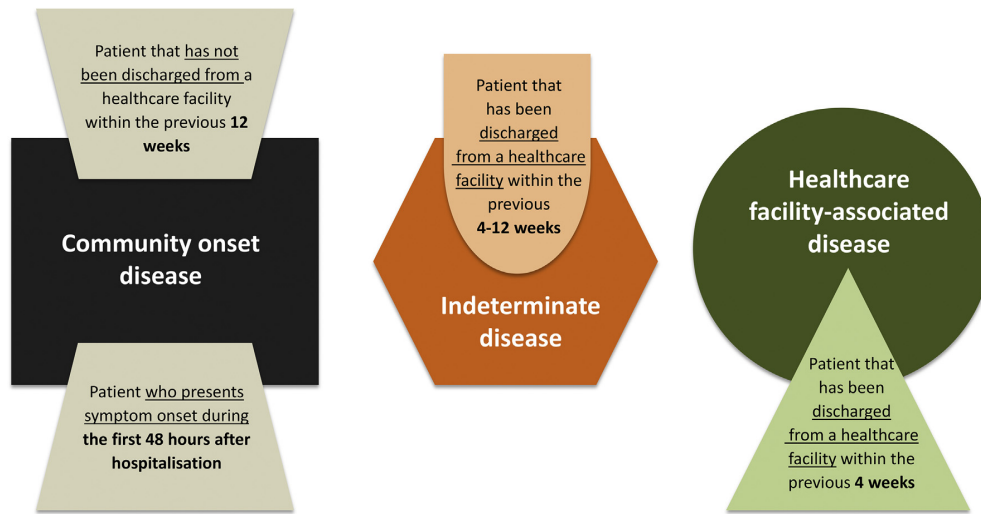
Fig. 2. Community onset CDI and healthcare facility-associated CDI definitions.

samples must be received in sterile close containers and kept at room temperature for a maximum of 2 h, and therefore, specimens of dubious quality must be rejected [151].

The culture of samples is recognised as the most sensitive method for the detection of *C. difficile*, but its specificity for CDI is low because the rate of asymptomatic carriage of *C. difficile* among hospitalised patients is so high. This method is not clinically practical for routine diagnosis because it does not distinguish between toxigenic and non-toxigenic isolates and requires 24—48 h to obtain the first results [150]. However, stool culture testing permits the molecular typing of the isolated strains and antibiotic susceptibility determination, making it essential for epidemiological surveys [8,150]. Therefore, stool culture testing can be coupled with a cell cytotoxicity assay or EIA (enzyme immunoassay) to detect toxin-producing *C. difficile* strains (known as toxigenic cultures), resulting in increased specificity [150].

Since it was first proposed by George et al. in 1979 [133], cycloserine-cefoxitin fructose (CCF) has been the most commonly used medium for *C. difficile* isolation. The original formulation has been extensively modified, including the replacement of egg yolk by blood [150]. The addition of 1 g/L of taurocholate, desoxycholate or cholate has also been shown to induce the germination of *C. difficile* spores when they are incorporated in CCF [152,153]. Sodium salt of cholic acid is more inexpensive than pure taurocholate but just as effective [150]. The concentration of the selective agents has also varied among studies, from 250 mg/L to 500 mg/L for cycloserine and from 8 mg/L to 16 mg/L for cefoxitin. Other modifications to improve this media have been proposed. Delmée et al. [154] included cefotaxime instead of cefoxitin, which increased the sensitivity and specificity of the medium. *C. difficile* colonies are easily recognised in this media when observed under the microscope: with an appearance similar to ground glass, they release an odour akin to horse manure and reveal a yellow-green fluorescence under ultraviolet illumination. Early identification of *C. difficile* colonies in this primary selective culture can be performed using an antigen latex agglutination assay. Latex particles are coated with IgG antibodies specific to *C. difficile* cell wall antigens. When the bacterium is present, the latex particles agglutinate into large visible clumps within 2 min. However, cross-reactions have been described, including with *C. sordellii, Clostridium glycollicum* and

Clostridium bifermentans [155].

Presently, several commercial selective media are available for the detection of *C. difficile* from stool specimens. The new chromogenic media seem to be effective as well as more rapid and sensitive than the classic selective media and have been shown to aid in the diagnosis of CDI [156—159]. However, pre-made agars are expensive and unaffordable for many research groups. Furthermore, they are used for the clinical recovery of *C. difficile* from faecal samples and not for the semi-quantification of viable spores [160].

Pre-treatment of samples with ethanol shock has been associated with an increase in sensitivity [161—164]. However, in the different studies conducted in our laboratory (unpublished data), ethanol shock or pre-heat treatment of samples does not improve the recovery of *C. difficile* from faecal or food samples. Rather, it seems that an increase in the time of enrichment is best for improving the sensitivity of the method. A bacterial competition in the enrichment broth has been observed [164]. In a previous study on carcasses and faecal samples [116], after 30 days of enrichment, different *C. difficile* types were identified, and colonies other than *C. difficile* were rarely present in the plate. However, the enrichment of samples is a time-consuming technique for laboratory purposes and might not be worth the slight increase in sensitivity observed.

Toxin detection is the most important clinical test [8]. It can be performed using cell lines to examine a stool filtrate (cell cytotoxicity assay) or by EIA [150]. Cell cytotoxicity is often considered the best standard test for identifying toxigenic *C. difficile* as it can detect toxins at picogram levels and is recognised as the most sensitive available test for the detection of toxin B [165]. A laboratory cell line (Vero, Hep2, fibroblasts, CHO or HeLa cells) is exposed to a filtrate of a stool suspension. If *C. difficile* toxins are present, a cytopathic effect is observed after 24—48 h (cell rounding via cytoskeleton disruption). The effect is mainly due to toxin B, which is more cytotoxic than toxin A. The presence of toxigenic *C. difficile* can be confirmed if a specific antiserum (added later) reverses the effect on the cells. This method is very sensitive and specific but is relatively slow and requires the maintenance of cell lines. If the antiserum does not neutralise the cytopathic effect, which is observed in 21% of cases, the results are inconclusive [150].

EIA is rapid but less sensitive than the cell cytotoxicity assay [8], missing 40% of diagnoses [165]. However, it is easy to perform and

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does not require technical training or special equipment. EIA can detect both toxin A and toxin B and may also detect glutamate dehydrogenase (GDH), a specific enzyme of *C. difficile* found in toxigenic and non-toxigenic isolates. *C. difficile* constitutively produces GDH in easily detectable levels, so tests based on GDH detection have good sensitivity (96%–100%). As GDH only identifies the bacterium and not the presence of toxins, the method is comparable with stool culture. Furthermore, it only takes 15–45 min and the cost is low (estimated at 8 USD); it can also be combined with a cell cytotoxicity assay, EIA for toxins, or culture with further toxin characterisation of the strains [165]. However, the results from GDH seem to differ based on the commercial kit used, and therefore, for some authors, this approach remains an interim recommendation [8]. EIA may be useful in laboratories without tissue culture facilities, but it must be combined with a positive culture. If the EIA results are negative and the culture test results are positive, it is recommended to isolate the strain from the plate and repeat EIA testing to determine if it is toxigenic [150]. Table 5 summarises the most-used tests for the diagnosis of *C. difficile* infection.

Delmée et al. (2001) [150] proposed the following scheme for the routine bacteriological diagnosis of CDI in humans. First, culture and toxin detection (by cytotoxicity assay or by EIA) should be performed directly from the stool specimen. If both tests are negative, a diagnosis of CDI is excluded. In contrast, if both are positive, the patient is diagnosed with CDI and requires immediate treatment. When the culture is positive but toxin detection is negative, an EIA test should be performed on several colonies removed from the culture plate. If the test is still negative, treatment is not necessary. If the test is positive, the patient should be considered positive for CDI. Finally, when the culture is negative but toxin detection is positive, a control specimen is requested and the culture must be repeated, which results in a patient testing positive for *C. difficile* in most cases. Repeat testing of patients who were previously positive as a "test of cure" is not appropriate [150].

Many studies have developed different real-time PCR (RT-PCR) methods for the detection of *C. difficile* toxin genes directly from stool samples, not only from humans [166,167] but also from animals [168], and for the quantitative detection of *C. difficile* in hospital environmental samples [169]. Various automated RT-PCR systems are commercially available, intended as diagnostic tools for CDI. These systems include BD GeneOhm™ Cdiff (Becton Dickenson)[2] and Xpert® *C. difficile* (Cepheid).[3] These commercial RT-PCRs have been shown to be rapid (<4 h for a result), sensitive and specific. Therefore, they have been largely proposed for the laboratory diagnosis of CDI [170–172]. In addition, a recent guide for the management of *C. difficile* infection in surgical patients suggests that PCR testing of perirectal swabs may be an efficient method for toxigenic *C. difficile* detection [173].

There are several other molecular genetic test systems commercially available for the identification of *C. difficile* from stool and culture samples. One example is Genotype Cdiff (Hain Lifescience), which is based on DNA strip technology.[4] This system is based on DNA amplification, hybridisation and visualisation using the enzyme alkaline phosphatase. The results are visible in a colorimetric reaction. The test is rapid in detecting *C. difficile*, its toxins, deletions in the *tcdC* gene, and mutations in the *gyrA* gene that are associated with moxifloxacin resistance. However, these

new technologies require considerable capital equipment, costly cartridges and experienced laboratory personnel. Furthermore, the results reported have not shown any significant improvement when compared with classic methods [150]. Therefore, some laboratories use these procedures to verify dubious results observed after rapid screening with other methods or to further process the samples for epidemiological purposes.

8. *C. difficile* typing methods

To characterise and compare the circulating strains and to identify emerging strains and those responsible for outbreaks worldwide, several typing methods have been applied. Table 6 summarises the available typing methods and their advantages and disadvantages. Lemée et al. (2004) [140] designed a multiplex PCR for the simultaneous identification and toxigenic type characterisation of *C. difficile* isolates. Several other studies have proposed different multiplex PCR primers and protocols not only to detect the genes encoding the major toxins A and B but also to detect binary toxin genes (*cdtA* and *cdtB*) and other deletions in the Paloc genes [174].

Pulsed-field gel electrophoresis (PFGE[5]) and restriction enzyme analysis (REA) are widely used in the United States and Canada. PFGE was one of the first molecular methods used for *C. difficile* and other food-borne pathogens in North America [175]. The method uses restriction enzymes that infrequently cut (such as *Sma*I or *Sac*II) to cleave bacterial DNA at different restriction sites. The use of these infrequently cutting restriction enzymes limits the number of restriction fragments (to between 7 and 20) and ensures that they are relatively large [176]. Generally, the frequency of cutting is inversely proportional to the number of nucleotides in the recognition site [177]. In North America, the isolates are designated with NAP and a number (e.g., NAP1: North America Pulsotype 1) [178]. The technique separates the large fragments of DNA generated based on size using a pulsed-field electrophoresis gel with resulting electrophoresis patterns that are highly discriminatory. However, large amounts of high-molecular-weight DNA have to be read, making the process labour-intensive (Table 6). *C. difficile* typing based on REA is performed using total cellular DNA, which is digested with a frequently cutting restriction enzyme (*Hin*dIII), and the resulting fragments are resolved by classical agarose electrophoresis. This method was shown to be reproducible, highly discriminatory and universally applicable. However, the visual assessment of the large number of fragments in a single gel can be difficult and may be confounded by the presence of extra-chromosomal DNA [175] (Table 6).

In Europe, *C. difficile* PCR-ribotyping has been recognised as the dominant typing method. PCR-ribotyping is based on the size variation of the 16S–23S rDNA intergenic spacer regions. A PCR-ribotype is defined as a group of strains that produce an identical band pattern. Therefore, a single band difference warrants a new ribotype [178]. Stubbs et al. (1999) [179] constructed a *C. difficile* PCR-ribotype reference library composed of 2030 isolates, with a total of 116 distinct types identified from environmental, hospital, community practitioner, veterinary and reference sources. The method was performed with agarose gel-based electrophoresis. Bidet et al. (1999) [180] improved the reading of the banding patterns by selecting a partial sequence of the rRNA genes (16S–23S) and the intergenic spacer region with a new set of primers located closer to this intergenic spacer region. The Public Health Laboratory

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Table 5
Laboratory test for *C. difficile*.

C. difficile detection	Test	Objective	Characteristics	Confirmation	Advantages	Disadvantages	Study
Toxin/enzyme detection	Cell cytotoxicity assay	Considered the best standard test for identifying toxigenic C. difficile	A laboratory cell line (Vero, Hep2, fibroblast, CHO or HeLa cells) is exposed to a filtrate of a stool suspension. If C. difficile toxins are present, a cytopathic effect is observed after 24–48 h (cell rounding via cytoskeleton disruption). The effect is mainly due to toxin B (which is more cytotoxic than toxin A)	The presence of toxigenic C. difficile can be confirmed if a specific antiserum (added later) reverses the effect on the cells	It can detect toxins at picogram levels. Most sensitive available test for detection of toxin B	Relatively slows and requires the maintenance of cell lines. If the antiserum does not neutralise the cytopathic effect, which is observed in 21% of cases, the results are inconclusive	[8,165,166]
	Enzyme immunoassay (EIA)	It can detect both toxins A and/or B, and may also detect glutamate dehydrogenase (GDH)[c]	C. difficile constitutively produces GDH in easily detectable levels, but only identifies the bacterium and not the presence of toxins (the method is comparable with stool culture)	The method can be combined with cell cytotoxicity assay, EIA for toxins, or culture with further toxin characterisation of the strains. If EIA results are negative and culture testing results are positive, it is recommended to isolate the strain from the plate and repeat EIA testing to determine if it is toxigenic	Rapid (15–45 min). Low cost (estimated at 8 USD). Easy to perform and does not require technical training or special equipment. Good sensitivity for detection of GDH (96%–100%). Useful in laboratories without culture facilities	Less sensitive than the cell cytotoxicity assay missing 40% of diagnosis. Results seem to differ based on the commercial kit used. It must be combined with a positive culture	
Culture	Stool culture	It permits molecular typing of the isolated strains and antibiotic susceptibility determination (essential for epidemiological surveys)	Cycloserine-cefoxitin fructose (CCF) has been the most commonly used medium for C. difficile isolation. The medium includes egg-yolk or blood and taurocholate, desoxycholate or cholate, which has been shown to induce germination of spores. Presently, several commercial selective media are available. The new chromogenic media improve the recovery of the bacterium. Pre-treatment of samples with ethanol shock has been associated with an increase in sensitivity	Molecular typing of the strains	Most sensitive method for detection of C. difficile. Pre-made agars are used for the clinical recovery of C. difficile from faecal samples and not for the semi-quantification of viable spores	Low specificity for CDI[d]. Not clinically practical for routine diagnosis: Slow (24–48 h) and its not give information about toxin production	[8,150,166–170]
Molecular methods	Real Time PCR (RT-PCR)	Detection of C. difficile toxin genes directly from stool samples[e]	BD GeneOhmTM Cdiff (Becton Dickenson)[a]. Xpert® C.difficile (Cepheid)[b]	Culture with further characterisation of the strains	Rapid (<4 h for a result), sensitive and specific	Expensive. Requires technical training and special equipment	[171–173]

[a] https://www.bd.com/resource.aspx?IDX=17953.
[b] http://www.cepheid.com/us/cepheid-solutions/clinical-ivd-tests/healthcare-associated-infections/xpert-c-difficile.
[c] Specific enzyme of C. difficile produced in both toxigenic and non-toxigenic isolates.
[d] Low specificity for C. difficile infection (CDI) because the rate of asymptomatic carriage of C. difficile among hospitalised patients is so high.
[e] They have been largely proposed for laboratory diagnosis of CDI but also for quantitative detection of C. difficile in hospital environmental samples.

Table 6
C. difficile typing methods.

Method	Characteristics	Advantages	Disadvantages	Reference
Multiplex PCR	The PCR can target: • *tpi* housekeeping gene[a] • an internal fragment of the toxin B (*tcdB* gene) • the 3' region, deleted or not, of the toxin A (*tcdA* gene) • binary toxin genes (*cdtA* and *cdtB*) • Other deletions in the Paloc genes	Low cost	Process labour-intensive	[140,174]
Pulsed-field gel electrophoresis (PFGE)	The method uses infrequently-cutting restriction enzymes (like *SmaI* or *SacII*) to cut bacterial DNA at different restriction sites	Highly discriminatory	Process labour-intensive Difficulty of lysing spores Difficulty of interpreting results and the inter-laboratory exchange of the results Equipment and time required	[175,176]
Restriction enzyme analysis (REA)	The method uses frequently-cutting restriction enzymes (*HindIII*) to cut total cellular DNA at different restriction sites	Reproducible and highly discriminatory power Universally applicable	Difficulty of interpreting results and the inter-laboratory exchange of the results	[175]
PCR-ribotyping	A PCR-ribotype is defined as a group of strains that produce an identical band pattern based on the size variation of the 16S–23S rDNA intergenic spacer regions	Dominant typing method Inter-laboratory comparisons easy if standard nomenclature is available or use of Webribo database	Time intensive Equipment and material costs Technical training required Inter-laboratory comparisons difficult if standard nomenclature is not available	[178,182]
QIAxcel® system	For C. difficile ribotyping, the detection of *tcdC*18 bp deletion, and toxin gene detection Based on an automated electrophoresis platform	Reduce the cost of hands-on time Allows analysis of up to 96 samples per run	Cost of the cartridges and equipment Limited sensitivity and discriminatory power (cannot distinguish between closely related PCR-ribotypes)	[184]
Serotyping - by slide agglutination - by polyacrylamide gel electrophoresis - by ELISA	The method distinguishes variations in C. difficile strains based on bacteria surface antigens	Good correlation between methods Reproducible, Rapid and reliable Inter-laboratory comparisons easy	Cross-agglutination caused by flagellar antigens (totally suppressed by ELISA)	[185,186]
Surface-layer protein A gene sequence typing (*slpAST*)	Sequencing of *slpA* gene, which encodes for a surface immuno protein Layer (S-layer)	Good discriminatory power It can be applied to direct typing (without culture) from DNA stool specimens	It has been showed that C. difficile genotype is no predictive of antigenic types	[187–189]
Repetitive sequence-based PCR typing (rep-PCR)	Specific repetitive PCR-primers complement the short repetitive sequences dispersed the bacterial genome The amplified DNA fragments provide a genomic fingerprint that can be employed for subspecies discrimination	Automated rep-PCR with a higher discriminatory power than traditional PCR-ribotyping	Requires visual interpretation and technical skills Inter-laboratory reproducibility has not been demonstrated	[190–192]
Random amplified polymorphic DNA (RAPD)	Random amplification of DNA segments by PCR reaction using a single primer of arbitrary nucleotide sequence	Inexpensive Does not require any specific knowledge of the DNA sequence	Must be combined with PCR-ribotyping to obtain higher discriminatory power	[193,194]
Amplified fragment length polymorphism (AFLP)	Genomic DNA is totally digested with two restriction enzymes. This step is followed by ligation of double-stranded oligonucleotide adaptors to the sticky ends of the restriction fragments followed by amplified by PCR	Low cost	Suboptimal reproducibility (variation in the precision of sizing of fragments) Limited application in C. difficile typing	[195]
Toxinotyping	Polymerase chain reaction-restriction fragment length polymorphism based method for differentiating strains according to changes in their toxin genes when compared to the reference VPI 10463 strain	Results of toxinotyping and PCR-ribotyping correlated well	Requires technical skills	[175]
Multilocus sequence typing (MLST)	The sequences of internal fragments of housekeeping genes (usually seven) are used to characterise the strains	Inter-laboratory comparisons easy	Time-consuming (several days) Costly and laborious technique Requires specific technical skills	[199,200]
Multilocus variable number tandem repeat analysis (MLVA)	The method utilizes the naturally occurring variation in the number of tandem repeated DNA sequences found in many different loci in the genome. The different lengths of variable number of tandem repeats (VNTR) regions are determined to distinguish among strains	High discriminatory power which allows tracking of outbreaks and determining phylogenetic relationships	Time-consuming Costly and laborious technique Requires specific technical skills Inter-laboratory comparisons difficult	[175,201]
Whole genome sequencing (WGS)	The method reveals the complete DNA of the bacterium at a single time	Provides the most comprehensive collection of an individual's genetic variation Increasingly low cost	Method still under development The large amount of data requires technical skills for further processing and analysis	[203]

[a] Species species-interspecific fragment of the triose phosphate isomerase (*tpi*) housekeeping gene.

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Service Anaerobe Reference Unit, Cardiff (UK) has established a ribotyping nomenclature reference database for *C. difficile*. This nomenclature is designated by a three-digit number starting from 001 (ex. PCR-ribotype 027). Currently, the collection of existing PCR-ribotypes and the assignment of new ones is performed by the Health Protection Agency-funded *C. difficile* Ribotype Network (CDRN) in Leeds (UK), which has more than 600 different PCR-ribotypes in the CDRN database [181]. However, in many laboratories, the standard nomenclature is not always available and a local nomenclature is used, making inter-laboratory comparisons difficult [178] (Table 6). Indra et al. (2008) [182] developed a *C. difficile* sequencer-based PCR-ribotyping method based on capillary gel electrophoresis that was proposed in order to solve the problems associated with inter-laboratory comparisons of typing results and to make PCR-ribotyping less time-intensive (Table 6). PCR amplification was performed using a fluorescent label in one of the primers, and the amplicon sizes were determined using an ABI genetic analyser [175]. A database and web-based software programme was created that allows the analysis and comparison of *C. difficile* capillary-sequencer-based PCR-ribotyping data by simply uploading sequencer data files.[6] Janezic et al. (2011) [183] described a modification to PCR-ribotyping that enables the detection of *C. difficile* in stool samples within hours. The designed primers were located partially within the *C. difficile* 16S—23S rRNA intergenic spacer regions and partially within 16S (forward primer) and 23S (reverse primer).

The QIAxcel® system has been proposed as a new method for *C. difficile* ribotyping, the detection of *tcdC18* bp deletion, and toxin gene detection (toxin A, toxin B and binary toxin CDT genes).[7] QIAxcel is based on an automated electrophoresis platform that processes samples in batches of 12 and allows the analysis of up to 96 samples per run. The system does not require the use of fluorescein-labelled primers and displays the data as both a gel-view format and an electropherogram. The system has the potential to reduce the cost of PCR-ribotyping by drastically reducing the hands-on time. The major costs are the purchase of cartridges, the setup of the QIAxcel system hardware, and the BioCalculator analysis. However, the method has limited sensitivity and discriminatory power. It cannot clearly distinguish between closely related ribotypes, such as 027 and 176 [184].

Serotyping distinguishes variations in *C. difficile* strains based on the bacterial surface antigens. Serogrouping by slide agglutination and by polyacrylamide gel electrophoresis have both been traditionally accepted as practical in routine typing [185]. Both methods have shown a good correlation in results and allow the differentiation of 10 major serogroups (A, B, C, D, F, G, H, I, K and X). Specific profiles have been associated with each of the 10 serogroups except for serogroup A. Strains from serogroup A have a common flagellar antigen that is responsible for cross-agglutination. The shearing of the flagella allows the differentiation of 12 different subgroups of serogroup A. Delmée et al. (1993) [186] used an enzyme-linked immunosorbent assay (ELISA) with antisera specific for the 10 *C. difficile* serogroups (A1, B, C, D, F, G, H, I, K, X) and the 12 serogroups within serogroup A (A2 to A12) for the serogrouping of *C. difficile* colonies for 314 positive faecal samples. The authors found that ELISA was a rapid and reliable method for *C. difficile* serotyping and that cross-agglutination caused by flagellar antigens in the slide agglutination method is totally suppressed by ELISA.

Surface-layer protein A gene sequence typing (*slpA*ST) has also been described for *C. difficile* characterisation. *C. difficile* has a surface immuno-protein Layer (S-layer) encoded by the *slpA* gene, and the typing of isolates is performed by the sequencing of this *slpA* gene. It can also be used for direct typing from DNA stool specimens without culture [187]. This method has been reported as a discriminative tool for *C. difficile* characterisation [188]. However, Dingle et al. (2013) [189] showed that the *C. difficile* genotype was not predictive of antigenic types. Therefore, S-layer typing could be useful for explaining the temporal changes and geographic differences in the epidemiology of CDI as well as the way in which isolates (and antigens) are selected for inclusion in *C. difficile* vaccines [189].

Repetitive sequence-based PCR typing (rep-PCR) is another method proposed for the characterisation of *C. difficile* strains [190]. Bacterial genomes contain multiple dispersed short repetitive sequences separating longer single-copy DNA sequences. Specific repetitive PCR primers complement these repetitive sequences, and the amplified DNA fragments provide a genomic fingerprint that can be employed for subspecies discrimination [191]. The Diversi-Lab system[8] is an automated rep-PCR typing method that has a high discriminatory power when compared to traditional PCR-ribotyping. However, this method requires the visual interpretation of rep-PCR fingerprint patterns and technical skills. Furthermore, interlaboratory reproducibility for this method must be demonstrated prior to its use for *C. difficile* surveillance [192].

Random amplified polymorphic DNA (RAPD) analysis is the 'random amplification' of DNA segments by PCR reaction using a single primer consisting of an arbitrary nucleotide sequence. RAPD is an inexpensive and powerful typing method and does not require any specific knowledge of the DNA sequence of the target microorganism. The amplification of a segment of DNA will be performed depending on positions that are complementary to the primer sequence. Green et al. (2011) [193] used PCR-ribotyping in conjunction with RAPD to further categorise different *C. difficile* types within defined PCR-ribotypes and therefore obtained a higher discriminatory power than either of the methods used alone. Barbut et al. (1993) [194] evaluated genomic fingerprinting of *C. difficile* strains using RAPD and suggested that this method could be an additional valuable tool for epidemiological studies.

In the amplified fragment length polymorphism (AFLP) technique, a small amount of purified genomic DNA is totally digested with two restriction enzymes, one with an average cutting frequency and the other with a higher cutting frequency. This step is followed by the ligation of double-stranded oligonucleotide adaptors to the sticky ends of the restriction fragments, followed by PCR amplification. After final amplification, the selectively amplified fragments are separated by gel electrophoresis and comparison of banding patterns is typically achieved using dedicated fingerprinting analysis software. AFPL has a relatively low cost, but variation in the precision of the sizing of fragments has been observed, leading to suboptimal reproducibility [195]. This method has seen limited application in *C. difficile* typing. Klaassen et al. (2002) [196] reported that AFLP analysis yielded high-resolution and highly reproducible fingerprinting patterns in a short time period (24 h) to evaluate epidemiological relatedness among hospital *C. difficile* isolates. A further study showed that AFLP is better able to discriminate between *C. difficile* reference strains (most of them toxin A+, toxin B+) than PCR-ribotyping. However, for toxin A−, toxin B+ isolates, both methods yielded similar results [138].

Toxinotyping is a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based method for differentiating *C. difficile* strains according to changes in their toxin genes

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[6] https://webribo.ages.at/.
[7] https://www.qiagen.com/be/products/catalog/automated-solutions/detection-and-analysis/qiaxcel-advanced-system/.

[8] http://www.biomerieux-diagnostics.com/diversilab.

when compared to the reference strain VPI 10463. Strains belonging to the same toxinotype have identical changes in the PaLoc region. Toxinotyping is performed by PCR amplification and restriction enzyme digestion of 10 regions of the PaLoc [175]. Currently, 31 different toxinotypes are identified and designated by Roman numerals from I to XXXII. Strains with toxin genes similar to VPI 10463 are classified as toxinotype 0. A total of 12 out of 20 toxinotypes with variant strains produce binary toxin, while most of the toxinotypes not producing binary toxin have toxin genes very similar to the reference strain, VPI 10463[9]. In this context, only one strain resembling VPI 10463 and positive for CDT has been previously described [197]. Toxinotype XXXII has been recently reported and corresponds to a new type of *C. difficile* strain (toxin A−, B+, CDT-) that completely lacks the *tcdA* gene and has an atypical organisation of the PaLoc integration site [198].

Multilocus sequence typing (MLST) analysis uses the sequences of internal fragments of housekeeping genes (usually seven) to characterise the strains. The internal fragments of each gene (450−500 bp) are sequenced on both strands using an automated DNA sequencer. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles, and for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). The data obtained are unambiguous, and the allelic profiles of the isolates can easily be compared to those in a large central database and therefore can be compared between laboratories[10]. There are two MLST databases available for *C. difficile*, each adapted to a different typing scheme: the first is organised according to the scheme described by Griffiths et al. (2010) [199], which is performed with the housekeeping genes *adk, atp, dxr, recA, sodA, tpi* and *glyA*[11]; and the second is organised according to the scheme described by Lemée et al. (2004) [200], which is performed with the housekeeping genes *aroE, dutA, gmk, groEL, recA, sodA* and *tpi*.[12] The primary problem with MLST is the time-consuming nature of the method, with the completion of analysis taking several days. In addition, MLST is a relatively costly and laborious technique that requires specific technical skills.

Multilocus variable-number tandem-repeat analysis (MLVA) has been suggested to have higher discriminatory power than other typing methods for investigating the relatedness between *C. difficile* strains [201]. It has been widely used in medical microbiology as an alternative or complement to other typing techniques such as PFGE, rep-PCR or MLST [202]. MLVA utilizes the naturally occurring variation in the number of tandem repeat DNA sequences found in many different loci in the genome. Therefore, the lengths of the variable number of tandem repeats (VNTR) regions are determined to distinguish among the strains. The technique is achieved by a multiplex PCR assay (with primers designed to target different VNTR regions in the genome) and visualised by electrophoresis or automated fragment analysis on a sequencer. The product size is used to calculate the number of repeat units of each locus. The calculated numbers of repeats of the VNTR loci (alleles) are combined, and this provides the MLVA profile. Each unique MLVA profile is given an MLVA type designation. The MLVA profile can be used for the comparison and clustering of the bacterial strains.[13] Compared with traditional PCR ribotyping, MLVA has increased discriminatory power, which allows for the more efficient tracking of outbreaks and has the potential to determine phylogenetic

relationships. In addition, MLVA produces digital data with a decreased turnaround time [175].

Whole genome sequencing (WGS) reveals the complete DNA of an organism at a single time and provides the most comprehensive collection of an individual's genetic variation [203]. Sanger sequencing and subsequently Roche 454 and Illumina next-generation sequencing technologies have been applied to study the evolutionary dynamics of *C. difficile* [175] at increasingly low cost [203]. Recent studies have applied WGS to determine the epidemiological relationships between *C. difficile* strains in healthcare settings or in the scientific community employing WGS [204]. Although the method is still under development and the large amount of data obtained requires technical skills for further processing and analysis, it is very probable that in the near future, WGS will replace all other current typing techniques.

Killgore et al. (2007) [205] compared seven different techniques (REA, PGE, PCR-ribotyping, MLST, MLVA, AFLP and *slpA*ST) for epidemic *C. difficile* strain typing. They found that all methods appeared to be adequate for detecting an outbreak strain in a particular institution. However, REA or MLVA showed the highest level of discrimination between strains, and they seem to be the most recommended methods to track outbreak strains geographically. However, as neither of the techniques are widely used and little data are available, there is currently no method with proven interlaboratory reproducibility for inter-institutional *C. difficile* tracking.

9. High-throughput sequencing analysis and CDI

16S metagenetics is a culture-independent strategy allowing the identification of bacterial populations present in a large panel of samples. It has been recently introduced to investigate the intestinal microbiota of healthy patients and patients suffering CDI. In the last year, most of the studies reported bacteria at the phylum and class level, but higher taxonomic resolution may reveal more differences in the population structure [206]. Preliminary results have shown that one of the taxa found in high proportions in patients with CDI is *Proteobacteria*, while *Bacteroidetes* proportions are lower in infected patients [207]. In this context, one study has proposed the use of *Bacteroidetes* and *Firmicutes* as probiotics to treat CDI [208]. However, the few available data regarding alterations to the intestinal microbiota of patients with CDI has been obtained in different patient conditions (age, antibiotic treatment, hospitalisation), which explains the high amount of variability between the studies. Despite this limitation, further studies exploring the diversity of the gut microbiota in CDI patients will be critical for further understanding the pathogenesis of *C. difficile* and for developing new approaches for the treatment and prophylaxis of the infection. In addition, recent studies in humans and animals have shown that many of the sequences were not identical to sequence entries present in the available databases [209,210]. Furthermore, among the sequences identical to known entries, the species name was seldom taxonomically defined. As previously reported, these findings underline the complexity of the gut microbiota, stressing the need for further research on taxonomy and functional microbiology [207].

10. Conclusions and perspectives

Eighty years after its discovery, *C. difficile* continues to be the focus of attention in hospitals and an important topic for many research groups worldwide. Recognised as the leading cause of nosocomial antibiotic-associated diarrhoea, the incidence of CDI remains high and in some years has increased, despite the efforts to improve prevention and reduce the spread of the bacterium in

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[9] http://www.mf.uni-mb.si/tox/.
[10] http://pubmlst.org/general.shtml.
[11] http://pubmlst.org/cdifficile/.
[12] http://www.pasteur.fr/recherche/genopole/PF8/mlst/Cdifficile2.html.
[13] http://www.mlva.net/.

healthcare settings. Outside of Europe and America, the incidence of CDI infection is also rising. The major research studies of the last decade have been focused on the control of the spread of the bacterium, the rapid diagnosis of CDI, and the efficacy of treatment and recurrence prevention. Different guidelines have been designed to improve the management of the infection. Diagnosis must consider both clinical and laboratory findings. Laboratory tests must be rapid and sensitive; therefore, stool culture is not clinically practical. However, the isolation of the strain is necessary for epidemiological studies. There is a need for highly discriminatory typing methods, and results should be comparable between laboratories. One potential alternative in the near future is whole genome sequencing, now considered as the next-generation typing tool.

The investigation of the gut's microbial communities by new metagenetic analyses will allow researchers to discern whether any alteration of the gut microbiota composition can favour *C. difficile* colonisation, as well as the microbes responsible for rendering individuals less susceptible to the infection. This approach will be critical in the future to further understand the pathogenesis of *C. difficile* and to develop new successful prevention and treatment measures.

References

[1] S. Fridkin, J. Baggs, R. Fagan, S. Magill, L.A. Pollack, P. Malpiedi, et al., Vital signs: improving antibiotic use among hospitalized patients, MMWR Morb. Mortal. Wkly. Rep. 63 (2014) 194–200.

[2] C. Rodriguez, N. Korsak, B. Taminiau, V. Avesani, J. Van Broeck, M. Delmée, et al., *Clostridium difficile* infection in elderly nursing home residents, Anaerobe 30 (2014) 184–187.

[3] E. Borali, G. Ortisi, C. Moretti, E.F. Stacul, R. Lipreri, G.P. Gesu, et al., Community-acquired *Clostridium difficile* infection in children: a retrospective study, Dig. Liver Dis. 47 (2015) 842–846.

[4] E.J. Kuijper, B. Coignard, P. Tüll, ESCMID study group for *C. difficile*, EU member States, european centre for disease prevention and control. Emergence of *Clostridium difficile*-associated disease in North America and Europe, Clin. Microbiol. Infect. 12 (Suppl 6) (2006) 2–18.

[5] E.J. Kuijper, F. Barbut, J.S. Brazier, N. Kleinkauf, T. Eckmanns, M.L. Lambert, et al., Update of *C. difficile* infection due to PCR-ribotype 027 in Europe, Eurosurveillance 2008 (13) (2008) 31.

[6] A. Goorhuis, D. Bakker, J. Corver, S.B. Debast, C. Harmanus, D.W. Notermans, et al., Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078, Clin. Infect. Dis. 47 (2008) 1162–1170.

[7] J. Freeman, J. Vernon, K. Morris, S. Nicholson, S. Todhunter, C. Longshaw, et al., Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes, Clin. Microbiol. Infect. 21 (248) (2015) e9–e16.

[8] S.H. Cohen, D.N. Gerding, S. Johnson, C.P. Kelly, V.G. Loo, L.C. McDonald, et al., Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for healthcare epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), Infect. Control Hosp. Epidemiol. 31 (2010) 431–455.

[9] I.C. Hall, E. O'Toole, Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*, Am. J. Dis. Child. 49 (1935).

[10] M.L. Snyder, Further studies on *Bacillus difficilis* (Hall and O'Toole), J. Infect. Dis. 60 (1937) 223–231.

[11] A. Prévot, Etudes de systematic bacterienne, Ann. Inst. Pasteur 61 (1938) 72–91.

[12] V. Skerman, V. McGowan, P. Sneath, Approved Lists of Bacterial Names (Amended), ASM Press, Washington (DC), 1989.

[13] M.L. Snyder, The normal fecal flora of infants between two weeks and one year of age, J. Infect Dis 1940 (66) (1940) 1–16.

[14] L.D. Smith, E.O. King, Occurrence of *Clostridium difficile* in infections of man, J. Bacteriol. 84 (1962) 65–67.

[15] D. Danielsson, D. Lambe, S. Persson, The immune response in patient to an infection with *Bacteroides fragilis spp. fragilis* and *Clostridium difficile*, Acta Path Microbiol. Scand. 80 (1972) 709–712.

[16] S. Gorbach, K. Menda, L. Thadepalli, L. Keith, Anaerobic flora of the cervix in healthy women, Am. J. Obstet. Gynecol. 15 (1973) 1053–1055.

[17] V. Sutter, S. Finegold, The effect of antimicrobial agents on human fecal flora: studies with cephamexin, cyclacillin and clindamycin, in: F. Skinner, J.G. Carr (Eds.), The Normal Microbial Flora of Man, Academic Press, London, 1974, pp. 229–240.

[18] S. Gorbach, H. Thadepalli, Isolation of *Clostridium* in humans infections: evaluation of 114 cases, J. Infect. Dis. 131 (1975) S81–S85.

[19] S. Hafiz, R.S. Morton, M.G. Mcentegart, S.A. Waitkins, *Clostridium difficile* in the urogenital tract of males and females, Lancet 305 (1975) 420–421.

[20] S. Hafiz, C. Oakley, *Clostridium difficile*: isolation and characteristics, J. Med. Microbiol. 9 (1976) 129–136.

[21] W.L. George, V.L. Sutter, S.M. Finegold, Antimicrobial agent-induced diarrhea–a bacterial disease, J. Infect. Dis. 136 (1977) 822–828.

[22] D. Hamre, G. Rake, C. McKee, H. MacPhillamy, The toxicity of penicillin as prepared for clinical use, Am. J. Med. Sci. 206 (1943) 642.

[23] R.H. Green, The association of viral activation with penicillin toxicity in guinea pigs and hamsters, Yale J. Bio Med. 47 (1974) 166–181.

[24] J.G. Bartlett, *Clostridium difficile* infection: historic review, Anaerobe 15 (2009) 227–229.

[25] J. Finney, Gastro-enterostomy for cicatrizing ulcer of the pylonus, Johs Hopkins Hosp. Bull. 4 (1893) 53–55.

[26] N.M. Thielman, R.L. Guerrant, *Clostridium difficile* and its toxins, in: J. Mogs, B. Iglewski, M. Vaughan, T.A. Tu (Eds.), Bacterial Toxins and Virulence Factors in Disease. Handbook of Natural Toxins, Marcel Dekker Inc, New York, 1995, p. 328.

[27] M.R.B. Keighley, Antibiotic-associated pseudomembranous colitis: pathogenesis and management, Drugs 20 (1980) 49–56.

[28] L. Reiner, M.J. Schlesinger, G.M. Miller, Pseudomembranous colitis following aureomycin and chloramphenicol, AMA Arch. Pathol. 54 (1952) 39–67.

[29] E. Benner, W. Tellman, Pseudomembranous colitis as a sequel to oral lincomycin, Am. J. Gastroenterol. 54 (1970) 55–58.

[30] A. Scott, G. Nicholson, A. Kerr, Lincomycin as a cause of pseudomembranous colitis, Lancet 2 (1973) 1232–1234.

[31] D.N. Gerding, *Clostridium difficile* 30 years on: what has, or has not, changed and why? Int. J. Antimicrob. Agents 33 (2009) S2–S8.

[32] H.E. Larson, A.B. Price, Pseudomembranous colitis: presence of clostridial toxin, Lancet 2 (1977) 1312–1314.

[33] J.G. Bartlett, A.B. Onderdonk, R.L. Cisneros, D.L. Kasper, Clindamycin-associated colitis due to a toxin-producing species of *Clostridium* in hamsters, J. Infect. Dis. 136 (1977) 701–705.

[34] J.G. Bartlett, T. Wen Chang, M. Gurwith, S.L. Gorbach, A.B. Onderdonk, Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia, N. Engl. J. Med. 298 (1978) 531–534.

[35] G.D. Rifkin, J. Silva, R. Fekety, Gastrointestinal and systemic toxicity of fecal extracts from hamsters with clindamycin-induced colitis, Gastroenterology 74 (1978) 52–57.

[36] G.D. Rifkin, F.R. Fekety, J. Silva, Antibiotic-induced colitis implication of a toxin neutralised by *Clostridium sordellii* antitoxin, Lancet 2 (1977) 1103–1106.

[37] R.H. George, J.M. Symonds, F. Dimock, J.D. Brown, Y. Arabi, N. Shinagawa, et al., Identification of *Clostridium difficile* as a cause of pseudomembranous colitis, Br. Med. J. 1 (1978) 695.

[38] W.L. George, V.L. Sutter, E.J. Goldstein, S.L. Ludwig, S.M. Finegold, Aetiology of antimicrobial-agent-associated colitis, Lancet 1 (1978) 802–803.

[39] H.E. Larson, A.B. Price, P. Honour, S.P. Borriello, *Clostridium difficile* and the aetiology of pseudomembranous colitis, Lancet 1 (1978) 1063–1066.

[40] M.R. Keighley, D.W. Burdon, Y. Arabi, J.A. Williams, H. Thompson, D. Youngs, et al., Randomised controlled trial of vancomycin for pseudomembranous colitis and postoperative diarrhoea, Br. Med. J. 16 (1978) 1667–1669.

[41] T.V. Riley, *Clostridium difficile*: a pathogen of the nineties, Eur. J. Clin. Microbiol. Infect. Dis. 17 (1998) 137–141.

[42] F. Barbut, G. Jones, C. Eckert, Epidemiology and control of *C. difficile* infections in healtcare settings: an update, Curr. Opin. Infect. Dis. 24 (2011) 370–376.

[43] M.P. Hensgens, E.C. Keessen, M.M. Squire, T.V. Riley, M.G. Koene, E. de Boer, et al., *Clostridium difficile* infection in the community, a zoonotic disease? Clin. Microbiol. Infect. 18 (2012) 635–645.

[44] N. Yutin, M.Y. Galperin, A genomic update on clostridial phylogeny: gram-negative spore formers and other misplaced clostridia, Environ. Microbiol. 15 (2013) 2631–2641.

[45] N.L. Crogan, B.C. Evans, *Clostridium difficile*: an emerging epidemic in nursing homes, Geriatr. Nurs. 28 (2007) 161–164.

[46] J.M. Keller, C.M. Surawicz, *Clostridium difficile* infection in the elderly, Clin. Geriatr. Med. 30 (2014) 79–93.

[47] N. Nanwa, T. Kendzerska, M. Krahn, J.C. Kwong, N. Daneman, W. Witteman, et al., The economic impact of *Clostridium difficile* infection: a systematic review, Am. J. Gastroenterol. 110 (2015) 511–519.

[48] A.M. Jones, E.J. Kuijper, M.H. Wilcox, Clostridium difficile: a European perspective, J. Infect. 66 (2013) 115–128.

[49] P. Tattevin, S. Buffet-Bataillon, P.Y. Donnio, M. Revest, C. Michelet, *Clostridium difficile* infections: do we know the real dimensions of the problem? Int. J. Antimicrob. Agents 42 (2013) S36–S40.

[50] M. Natarajan, S.T. Walk, V.B. Young, D.M. Aronoff, A clinical and epidemiological review of non-toxigenic *Clostridium difficile*, Anaerobe 22 (2013) 1–5.

[51] M.M. Riggs, A.K. Sethi, T.F. Zabarsky, E.C. Eckstein, R.L.P. Jump, C.J. Donskey, Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic *Clostridium difficile* strains among long-term care facility residents, Clin. Infect. Dis. 45 (2007) 992–998.

[52] J. Ryan, C. Murphy, C. Twomey, C. Paul-Ross, M.C. Rea, J. MacSharry, et al., Asymptomatic carriage of *Clostridium difficile* in an Irish continuing care institution for the elderly: prevalence and characteristics, Ir. J. Med. Sci. 179 (2010) 245–250.

[53] M. Arvand, V. Moser, C. Schwehn, G. Bettge-Weller, M.P. Hensgens,

E.J. Kuijper, High prevalence of *Clostridium difficile* colonization among nursing home residents in Hesse, Germany, PloS One 7 (2012) e30183.

[54] D.W. Eyre, D. Griffiths, A. Vaughan, T. Golubchik, M. Acharya, L. O'Connor, et al., Asymptomatic *Clostridium difficile* colonisation and onward transmission, PloS One 8 (2013) e78445.

[55] J.H. Boone, M. Goodykoontz, S.J. Rhodes, K. Price, J. Smith, K.N. Gearhart, et al., *Clostridium difficile* prevalence rates in a large healthcare system stratified according to patient population, age, gender, and specimen consistency, Eur. J. Clin. Microbiol. 31 (2012) 1551–1559.

[56] C. Rousseau, I. Poilane, L. De Pontual, A.C. Maherault, A. Le Monnier, A. Collignon, *Clostridium difficile* carriage in healthy infants in the community: a potential reservoir for pathogenic strains, Clin. Infect. Dis. 55 (2012) 1209–1215.

[57] M. Delmée, G. Verellen, V. Avesani, G. Francois, *Clostridium difficile* in neonates: serogrouping and epidemiology, Eur. J. Pediatr. 147 (1988) 36–40.

[58] H. Kato, N. Kato, K. Watanabe, K. Ueno, H. Ushijima, S. Hashira, et al., Application of typing by pulsed-field gel electrophoresis to the study of *Clostridium difficile* in a neonatal intensive care unit, J. Clin. Microbiol. 32 (1994) 2067–2070.

[59] C. Simango, S. Uladi, Detection of *Clostridium difficile* diarrhoea in Harare, Zimbabwe, Trans. R. Soc. Trop. Med. Hyg. 108 (2014) 354–357.

[60] T. Jaeggi, G.A.M. Kortman, D. Moretti, C. Chassard, P. Holding, A. Dostal, et al., Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants, Gut 64 (2014) 731–742.

[61] D.A. Collins, P.M. Hawkey, T.V. Riley, Epidemiology of *Clostridium difficile* infection in Asia, Antimicrob. Resist Infect. Control 2 (2013) 21.

[62] P. Putsathit, P. Kiratisin, P. Ngamwongsatit, T.V. Riley, *Clostridium difficile* infection in Thailand, Int. J. Antimicrob. Agents 45 (2015) 1–7.

[63] I.T. Balassiano, E.A. Yates, R.M.C.P. Domingues, E.O. Ferreira, *Clostridium difficile*: a problem of concern in developed countries and still a mystery in Latin America, J. Med. Microbiol. 61 (2012) 169–179.

[64] C. Garcia, F. Samalvides, M. Vidal, E. Gotuzzo, H.L. Dupont, Epidemiology of *Clostridium difficile*-associated diarrhea in a Peruvian tertiary care hospital, Am. J. Trop. Med. Hyg. 77 (2007) 802–805.

[65] N.J. Ashbolt, Microbial contamination of drinking water and disease outcomes in developing regions, Toxicology 20 (2004) 229–238.

[66] A.K. Jha, B. Uppal, S. Chadha, P. Bhalla, R. Ghosh, P. Aggarwal, et al., Clinical and microbiological profile of HIV/AIDS cases with diarrhea in North India, J. Pathog. 2012 (2012) 97.

[67] K. Onwueme, Y. Fadairo, L. Idoko, J. Onuh, O. Alao, P. Agaba, et al., High prevalence of toxinogenic *Clostridium difficile* in Nigerian adult HIV patients, Trans. R. Soc. Trop. Med. Hyg. 105 (2011) 667–669.

[68] A. Neuberger, T. Saadi, A. Shetern, E. Schwartz, *Clostridium difficile* Infection in travelers—a neglected pathogen? J. Travel Med. 20 (2013) 37–43.

[69] A. Kramer, I. Schwebke, G. Kampf, How long do nosocomial pathogens persist on inanimate surfaces? A systematic review, BMC Infect. Dis. 6 (2006) 130.

[70] M. Sjörberg, M. Eriksson, J. Andersson, T. Noren, Transmission of *Clostridium difficile* spores in isolation room environments and through hospital beds, APMIS 30 (2014).

[71] P. Verity, M.H. Wilcox, W. Fawley, P. Parnell, Prospective evaluation of environmental contamination by *Clostridium difficile* in isolation side rooms, J. Hosp. Infect. 49 (2001) 204–209.

[72] G.W. Kaatz, S.D. Giftin, D.R. Schaberg, K.H. Wilson, C.A. Kauffman, S.M. Seo, et al., Acquisition of *Clostridium difficile* from the hospital environment, Am. J. Epidemiol. 127 (1988) 1289–1294.

[73] K.H. Kim, R. Fekety, D.H. Batts, D. Brown, M. Cudmore, J. Silva Jr., et al., Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic associated colitis, J. Infect. Dis. 143 (1981) 42–50.

[74] M.E. Mulligan, W.L. George, R.D. Rolfe, S.M. Finegold, Epidemiological aspects of *Clostridium difficile*-induced diarrhea and colitis, Am. J. Clin. Nutr. 33 (11 Suppl) (1980) 2533–2538.

[75] M.H. Wilcox, W.N. Fawley, P. Wigglesworth, P. Parnell, P. Verity, J. Freeman, Comparison of the effect of detergent versus hypochlorite cleaning on environmental contamination and incidence of *C. difficile* infection, J. Hosp. Infect. 54 (2003) 109–114.

[76] W.N. Fawley, M.H. Wilcox, Molecular epidemiology of endemic *Clostridium difficile* infection, Epidemiol. Infect. 126 (2001) 343–350.

[77] B.A. Walters, R. Stafford, R.K. Roberts, E. Seneviratne, Contamination and crossinfection with *Clostridium difficile* in an intensive care unit, Aust. N. Z. J. Med. 12 (1982) 255–258.

[78] S.E. Brooks, R.O. Veal, M. Kramer, L. Dore, N. Schupf, M. Adachi, Reduction in the incidence of *Clostridium-difficile* associated diarrhea in an acute care hospital and a skilled nursing facility following replacement of electronic thermometers with single use disposables, Infect. Control Hosp. Epidemiol. 13 (1992) 98–103.

[79] J.A. Jemigan, Slegman-Igra, R.C. Guerrant, B.M. Farr, A randomized crossover study of disposable thermometers for prevention of *Clostridium difficile* and other nosocomial infections, Infect. Control Hosp. Epiemiol 19 (1998), 794–499.

[80] S. Brooks, A. Khan, D. Stoica, J. Griffith, L. Friedeman, R. Mukherji, et al., Reduction in vancomycin-resistant Enterococcus and *Clostridium difficile* infection following change to tympanic thermometers, Infect. Control Hosp. Epidemiol. 19 (1998), 336–336.

[81] C. Rodriguez, N. Korsak, B. Taminiau, V. Avesani, J. Van Broeck, P. Brach, et al., *Clostridium difficile* from food and surface samples in a Belgian nursing home; an unlike source of contamination, Anaerobe 32 (2015) 87–89.

[82] L.M. Kiedrowski, A. Perisetti, M.H. Loock, M.L. Khaitsa, D.M. Guerrero, Disinfection of iPad to reduce contamination with *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus*, Am. J. Infect. Control 41 (2013) 1136–1137.

[83] M.A. Alqumber, *Clostridium difficile* in retail baskets, trolleys, conveyor belts, and plastic bags in Saudi Arabia, Saudi Med. J. 35 (2014) 1274–1277.

[84] V. Pasquale, V.J. Romano, M. Rupnik, S. Dumonlet, I. Ciznar, F. Aliberti, et al., Isolation and characterization of *Clostridium difficile* from shellfish and marine environments, Folia Microbiol. 56 (2011) 431–437.

[85] M. del Mar Gamboa, E. Rodriguez, P. Vargas, Diversity of mesophilic clostridia in Costa Rica soils, Anaerobe 11 (2005) 322–326.

[86] V. Zidaric, S. Beigot, S. Lapajne, M. Rupnik, The occurrence and high diversity of *Clostridium difficile* genotypes in rivers, Anaerobe 16 (2010) 371–375.

[87] C. Simango, Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe, Trans. R. Soc. Trop. Med. Hyg. 100 (2006) 1146–1150.

[88] V. Baverud, A. Gustafsson, A. Franklin, A. Aspan, A. Gunnarsson, *Clostridium difficile*: prevalence in horses and environment, and antimicrobial susceptibility, Equine Vet. J. 35 (2003) 465–471.

[89] A.M. Bojesen, K.E.P. Olsen, M.F. Bertelsen, Fatal enterocolitis in Asian elephants (Elephas maximus) caused by *Clostridium difficile*, Vet. Microbiol. 116 (2006) 329–335.

[90] R.O.S. Silva, M.L. D'Elia, D.F. de Magalhanes Soares, A.R. Cavalcanti, R.C. Leal, C. Cavalcanti, et al., *Clostridium difficile*-associated diarrhea in an ocelot (Leopardus pardalis), Anaerobe 20 (2013) 82–84.

[91] E. French, A. Rodriguez-Palacios, J.T. LeJeune, Enteric bacterial pathogens with zoonotic potential isolated from farm-raised deer, Foodborne Pathog. Dis. 7 (2010) 1031–1037.

[92] R.O. Silva, M.L. D'Elia, E.P. Tostes Teixeira, P.L. Pereira, D.F. de Magalhanes Soares, A.R. Cavalcanti, et al., *Clostridium difficile* and *Clostridium perfringens* from wild carnivores species in Brazil, Anaerobe 28 (2014) 207–211.

[93] P. Bandelj, T. Trilar, R. Blagus, M. Ocepek, J. Rousseau, J.S. Weese, et al., Prevalence and molecular characterization of *Clostridium difficile* isolated from European Barn Swallows (Hirundo rustica) during migration, BMC Vet. Res. 10 (2014) 40.

[94] P. Bandelj, T. Trilar, J. Racnik, M. Zadrvec, T. Pirs, J. Avbersek, et al., Zero prevalence of *Clostridium difficile* in wild passerine birds in Europe, FEMS Microb. Lett. 321 (2011) 183–185.

[95] S. Alvarez-Perez, J.L. Blanco, E. Martinez-Nevado, T. Pelaez, C. Harmanus, E. Kuijper, M.E. Garcia, Shedding of *Clostridium difficile* PCR-ribotype 078 by zoo animals, and report of an unstable metronidazole-resistant from a zebra foal (Equus quagga burchellii), Vet. Microbiol. 169 (2014) 218–222.

[96] C.E. Anderson, M. Haulena, E. Zabek, G. Habing, S. Raverty, Clinical and epidemiologic considerations of *Clostridium difficile* in harbor seals (Phoca Vitulina) at a marine mammal rehabilitation center, J. Zoo. Wildl. Med. 46 (2015) 191–197.

[97] R.O. Silva, L. Ribeiro de Almeida, C.A. Oliveira Junior, D.F. de Magalhaes Soares, P.L. Pereira, M. Rupnik, et al., Carriage of *Clostridium difficile* in free-living South American coati (Nasua nasua) in Brazil, Anaerobe 30 (2014) 99–101.

[98] C.M. Jardine, R.J. Reid-Smith, J. Rousseau, J.S. Weese, Detection of Clostridium difficile in samlet and medium-sized wild mammals in Southern Ontario, Canada, J. Wildl. Dis. 49 (2013) 418–421.

[99] C.G. Himsworth, D.M. Patrick, S. Mak, C.M. Jardine, P. Tang, J.S. Weese, Carriage of *Clostridium difficile* by wild urban Norway rats (Rattus norvegicus) and black rats (Rattus rattus), Appl. Environ. Microbiol. 80 (2014) 1299–1305.

[100] S. Thakur, M. Sandfoss, S. Kennedy-Stoskopf, C.S. DePerno, Detection of *Clostridium difficile* and *Salmonella* in feral swine population in North Carolina, J. Wildl. Dis. 47 (2011) 774–776.

[101] S. Alvarez-Perez, J.L. Blanco, T. Pelaez, R.J. Astorga, C. Harmanus, E. Kuijper, et al., High prevalence of the epidemic *Clostridium difficile* PCR-ribotype 078 in Iberian free-range pigs, Res. Vet. Sci. 95 (2013) 358–361.

[102] J.S. Weese, R. Finley, R.R. Reid-Smith, N. Janecko, J. Rousseau, Evaluation of *Clostridium difficile* in dogs and the household environment, Epidemiol. Infect. 138 (2010) 1100–1104.

[103] M. Usui, K. Suzuki, K. Oka, K. Miyamoto, M. Takahashi, T. Inamatsu, et al., Distribution and characterization of Clostridium difficile isolated from dogs in Japan, Anaerobe 37 (2016) 58–61.

[104] I. Hussain, R.K. Sharma, P. Borah, S. Rajkhowa, I. Hussain, L.M. Barkalita, et al., Isolation and characterization of Clostridium difficile from pet dogs in Assam, India, Anaerobe 36 (2015) 9–13.

[105] S. Alavarez-Pérez, J.L. Blanco, T. Pelaez, M.P. Lanzarot, C. Harmanus, E. Kuijper, et al., Faecal shedding of antimicrobial-resistant *Clostridium difficile* strains by dogs, J. Small Anim. Pract. 56 (2015) 190–195.

[106] K.J. Wetterwik, G. Trowald-Wigh, L.L. Fernstrom, K. Krovacek, *Clostridium difficile* in faeces from healthy dogs and dogs with diarrhea, Acta Vet. Scand. 55 (2013) 23.

[107] J.S. Weese, J. Amstrong, Outbreak of *Clostridium difficile*-associated disease in a small animal veterinary teaching hospital, J. Ver. Intern Med. 17 (2003) 813–816.

[108] A. Schneeberg, M. Rupnik, H. Neubauer, C. Seyboldt, Prevalence and

distribution of *Clostridium difficile* PCR-ribotypes in cats and dogs from animal shelters in Thuringia, Germany, Anaerobe 18 (2012) 484–488.

[109] J.S. Weese, H.E. Weese, T.L. Bourdeau, H.R. Staempfli, Suspected *Clostridium difficile*-associated diarrhea in two cats, J. Am. Med. Assoc. 121 (2001) 1436–1439.

[110] B.R. Madewell, J.K. Bea, S.A. Kraegel, M. Winthrop, Y.J. Tang, J. Silva Jr., *Clostridium difficile*: a survey of fecal carriage in cats in a veterinary medical teaching hospital, J. Vet. Diagn Invest 11 (1999) 50–54.

[111] S.S. Diab, G. Songer, F.A. Uzal, *Clostridium difficile* infection in horses: a review, Vet. Microbiol. 167 (2013) 42–49.

[112] C. Rodriguez, B. Taminiau, B. Brévers, V. Avesani, J. Van Broeck, A. Leroux, et al., Faecal microbiota characterisation of horses using 16 rda barcoded pyrosequencing, and carriage rate of *Clostridium difficile* at hospital, BMC Microbiol. 15 (2015) 181.

[113] C. Rodriguez, B. Taminiau, B. Brévers, V. Avesani, J. Van Broeck, A.A. Leroux, et al., Carriage and acquisition rates of *Clostridium difficile* in hospitalized horses, including molecular characterization, multilocus sequence typing and antimicrobial susceptibility of the bacterial isolates, Vet. Microbiol. 172 (2014) 309–317.

[114] C.E. Medina-Torres, J.S. Weese, H.R. Staempfli, Prevalence of *Clostridium difficile* in horses, Vet. Microbiol. 152 (2011) 212–215.

[115] S. Thean, B. Elliott, T.V. Riley, *Clostridium difficile* in horses in Australia-a preliminary study, J. Med. Microbiol. 60 (2011) 1188–1192.

[116] C. Rodriguez, V. Avesani, J. Van Broeck, B. Taminiau, M. Delmée, G. Daube, Presence of *Clostridium difficile* in pigs and cattle intestinal contents and carcass contamination slaughterhouse in Belgium, Int. J. Food Microbiol. 166 (2013) 256–262.

[117] T. Norén, K. Johansson, M. Unemo, *Clostridium difficile* PCR-ribotype 046 is common among neonatal pigs and humans in Sweden, Clin. Microbiol. Infect. 20 (2014) O2–O6.

[118] A. Indra, H. Lassnig, N. Baliko, P. Much, A. Fiedler, S. Huhulescu, et al., Clostridium difficile: a new zoonotic agent? Wien Kin. Wochensr 121 (2009) 027–91.

[119] E.C. Keessen, A.J. van den Berkt, N.H. Haasjes, C. Hermanus, E.J. Kuijper, L.J. Lipman, The relation between farm specific factors and prevalence of *Clostridium difficile* in slaughter pigs, Vet. Microbiol. 154 (2011) 130–134.

[120] E. Hoffer, H. Haechler, R. Frei, R. Stephan, Low occurrence of *Clostridium difficile* in faecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland, J. Food Prot. 73 (2010) 973–975.

[121] A.A. Bakker, E. Davis, T. Rehberger, D. Rosener, Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the Midwest, Appl. Environ. Microbiol. 76 (2010) 2961–2967.

[122] J.S. Weese, J. Rousseau, A. Deckert, S. Gow, Reid-Smith RJ.*Clostridium difficile* and methicillin-resistant Staphylococcus aureus shedding by slaughter-age pigs, BMC Vet. Res. 7 (2011) 41.

[123] M.M. Squire, G.P. Carter, K.E. Mackin, A. Chakravorty, T. Noren, B. Elliot, et al., Novel molecular type of *Clostridium difficile* in neonatal pigs, Western Australia, Emerg. Infect. Dis. 19 (2013) 790–792.

[124] T. Asai, M. Usui, M. Hiki, M. Kawanishi, H. Nagai, Y. Sasaki, *Clostridium difficile* isolated from the faecal contents of swine in Japan, J. Vet. Med. Sci. 75 (2013) 539–541.

[125] J. Avbersek, S. Janezic, M. Pate, M. Rupnik, V. Zidaric, et al., Diversity of *Clostridium difficile* in pigs and other animals in Slovenia, Anaerobe 15 (2009) 252–255.

[126] A. Schneeberg, H. Neubauer, G. Schmoock, E. Grossmann, C. Seyboldt, Presence of *Clostridium difficile* PCR-ribotype clusters related to 033, 078 and 045 in diarrhoeic calves in Germany, J. Med. Microbiol. 62 (2013) 1190–1198.

[127] A. Doosti, A. Mokhtari-Farsani, Study of the frequence of *Clostridium difficile* tcdA, tcdB, cdtA and cdtB genes in feces of calves in soyjt west of Iran, Ann. Clin. Microbiol. 13 (2014) 21.

[128] A. Rodriguez-Palacios, T. Barman, J.T. LeJeune, Three-week summer period prevalence of Clostridium difficile in farm animals in a temperate region of the United States (Ohio), Can. Vet. J. 55 (2014) 786–789.

[129] A. Rodriguez-Palacios, S. Borgmann, T. LeJeune, *Clostridium difficile* in foods and animals: history and measures to reduce exposure, Anim. Health Res. Rev. 14 (2013) 11–29.

[130] D.R. Bonne, R.W. Castenholz, Bergey's Manual of Systematic Bacteriology, 2th ed., Springer-Verlag, New York, 2001.

[131] L.J. Wheeldon, T. Worthington, P.A. Lambert, Histidine acts as a co-germinant with glycine and taurocholate for *Clostridium difficile* spores, J. Appl. Microbiol. 110 (2011) 987–994.

[132] P.G. Engelkirk, J.L. Duben-Engelkirk, Laboratory Diagnosis of Infectious Diseases: Essentials of Diagnostic Microbiology, first ed., Wolters Kluwer Health/Lippincott Williams & Wilkins, Baltimore, 2008.

[133] W.L. George, V.L. Sutter, D. Citron, S.M. Finegold, Selective and differential medium for isolation of *Clostridium difficile*, J. Clin. Microbiol. 9 (1979) 214–219.

[134] L.F. Dawson, R.A. Stabler, B.W. Wren, Assessing the role of p-cresol tolerance in *Clostridium difficile*, J. Med. Microbiol. 57 (2008) 745–749.

[135] D. Drudy, S. Fanning, L. Kyne, Toxin A-negative, toxin B-positive *Clostridium difficile*, Int. J. Infect. Dis. 11 (2007) 5–10.

[136] D. Lyras, J.R. O'Connor, P.M. Howarth, S.P. Sambol, G.P. Carter, T. Phumoonna, et al., Toxin B is essential for virulence of *Clostridium difficile*, Nature 458 (2009) 1176–1179.

[137] S.A. Kuehne, S.T. Cartman, N.P. Minton, Both, toxin A and toxin B, are important in *Clostridium difficile* infection, Gut microbes 2 (2011) 252–255.

[138] R.J. Van den Berg, E.C.J. Claas, D.H. Oyib, C.H.W. Klaassen, L. Dijkshoorn, Brazier, et al., Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping, J. Clin. Microbiol. 42 (2004) 1035–1041.

[139] M.M. Squire, G.P. Carter, K.E. Mackin, A. Chakravorty, T. Norén, B. Elliott, D. Lyras, et al., Novel molecular type of *Clostridium difficile* in neonatal pigs, Western Australia, Emerg. Infect. Dis. 19 (2013) 790–792.

[140] L. Lemée, A. Dhalluin, S. Testelin, M.A. Mattrat, K. Maillard, J.F. Lemeland, et al., Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (Toxin A), and tcdB (Toxin B) genes for toxigenic culture of *Clostridium difficile*, J. Clin. Microbiol. 42 (2004) 5710–5714.

[141] D.E. Voth, J.D. Ballard, *Clostridium difficile* toxins: mechanism of action and role in disease, Clin. Microbiol. Rev. 18 (2005) 247–263.

[142] J.L. Trudel, *Clostridium difficile* colitis, Clin. Colon Rectal Surg. 20 (2007) 13–17.

[143] S. Stubbs, M. Rupnik, M. Gibert, J. Brazier, B. Duerden, M. Popoff, Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*, FEMS Microbiol. Lett. 186 (2000) 307–312.

[144] S. Hemmasi, B.A. Czulkies, B. Schorch, A. Veit, K. Aktories, P. Papatheodorou, Interaction of the *Clostridium difficile* binary toxin CDT and its host cell receptor, lipolysis-stimulated lipoprotein receptor (LSR), J. Biol. Chem. 290 (2015) 14031–14044.

[145] C. Schwan, B. Stecher, T. Tzivelekidis, M. van Ham, M. Rohde, W.D. Hardt, et al., *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria, PLoS Pathog. 5 (2009) e1000626.

[146] S. Bacci, K. Mølbak, M.K. Kjeldsen, K.E.P. Olsen, Binary toxin and death after *Clostridium difficile* infection, Emerg. Infect Dis. 17 (2011) 976–982.

[147] Y. Tagashira, H. Kato, M. Senoh, A. Nakamura, Two cases of fulminant colitis due to binary toxin-positive *Clostridium difficile* that are not PCR ribotype 027 or type 078, J. Med. Microbiol. 62 (2013) 1486–1489.

[148] D.B. Stewart, A. Berg, J. Hegarty, Predicting recurrence of *C. difficile* colitis using bacterial virulence factors: binary toxin is the key, J. Gastrointest. Surg. 17 (2013) 118–124.

[149] C. Eckert, A. Emirian, A. Le Monnier, L. Cathala, H. De Montclos, J. Goret, et al., Prevalence and pathogenicity of binary toxin-positive *Clostridium difficile* strains that do not produce toxins A and B, New Microbes New Infect. 3 (2015) 12–17.

[150] M. Delmée, Laboratory diagnosis of *Clostridium difficile* disease, Clin. Microbiol. Infect. 7 (2001) 411–416.

[151] E.J. Baron, J.M. Miller, M.P. Weinstein, S.S. Richter, P.H. Gilligan, B. Richard, et al., A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 213 recommendations by the Infectious Disease Society of America (IDSA) and the America Society for Microbiology (ASM), Clin. Microbiol. Infect. Dis. 57 (2013) e22–e121.

[152] K.H. Wilson, Efficiency of various bile salt preparations for stimulation of *Clostridium difficile* spore germination, J. Clin. Microbiol. 18 (1983) 1017–1019.

[153] K.L. Tyrrell, D.M. Citron, E.S. Leoncio, C.V. Merriam, E.J.C. Goldstein, Evaluation of cycloserine-cefoxitin fructose agar (CCFA), CCFA with horse blood and taurocholate, and cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme for recovery of *Clostridium difficile* isolates from fecal samples, J. Clin. Microbiol. 51 (2013) 3094–3096.

[154] M. Delmée, G. Wauters, The role of *Clostridium difficile* in diarrhea appearing after antibiotic treatment: a study of 87 cases, Acta Clin. Belg 36 (1981) 178–184.

[155] DR1107, C. difficile Test Kit | Oxoid – Product Detail n.d. http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=DR1107&minfo=Y (accessed 10.12.15).

[156] S.B. Han, J. Chang, S.H. Shin, K.G. Park, G.D. Lee, Y.G. Park, et al., Performance of chromID *Clostridium difficile* agar compared with BBL *C. difficile selective* agar for detection of *C. difficile* in stool specimens, Ann. Lab. Med. 34 (2014) 376–379.

[157] J.J. Yang, Y.S. Nam, M.J. Kim, S.Y. Cho, E. You, Y.S. Soh, et al., Evaluation of a chromogenic culture medium for the detection of *Clostridium difficile*, Yonsei Med. J. 55 (2014) 994–998.

[158] B.-M. Shin, E.J. Lee, Comparison of ChromID agar and *Clostridium difficile* selective agar for effective isolation of *C. difficile* from stool specimens, Ann. Lab. Med. 34 (2014) 15–19.

[159] K.C. Carson, L.V. Boseiwaqa, S.K. Thean, N.F. Foster, T.V. Riley, Isolation of *Clostridium difficile* from faecal specimens – a comparison of chromID *C. difficile* agar and cycloserine-cefoxitin-fructose agar, J. Med. Microbiol. 62 (2013) 1423–1427.

[160] M. Lister, E. Stevenson, D. Heeg, N.P. Minton, S.A. Kuehne, Comparison of culture based methods for the isolation of *Clostridium difficile* from stool samples in a research setting, Anaerobe 28 (2014) 226–229.

[161] B. Limbago, A.D. Thompson, S.A. Greene, D. MacCannell, C.E. MacGowan, B. Jolbitado, et al., Development of a consensus method for culture of *Clostridium difficile* from meat and its use in a survey of U.S. retail meats, Food Microbiol. 32 (2012) 448–451.

[162] J.L. Blanco, S. Álvarez-Pérez, M.E. García, Is the prevalence of *Clostridium difficile* in animals underestimated? Vet. J. 197 (2013) 694–698.

[163] S.P. Borriello, P. Honour, Simplified procedure for the routine isolation of

78   *C. Rodriguez et al. / Microbial Pathogenesis 97 (2016) 59–78*

Clostridium difficile from faeces, J. Clin. Pathol. 34 (1981) 1124–1127.

[164] J.S. Weese, B.P. Avery, J. Rousseau, R.J. Reid-Smith, Detection and enumeration of *Clostridium difficile* spores in retail beef and pork, Appl. Environ. Microbiol. 75 (2009) 5009–5011.

[165] J.G. Bartlett, D.N. Gerding, Clinical recognition and diagnosis of *Clostridium difficile* infection, Clin. Infect. Dis. 46 (Suppl 1) (2008). S12–S8.

[166] L.R. Peterson, R.U. Manson, S.M. Paule, D.M. Hacek, A. Robicsek, R.B. Thomson, et al., Detection of toxigenic *Clostridium difficile* in stool samples by real-time polymerase chain reaction for the diagnosis of *C. difficile*-associated diarrhea, Clin. Infect. Dis. 45 (2007) 1152–1160.

[167] J. Avbersek, M. Cotman, M. Ocepek, Detection of *Clostridium difficile* in animals: comparison of real-time PCR assays with the culture method, J. Med Microbiol 60 (2011) 1119–1125.

[168] B.A. Houser, A.L. Hattel, B.M. Jayarao, Real-time multiplex polymerase chain reaction assay for rapid detection of *Clostridium difficile* toxin-encoding strains, Foodborne Pathog. Dis. 7 (2010) 719–726.

[169] R. Mutters, C. Nonnenmacher, C. Susin, U. Albrecht, R. Kropatsch, S. Schumacher, Quantitative detection of *Clostridium difficile* in hospital environmental samples by real-time polymerase chain reaction, J. Hosp Infect. 71 (2009) 43–48.

[170] F. Barbut, M. Braun, B. Burghoffer, V. Lalande, C. Eckert, Rapid detection of toxigenic strains of *Clostridium difficile* in diarrheal stools by real-time PCR, J. Clin. Microbiol. 47 (2009) 1276–1277.

[171] G. Terhes, E. Urbán, J. Sóki, E. Nacsa, E. Nagy, Comparison of a rapid molecular method, the BD GeneOhm Cdiff assay, to the most frequently used laboratory tests for detection of toxin-producing *Clostridium difficile* in diarrheal feces, J. Clin. Microbiol. 47 (2009) 3478–3481.

[172] S.D. Goldenberg, T. Dieringer, G.L. French, Detection of toxigenic *Clostridium difficile* in diarrheal stools by rapid real-time polymerase chain reaction, Diagn Microbiol. Infect. Dis. 67 (2010) 304–307.

[173] M. Sartelli, A. Malangoni, F.M. Abu-Zidan, E.W. Griffiths, S. Di Bella, L. McFarland, et al., WSES guidelines for management of *Clostridium difficile* infection in surgical patients, World J. Emerg. Surg. 10 (2015) 38.

[174] J. Antikainen, T. Pasanen, S. Mero, E. Tarkka, J. Kirveskari, S. Kotila, et al., Detection of virulence genes of *Clostridium difficile* by multiplex PCR, APMIS 117 (2009) 607–613.

[175] C.A. Huber, N.F. Foster, T.V. Riley, D.L. Paterson, Challenges for standardization of *Clostridium difficile* typing methods, J. Clin. Microbiol. 51 (2013) 2810–2814.

[176] G.H. Mazurek, V. Reddy, B.J. Marston, W.H. Haas, J.T. Crawford, DNA fingerprinting by infrequent-restriction-site amplification, J. Clin. Microbiol. 34 (1996) 2386–2390.

[177] M.M. Read, Trends in DNA Fingerprinting Research, first ed., Nova Science Publisher Inc, New York, 2005.

[178] I. Filippis, M.L. McKee, Molecular Typing in Bacterial Infections, Springer Science & Business Media, New York, 2012.

[179] S.L. Stubbs, J.S. Brazier, G.L. O'Neill, B.I. Duerden, PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes, J. Clin. Microbiol. 37 (1999) 461–463.

[180] P. Bidet, F. Barbut, V. Lalalnde, B. Burghoffer, J.C. Petit, Development of a new PCR-ribotyping for *Clostridium difficile* based on ribosomal RNA gene sequencing, FEMS Microbiol. Lett. 175 (1999) 261–266.

[181] D.R. Knight, B. Elliott, B.J. Chang, T.T. Perkins, T.V. Riley, Diversity and evolution in the genome of *Clostridium difficile*, Clin. Microbiol. Rev. 28 (2015) 721–741.

[182] A. Indra, S. Huhulescu, M. Schneeweis, P. Hasenberger, S. Kernbichler, A. Fiedler, et al., Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping, J. Med. Microbiol. 57 (2008) 1377–1382.

[183] S. Janezic, I. Strumbelj, M. Rupnik, Use of modified PCR ribotyping for direct detection of *Clostridium difficile* ribotypes in stool samples, J. Clin. Microbiol. 49 (2011) 3024–3025.

[184] S. Janezic, I. Strumbelj, M. Rupnik, Use of modified PCR ribotyping for direct detection of *Clostridium difficile* ribotypes in stool samples, J. Clin. Microbiol. 49 (2011) 3024–3025.

[185] M. Xiao, F. Kong, P. Jin, Q. Wang, K. Xiao, N. Jeoffreys, et al., Comparison of two capillary gel electrophoresis systems for *Clostridium difficile* ribotyping, using a panel of ribotype 027 isolates and whole-genome sequences as a reference standard, J. Clin. Microbiol. 50 (2012) 2755–2760.

[186] M. Delmée, C. Depitre, G. Corthier, A. Ahoyo, V. Avesani, Use of an enzyme-linked immunoassay for *Clostridium difficile* serogrouping, J. Clin. Microbiol. 31 (1993) 2526–2528.

[187] H. Kato, H. Kato, Y. Ito, T. Akahane, S. Izumida, T. Yokoyama, et al., Typing of *Clostridium difficile* isolates endemic in Japan by sequencing of slpA and its application to direct typing, J. Med. Microbiol. 59 (2010) 556–562.

[188] I. Joost, K. Speck, M. Herrmann, L. von Müller, Characterisation of *Clostridium difficile* isolates by slpA and tcdC gene sequencing, Int. J. Antimicrob. Agents

33 (Suppl 1) (2009) S13–s18.

[189] K.E. Dingle, X. Didelot, M.A. Ansari, D.W. Eyre, A. Vaughan, D. Griffiths, et al., Recombinational switching of the *Clostridium difficile* S-layer and a novel glycosylation gene cluster revealed by large-scale whole-genome sequencing, J. Infect. Dis. 207 (2013) 675–686.

[190] G. Northey, M. Gal, A. Rahmati, J.S. Brazier, Subtyping of *Clostridium difficile* PCR ribotype 001 by REP-PCR and PFGE, J. Med. Microbiol. 54 (2005) 543–547.

[191] K.L. Hiett, B.S. Seal, Use of repetitive element palindromic PCR (rep-PCR) for the epidemiologic discrimination of foodborne pathogens, Methods Mol. Biol. 551 (2009) 49–58.

[192] C. Eckert, J. Van Broeck, P. Spigaglia, B. Burghoffer, M. Delmée, Mastrantonio, et al., Comparison of a commercially available repetitive-element PCR system (DiversiLab) with PCR ribotyping for typing of *Clostridium difficile* strains, J. Clin. Microbiol. 49 (2011) 3352–3354.

[193] L.M. Green, T. Worthington, A.C. Hilton, P.A. Lambert, Genetic characterization of clinical isolates of *Clostridium difficile* using an optimized RAPD protocol and PCR ribotyping reveals strain diversity between two tertiary referral Trusts in the West Midlands, UK, J. Med. Microbiol. 60 (2001) 1287–1291.

[194] F. Barbut, N. Mario, M. Delmée, J. Gozian, Genomic fingerprinting of *Clostridium difficile* isolates by using a random amplified polymorphic DNA (RAPD) assay, FEMS Microbiol. Lett. 114 (1993) 161–166.

[195] N.K. Fry, P.H.M. Savelkoul, P. Visca, Amplified fragment-length polymorphism analysis, Methods Mol. Biol. 551 (2009) 89–104.

[196] C.H.W. Klaassen, H.A. van Haren, A.M. Horrevorts, Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism, J. Clin. Microbiol. 40 (2002) 101–104.

[197] P. Spigaglia, P. Mastrantonio, Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates, J. Clin. Microbiol. 40 (2002) 3470–3475.

[198] S. Janezic, M. Marín, A. Martín, M. Rupnik, A new type of toxin A-negative, toxin B-positive *Clostridium difficile* strain lacking a complete tcdA gene, J. Clin. Microbiol. 53 (2015) 692–695.

[199] D. Griffiths, W. Fawley, M. Kachrimanidou, R. Bowden, D.W. Crook, R. Fung, et al., Multilocus sequence typing of *Clostridium difficile*, J. Clin. Microbiol. 48 (2010) 770–778.

[200] L. Lemée, A. Dhalluin, M. Pestel-Caron, J.F. Lemeland, J.L. Pons, Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types, J. Clin. Microbiol. 42 (2004a) 2609–2617.

[201] A. Schneeberg, H. Neubauer, G. Schmoock, E. Grossmann, C. Seyboldt, Presence of *Clostridium difficile* PCR ribotype clusters related to 033, 078 and 045 in diarrhoeic calves in Germany, J. Med. Microbiol. 62 (2013) 1190–1198.

[202] J. Zaluga, P. Stragier, J. Van Vaerenbergh, M. Maes, P. De Vos, Multilocus variable-number-tandem-repeats analysis (MLVA) distinguishes a clonal complex of *Clavibacter michiganensis subsp. michiganensis* strains isolated from recent outbreaks of bacterial wilt and canker in Belgium, BMC Microbiol. 13 (2013) 126.

[203] P.C. Ng, E.F. Kirkness, Whole genome sequencing, Methods Mol. Biol. 628 (2010) 215–226.

[204] D.W. Eyre, M.L. Cule, D.J. Wilson, D. Griffiths, A. Vaughan, L. O'Connor, et al., Diverse sources of *C. difficile* infection identified on whole-genome sequencing, N. Engl. J. Med. 369 (2013) 1195–1205.

[205] G. Killgore, A. Thompson, S. Johnson, J. Brazier, E. Kuijper, J. Pepin, et al., Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat an, J. Clin. Microbiol. 46 (2007) 431–437.

[206] C. Rodriguez, B. Taminiau, B. Brévers, V. Avesani, J. Van Broeck, A. Leroux, et al., Faecal microbiota characterisation of horses using rDNA barcoded pyrosequencing, and carriage rate of *Clostridium difficile* at hospital admission, BMC Microbiol. 15 (2015) 181.

[207] C. Rodriguez, B. Taminiau, J. Van Broeck, M. Delmée, G. Daube, *Clostridium difficile* infection and intestinal microbiota interactions, Microb. Pathog. 89 (2015) 201–209.

[208] D. Shahinas, M. Silverman, T. Sittler, C. Chiu, P. Kim, E. Allen-Vercoe, et al., Toward an understanding of changes in diversity associated with fecal microbiome transplantation based on 16S rRNA gene deep sequencing, MBio 23 (2012), 3(5).

[209] K. Daly, C.S. Stewart, H.J. Flint, S.P. Shirazi-Beechey, Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes, FEMS Microbiol. Ecol. 38 (2011) 141–151.

[210] H.B. Nielsen, M. Almeida, A.S. Juncker, S. Rasmussen, J. Li, S. Sunagawa, et al., Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes, Nat. Biotech. 32 (2014) 822–828.

1.2 *C. difficile* in animals, foods and their environments

C. difficile is able to colonise the intestinal tract of human beings and different animals, which excrete the bacterium in their feces. To be transmitted from host to host and survive in anaerobic conditions, the bacterium must form a dormant spore. These spores are ubiquitous in the environment as they are extremely resistant to desiccation, heating, alcohol and routine environmental cleaning with detergents, surviving on hard surfaces for months. Transmission of *C. difficile* occurs via faecal-oral route, when there is a direct or indirect contact with contaminated surfaces or other sources (water, animals or foods) and spores are ingested. It is largely accepted that, when intestinal conditions are changed, *C. difficile* can be established and may produce clinical signs of infection. But, with a normal gut microbiota, intestinal colonisation may be transient (in the sense that shedding can result from short-term successful bacterial colonisation or from intestinal passage of dormant spores ingested), without associated pathology. The problem is that apparently healthy animals can carry *C. difficile* spores at slaughter, and therefore, there is a potential risk of meat contamination during processing. Furthermore, close contact with colonised animals, including household pets, are probably involved in the epidemiology of *C. difficile* in the community. A large number of reports have described the presence of *C. difficile* spores in foods, animals and their environments. For this reason, several studies have considered food and animals as potential sources for human community-acquired *C. difficile* infection (CA-CDI).

The second part of the introduction of this dissertation describes the current knowledge regarding *C. difficile* in animals, foods and the environment, starting from the first description up to the present. The available data about the role of animals and foods as reservoirs of CDI has also been reviewed.

C. difficile in foods and animals: A comprehensive review

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Clostridium difficile in Food and Animals: A Comprehensive Review

C. Rodriguez, B. Taminiau, J. Van Broeck, M. Delmée, and G. Daube

Abstract

Zoonoses are infections or diseases that can be transmitted between animals and humans through direct contact, close proximity or the environment. *Clostridium difficile* is ubiquitous in the environment, and the bacterium is able to colonise the intestinal tract of both animals and humans. Since domestic and food animals frequently test positive for toxigenic *C. difficile*, even without showing any signs of disease, it seems plausible that *C. difficile* could be zoonotic. Therefore, animals could play an essential role as carriers of the bacterium. In addition, the presence of the spores in different meats, fish, fruits and vegetables suggests a risk of foodborne transmission. This review summarises the current available data on *C. difficile* in animals and foods, from when the bacterium was first described up to the present.

Keywords

Clostridium difficile • Epidemiology • Animals • Food • Transmission

1 Introduction

Clostridium difficile is a spore-forming anaerobic bacterium recognised as the leading cause of

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antibiotic-associated diarrhoea in hospitalised patients. However, in recent years *C. difficile* infection (CDI) is increasingly common in the community, in younger patients without a previous history of hospitalisation or antibiotic treatment (Gupta and Khanna 2014). Studies worldwide have reported the presence of the bacterium in animals and foods (Songer and Anderson 2006; Hoover and Rodriguez-Palacios 2013; Rodriguez-Palacios et al. 2013) with a prevalence that varies according to the methodology used, the geographical area, the age and the animal species studied. While *C. difficile* is

well known as enteric pathogen in some food producing, wild and companion animal species (Donaldson and Palmer 1999; Songer and Uzal 2005), there are several reports describing the presence of the bacterium in the intestinal contents of apparently healthy animals (Rodriguez et al. 2012; Hawken et al. 2013). Moreover, data recently published suggests that besides the nosocomial transmission, animals are an important source of human CDI, whether through environmental contamination, direct or indirect contact, or food contamination, including carcass and meat contamination at slaughter – or in the case of vegetables and other fruits, by the use of organic fertilizer or contaminated water (Rupnik and Songer 2010; Hoover and Rodriguez-Palacios 2013; Rodriguez-Palacios et al. 2013).

The European Food Safety Authority (EFSA) defines zoonoses as infections or diseases that can be transmitted directly or indirectly between animals and humans (through direct contact or close proximity with infected animals, or through the environment). As noted before (Rodriguez-Palacios et al. 2013), the relevance of the presence of *C. difficile* in some environments, animals and foods is little understood. This review describes the current knowledge regarding *C. difficile* in animals, foods, and the environment, as well as the prevalence among animals with and without signs of disease. The available data about animals and foods as vectors of CDI in humans has also been reviewed.

2 The Evolutionary History of *C. difficile* Detection in Animals and the Natural Environment

C. difficile was first reported in animals in 1960 (McBee 1960). The bacterium was isolated from a sample of a Weddell seal's large intestine contents, obtained during the course of a brief biological survey in the Ross Sea area of Antarctica. In 1974, a doctoral thesis described for the first time the presence of *C. difficile* in hay, soil, sand, and mud from the bank of the

river, and in stools from diverse animals such as donkeys, horses, cows and camels, in Pakistan (Hafiz 1974). In an experimental study conducted in 1979 to reproduce neonatal diarrhoea in young gnotobiotic hares, the authors concluded that *C. difficile* was the causal agent of neonatal diarrhoea and that other strains of *Clostridium* enhanced its pathogenic effect (Dabard et al. 1979). CDI in pigs was first confirmed in 1980 when gnotobiotic pigs were accidentally exposed to *C. difficile* and accordingly suffered dehydration and excreted mucoid faeces containing specks of blood (Nagy and Bilkei 2003). In 1981 *C. difficile* was isolated from a goat (Hunter et al. 1981) and in 1982 the bacterium was obtained from rectal samples of healthy cattle in Nigeria of different breeds aged 6 months and above (Princewell and Agba 1982). Borriello et al. (1983) were the first to report the carriage of *C. difficile* in household pets and their immediate environment, including dogs, cats, ducks, geese, chicken, ring-necked parakeets, rabbits, goats, hedgehogs and guinea pigs. However, most of the recovered isolates were identified as non-cytotoxigenic. In the same year, *C. difficile* was recovered from pigs (Jones and Hunter 1983) and identified as the causative agent of antibiotic-associated colitis in a Kodiak bear (Orchard et al. 1983). Interest in the study of *C. difficile* in animals continued to increase during this period. From 1984 to 1987 three new studies described the bacterium as causal agent of enteric disease and diarrhoea in hares, European and cottontail rabbits (Carman and Evans 1984), horses (Ehrich et al. 1984) and foals (Jones et al. 1987). These findings raised the first concerns that domestic animals might be vectors of *C. difficile* among humans (Weber et al. 1988). From 1978 onwards, several studies focused on the isolation procedures and characterisation of *C. difficile* from healthy and diarrhoeic animals, including not only domestic animals such as foals (Jones 1989), cats, dogs (Weber et al. 1989; Riley et al. 1991; Martirossian et al. 1992) and captive ostriches (Frazier et al. 1993), but also wild animals such as cotton-top tamarinds (Snook et al. 1989). In 1995, *C. difficile* toxins were detected in the

small intestine and cecum of three juveniles and one adult rabbit with clinical signs of anorexia, decreased faecal output, nasal exudate and laboured breathing before death (Perkins et al. 1995). A later study in 1996 also reported the presence of *C. difficile* in animals (dogs, cats, horses, sheep and poultry) and in the environment: in soils, in river, sea and lake waters, and in swimming pool and tap waters (al Saif and Brazier 1996). Waters et al. (1998) described an outbreak of *C. difficile* in suckling piglets, and in 1999, Rieu-Lesme and Fonty isolated the bacterium from the ruminal reservoir of newborn lambs (Rieu-Lesme and Fonty 1999).

Besides clinical reports of CDI in exotic animals, such as Asian elephants (Bojesen et al. 2006) and ocelots (Silva et al. 2013a), *C. difficile* has been also isolated from faecal samples of captive white-tailed deer (*Odocoileus virginianus*) in confinement facilities in Ohio, USA, with a prevalence of 36.7 % (French et al. 2010). Furthermore, different studies have investigated the presence of the bacterium in wild animals, including wild passerine birds (Bandelj et al. 2011) and barn swallows (Bandelj et al. 2014); zoo animals (chimpanzees, dwarf goats, Iberian ibexes and plains zebras) (Álvarez-Pérez et al. 2014); sea otters (Miller et al. 2010); free-living South America coatis (Silva et al. 2014); small and medium-size wild mammals (raccoons, shrews, deer and house mice, rats, voles, opossum and groundhogs) (Jardine et al. 2013); black and Norway rats (Firth et al. 2014; Himsworth et al. 2014); feral pigs (Thakur et al. 2011) and Iberian free-range pigs (Álvarez-Pérez et al. 2013).

In the natural environment, *C. difficile* has recently been described in soils of studfarms and farms with mature horses in Sweden (Båverud et al. 2003), in homestead soils and household-stored water in Zimbabwe (Simango 2006), in tropical soils in Costa Rica (del Mar Gamboa et al. 2005) and in Slovenian rivers (Zidaric et al. 2010). In a study conducted in marine environments in the South of Italy, toxigenic *C. difficile* was also detected in seawater and zooplankton (Pasquale et al. 2011).

3 *Clostridium difficile* in Household Pets: Dogs and Cats

Rodriguez-Palacios et al. (2013) refer to the importance of household pets as common transmission routes for human infections of *C. difficile*: in modern lifestyles dogs and cats are considered family members and have access to all parts of the house, including beds, sofas, kitchens and dining rooms. Children under 16 years old often have close contact with their pets, as dogs often licked their faces and both cats and dogs usually sleep in the child's bed. In a study conducted in Canada, it was reported that very few of these children (2.9–4.4 %) recognised the need for washing their hands after contact with pets (Stull et al. 2013). A further study evaluating *C. difficile* in dogs and in the household environment indicated that 10 % of dogs were colonised by the bacterium and 31 % of households were contaminated with its spores, suggesting that exposure to this pathogen may be common (Weese et al. 2010a). In this environment, children, elderly and immune-compromised people could be more at risk of being colonised and developing CDI. In the same study, molecular characterisation of the isolates revealed that household and dog strains were different, concluding that there are sources of household *C. difficile* contamination other than dogs (Weese et al. 2010a). In any case, all dog isolates were indistinguishable from those circulating in human hospitals in the same geographical area (Rodriguez-Palacios et al. 2013). Therefore, the potential transmission of *C. difficile* between pets and humans is currently unclear.

Conversely, it has been reported that pets owned by an immune-compromised person or dogs living with a human receiving antimicrobial treatment were at greater risk of being colonised, presumably because the owner is at greater risk of developing the disease and in turn becoming a source of infection for the pet (Rodriguez-Palacios et al. 2013; Weese 2011). *C. difficile* has been detected in very high rates in healthy

dogs that visit human hospitals (58 %) (Lefebvre et al. 2006a). The risk seems to be particularly high when they accepted treats during the visit or licked patients (Lefebvre et al. 2009). However, it is not yet clear whether the contamination comes from patients or the hospital environment (Weese and Fulford 2011). Lefebvre et al. (2006b) reported the first human epidemic strain PCR-ribotype 027 in a healthy 4-year-old toy poodle that visited patients in healthcare settings in Ontario on a weekly basis. In 2009, Lefebvre and Weese (2009) reported the acquisition of toxigenic *C. difficile* by a therapy dog on its paws during a visit to an acute care facility. In this visit, the dog had been encouraged to 'shake paws' with patients. With these findings authors demonstrated that transient contamination of pet therapy animals (without colonisation) could be a source of pathogen transmission.

Regarding *C. difficile* as a cause of disease in pets, it seems that infection is more commonly community-associated rather than acquired at veterinary hospitals or after antimicrobial therapy (Weese 2011). However, the prevalence and causes of infections acquired in veterinary practices is largely unknown. A previous study identified administration of antimicrobials prior to admission, or administration of immunosuppressive drugs during hospitalisation, as risk factors for veterinary hospital-associated colonisation (Clooten et al. 2008). Murphy et al. (2010) described an important proportion of veterinary hospitals (58 %) with positive environmental swabs for *C. difficile*. While signs of disease could range from mild self-limiting diarrhoea to chronic or fatal diarrhoea (Berry and Levett 1986), the relevance of the bacterium in small veterinary clinics is still uncertain (Weese 2011; Busch et al. 2014). Different other studies have associated the presence of *C. difficile* in faeces with diarrhoea in dogs and cats (Weese et al. 2001a; 2001b; Weese and Armstrong 2003; Koene et al. 2012; Wetterwik et al. 2013). However, dogs can also be healthy carriers of *C. difficile* strains belonging to human epidemic PCR-ribotypes (Schneeberg et al. 2012; Silva et al. 2013b; Spigaglia et al. 2015), with a high

colonisation in the first period of live (Perrin et al. 1993; Álvarez-Pérez et al. 2015).

Regarding CDI in cats, little information is available. It seems that colonisation rates are relatively low in the general population (0–21 %), but slightly higher among cats in veterinary hospitals (9.4–31 %) (Marks et al. 2011). The same *C. difficile* strains were recovered from cats and floor drains in the same veterinary hospital, suggesting the clinical environment was a possible source of contamination (Madewell et al. 1999).

Pet nutrition has been identified as a possible source of *C. difficile*, via pet treats (as bully sticks for dogs) and other raw or processed foods (Freeman et al. 2013; Rodriguez-Palacios et al. 2013). In a study conducted in France, *C. difficile* was not detected in any feline raw foods (n = 20) purchased from 20 Paris stores (Bouttier et al. 2010). However, a further study conducted in Ontario reported the presence of toxigenic *C. difficile* in turkey-based pet food. In the same study the authors recommended disinfecting food and water bowls daily with a 10 % bleach solution to reduce the potential burden of bacteria. Furthermore, it was proposed owners should not feed pets with raw diets in households with young children or immunosuppressed or elderly individuals (Weese et al. 2005).

4   Clostridium difficile in Horses

C. difficile toxins were associated with equine diarrhoea for the first time in 1984, in a study of horses in Potomac River area. In this study, Ehrich et al. (1984) concluded that toxins appeared not to be primary determinants of diarrhoea but they may have contributed to the disease. Currently, *C. difficile* is considered one of the most important causes of diarrhoea and enterocolitis in foals and horses (Arroyo et al. 2006; Weese et al. 2006; Uzal et al. 2012; Diab et al. 2013b). The prevalence of *C. difficile* in foals and adult horses with gastrointestinal disease varies considerably among studies, ranging between 5 % and 63 % (Diab et al. 2013b).

In newborn foals, *C. difficile* has been associated with spontaneous watery or bloody diarrhoea immediately after birth, depression, dehydration, toxaemia and finally death (Diab et al. 2013a). While in some cases the disease can occur without a history of antibiotic therapy or hospitalisation (Diab et al. 2013b), the major risk factors for the development of CDI in horses are antimicrobial treatment, hospitalisation, pre- or post-surgical feed withdrawal or changes in diet. The antimicrobials that have been most frequently associated with *C. difficile* diarrhoea in horses are erythromycin, clindamycin, rifampicin and gentamicin (Diab et al. 2013b).

Like other species, horses can carry *C. difficile* without showing signs of disease. In healthy foals the reported prevalence can vary between 0 and 29 % depending on different factors such the type of the study, the diagnostic test used and the method of sample collection (Diab et al. 2013b). A colonisation rate of up to 44 % has been reported in non-diarrhoeic foals under antibiotic treatment (Båverud et al. 2003). Mare-foal pairs can harbour *C. difficile* subclinically and potentially serve as reservoirs for cross-colonisation (Magdesian and Leutenegger 2011). In hospitalised horses without clinical signs of *C. difficile* disease, the observed prevalence ranged from 4.8 to 11 % (Medina-Torres et al. 2011; Rodriguez et al. 2014a), possibly under the influence of stresses that alter the intestinal flora (such as change of diet, transportation to the hospital, hospitalisation, and surgical or medical treatments) (Båverud 2004). Some studies have suggested a transient shedding of *C. difficile* in adult horses (Schoster et al. 2012) but also in other animal species including cattle (Rodriguez-Palacios et al. 2011b) and humans (Ozaki et al. 2004).

A recent study has evaluated the effect of probiotics on foals developing diarrhoea within 6 months of birth. The authors concluded that there was no benefit observable of administering a 3-week course of probiotics. Furthermore, a significantly higher incidence of diarrhoea in foals receiving probiotics than in control groups suggested a negative impact of probiotics (Schoster et al. 2015), although in vitro inhibition of *C. difficile* and *C. perfringens* by commercial probiotic strains has also been reported (Schoster et al. 2013).

5 *C. difficile* in Food-Producing Animals

In the twenty-first century the possibility of human exposure to *C. difficile* spores via environments and foods contaminated with feces of colonised animals has aroused considerable interest. Furthermore, besides the concern for zoonotic transmission, *C. difficile* is also a costly disease on companion animals and livestock production. There are no financial loss estimates for the treatment of household pets, but veterinary services and medical treatment for a case of acute diarrhoea without further complications costs between 100 and 200 euros in Europe. In production animals, *C. difficile* losses and treatment costs have also not been estimated, but *C. difficile* can produce mortality in breeding, weight loss, and delayed weight gain in animals (Rodriguez-Palacios et al. 2013; Squire and Riley 2013).

5.1 Food-Producing Animals: Swine

C. difficile has been widely described in both healthy pigs and pigs with diarrhoea (Table 1). In neonatal piglets (<15 days old), *C. difficile* has been proposed as the most common cause of diarrhoea (Songer and Anderson 2006) with a mortality rate of up to 50 % in suckling piglets (Songer 2000). Previous studies reported spore or toxin detection ranging between 23 and 93 % in faeces of diarrhoeic piglets and between 1.4 and 96 % in piglets with normal faeces (Table 1). The presence of *C. difficile* toxins in the colon of neonatal swine has been associated with: profuse non-haemorrhagic yellow pasty-to-watery diarrhoea, colitis, typhocoloitis, severe mesocolonic edema, other microscopic lesions such as erosive or ulcerative colonic lesions, infiltration of neutrophils in the lamina propia, and exudation of fibrin into the lumen, resulting

Table 1 Presence of *C. difficile* in piglets and adult pigs at farms, slaughterhouses and clinics

Area	Country/State	Year[a] From 2000	Age or situation	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Europe	Slovenia	08	1–10 days	D and ND	133/257 (51.8)	T (100)	–	Pirs et al. (2008)
	Spain	09	<10 days	D (77.7 of litters)	247/485 (50.9)	T (99.6)	066 (68.3)	Avbersek et al. (2009)
	Spain	09	1–7 days	D (49.7)	58/254 (22.8)	T (100)	–	Alvarez-Perez et al. (2009)
				ND (50.3)	76/257 (29.6)	T (90.8)		
			1–2 months	D (6)	0/12 (0)	–		
				ND (94)	0/187 (0)			
	Belgium	11	<15 days	ND (100)	18/23 (78.3)	T (100)	078 (66.7) 002 (16.7)	Rodriguez et al. (2012)
			At slaughter (5–6 months)	ND (100)	0/194 (0)	–	–	
		11–12	At slaughter (5–6 months)	ND (100)	1/100 (1)	T (100)	078 (100)	Rodriguez et al. (2013)
	Sweden	12	Neonatal	D and ND	45/67 (67)	T (100)	046 (100)	Norén et al. (2014)
	Germany	12	0–1 days	D (70.5)	19/31 (61)	T (100)	078 (55) 126 (20)	Schneeberg et al. (2013a)
				ND (29.5)	11/13 (85)			
			2–14 days	D (77.8)	78/84 (93)			
				ND (22.2)	23/24 (96)			
			15–77 days	D (71.4)	11/35 (31)			
				ND (28.6)	5/14 (36)			
	Austria	08	At slaughter	ND (100)	2/61 (3.3)	T (100)	–	Indra et al. (2009)
	The Netherlands	09	At slaughter	ND (100)	14/50 (28)	T (100)	015 (35.7)	Hopman et al. (2011b)
		09–10	At slaughter	ND (100)	0/100 (0)	–	–	Koene et al. (2012)
			In clinics	D (100)	9/25 (36)	T (100)	078 (77.8) 023 (11.1) 005 (11.1)	
		09–10	At slaughter	ND (100)	58/677 (8.6)	–	078 (31) 014 (15.5) 013 (12.1)	Keessen et al. (2011b)
	Switzerland	10	At slaughter	ND (100)	0/165 (0)	–	–	Hoffer et al. (2010)

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Country	Region	Year	Category	Method	Positive/Total (%)	Toxin	078	Reference
USA	Texas	04–07	At farrowing	–	175/702 (24.9)	T (97.2)	078 (26.2)	Norman et al. (2011)
			Nursery		14/274 (5.1)			
			Breeding		26/604 (4.3)			
			Growth or finishing (at farm)		37/1370 (2.7)			
		06–07	Suckling	ND (100)	61/122 (50)	T93	–	Norman et al. (2009)
			Nursery		10/119 (8.4)			
			Growth or finishing (at farm)		15/382 (3.9)			
	Midwest	06	Neonatal	D (1000)	241/513 (47)	T [578 isolates]	–	Baker et al. (2010)
	Michigan	08	At farm	–	1/56	T (100)	–	McNamara et al. (2011)
	Ohio	08	16–20 weeks (at farm)	–	1/150 (0.67)	T (100)	–	Rodriguez-Palacios et al. (2014)
	Ohio	10	At farrowing	ND (100)	183/251 (73)	T (83.6)	–	Thakur et al. (2010)
	North Carolina		Sows after farrowing		32/68 (47)	T (90.6)		
	North Carolina	08–10	At farrowing (conventional farms)	ND (100)	120/350 (34.3)	T (97.8)	–	Susick et al. (2012)
			At farrowing (antimicrobial-free farms)		56/241 (23)			
			Nursing (conventional farms)		34/651 (5.2)			
			Nursing (antimicrobial-free farms)		7/491 (1.4)			
			Conventional sows in the farrowing barns		24/70 (34.3)			
			Antimicrobial-free sows in the farrowing barns		2/39 (5.1 %)			
			Finishing at conventional farms		2/579 (0.3)			

Table 1 (continued)

Area	Country/State	Year[a] From 2000	Age or situation	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Canada	Ontario	10	On day 2	–	90/121 (74)	T (100)	078 (94)	Weese et al. (2010c)
			On day 7		66/117 (56)			
			On day 30		45/113 (40)			
			On day 44		23/101 (23)			
			On day 62		2/54 (3.7)			
			Sows prior to farrowing		4/10 (40)	T (100)	078 (12)	
	Ontario	13	1 day	–	28/30 (93)	T (100)	078	Hawken et al. (2013)
			Market age (188 days)		1/26 (3.8)	T (100)	078 (100)	
	Various provinces	11	At slaughter	–	30/436 (6.9)	T (93.3)	078 (67)	Weese et al. (2011)
Australia	Western Australia	09	Neonatal	D (94.1)	103/174 (59.19)	T (100)	273 (100)	Squire et al. (2013)
				ND (5.9)	11/11 (100)			
	Five states	12–13	<7 days	D (12 farms with idiopathic diarrhoea) ND (9 farms without idiopathic diarrhoea)	154/229 (67.2)	T (87)	014 (23)	Knight et al. (2015b)
	Georgia	11	Before slaughter	–	55/345 (15.9)	–	–	Thitaram et al. (2011)
Japan	Kanto-Tokai	12	Finishing at farm (13–27 weeks)	–	2/250 (0.8)	T (50)	–	Asai et al. (2013)

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(–) Data not available or not applicable

in 'volcano lesions' (Lizer 2010). Scrotal edema, dyspnoea, mild abdominal distension, hydrothorax, ascites, anorexia and dehydration are other extra-intestinal symptoms probably caused by systemic sepsis (Squire and Riley 2013). However, an absence of diarrhoea does not discount possible *C. difficile* colonisation (Yaeger et al. 2007). Why some colonised piglets with toxigenic strains of *C. difficile* do not develop any signs of disease remains unclear and may be explained by the variability in colostrum intake and colostrum antibody concentration (Squire and Riley 2013). Similarly, the presence of *C. difficile*-negative piglets has been described in litters where most of the members carried the bacterium. The reason why these piglets were negative despite being constantly exposed to the bacterium is also unknown (Weese et al. 2010c). The prevalence of the bacterium decreases with age, varying from 0 to 23 % at finishing in the farm or at slaughter (Table 1). Furthermore, outbreaks in adult pigs have only been reported in periparturient sows (Kiss and Bilkei 2005). It appears that sows are more likely to be colonised by *C. difficile* before or after farrowing (Thakur et al. 2010; Weese et al. 2010c; Susick et al. 2012), which may be due to environmental stress or the administration of antibiotics (Kiss and Bilkei 2005). While it seems sows would pose an obvious contamination source for piglets during farrowing, one study describes the predominance of different PCR-ribotypes in each group, suggesting that external sources other than sows could be responsible for CDI in piglets (Weese et al. 2010c; Hopman et al. 2011a). Widespread aerial dissemination of *C. difficile* on a pig farm was demonstrated and associated with personnel activity. Furthermore, possible aerial dispersal of the bacterium between farrowing pens was revealed by the detection of spores in the hallway following relocation of piglets (Keessen et al. 2011a). On pig farms, vermin such as house mice, drain flies, lesser houseflies and yellow mealworms were found positive for *C. difficile* and proposed as vectors for bacteria transmission (Burt et al. 2012). Despite the progress made in these studies, the sources of *C. difficile* in pig farms and aspects of

the infection cycle still remain unclear. Several procedures, like surface disinfection and the use of gloves, have been proposed to reduce disease-associated mortality in piggeries (Squire and Riley 2013).

5.2 Food-Producing Animals: Cattle

As in the case of swine, the reported prevalence of *C. difficile* in cattle can vary wildly from one study to another depending on the geographical location studied, with percentages as diverse as 0 % in farms in North America and 60 % in Iran (Doosti and Mokhtari-Farsani 2014; McNamara et al. 2011) (Table 2). Furthermore, the pathogenicity of *C. difficile* in cattle is not fully understood. The bacterium and its toxins have been associated with diarrhoea in calves and dairy cows (Table 2). Using post-mortem analysis of calves infected with *C. difficile*, it has been showed that the bacterium was more frequently encountered in the cecum, where histologic lesions were also more severe (Rodriguez-Palacios et al. 2007b).

A higher prevalence (up to 56 %) has been reported in apparently healthy calves aged less than three months old (Table 2). One experimental study investigated the infection of neonatal calves by oral inoculation (in the colostrum) of toxigenic *C. difficile* spores. Results showed faecal shedding but did not detect toxins or the induction of enteric disease, and suggested that simple exposure to *C. difficile* could not cause disease in calves (Rodriguez-Palacios et al. 2007b). Colostrum can also play a protective role, providing passive immunity in neonatal calves. A natural protective effect of this first milk when ingested by calves immediately after birth is plausible (Rodriguez-Palacios et al. 2007b) and merits further investigation. In the literature, many studies have investigated hyperimmune bovine colostrum (obtained by repeated immunisation of pregnant cows) as an effective treatment for CDI in human patients (Steele et al. 2013). However, with or without signs of enteric disease, a decrease in the prevalence rate of *C. difficile* is observed in adult

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Table 2 Presence of *C. difficile* in calves, dairy cattle and beef cattle at farms and slaughterhouses

Area	Country/State	Year[a] From 2000	Age	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Europe	Slovenia	08	>21 days	D (100)	1/56 (1.8)	T (100)	033 (100)	Pirs et al. (2008)
		09	<12 weeks	D (76.1)	4/42 (9.5)	T (100)	077 (50) 038 (25) 002 (25)	Avbersek et al. (2009)
	Belgium	10	14 days (at arrival)	D (60)	5/50 (10)	T (95)	126 (36.8) 078 (31.6) 045 (10.5) 033 (7.9) 012 (7.9)	Zidaric et al. (2012)
			18 days		8/50 (16)			
			25 days		6/50 (12)			
			32 days		1/50 (2)			
			46 days		1/50 (2)			
			194 days (just before slaughter)		6/50 (0)			
		11	<3 months	ND (100)	4/18 (22.2)	T (100)	078 (75) 015 (25)	Rodriguez et al. (2012)
			At slaughter (11–52 months)	ND (100)	14/202 (6.9)	T (71.4)	002 (7.1) 014 (7.1) 081 (7.1)	
		11–12	At slaughter (15–56 months)	ND (100)	10/101 (9.9)	T (80)	078 (54.5) 029 (18.2)	Rodriguez et al. (2013)
	The Netherlands	09–10	Veal calves	ND (100)	6/100 (6)	T (100)	012 (83.3) 033 (16.6)	Koene et al. (2012)
			Dairy cows	D (100)	0/5 (0)	–	–	
				ND (100)	1/100 (1)	T (100)	012 (100)	
	Austria	08	At slaughter	ND (100)	3/67 (4.5)	T (3)	–	Indra et al. (2009)
	Switzerland	10	At slaughter	ND (100)	1/204 (4.2)	T (100)	078 (100)	Hoffer et al. (2010)
		10	Cows	–	1/63 (1.6)	T (100)	137 (100)	Romano et al. (2012)
			Calves		6/47 (12.7)	T (83.3)	033 (16.7) 003 (16.7) 066 (16.7) 070 (16.7)	

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Germany	10–12	Calves		D (100)	176/999 (17.6)	–	033 (57) 078 (17) 045 (9)	Schneeberg et al. (2013b)
	11–12	Dairy cattle	–		25/29 (86.2)	T (17)	–	Schmid et al. (2013)
		Beef cattle			4/29 (13.8)			
Asia	Iran	13	3–25 days	–		90/150 (60)	T (41)	–	Doosti and Mokhtari-Farsani (2014)
USA	Georgia	11	Dairy cattle	ND (100)	32/1325 (2.4)	–	–	Thitaram et al. (2011)
		Beef cattle			188/2965 (6.3)			
	Ohio	08	Dairy cow	–	2/330 (0.61)	T (100)	027 (50)	Rodriguez-Palacios et al. (2014)
			Beef cow					
		07	On arrival	ND (100)	24/186 (12.9)	T (92.8)	078	Rodriguez-Palacios et al. (2011b)
			Week 1		0/176 (0)			
			Week 4		3/176 (1.7)			
			Week 12		0/168 (0)			
			Week 20		5/168 (3.6)			
			Prior to slaughter		2/167 (1.2)			
			At slaughter (intestinal contents)		2/168 (1/2)			
		11	At harvest (meat processing plants)	ND (100)	17/944 (1.8)	T (0.4)	078 (5.9)	Rodriguez-Palacios et al. (2011a)
	Michigan	08	At farm	–	0/50 (0)	–	–	McNamara et al. (2011)
	South-western	08	1–6 weeks	D (82.7) ND (17.3)	64/253 (25.3) 7/53 (13.2)	T (23) T (30.2)	–	Hammitt et al. (2008)
	Pennsylvania	12	<2 weeks	ND (100)	8/200 (4)	T (7.1)	–	Houser et al. (2012)
			4–6 weeks		18/200 (9)			
			8–10 weeks		6/200 (3)			
			12–18 weeks		10/200 (5)			
			20–22 weeks		18/200 (9)			

(continued)

Table 2 (continued)

Area	Country/ State	Year[a] From 2000	Age	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Canada	Ontario	04	<1 month	D (51.8)	11/144 (7.6)	T (100)	078 (25.8)	Arroyo et al. (2005)
				ND (48.2)	20/134 (14.8)		017 (29)	
				014 (12.9)				
				027 (12.9)				
				033 (9.7)				
	Ontario	08–09	2–10 days (48 h after arrival)	–	56/174 (32)	T (98.7)	078 (67)	Costa et al. (2011)
			1 week		88/172 (51.1)			
			17 weeks		4/183 (2)			
			21 weeks		4/156 (2)			
	Alberta	09	At feedlot on arrival	–	18/539 (3.3)	T (100)	078 (100)	Costa et al. (2012)
			Mid-feeding period		18/335 (5.4)			
Australia	Western Australia	07–08	Adult cattle at slaughterhouse (intestinal contents)	ND (100)	0/158 (0)	T (98.6)	127 (50.2)	Knight et al. (2013)
							033 (19.6)	
							16 (7.7)	
	New South Wales	08–09	Adult cattle at slaughterhouse (faeces)		5/280 (1.8)		126 (5.7)	
	Queensland						3 (1.4)	
	South Australia						103 (1.4)	
	Victoria						002 (1)	
	Western Australia						137 (0.5)	
	Victoria	12	Calves aged < 7 days at slaughterhouse		203/360 (56)		7 (3.3)	
	Queensland		Calves 2–6 months at slaughterhouse		1/26 (3.8)			

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature (−) Data not available or not applicable

animals (Table 2). While the reason for this age effect is still unknown, a probable explanation is that the bacterium is better able to colonise and proliferate in the intestinal tract of younger animals, where the gut microbiota is less developed (Rodriguez-Palacios et al. 2006).

5.3 Food-Producing Animals: Poultry

A wide variety of zoonotic diseases can be transmitted by poultry. However, few studies have focused on the study of *C. difficile* in these animals. The limited data available shows that the situation is similar to other species, with prevalence decreasing with increasing age (ranging from 100 % in faecal samples of 14-day-old birds to 0.29 % in mature farm animals), and with bacterial colonisation observable with or without development of disease (Table 3).

Only one outbreak of *C. difficile* has been described in newly hatched ostriches (Cooper et al. 2013). In this outbreak, more than 90 % of birds died within three days of the onset of diarrhoea. At necropsy, the colon and rectum were dilated and diffusely haemorrhagic. Microscopic examination also revealed necrotizing typhilitis and colitis in all the birds. After this report, 300 additional birds from a subsequent hatching were also affected by an epidemic of necrotic enteritis. Identical symptoms were observed which may suggest that CDI is a common and important problem in captive ostrich chicks (Frazier et al. 1993).

In rural communities in Zimbabwe, chickens were identified as major reservoirs of *C. difficile*. Water probably acted as a source of the bacterium for these chickens, as spores were detected in well water and household-stored water. Sources of water contamination may be faeces of domestic animals or humans, although this was not investigated in the study. In addition, soils were also heavily contaminated with *C. difficile* by chicken faeces. The free movement of chickens between neighbouring homesteads highlights the importance of these colonised animals as vectors for widespread distribution

of *C. difficile* in rural communities (Simango 2006).

5.4 Food-Producing Animals: Sheep and Goats

Other production animals such as lambs, sheep and goats have been also described as carriers of the bacterium, with a prevalence varying between 0.6 and 10.1 % (Table 3). As in other animal species, the rate of *C. difficile* detection seems to decrease with age.

On average, a lower prevalence has been reported in sheep and lambs than in swine. This may be associated with the greater use of antimicrobials in production of pigs than in sheep (Knight and Riley 2013). However, as stated before, the few studies available in the literature studying the effect of antibiotics did not find a direct relation between the use of antimicrobials and *C. difficile* colonisation or infection (Romano et al. 2012; Susick et al. 2012). While the presence of *C. difficile* in apparently healthy sheep and goats in farms and at slaughter could play a role in animal-to-animal, environmental or zoonotic transmission, there are no reports identifying the bacterium as responsible for outbreaks of enteropathogen in these animal species.

6 Clostridium difficile in Foods

Recent studies have described the presence of *C. difficile* spores in a variety of food products of both animal and plant origin. These findings highlight the potential risk of infection associated with consuming foods, particularly if they are not cooked prior to eating (Lund and Peck 2015).

6.1 Prevalence and Food Products Concerned

The contamination by *C. difficile* spores has been detected in different types of food products,

Table 3 Presence of *C. difficile* in other food-producing animals

Animal species (Origin)	Area	Country	Year[a] From 2000	Age	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Poultry	Europe	Slovenia	07–08	14 weeks (flock 1)	–	5/7 (71.4)	T (100)	023 (6.8)	Zidaric et al. (2008)
				1 day (flock 2)		0/8 (0)	–	–	
				15 days (flock 2)		24/24 (100)	T (96.3)		
				18 weeks (flock 2)		9/22 (40.9)	T (90)		
		The Netherlands	09–10	Clinics	D (100)	2/21 (9.5)	T (57.1)	014 (28.6) 010 (28.6)	Koene et al. (2012)
				At slaughter	ND (100)	5/100 (5)			
		Austria	08	Broiler chicken at slaughter	ND (100)	3/59 (5)	T (66.7)	001 (33.3) 446 (33.3)	Indra et al. (2009)
	Africa	Zimbabwe	06	Chicken faeces (home leads rural community)	–	20/115 (17.4)	T (55)	–	Simango (2006)
			08	Broiler chickens at market places	–	29/100 (29)	T (89.7)	–	Simango and Mwakurudza (2008)
	USA	Ohio	08	At farm	–	1/340 (0.29)	T (0)	–	Rodriguez-Palacios et al. (2014)
		Texas	09	Broiler chicken 42 days-old at barns	–	6/300 (2.3)	T (100)	078	Harvey et al. (2011a)
Sheep	Europe	The Netherlands	09–10	Clinics	D (100)	2/11 (18.2)	T (100)	015 (50) 097 (50)	Koene et al. (2012)
		Slovenia	09–11	Adult sheep >1 year at farms	D (12.4)	0/27 (0)	T (100)	056 (16.7) 061 (16.7)	Avberšek et al. (2014)
				Lambs between 1 day and 4 months	D (7.6)	6/78 (7.7)			
	Australia	South Australia	11–12	Sheep at slaughterhouse	ND (100)	1/156 (0.6)	T (93.3)	056 (40)	Knight and Riley (2013)
		New South Wales						101 (40)	
		Victoria		Lambs at slaughterhouse or farm	–	14/215 (6.5)		m	
		Western Australia							
	USA	Michigan	08	At farm	–	0/57 (0)	–	–	McNamara et al. (2011)

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Goats	Europe	Slovenia	09–11	Adult goats >1 year at farms	ND (100)	0/10 (0)	T (90)	045 (40) 010 (10) 014 (10) 020 (10)	Avberšek et al. (2014)
				Goats between 1 day and 4 months		10/99 (10.1)			
		Switzerland	10	At farm	–	3/40 (7.5)	T (100)	001 (66.7) 066 (33.3)	Romano et al. (2012)
	USA	Michigan	08	At farm	–	0/14 (0)	–	–	McNamara et al. (2011)

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(−) Data not available or not applicable

including seafood, vegetables and meats, with a prevalence ranging between 2.9 and 66.7 % (Tables 4 and 5). Considering that *C. difficile* is present in healthy food-producing animals at slaughter, it is not surprising that its spores have also been found in meats (Table 4). The mean prevalence of *C. difficile* spores in these products ranges between 0 and 15 %. While early studies conducted in North America reported a much higher contamination rate than elsewhere (Rupnik and Songer 2010), recent studies show the situation to be similar to other countries (Table 4). Rodriguez-Palacios et al. (2009), noting an increased recovery of the bacterium from ground beef and chops in winter in Canada, suggested a seasonal component in *C. difficile* contamination in meats, and also hypothesised a possible epidemiological connection between the prevalence of *C. difficile* in food animals, some foods and humans (Rodriguez-Palacios et al. 2013).

If the initial contamination of food products with *C. difficile* is low, the preservation method used may play a fundamental role in the spores' survival. One of the key features of *C. difficile* in foods is if the pathogen grows or resides in the dormant state, especially if there are anaerobic conditions and the cool chain is not respected. *C. difficile* has been reported in vacuum-packaged meat in France (Bouttier et al. 2010) and in New Zealand, where the bacterium was isolated from chilled vacuum-packed meats in which 'blown pack' spoilage had been observed (Broda et al. 1996). The impact of *C. difficile* survival in these storage conditions clearly demands further study.

There has also been interest with respect to thermal inactivation of *C. difficile* spores by thermal treatment. Rodriguez-Palacios and Lejeune (2011) reported that cooking food at a minimum of 96 °C for 15 min produced an inhibitory effect on *C. difficile* spores. However, minimally-processed fruits and vegetables are treated below these temperatures and therefore could be potential vectors of human infection (Rodriguez-Palacios et al. 2013). The contamination source of these fruits and vegetables could be the use of organic fertilizer containing

C. difficile spores, or irrigation or washing with contaminated water.

6.2   Routes of Food Contamination

As stated before, *C. difficile* is present in the intestinal contents of apparently healthy food-producing animals, suggesting carcasses and meats could be contaminated during the slaughter process. A few studies have addressed the contamination of carcasses at slaughter. In pigs, *C. difficile* was detected in a total of 3 out of 20 carcasses (15 %) sampled at post-bleed and a further 3 out of 20 (15 %) at post-evisceration in a processing facility in Canada (Hawken et al. 2013). A further study reported a prevalence of 2.2 % and 2.5 % in antimicrobial-free pigs at post-evisceration and post-chill respectively (Susick et al. 2012). Harvey et al. (2011b) detected 3 positive samples from a total of 10 sponge swabs collected from carcass hide, post-excision hides and ears from pigs in a processing plant in Texas. In Belgium, the prevalence reported in carcasses from slaughter pigs was 7 % (7/100) (Rodriguez et al. 2013).

C. difficile has also been described in cattle carcasses. In Belgium, the observed prevalence in cattle carcasses reached up to 7.9 % (8/101) (Rodriguez et al. 2013). In a study conducted in Pennsylvania, Houser et al. (2012) detected the *tpi* housekeeping gene in 4 out of 100 cattle carcass swabs by PCR, but *C. difficile* was not isolated using culture techniques. The same data has been reported in an Australian study of cattle carcasses sampled in the processing area of the slaughter line where none of the samples taken (n = 151) were positive for *C. difficile* (Knight et al. 2013). Rodriguez-Palacios et al. (2011b) reported 0 positive carcasses from a total of 168 samples analysed.. In a further study conducted in the USA, samples were collected from pig hides, pre-evisceration carcasses, post-intervention carcasses and ground beef. The bacterium was detected in hides with a prevalence of 3.2 %. However, none of the carcass or meat samples tested positive, evidencing a low

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Table 4 Presence of *C. difficile* in meats (in processing plants or the retail trade) and other foods (at farms or markets)

Area	Country/State	Year[a]	Sample Type	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b] (%)	Study
Asia	Iran (Isfahan)	2014	Chopped beef	1/35 (2.8)	T (100)	–	Esfandiari et al. (2014a)
			Ground beef	1/46 (2.1)			
			Chopped mutton	2/55 (3.6)			
			Ground mutton	4/64 (6.2)			
		2012	Beef meat samples	3/54 (5.6)	T (100)	–	Esfandiari et al. (2014b)
			Beef hamburger	4/56 (7.1)			
	Iran (Isfahan/Khuzestan)	2012	Buffalo meat	6/67 (9)	T (92.3)	078 (53.8)	Rahimi et al. (2014)
			Goat meat	3/92 (3.3)			
			Beef meat	2/121 (1.7)			
			Cow meat	1/106 (0.9)			
			Sheep meat	1/150 (0.7)			
			Camel meat	0/124 (0)			
USA	Connecticut	2015	Ground beef	0/100 (0)	T (0)	–	Mooyottu et al. (2015)
			Ground pork	2/100 (2)			
			Chicken wings	0/100 (0)			
	Pennsylvania	2011–2012	Beef meat samples	5/72 (6.9)	T (100)	027 (40)/078 (40)	Varshney et al. (2014)
			Pork meat samples	9/78 (11.5)	T (66.7)	078 (44)	
			Turkey meat samples	11/76 (14.5)	T (81.8)	027 (9.1)/078 (18.2)	
			Chicken meat samples	6/77 (7.8)	T (33.3)	–	
		2012	Pork sausages	2/102 (2)	T (100)	078 (100)	Curry et al. (2012)
		2012	Ground veal products	4/50 (8)	T (100)	–	Houser et al. (2012)
	Nine different states	2009–2011	Retail meat products	0/1755 (0)	–	–	Limbago et al. (2012)
	Nebraska	2013	Ground beef	0/956 (0)	–	–	Kalchayanand et al. (2013)
	Texas	2004–2009	Ground pork and turkey	23/243 (9.5)	T (100)	078 and variants (95.7)	Harvey et al. (2011b)
			Pork chorizo and trim				
	Arizona	2007	Ground beef uncooked	13/26 (50)	T (100)	078 (2.2)/027 (4.4)	Songer et al. (2009)
			Summer sausage (cooked)	1/7 (14.3)			
			Ground pork (uncooked)	3/7 (42.9)			

(continued)

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Table 4 (continued)

Area	Country/State	Year[a]	Sample Type	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b] (%)	Study
			Braunschweiger (cooked)	10/16 (62.5)			
			Chorizo (uncooked)	3/10 (30)			
			Pork sausage (uncooked)	3/13 (23.1)			
			Ground turkey (uncooked)	4/9 (44.4)			
		2010	Poultry meats	4/32 (12.5)	T (100)	078 and variants (100)	Harvey et al. (2011a)
Africa	Abidjan	2009–2010	Cooked kidney beef	19/172 (11)	–	–	Kouassi et al. (2014)
			Cooked flesh beef	30/223 (13.4)			
Europe	Belgium	2012	Ground and burger beef	3/133 (2.3)	T (100)	078 (33.3)/014 (66.7)	Rodriguez et al. (2014b)
			Ground and sausage pork	5/107 (4.7)	T (80)	078 (20)/014 (40)	
	The Netherlands	2008–2009	Beef meat	0/145 (0)	–	–	de Boer et al. (2011)
			Pork meat	0/63 (0)	–	–	
			Calf meat	0/19 (0)	–	–	
			Lamb meat	1/16 (6.3)	T (100)	045 (100)	
			Chicken meat	7/257 (2.7)	T (2.7)	001/003/087/071	
	Switzerland	2010	Minced meat products	0/46 (0)	–	–	Hoffer et al. (2010)
	France	2007–2008	Ground beef	2/105	T (100)	012 (100)	Bouttier et al. (2010)
			Pork sausage	0/59			
	Austria	2007–2008	Ground meat	3/100	T (66.7)	053 (33.3)	Jöbstl et al. (2010)
		2008	Beef meat	0/51 (0)	–	–	Indra et al. (2009)
			Pork meat	0/27 (0)			
			Chicken meat	0/6 (0)			
	Sweden	2008	Ground meat	2/82 (2.4)	T (100)	–	Von Abercron et al. (2009)

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Canada	Manitoba	2007	Ground beef	2/24 (8.3)	T (100)	–	Visser et al. (2012)
			Ground pork	1/24 (4.2)			Metcalf et al. (2010a)
			Ground pork				
			Chopped pork				
	Ontario	2008–2009	Chicken thighs	10/11 (9)	T (100)	078 (100)[2]	Weese et al. (2010b)
			Chicken wings	13/72 (18)			
			Chicken legs	3/20 (15)			
	Four provinces	2008	Ground beef	14/115 (12)	T (100)	078 (71.4)/027 (7.1)	Lefebvre and Weese (2009)
			Ground pork	14/115 (12)	T (100)	078 (85.7)/027 (7.1)	
	Various provinces	2006	Ground beef	22/149 (14.8)	T (89.3)	027 (30.8)/077 (23.1)/014 (15.4)	Rodriguez-Palacios et al. (2009)
			Veal chops	6/65 (9.2)			
	Ontario and Quebec	2005	Beef and veal ground meat	12/60 (20)	T (91.7)		Rodriguez-Palacios et al. (2007a)
Central America	Costa Rica	2013	Beef meat	1/67 (1.5)	T (100)	–	Quesada-Gómez et al. (2013)
			Pork meat	2/66 (3)			
			Poultry meat	1/67 (1.5)			

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(–) Data not available or not applicable

Table 5 Presence of *C. difficile* in other foods sampling from farms, wholesalers or markets

Food Type	Area	Country/ State	Year[a]	Sample Type	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes [b] (%)	Study
Seasoned ingredients	Asia	Iran	2015	Defrosted onions	0/14 (0)	–	–	Esfandiari et al. (2014b)
				Textured soy proteins	0/14 (0)			
				17 seasoning	0/17 (0)			
Seafood	USA	Texas	2012	Fresh mussel	3/67 (4.5)	T (100)	078 (66.7)	Norman et al. (2014)
				Frozen salmon/ shrimp				
	Europe	Italy	2010–2011	*Mytilus galloprovincialis*	16/33 (48.5)	T (43.7)	014/020/078/045/ 012/	Pasquale et al. (2012)
				Tapes philippinarum	10/19 (52.6)	T (80)	002/001/003/106	
				Venus verrucosum	0/1 (0)	–	–	
			2009	*Mytilus galloprovincialis*	2/3 (66.7)	T (50)	066 (50)/010 (50)	Pasquale et al. (2011)
				Tapes philippinarum	1/1 (100)	T (0)	010 (100)	
				Callista chione	1/2 (50)	T (100)	005 (100)	
	Canada	Ontario	2010	Frozen scallop and shrimp	5/119 (4.8)	T (80)	078 (80)	Metcalf et al. (2011)
				Fresh perch and salmon				
				Cooked shrimp				
Ready-to-eat/raw vegetables	Europe	France	2013	Heard of lettuce	3/104 (2.9)	T (100)	001 (33.3)	Eckert et al. (2013)
				Lambs lettuce salad			014/020/077 (33.3)	
				Peat sprouts			015 (33.3)	
	Canada	Ontario	2008	Ginger	5/111 (4.5)	T (100)	078 (60)	Metcalf et al. (2010b)
				Carrot				
				Eddoes				
Milk	Europe	Austria	2008	Bactofugates	0/50 (0)	–	–	Jöbstl et al. (2010)

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(–) Data not available or not applicable

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contamination of the production chain (Kalchayanand et al. 2013).

Regarding the environmental shedding of *C. difficile* in processing facilities, little data is available. In seven hamburger processing plants in Iran, *C. difficile* was detected in 3.5 % (2/56) of swabs taken from the environment. The authors suggested that this environmental contamination might be due to biofilm formation which could facilitate the attachment of spores (Esfandiari et al. 2014b). In contrast, in a further study conducted in three sausage-manufacturing plants, sponge swabs collected from equipment and facilities yielded no *C. difficile* isolates (Harvey et al. 2011b), while meat samples tested positive for the bacterium, indicating meat contamination with *C. difficile* from the intestinal contents of food animals.

The hands of food handers, especially of those who produce ready-to-eat food, are well-known vectors of foodborne pathogens, in most cases due to poor hygiene. However the impact of contamination of *C. difficile* by humans who handle foods without washing their hands has not yet been evaluated. In a previous study investigating the *C. difficile* contamination of foods prepared in-house at a Belgian nursing hom, only 1 out of 188 food samples tested positive for *C. difficile*. This positive sample was recovered from a meal composed of carrot salad, mustard sauce and pork sausage. However, as they were analysed together, contamination could have originated from any of the ingredients or as a result of manipulation (Rodriguez et al. 2015).

7 The Threat of Zoonotic and Foodborne Transmission

The literature of the last decade has presented several hypotheses about *C. difficile* transmission (Bauer and Kuijper 2015). Weese et al. (2002) reported a risk of zoonotic transmission of some animal diseases, including *C. difficile*, especially in small veterinary hospitals. Goorhuis et al. (2008) described PCR-ribotype 078 as frequently encountered in human CDI and in pigs

with diarrhoea in The Netherlands. A further study reported that this ribotype was the most prevalent type in pig, cattle and horse species worldwide, and also reported an increase in its prevalence in humans in different countries (Rupnik et al. 2008). Other studies conducted in 2008 (Jhung et al. 2008) and in 2009 (Debast et al. 2009) showed a high degree of similarity between pig and animal *C. difficile* PCR-ribotype 078 toxinotype V strains, suggesting a common origin. Recently, Janezic et al. (2014) showed that the most prevalent *C. difficile* types in humans are also prevalent in different animals from different geographic areas, evidencing the potential for global dissemination of some strains.

In the twenty-first century, the development of different typing methods has allowed genome analysis and the comparison of animal, food and human strains (Griffiths et al. 2010). The first study investigating the phylogeny of *C. difficile* by multilocus sequence typing (MLST) analysis reported that differences between phylogenetic lineages do not correlate with the type of host (human or animal) (Pons 2004). Lemée et al. (2004) studied the genetic relationships and population structures of 72 *C. difficile* isolates from various hosts and geographic sources, including human, dog, horse, cow and rabbit stools. Results obtained in the study showed that animal isolates did not constitute a distinct lineage from human isolates. In subsequent works, the same study group (Lemée et al. 2005; Lemée and Pons 2010) observed that animal isolates were intermixed with human isolates. In the recent years, clade 5 has been largely studied as it contains *C. difficile* PCR-ribotype 078 (Knight et al. 2015a). This type was classically associated with animals, especially pigs (Álvarez-Pérez et al. 2013). However, lately it has been also reported in hospitals (Indra et al. 2015). At present, clade 5 seems to be highly heterogeneous and divergent from the rest of population (Janezic and Rupnik 2015).

Marsh et al. (2010) used multiple-locus variable number tandem repeat analysis (MLVA) to show that toxinotype V (REA group BK) human

and animal isolates were highly related but differentiated. In another study conducted in the Netherlands (Koene et al. 2012), faecal samples from healthy and diarrhoeic animals were compared with human strains isolated from patients with diarrhoea and hospitalised patients. MLVA analysis showed a genotypic correlation between animal and human PCR-ribotype 078, but a distinction between human and animal PCR-ribotypes 012 and 014.

Whole genome sequencing (WGS) has recently been used to study the epidemiology of CDI and the genetics of *C. difficile* (Knight et al. 2015a). One such study investigated the evolutionary relatedness of *C. difficile* PCR-ribotype 078 isolated from humans and pigs (in farms) (Knetsch et al. 2014). Results revealed that farmers and pigs were colonised with identical or nearly identical *C. difficile* clones (with zero or less than two single nucleotide polymorphism differences). These results supported the hypothesis of interspecies transmission between animals and humans; however, the existence of a common contamination source (in the environment) was also possible.

It seems that *C. difficile* occurs as a low-level contaminant in meats and other food products. Therefore foodborne transmission may be responsible for only a small proportion of human CDI cases (Curry et al. 2012). However, other authors have reported no molecular relationship between clinical human and meat isolates and, therefore, that sources other than meat are responsible for CDI (Esfandiari et al. 2014a). At present, the human infectious dose for *C. difficile* is not known (Hoover and Rodriguez-Palacios 2013) and the risk posed by the presence of its spores in meat and other foods is still not clarified. Among healthy people with normal intestinal flora, the ingestion of low quantities of spores may not have major repercussions. However, the consumption of these contaminated foods by vulnerable populations with gastrointestinal perturbations could lead to *C. difficile* colonisation and infection, or can contribute to the asymptomatic *C. difficile* carriage and transmission in the community.

8   Conclusions and Perspectives

Eighty years after its discovery, *C. difficile* continues to be the focus of attention in hospitals and an important topic for many research groups worldwide. Comparisons of strains have revealed that in some regions animals and humans are colonised with identical *C. difficile* clones or these strains cluster in the same lineage. Therefore, it is suggested that *C. difficile* should be considered as a zoonotic pathogen and that animals play an important role as reservoirs of the bacterium.

While many questions remain unanswered, next generation typing techniques must be applied in the future to study the relatedness of strains of human and animal origins. In this context, it will be interesting to assess the presence of *C. difficile* in close related human and animal populations, like pets and their owners or farmers in close contact with their animals. The analysis of the isolates by WGS analysis will definitively confirm the absence of host tropism of certain strains and the zoonotic transmission of the bacterium.

References

Al Saif N, Brazier JS (1996) The distribution of Clostridium difficile in the environment of South Wales. J Med Microbiol 45:133–137

Alvarez-Perez S, Blanco JL, Bouza E, Alba P, Gibert X, Maldonado J, Garcia ME (2009) Prevalence of Clostridium difficile in diarrhoeic and non-fdiarrhoeic piglets. Vet Microbiol 137:302–305

Álvarez-Pérez S, Blanco JL, Peláez T et al (2013) High prevalence of the epidemic *Clostridium difficile* PCR ribotype 078 in Iberian free-range pigs. Res Vet Sci 95:358–361

Álvarez-Pérez S, Blanco JL, Martínez-Nevado E et al (2014) Shedding of *Clostridium difficile* PCR ribotype 078 by zoo animals, and report of an unstable metronidazole-resistant isolate from a zebra foal (Equus quagga burchellii). Vet Microbiol 169:218–222

Álvarez-Pérez S, Blanco JL, Peláez T et al (2015) Faecal shedding of antimicrobial-resistant *Clostridium difficile* strains by dogs. J Small Anim Pract 56:190–195

Arroyo LG, Kruth SA, Willey BM et al (2005) PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. J Med Microbiol 54O:163–166

Arroyo LG, Stämpfli HR, Weese JS (2006) Potential role of *Clostridium difficile* as a cause of duodenitis-proximal jejunitis in horses. J Med Microbiol 55:605–608

Asai T, Usui M, Hiki M et al (2013) *Clostridium difficile* isolated from the fecal contents of swine in Japan. J Vet Med Sci 75:539–541

Avbersek J, Janezic S, Pate M et al (2009) Diversity of *Clostridium difficile* in pigs and other animals in Slovenia. Anaerobe 15:252–255

Avberšek J, Pirš T, Pate M et al (2014) *Clostridium difficile* in goats and sheep in Slovenia: characterisation of strains and evidence of age-related shedding. Anaerobe 28:163–167

Baker AA, Davis E, Rehberger T et al (2010) Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among Swine Herds in the Midwest. Appl Environ Microbiol 76:2961–2967

Bandelj P, Trilar T, Racnik J et al (2011) Zero prevalence of *Clostridium difficile* in wild passerine birds in Europe. FEMS Microbiol Lett 321:183–185

Bandelj P, Trilar T, Blagus R et al (2014) Prevalence and molecular characterization of *Clostridium difficile* isolated from European Barn Swallows (Hirundo rustica) during migration. BMC Vet Res 10:40

Bauer MP, Kuijper EJ (2015) Potential sources of *Clostridium difficile* in human infection. Infect Dis Clin North Am 29:29–35

Båverud V (2004) *Clostridium difficile* diarrhea: infection control in horses. Vet Clin North Am Equine Prac 20:615–630

Båverud V, Gustafsson A, Franklin A et al (2003) *Clostridium difficile*: prevalence in horses and environment, and antimicrobial susceptibility. Equine Vet J 35:465–471

Berry AP, Levett PN (1986) Chronic diarrhoea in dogs associated with *Clostridium difficile* infection. Vet Rec 118:102–103

Bojesen AM, Olsen KEP, Bertelsen MF (2006) Fatal enterocolitis in Asian elephants (Elephas maximus) caused by *Clostridium difficile*. Vet Microbiol 116:329–335

Borriello SP, Honour P, Turner T et al (1983) Household pets as a potential reservoir for *Clostridium difficile* infection. J Clin Pathol 36:84–87

Bouttier S, Barc MC, Felix B et al (2010) *Clostridium difficile* in ground meat, France. Emerg Infec Dis 16:733–735

Broda DM, DeLacy KM, Bell RG et al (1996) Psychrotrophic *Clostridium spp.* associated with "blown pack" spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int J Food Micro 29:335–352

Burt SA, Siemeling L, Kuijper EJ et al (2012) Vermin on pig farms are vectors for *Clostridium difficile* PCR ribotypes 078 and 045. Vet Microbiol 160:256–258

Busch K, Suchodolski JS, Kühner KA et al (2014) *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxin A/B do not play a role in acute haemorrhagic diarrhoea syndrome in dogs. Vet Rec 176:253

Carman RJ, Evans RH (1984) Experimental and spontaneous clostridial enteropathies of laboratory and free living lagomorphs. Lab Anim Sci 34:443–452

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

Cooper KK, Songer JG, Uzal FA (2013) Diagnosing clostridial enteric disease in poultry. J Vet Diagn Invest 25:314–327

Costa MC, Stämpfli HR, Arroyo LG (2011) Epidemiology of *Clostridium difficile* on a veal farm: prevalence, molecular characterization and tetracycline resistance. Vet Microbiol 152:379–384

Costa MC, Reid-Smith R, Gow S et al (2012) Prevalence and molecular characterization of *Clostridium difficile* isolated from feedlot beef cattle upon arrival and mid-feeding period. BMC Vet Res 8:38

Curry SR, Marsh JW, Schlackman JL et al (2012) Prevalence of *Clostridium difficile* in uncooked ground meat products from Pittsburgh, Pennsylvania. Appl Environ Microbiol 78:4183–4186

Dabard J, Dubos F, Martinet L et al (1979) Experimental reproduction of neonatal diarrhea in young gnotobiotic hares simultaneously associated with *Clostridium difficile* and other *Clostridium* strains. Infect Immun 24:7–11

de Boer E, Zwartkruis-Nahuis A, Heuvelink AE et al (2011) Prevalence of *Clostridium difficile* in retailed meat in the Netherlands. Int J Food Microbiol 144:561–564

Debast SB, van Leengoed LAMG, Goorhuis A et al (2009) *Clostridium difficile* PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. Environ Microbiol 11:505–511

del Mar Gamboa M, Rodríguez E, Vargas P (2005) Diversity of mesophilic clostridia in Costa Rican soils. Anaerobe 11:322–326

Diab SS, Rodriguez-Bertos A, Uzal FA (2013a) Pathology and diagnostic criteria of *Clostridium difficile* enteric infection in horses. Vet Pathol 50:1028–1036

Diab SS, Songer G, Uzal FA (2013b) *Clostridium difficile* infection in horses: a review. Vet Microbiol 167:42–49

Donaldson MT, Palmer JE (1999) Prevalence of *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxin A in feces of horses with diarrhea and colic. J Am Vet Med Assoc 215:358–361

Doosti A, Mokhtari-Farsani A (2014) Study of the frequency of *Clostridium difficile tcdA, tcdB, cdtA* and *cdtB* genes in feces of Calves in south west of Iran. Ann Clin Microbiol Antimicrob 13:21

Eckert C, Burghoffer B, Barbut F (2013) Contamination of ready-to-eat raw vegetables with *Clostridium difficile* in France. J Med Microbiol 62:1435–1438

Ehrich M, Perry BD, Troutt HF et al (1984) Acute diarrhea in horses of the Potomac River area: examination for clostridial toxins. J Am Vet Med Assoc 185:433–435

Esfandiari Z, Jalali M, Ezzatpanah H et al (2014a) Prevalence and characterization of *Clostridium difficile* in beef and mutton meats of Isfahan region, Iran. Jundishapur J Microbiol 7, e16771

Esfandiari Z, Weese S, Ezzatpanah H et al (2014b) Occurrence of *Clostridium difficile* in seasoned hamburgers and seven processing plants in Iran. BMC Microbiol 14:283

Firth C, Bhat M, Firth MA, Williams SH et al (2014) Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. MBio 5:e01933-14

Frazier KS, Herron AJ, Hines ME et al (1993) Diagnosis of enteritis and enterotoxemia due to *Clostridium difficile* in captive ostriches (Struthio camelus). J Vet Diagn Invest 5:623–625

Freeman LM, Janecko N, Weese JS (2013) Nutritional and microbial analysis of bully sticks and survey of opinions about pet treats. Can Vet J 54:50–54

French E, Rodriguez-Palacios A, LeJeune JT (2010) Enteric bacterial pathogens with zoonotic potential isolated from farm-raised deer. Foodborne Pathog Dis 7:1031–1037

Goorhuis A, Debast SB, van Leengoed LAMG et al (2008) *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and in pigs? J Clin Microbiol 46:1157, author reply 1158

Griffiths D, Fawley W, Kachrimanidou M et al (2010) Multilocus sequence typing of *Clostridium difficile*. J Clin Microbiol 48:770–778

Gupta A, Khanna S (2014) Community-acquired *Clostridium difficile* infection: an increasing public health threat. Infect Drug Resist 7:63–72

Hafiz S (1974) *Clostridium difficile* and its toxins. (Thesis Ph.D) Department of Microbiology, University of Leeds.

Hammitt MC, Bueschel DM, Keel MK et al (2008) A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet Microbiol 127:343–352

Harvey RB, Norman KN, Andrews K et al (2011a) *Clostridium difficile* in poultry and poultry meat. Foodborne Pathog Dis 8:1321–1323

Harvey RB, Norman KN, Andrews K et al (2011b) *Clostridium difficile* in retail meat and processing plants in Texas. J Vet Diagn Invest 23:807–811

Hawken P, Weese JS, Friendship R (2013) Longitudinal Study of *Clostridium difficile* and Methicillin-Resistant *Staphylococcus* Associated with Pigs from Weaning through to the End of Processing. J Food Prot 76:624–630

Himsworth CG, Patrick DM, Mak S et al (2014) Carriage of *Clostridium difficile* by wild urban Norway rats (Rattus norvegicus) and black rats (Rattus rattus). Appl Environ Microbiol 80:1299–1305

Hoffer E, Haechler H, Frei R et al (2010) Low occurrence of *Clostridium difficile* in fecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. J Food Prot 73:973–975

Hoover DG, Rodriguez-Palacios A (2013) Transmission of *Clostridium difficile* in foods. Infect Dis Clin North Am 27:675–685

Hopman NEM, Keessen EC, Harmanus C et al (2011a) Acquisition of *Clostridium difficile* by piglets. Vet Microbiol 149:186–192

Hopman NEM, Oorburg D, Sanders I et al (2011b) High occurrence of various *Clostridium difficile* PCR ribotypes in pigs arriving at the slaughterhouse. Vet Q 31:179–181

Houser BA, Soehnlen MK, Wolfgang DR et al (2012) Prevalence of *Clostridium difficile* toxin genes in the feces of veal calves and incidence of ground veal contamination. Foodborne Pathog Dis 9:32–36

Hunter D, Bellhouse R, Baker K (1981) *Clostridium difficile* isolated from a goat. Vet Rec 109:291–292

Indra A, Lassnig H, Baliko N et al (2009) *Clostridium difficile*: a new zoonotic agent? Wien Klin Wochensr 121:91–95

Indra A, Schmid D, Huhulescu S et al (2015) *Clostridium difficile* ribotypes in Austria: a multicenter, hospital-based survey. Wien Klin Wochensr 127:587–593

Janezic S, Rupnik M (2015) Genomic diversity of *Clostridium difficile* strains. Res in Microbiol 166:353–360

Janezic S, Zidaric V, Pardon B et al (2014) International *Clostridium difficile* animal strain collection and large diversity of animal associated strains. BMC Microbiol 14:173

Jardine CM, Reid-Smith RJ, Rousseau J et al (2013) Detection of *Clostridium difficile* in small and medium-sized wild Mammals in Southern Ontario, Canada. J Wildl Dis 49:418–421

Jhung MA, Thompson AD, Killgore GE et al (2008) Toxinotype V *Clostridium difficile* in humans and food animals. Emerg Infect Dis 14:1039–1045

Jöbstl M, Heuberger S, Indra A et al (2010) *Clostridium difficile* in raw products of animal origin. Int J Food Microbiol 138:172–175

Jones RL (1989) Diagnostic Procedures for Isolation and Characterization of *Clostridium difficile* Associated with Enterocolitis in Foals. J Vet Diagn Invest 1:84–86

Jones MA, Hunter D (1983) Isolation of *Clostridium difficile* from pigs. Vet Rec 112:253

Jones RL, Adney WS, Shideler RK (1987) Isolation of *Clostridium difficile* and detection of cytotoxin in the feces of diarrheic foals in the absence of antimicrobial treatment. J Clin Microbiol 25:1225–1227

Clostridium difficile in Food and Animals: A Comprehensive Review

Kalchayanand N, Arthur TM, Bosilevac DM et al (2013) Isolation and characterization of *Clostridium difficile* associated with beef cattle and commercially produced ground beef. J Food Prot 76:256–264

Keessen EC, Donswijk CJ, Hol SP et al (2011a) Aerial dissemination of *Clostridium difficile* on a pig farm and its environment. Environ Res 111:1027–1032

Keessen EC, van den Berkt AJ, Haasjes NH et al (2011b) The relation between farm specific factors and prevalence of *Clostridium difficile* in slaughter pigs. Vet Microbiol 154:130–134

Kiss D, Bilkei G (2005) A new periparturient disease in Eastern Europe, *Clostridium difficile* causes postparturient sow losses. Theriogenology 63:17–23

Knetsch CW, Connor TR, Mutreja A et al (2014) Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011. Euro Surveill 19:20954

Knight DR, Riley TV (2013) Prevalence of gastrointestinal *Clostridium difficile* carriage in Australian sheep and lambs. Appl Environ Microbiol 79:5689–5692

Knight DR, Thean S, Putsathit P et al (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

Knight DR, Elliott B, Chang BJ et al (2015a) Diversity and Evolution in the Genome of *Clostridium difficile*. Clin Microbiol Rev 28:721–741

Knight DR, Squire MM, Riley TV (2015b) Nationwide surveillance study of *Clostridium difficile* in Australian neonatal pigs shows high prevalence and heterogeneity of PCR ribotypes. Appl Environ Microbiol 81:119–123

Koene MGJ, Mevius D, Wagenaar JA et al (2012) *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. Clin Microbiol Infect 18:778–784

Kouassi KA, Dadie AT, N'Guessan KF et al (2014) *Clostridium perfringens* and *Clostridium difficile* in cooked beef sold in Côte d'Ivoire and their antimicrobial susceptibility. Anaerobe 28:90–94

Lefebvre SL, Weese JS (2009) Contamination of pet therapy dogs with MRSA and *Clostridium difficile*. J Hosp Infect 72:268–269

Lefebvre SL, Arroyo LG, Weese JS (2006a) Epidemic *Clostridium difficile* strain in hospital visitation dog. Emerg Infect Dis 12:1036–1037

Lefebvre SL, Waltner-Toews D, Peregrine AS et al (2006b) Prevalence of zoonotic agents in dogs visiting hospitalized people in Ontario: implications for infection control. J Hosp Infect 62:458–466

Lefebvre SL, Weese JS (2009) Contamination of pet therapy dogs with MRS and Clostridium difficile. J Hosp Infect 72:268–269

Lemée L, Pons JL (2010) Multilocus sequence typing for *Clostridium difficile*. Methods Mol Biol 646:77–90

Lemée L, Dhalluin A, Pestel-Caron M et al (2004) Multilocus Sequence Typing Analysis of Human and Animal *Clostridium difficile* Isolates of Various Toxigenic Types. J Clin Microbiol 42:2609–2617

Lemée L, Bourgeois I, Ruffin E et al (2005) Multilocus sequence analysis and comparative evolution of virulence-associated genes and housekeeping genes of *Clostridium difficile*. Microbiology 151:3171–3180

Limbago B, Thompson AD, Greene SA et al (2012) Development of a consensus method for culture of *Clostridium difficile* from meat and its use in a survey of U.S. retail meats. Food Microbiol 32:448–451

Lizer J (2010) Development of a conventional pig model for *Clostridium difficile* infection and associated disease in neonatal pigs. Iowa State University, Graduate Theses and Dissertations

Lund BM, Peck MW (2015) A possible route for foodborne transmission of *Clostridium difficile*? Foodborne Pathog Dis 12:177–182

Madewell BR, Bea JK, Kraegel SA et al (1999) *Clostridium difficile*: a survey of fecal carriage in cats in a veterinary medical teaching hospital. J Vet Diagn Invest 11:50–54

Magdesian KG, Leutenegger CM (2011) Real-time PCR and typing of *Clostridium difficile* isolates colonizing mare-foal pairs. Vet J 190:119–123

Marks SL, Rankin SC, Byrne BA et al (2011) Entero-pathogenic Bacteria in Dogs and Cats: Diagnosis, Epidemiology, Treatment, and Control. J Vet Intern Med 25:1195–1208

Marsh JW, O'Leary MM, Shutt KA et al (2010) Multilocus variable-number tandem-repeat analysis and multilocus sequence typing reveal genetic relationships among *Clostridium difficile* isolates genotyped by restriction endonuclease analysis. J Clin Microbiol 48:412–418

Martirossian G, Sokół-Leszczyńska B, Mierzejewski J et al (1992) Occurrence of *Clostridium difficile* in the digestive system of dogs. Med Dosw Mikrobiol 44:49–54

McBee RH (1960) Intestinal flora of some antarctic birds and mammals. J Bacteriol 79:311–312

McNamara SE, Abdujamilova N, Somsel P et al (2011) Carriage of *Clostridium difficile* and other enteric pathogens among a 4-H avocational cohort. Zoonoses Public Health 58:192–199

Medina-Torres CE, Weese JS, Staempfli HR (2011) Prevalence of *Clostridium difficile* in horses. Vet Microbiol 152:212–215

Metcalf D, Reid-Smith RJ, Avery BP, Weese JS (2010a) Prevalence of *Clostridium difficile* in retail pork. Can Vet J 51:873–876

Metcalf D, Costa MC, Dew WMV et al (2010b) *Clostridium difficile* in vegetables, Canada. Lett Appl Microbiol 51:600–602

Metcalf D, Avery BP, Janecko N et al (2011) *Clostridium difficile* in seafood and fish. Anaerobe 17:85–86

Miller MA, Byrne BA, Jang SS et al (2010) Enteric bacterial pathogen detection in southern sea otters (Enhydra lutris nereis) is associated with coastal urbanization and freshwater runoff. Vet Res 41:1

Mooyottu S, Flock G, Kollanoor-Johny A et al (2015) Characterization of a multidrug resistant *C. difficile* meat isolate. Int J Food Microbiol 192:111–116

Murphy CP, Reid-Smith RJ, Boerlin P et al (2010) *Escherichia coli* and selected veterinary and zoonotic pathogens isolated from environmental sites in companion animal veterinary hospitals in southern Ontario. Can Vet J 51:963–972

Nagy J, Bilkei G (2003) Neonatal piglet losses associated with *Escherichia coli* and *Clostridium difficile* infection in a Slovakian outdoor production unit. Vet J 166:98–100

Norén T, Johansson K, Unemo M (2014) *Clostridium difficile* PCR ribotype 046 is common among neonatal pigs and humans in Sweden. Clin Microbiol Infect 20: O2–O6

Norman KN, Harvey RB, Scott HM et al (2009) Varied prevalence of *Clostridium difficile* in an integrated swine operation. Anaerobe 15:256–260

Norman KN, Scott HM, Harvey RB et al (2011) Prevalence and genotypic characteristics of *Clostridium difficile* in a closed and integrated human and swine population. Appl Environ Microbiol 77:5755–5760

Norman KN, Harvey RB, Andrews K et al (2014) Survey of *Clostridium difficile* in retail seafood in College Station, Texas. Food Addit Contam Part A Chem Anal Control Exp Risk Assess 31:1127–1129

Orchard JL, Fekety R, Smith JR (1983) Antibiotic-associated colitis due to *Clostridium difficile* in a Kodiak bear. Am J Vet Res 44:1547–1548

Ozaki E, Kato H, Kita H et al (2004) *Clostridium difficile* colonization in healthy adults: transient colonization and correlation with enterococcal colonization. J Med Microbiol 53:167–172

Pasquale V, Romano VJ, Rupnik M et al (2011) Isolation and characterization of *Clostridium difficile* from shellfish and marine environments. Folia Microbiol 56:431–437

Pasquale V, Romano V, Rupnik M et al (2012) Occurrence of toxigenic *Clostridium difficile* in edible bivalve molluscs. Food Microbiol 31:309–312

Perkins SE, Fox JG, Taylor NS (1995) Detection of *Clostridium difficile* toxins from the small intestine and cecum of rabbits with naturally acquired enterotoxemia. Lab Anim Sci 45:379–384

Perrin J, Buogo C, Gallusser A et al (1993) Intestinal carriage of *Clostridium difficile* in neonate dogs. Zentralbl Veterinarmed B 40:222–226

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

Pons JL (2004) *Clostridium difficile*, nosocomial enteropathogen: phylogeny and virulence. Ann Pharm Fr 62:304–309

Princewell TJT, Agba MI (1982) Examination of bovine faeces for the isolation and identification of *Clostridium* species. J Appl Bacteriol 52:97–102

Quesada-Gómez C, Mulvey MR, Vargas P et al (2013) Isolation of a toxigenic and clinical genotype of *Clostridium difficile* in retail meats in Costa Rica. J Food Prot 76:348–351

Rahimi E, Jalali M, Weese JS (2014) Prevalence of *Clostridium difficile* in raw beef, cow, sheep, goat, camel and buffalo meat in Iran. BMC Public Health 14:119

Rieu-Lesme F, Fonty G (1999) Isolation of *Clostridium difficile* from the ruminal reservoir of newborn lambs. Vet Rec 145:501

Riley TV, Adams JE, O'Neill G et al (1991) Gastrointestinal carriage of *Clostridium difficile* in cats and dogs attending veterinary clinics. Epidemiol Infect 107:659–665

Rodriguez C, Taminiau B, Van Broeck J et al (2012) *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 18:621–625

Rodriguez C, Avesani V, Van Broeck J et al (2013) Presence of *Clostridium difficile* in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium. Int J Food Microbiol 166:256–262

Rodriguez C, Taminiau B, Brévers B et al (2014a) Carriage and acquisition rates of *Clostridium difficile* in hospitalized horses, including molecular characterization, multilocus sequence typing and antimicrobial susceptibility of bacterial isolates. Vet Microbiol 172:309–317

Rodriguez C, Taminiau B, Avesani V et al (2014b) Multilocus sequence typing analysis and antibiotic resistance of *Clostridium difficile* strains isolated from retail meat and humans in Belgium. Food Microbiol 42:166–171

Rodriguez C, Korsak N, Taminiau B et al (2015) *Clostridium difficile* from food and surface samples in a Belgian nursing home: An unlikely source of contamination. Anaerobe 32:87–89

Rodriguez-Palacios A, Lejeune JT (2011) Moist-heat resistance, spore aging, and superdormancy in *Clostridium difficile*. Appl Environ Microbiol 77:3085–3091

Rodriguez-Palacios A, Stämpfli HR, Duffield T et al (2006) *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg Infect Dis 12:1730–1736

Rodriguez-Palacios A, Stämpfli HR, Duffield T et al (2007a) *Clostridium difficile* in retail ground meat, Canada. Emerg Infect Dis 13:485–487

Rodriguez-Palacios A, Stämpfli HR, Stalker M et al (2007b) Natural and experimental infection of neonatal calves with *Clostridium difficile*. Vet Microbiol 124:166–172

Rodriguez-Palacios A, Reid-Smith RJ, Staempfli HR et al (2009) Possible seasonality of *Clostridium difficile* in retail meat, Canada. Emerg Infect Dis 15:802–805

Rodriguez-Palacios A, Koohmaraie M, LeJeune JT (2011a) Prevalence, enumeration, and antimicrobial agent resistance of *Clostridium difficile* in cattle at harvest in the United States. J Food Prot 74:1618–1624

Rodriguez-Palacios A, Pickworth C, Loerch S et al (2011b) Transient fecal shedding and limited animal-to-animal transmission of *Clostridium difficile*

Von Abercron SMM, Karlsson F, Wigh GT et al (2009) Low occurrence of *Clostridium difficile* in retail ground meat in Sweden. J Food Prot 72:1732–1734

Waters EH, Orr JP, Clark EG et al (1998) Typhlocolitis caused by *Clostridium difficile* in suckling piglets. J Vet Diagn Invest 10:104–108

Weber A, Kroth P, Heil G (1988) Domestic animals as excreters of *Clostridium difficile*. Deutsch Med Wochenschr 113:1617–1618

Weber A, Kroth P, Heil G (1989) The occurrence of *Clostridium difficile* in fecal samples of dogs and cats. Zentralbl Veterinarmed B 36:568–576

Weese JS (2011) Bacterial enteritis in dogs and cats: diagnosis, therapy, and zoonotic potential. Vet Clin North Am Small Anim Pract 41:287–309

Weese JS, Armstrong J (2003) Outbreak of *Clostridium difficile*-associated disease in a small animal veterinary teaching hospital. J Vet Intern Med 17:813–816

Weese JS, Fulford BM (2011) Companion animal zoonoses. In: Weese JS, Fulford MB (eds) Companion Animal Zoonoses. Wiley-Blackwell, Oxford, UK, pp 234–295

Weese JS, Staempfli HR, Prescott JF et al (2001a) The roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in diarrhea in dogs. J Vet Intern Med 15:374–378

Weese JS, Weese HE, Bourdeau TL et al (2001b) Suspected *Clostridium difficile*-associated diarrhea in two cats. J Am Vet Med Assoc 218:1436–1439

Weese JS, Peregrine AS, Armstrong J (2002) Occupational health and safety in small animal veterinary practice: Part I--nonparasitic zoonotic diseases. Can Vet J 43:631–636

Weese JS, Rousseau J, Arroyo L (2005) Bacteriological evaluation of commercial canine and feline raw diets. Can Vet J 46:513–516

Weese JS, Toxopeus L, Arroyo L (2006) *Clostridium difficile* associated diarrhoea in horses within the community: predictors, clinical presentation and outcome. Equine Vet J 38:185–188

Weese JS, Finley R, Reid-Smith RR et al (2010a) Evaluation of *Clostridium difficile* in dogs and the household environment. Epidemiol Infect 138:1100–1114

Weese JS, Reid-Smith RJ, Avery BP et al (2010b) Detection and characterization of *Clostridium difficile* in retail chicken. Lett Appl Microbiol 50:362–365

Weese JS, Wakeford T, Reid-Smith R et al (2010c) Longitudinal investigation of *Clostridium difficile* shedding in piglets. Anaerobe 16:501–504

Weese JS, Rousseau J, Deckert A et al (2011) *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus* shedding by slaughter-age pigs. BMC Vet Res 7:41

Wetterwik KJ, Trowald-Wigh G, Fernström LL et al (2013) *Clostridium difficile* in faeces from healthy dogs and dogs with diarrhea. Acta Vet Scand 55:23

Yaeger MJ, Kinyon JM, Songer J (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

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

Zidaric V, Beigot S, Lapajne S et al (2010) The occurrence and high diversity of *Clostridium difficile* genotypes in rivers. Anaerobe 16:371–375

Zidaric V, Pardon B, Dos Vultos T et al (2012) Different antibiotic resistance and sporulation properties within multiclonal *Clostridium difficile* PCR ribotypes 078, 126, and 033 in a single calf farm. Appl Environ Microbiol 78:8515–8522

1.3 Gut microbial communities and *C. difficile* colonisation

As previously reviewed, in both humans and animals *C. difficile* colonisation can occur without development of any sign of disease, or the bacterium can cause disorders that range from mild-to-severe forms of gastrointestinal infections.

The gastrointestinal ecosystem is a fundamental component of health and a new point for the diagnosis of several diseases, including CDI, thanks to the emergence of metagenomic and multi-omic approaches. The gut community has been reported to play a critical role in resistance to colonisation by other pathogenic organisms. But the intestinal microbiota can be influenced by factors such as antibiotic use or comorbidities. Broad-spectrum antibiotic treatment has been associated with a decrease in overall diversity of gut bacterial communities and therefore, the total number and the proportions of metabolites in the intestine can be altered. It has been hypothesised, although not demonstrated, that changes in microbial composition with age will also modify the metabolic capacity of the gut microbiota and the resistance against pathogens colonisation, producing a favourable niche to lead to disease.

The third part of this introduction explores the current available data in humans about the differences in *C. difficile* colonisation among individuals of different ages and the role of the microbiota in CDI for each group of patients.

C. difficile infection and intestinal microbiota interactions

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Review

Clostridium difficile infection and intestinal microbiota interactions

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ABSTRACT

Clostridium difficile remains the leading cause of healthcare-associated diarrhoea and outbreaks continue to occur worldwide. Aside from nosocomial *C. difficile* infection, the bacterium is also increasingly important as a community pathogen. Furthermore, asymptomatic carriage of *C. difficile* in neonates, adults and animals is also well recognised. The investigation of the gut's microbial communities, in both healthy subjects and patients suffering *C. difficile* infection (CDI), provides findings and information relevant for developing new successful approaches for its treatment, such as faecal microbiota transplantation, or for the prophylaxis of the infection by modification of the gut microbiota using functional foods and beverages. The analysis of all available data shows new insights into the role of intestinal microbiota in health and disease.

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Contents

1. *C. difficile*: the scope of the problem

Clostridium difficile is a Gram-positive, anaerobic spore-forming bacterium considered as the leading cause of infectious diarrhoea and antibiotic-associated pseudomembranous colitis in hospitals. *C. difficile* is largely known as a nosocomial infectious agent in industrialised countries [1]. However recent studies have described outbreaks in other, developing regions [2,3], highlighting the

potential worldwide spread of this bacterium [4]. *C. difficile* has been isolated from the gastrointestinal tract of many animals, including wild, companion and food animals [5–9], soil [10], rivers [11] and foods [12–14]. These findings suggest the possibility of zoonotic and foodborne transmission of the bacterium, although this hypothesis remains unproven. Furthermore, several recent studies have shown that isolates of *C. difficile* from human beings, animals and foods were epidemiologically-linked [15], especially for PCR ribotype 078 isolates [16].

Direct or indirect contacts with animals and ingestion of contaminated foods have both been proposed as possible sources of *C. difficile* infection in the community. CDI acquired in the

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community accounts for one-quarter of all diagnosed CDI patients, and usually these subjects do not have the classic risk profile of patients who develop the infection in a healthcare facility [17]. Community-acquired CDI is defined as a case patient who had symptom onset in the community and/or within 48 h of admission to a healthcare setting, if the patient had not been discharged from a healthcare facility in the previous 12 weeks [18]. Changing epidemiology of CDI has been described not only in the community but also in hospitals. The disease is now frequently described among healthy peripartum women, who have been previously considered at very low risk for the infection [18,19]. The incidence of CDI in hospitalised patients has risen dramatically in the last 20 years; from 31 cases per 100,000 patients in 1996 [20] to 30—120 cases per 100,000 patients in 2011, according to one study conducted in the US [21]. Furthermore, it has been suggested that the current true incidence is substantially higher, reaching 390—730 per 100,000 person-years as estimated in the Netherlands [21,22]. Mortality rates also have risen dramatically worldwide, account 4.5 times more in hospitalised patients and 7 times in nursing homes in 2005 in Texas in 2005 than in 1999 [23], with an estimated 14,000 deaths annually in the US [24]. Attributable costs for CDI in children are estimated at €3545 per patient. In adults costs per patient range from €4396 to €14,023 in different countries in Europe, with the major cost being hospitalisation [25].

Standard treatment of CDI involves the administration of antibiotics. Metronidazole is the drug of choice (500 mg orally 3 times per day for 10—14 days) for the initial episode, mild or moderate diarrhoea, due to *C. difficile*. Vancomycin is the drug of choice for an initial episode of severe CDI (125 mg orally 4 times per day for 10—14 days). Vancomycin administered orally or per rectum with or without intravenously administered metronidazole is the treatment of choice for severe and complicate CDI [18]. Recurrence after discontinuation of antibiotic treatment occurs in 20%—30% of patients [26], leading to other interventions, like faecal microbiota transplantation. It has been described that faecal bacteriotherapy is capable of restoring the microbiota and promoting resistance to colonisation. Therefore, a new concern of several studies has been the identification of the microbial communities implicated in the infection.

Despite the interindividual and environmental variability, new sequencing techniques have made it possible to discern whether any alteration of the gut microbiota composition can favour *C. difficile* colonisation, infection and recurrence, as well as the microbes responsible for restoring the gut and those which render an individual less susceptible to the infection. The purpose of this review is to explore the current data about the differences in *C. difficile* colonisation among individuals of different ages and to analyse the microbiota's role in CDI for each group of patients.

2. *C. difficile* infection

C. difficile is an opportunistic pathogen that is able to proliferate, and to produce toxins and disease, when there is an event that causes a disruption of the normal flora. The normal human gut microbiota of adults and children over two years old is considered to be capable of preventing *C. difficile* colonisation. Within this natural barrier, anaerobic species, including *Bacteroides*, seem to be especially important [27].

To become infected, it is necessary to ingest spores. Spores are resistant to a wide range of cleaning products and are especially prevalent in hospitals and other healthcare settings [28]. *C. difficile* spores are transmitted via the faecal-oral pathway. If there is a disruption of the gut microbiota, they are able to germinate into the vegetative state once they have reached the gut [20]. *In vitro* studies have shown that germination depends on the presence of primary

bile acids, including taurocholate [29]. After the attachment to intestinal epithelial cells, *C. difficile* can express several virulence factors that include adherence to mucosa, expression of fimbriae, capsule production and secretion of tissue degradative enzymes. However, the most critical step is the production of toxins [30]. CDI is associated with isolates that produce at least one of the two toxins denoted as TcdA and TcdB. While most pathogenic strains have been classically associated with the production of both toxins, the toxin variant strain TcdA-negative TcdB-positive appears to be isolated with increasing frequency worldwide [31]. The presence of a third toxin, known as binary toxin (CDT) has been frequently associated with increased severity of CDI and increased 30-day mortality [32]. Recently, binary-toxin positive *C. difficile* strains that do not produce either toxins TcdA or TcdB have been reported in different and independent cases of patients with diarrhoea suspected of having CDI. Thus some literature has raised the question of the role of binary toxin as a virulence factor and its implications for laboratory diagnostics [33].

Enteric *C. difficile* colonisation of humans and animals can have a range of outcomes, from bacterial carriage without signs of disease, to mild or moderate diarrhoea typically non-haemorrhagic, to colitis or fulminant life-threatening pseudomembranous colitis in extreme cases [18]. Fever, abdominal pain and leucocytosis commencing 48—72 h post infection are other common clinical signs [34]. *C. difficile* infection (CDI) is essentially a disease of the colon and involvement of the small intestine is rare [15]. A case definition for CDI in humans is the emission of more than three unformed stools in less than 24 h, a stool test positive for toxigenic *C. difficile*, its toxins or its toxin's genes, or the presence of pseudomembranous colitis observed after colonoscopy or histopathology. The same criteria are used to diagnose recurrent cases of infection [18,35]. Recurrent CDI within six months after completion of the initial CDI case has been associated with an increased risk of death in human hospitalised patients [36]. Non-diarrhoeal presentation with acute abdomen pain has been described, which occurs with gastrointestinal ileus where faecal fluid collects in loops of dilated and atonic colon [15,37]. A history of antimicrobial treatment within the previous eight weeks is present for the majority of patients suffering CDI [18,38]. It has been reported that more than 90% of CDI cases occur in conjunction with antimicrobial therapy, making this antibiotic therapy the most important risk factor for CDI in humans [15]. Resistance of *C. difficile* to many classes of antibiotics allows the pathogen to survive antibiotic administration better than other commensal species. The decrease of these commensals in the gut creates conditions favourable for overgrowth and *C. difficile* colonisation [37,39]. The list of antibiotics associated with an increased risk of acquiring CDI includes clindamycin, cephalosporins, penicillins and fluoroquinolones [20]. Other risk factors associated with the disease are hospitalisation and the use of proton pump inhibitors and H2 blockers, which decrease the acidity of the stomach and allow *C. difficile* spores to transit through the stomach into the gut. *In vitro* studies show that proton pump inhibitors also affect the growth of other bacterial communities, including *Lactobacillus* [40]. The anaerobic environment of the gut and the presence of bile salts allow the spores to germinate into the vegetative state and produce the toxins [20,41]. Advanced age is known to be associated with CDI. Age-related changes in the gastrointestinal tract, changes in diet and host immune system reactivity inevitably affect microbial population composition [42]. Therefore, the gastrointestinal tract becomes more susceptible to *C. difficile* colonisation [30]. Other factors that have been associated with disease severity and complications are faecal incontinence, debility and cognitive impairment, and the use of gastrointestinal instrumentation such as endoscopy, oesphago-gastroduodenoscopy, left-sided colonoscopy, total colonoscopy and nasogastric or

percutaneous endoscopic gastrostomy feeding [43].

3. Gut microbiota interactions

The gastrointestinal ecosystem is a fundamental component of health and a new focal point for the diagnosis of several diseases through new DNA sequencing technologies. A rich community of an estimated 100–200 different bacterial species comprises the gut microbiota, although some studies have estimated up to 1000 species per individual [44]. The gut microbes are estimated to contain 100-fold more genetic potential than a human's own genome and, therefore, they can provide functions the host alone cannot supply [26]. This community has been reported to comprise 70% of the total microbiota found in the human body, with a total of 10^{14} bacteria that play a critical role in resistance to colonisation by other pathogenic organisms [45]. One previous study reported two predominant phylotypes in the distal gut of healthy adults, which include *Firmicutes* and *Actinobacteria* [46]. Other less prevalent phyla also described in the lower intestine are *Proteobacteria* and *Fusobacteria* [20]. The description of *Firmicutes* as one of the dominant phylum of gut bacterial populations is in agreement with other molecular analyses of intestinal communities [47]. Among this phylum, the dominant members seem to be associated with the *Clostridia* class [46,47]. Regarding *Actinobacteria*, the study of Eckbourg et al. [47] described relatively few sequences belonging to this phylum as well as fewer populations of *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* in the gut of healthy individuals. The authors reported that the majority of the detected species belonged to *Firmicutes* and *Bacteroidetes* phylum [47–49]. *Bacteroidetes* have been suggested as a providing protection against pathogen-mediated intestinal inflammation and to confer resistance to infectious colitis [50]. In addition, these studies also reported a high percentage of sequences from species that have not been cultured with the currently available methods and novel phylotypes not previously described in the available databases.

Direct microbe interactions, production of bacteriocins and competition for nutrients and niches are some of the mechanisms that the indigenous gut microbiota uses to resist colonisation [20]. The impact of the gut microbiota in the intestinal metabolism is well described, playing an important role in many metabolic functions that convert luminal compounds into secondary metabolites, which can be either beneficial or harmful to the host. These metabolic functions include carbohydrate fermentation, regulation of amino acid metabolism, protein digestion and lipid metabolism [45,51]. The genus *Eubacterium* has been implicated in steroid and bile transformation, creating potentially toxic metabolites [52]. Butyrate-producing bacteria, including *Lachnospiraceae* taxa, play a major role in the host metabolism. Butyrate acts as an energy source of colonic epithelial cells [53] and it is implicated in the hormo-neuro-inmuno-system as well as in tissue development and repair, and in down-regulation of bacteria virulence, both by direct effects on virulence gene expression and by acting on cell proliferation of the host cells [54]. Therefore, depletion of butyrate-producing bacteria and lactate accumulation can result in a high susceptibility to *C. difficile* infection in asymptomatic carriers, or could potentially lead to epithelial dysfunction and higher osmotic load in the intestinal lumen of patients suffering *C. difficile* infection; increasing the severity of the infection [55–57]. *Bacteroides* are thought to be responsible for the majority of polysaccharide digestion that occurs in the large intestine. Changes in this population at species level could produce alterations in microbiota metabolic profiles and in other bacterial communities, such as hydrogen-utilising syntrophs [52].

The intestinal microbiota can be influenced by factors such as antibiotic use or comorbidities. Antibiotic treatment has been associated with a decrease in overall diversity, and more specifically with a decrease in the abundance of the *Firmicutes* phylum and an increase of *Proteobacteria* [45]. Broad-spectrum antibiotic treatment may result in the decreased abundance of *Bacteroides*, which would favour germination and growth of other pathogens, like *C. difficile* [55,58]. Oral administration of vancomycin has been reported to significantly decrease Gram-positive bacteria (*Firmicutes*), bile acid dehydroxylation and peripheral insulin sensitivity in patients with metabolic syndrome. These data further suggest the important role of *Firmicutes* in glucose and bile acid metabolism [59]. In the case of metronidazole, a 1000-fold reduction in cell counts only 12 h post-treatment was observed. *Clostridium butyricum* and *Clostridium innocuum* have also been described as highly susceptible to this drug. *Bifidobacteria*, including *Bifidobacterium adolescentis* and *Bifidobacterium longum*, and *Clostridium perfringens* have been reported to be unaffected after therapy with metronidazole. Ampicillin treatment also reduced *Bacteroides* and *C. perfringens* populations at different dose rates [60]. Cephalosporin and fluoroquinole exposure have been described as decreasing the abundance of *Clostridiales* incertae sedis IX. This bacterial group has been hypothesised as competing with *C. difficile* for similar nutrient sources or ecological niches in the gut and, therefore, their depletion will increase the probability of developing CDI [61] (Table 1).

By altering the gut microbiota, antibiotics affect the total number and the proportions of metabolites in the intestine. Bacterial fermentation is particularly modified, which results in a decrease of short-chain fatty acids and an excess of carbohydrates and amino acids. *C. difficile* would be able to utilise these metabolites for germination and, therefore, would also have a favourable niche to colonise and grow [45,55].

4. Asymptomatic colonisation versus *C. difficile* infection

4.1. Asymptomatic colonisation of C

difficile without any signs of disease has been described in human beings and animals [8,62,63]. Testing of stools from human patients without symptoms of *C. difficile* infection is not clinically useful and therefore not recommended [18]. However, several studies have showed that intestinal *C. difficile* colonisation rates of healthy adults can range from 2.4% to 17.5% [62,64]. Zhang et al. [55] considered that asymptomatic *C. difficile* colonisation was associated with the intestinal microbial communities but also with other extrinsic factors, like living environment as well as the host immune state. Ozaki et al. [62] reported that *C. difficile* colonisation was relatively common among healthy individuals belonging to the same employee group, and therefore a common contamination source in the work environment was possible. In the same study, the authors also examined possible host-related factors that may affect colonisation by *C. difficile* and reported a significant number of enterococci in faeces among *C. difficile*-positive subjects compared with non-colonised individuals. Interestingly, high proportions of enterococci have also been described in the gut of infants [62,65], which are more frequently colonised by *C. difficile* strains than adults [66]. Hopkins and Macfarlane [52] detected high levels of enterococci in colonised individuals, however in this case in patients suffering CDI. According to these studies, there could be an association between enterococci and *C. difficile* colonisation and/or infection [62]. A further study reported that colonised but asymptomatic subjects showed no major differences at phylum or family level compared to those that were culture negative. Therefore, the authors suggest that the commensal flora in such subjects could protect the host by preventing potentially pathogenic *C. difficile* colonisation, multiplication and toxin production [67].

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Table 1
Summary of gut microbiota alterations in relation to *C. difficile* infection or other risk factors classically associated with the development of the disease.

Subjects/Type of study	*C. difficile* colonization/Study parameters	Gut microbiota characteristics or changes	References
Adults (different ages)	Healthy	*Bacteroides* - especially important as natural barrier to CDI	27
		Predominant phyla *Firmicutes* (*Clostridia* class), *Actinobacteria* and *Bacteroidetes*	46–49
		Less prevalent phyla *Proteobacteria, Fusobacteria* and *Verrumicrobia*	20, 47
	Asymptomatic colonization	[a]P *Enterococci*	62
		No major differences at phylum or family level compared to negative individuals	67
		[a]P *Enterococcus* and *Lactobacillus*	55
		[b]P *Bacteroides, Lachnospiraceae* and *Ruminococcaceae*	
	CDI	[a]P *Enterococci*	52
		Similar richness and microbial diversity compared to asymptomatic *C. difficile* carriers but different microbial communities	55
		Decrease in richness and diversity	57, 61
		Fewer taxa and [a]P *Proteobacteria[1]*	57
		[a]P *Proteobacteria* and low proportions of *Bacteroidetes* and *Firmicutes[2]*	55, 106, 105
		[b]P *Bacteroidetes, Bacteroidaceae, Bacteroides Porphyromonas-Prevotella* group and *Clostridiales incertae sedis IX.*	61
		[a]P *Enterococcaceae* and facultative anaerobes	
		[b]P *Ruminococcaceae* and *Lachnospiraceae*	55, 57, 107
		[b]P *Bacteroides, Lachnospiraceae* and *Ruminococcaceae*	55
		[a]P *Enterococcus* and *Lactobacillus*	
		No association between CDI and enterobacteria, bifidobacteria or lactobacilli groups	61
		[a]P *Gammaproteobacteria* and *Bacilli, Lactobacillus, Streptococcus* and *Enterobacter*	39
Infants	Healthy	*Bifidobacterium* antagonize adhesion of *C. difficile*	87
		[a]P *Bifidobacterium longum*	92
		Lactobacillus paracasei and *Lactobacillus plantarum* protective effect against *C. difficile*	93, 94
	Asymptomatic colonization	[a]P *Enterococci*	62, 65
		[a]P *Ruminococcus* gnavus and *Klebsiella pneumoniae*	92
	Asymptomatic colonization and born by caesarean	[b]P *Bifidobacterium* and *Bacteroides*	76
	Asymptomatic colonization in formula-fed infants	[a]P *Escherichia coli, Bacteroides* and *lactobacilli*	76
	Colonised SIDS	Triple colonization by *C. perfringens, C. difficile* and *C. innocuum*	95
	Healthy breast-fed infants	[a]P *Bifidobacterium* and suppression of *Bacteroides* and *Clostridium*	71, 86
		[b]P *Peptostreptococcaceae* family and *Verrucomicrobiaceae* family	80
Elderly	Healthy	[a]P *Bacteroides*, enterobacteria, clostridia and eubacteria,[b]P bifidobacteria	52, 100
	CDI	Decrease in taxa assignment compared with asymptomatic patients.	67
		[a]P *Bacteroidetes* phylum, Lactobacillaceae family and *Clostridium* spp.	
		[b]P *Bacteroides, Prevotella* and *Bifidobacterium*	
		[a]P eubacteria, *Bacteroides, Enterobacteriaceae,* enterococci, lactobacilli and clostridia (*C. difficile, C. ramosum, C. sporosphaeroides*).	52
		[b]P anaerobic facultative species	
Faecal microbiota transplantation in CDI patients (different ages)	After fecal transplantation	Replenishment *Roseburia* and *Bacteroides*	32
		Increase of richness and diversity, eradication of *Proteobacteria* species and restoration of *Firmicutes* and *Bacteroidetes*	57, 26
Healthy donors in faecal microbiota transplantation	Predominantly microbiota	*Bacteroidetes* desired	57
In vitro studies	Lactulose supplementation	Suppression of *C. difficile* and *Bacteroides* spp	102
	Fructo-oligosaccharides supplementation	Modulation of intestinal inflammatory responses to *C. difficile*	103
	Primary bile acids (taurocholate)	Germination of *C. difficile*	29
Drug treatment	Proton pump inhibitors	Affect the growth of *Lactobacillus*	40
	Broad-spectrum antibiotic treatment	Decrease in overall diversity, [a]P *Proteobacteria* [b]P the *Firmicutes* and *Bacteroides*	45, 55, 58
	Vancomycin	[b]P *Firmicutes*	59
	Metrodinazole	[b]P cell counts including *C. butyricum, C. innocuum*	60
	Ampicillim	[b]P *Bacteroides* and *C. perfringens*	60
	Cephalosporin and fluoroquinole	[b]P *Clostridiales* incertae sedis IX	61

CDI = *C. difficile* infection.
SIDS = Sudden infant death syndrome.
1 = patients have been treated with vancomycin.
2 = patients have NOT been treated with vancomycin.
[a] P = high proportions of.
[b] P = low proportions of.

Other previous studies [57,61] have described a decrease in richness and diversity in the microbiota of patients suffering CDI in comparison with healthy individuals. Zhang et al. [55] reported that species richness and microbial diversity of asymptomatic *C. difficile* carriers is similar to that of CDI patients, however the microbial community of the two groups is significantly different. Therefore, asymptomatic or transient colonisation could be explained by the fact that the infecting strain is poorly virulent, or because the degree of compromise of the intestinal flora is insufficient to permit establishment and full expression of virulence [30], which means that colonisation by *C. difficile* does not directly lead to *C. difficile*-associated disease. Changes in the intestinal microbiome may contribute to the development of CDI in asymptomatic patients [55]. It has been suggested that certain patients have a predisposition to acquire CDI after hospital admission due to a more permissive or less resilient intestinal microbiota composition, which enables the invasion of *C. difficile* when they are under antibiotic treatment. In contrast, neonates younger than two years are frequently colonised by the bacterium with absence of any clinical sign of disease, which may be due to the immaturity of toxin A receptors on intestinal epithelium, the protection by maternally derived toxin-neutralising antibodies or to the presence of non-toxigenic strains [15,68].

Intestinal *C. difficile* carriage by healthy infants and adults may play an important role as a reservoir for community-acquired CDI. However, a previous study suggested that cross-contamination of *C. difficile* does not occur frequently among family members at home [64]. In long-term care facilities, asymptomatic carriers had higher percentages of skin and environmental contamination, suggesting a potential source of transmission in these establishments [69,70]. Regarding neonates, the carrier state seems to be well tolerated, and the immunoglobulin G antitoxin response that infants develop during this carriage appears to provide subsequent protection for the disease [71]. However, a concern around *C. difficile* colonisation in infants is that pathogenic strains are circulating in asymptomatic individuals and may form a reservoir contributing to the spread of the bacterium in the community [72].

5. Further analysis of *C. difficile* colonisation in infants

C. difficile was first isolated from stool samples of healthy neonates [73]. Since this report in 1935, the presence of the bacterium in faecal samples of new born infants has been widely reported, with a prevalence that can reach 71% [66,74,75]. The acquisition of the bacterium by neonates and infants seems to be environmental [71] and hospitalisation and prematurity have been associated with higher prevalence counts of *C. difficile* [76]. Contamination of maternal genital tract and vaginal delivery has not been shown to be a risk factor for neonatal acquisition. In addition, rates of colonisation reported are near zero per cent in the first two days of life and they only rise when babies are seven days old or more [66,71,74,75]. One study examining vaginal swabs collected from mothers just before delivery they were all found negative for *C. difficile* by culture. However, their infants had the bacterium in their stools. This finding allowed the authors to conclude that infection is mainly from environmental sources rather than maternal transmission [66]. The findings of positive environmental cultures (including baby baths, oximeters and hospital floors) [74,77–79], and similar strains of *C. difficile* in different babies from the same ward support this hypothesis [74,75]. Furthermore, vaginal delivery has not been shown to predispose to higher rates of *C. difficile* colonisation in babies [71,75,80]. In contrast, birth by caesarean delivery was associated with a 100-fold increased colonisation with *C. difficile* [81]. In this sense, it seems that the mode of delivery has a major role in the composition of the neonatal gut microbiota [82]. Penders et al. [76] describe infants born by caesarean that had lower numbers of *Bifidobacterium* and *Bacteroides* and that were more often colonised with *C. difficile* compared with vaginal delivery infants.

A recent study conducted in a Japanese hospital reported that none of the neonates studied were positive for the bacterium [83]. Authors suggest that there was no environmental contamination or staff hand contamination with *C. difficile* in the neonatal care units of the hospital. Another explanation of these negative results was that the majority of the neonates were breast-fed, which may be associated with lower rates of *C. difficile* colonisation [71]. Type of feeding method has also been reported to influence the overall intestinal bacteria composition of infants. Factors preventing *C. difficile* colonisation in breast-fed infants are probably related to the reduced buffering capacity of breast milk versus formula milk and an increase in the acidity in the intestine contents, which may facilitate sporulation and reduce vegetative forms [71,84]. Proteins of human milk have shown an inhibitory effect in toxin TcdA binding to colonic receptors in animal models [85]. Secretory IgA has shown neutralising activity against toxin A because IgA contains a domain similar to the intestinal receptor for toxin A [71]. On the other hand, formula-fed infants seems to be more often colonised with *Escherichia coli*, *C. difficile*, *Bacteroides* and lactobacilli compared with breast-fed infants [76]. Breast-fed infants have a reduced faecal pH (5.29 in average), which could favour growth of *Bifidobacterium* and suppression of *Bacteroides* and *Clostridium* [71,86]. In this context, a further study investigated the gut microbiota of healthy infants in Canada and also reported the overrepresentation of *C. difficile* (Peptostreptococcaceae family) in formula-fed infants compared with breast-fed infants. The *Verrucomicrobiaceae* family was also more abundant in infants not receiving breast milk. In contrast, no relation could be established between the diet and the presence of the genus *Bifidobacterium* [80]. However, the ability of bifidobacteria strains isolated from healthy infants to antagonise adhesion of *C. difficile* to enterocytes has also been demonstrated, including *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum* and *Bifidobacterium pseudolongum* [87].

Both toxigenic and non-toxigenic *C. difficile* isolates are common in infants during the first two years of life [88,89]. However, some studies have reported a higher percentage of non-toxigenic strains in this population group [90]. Furthermore, even if toxigenic *C. difficile* strains are present, in most cases the colonisation in early stages of life seems to be transient and rarely associated with CDI [75]. The infant gut appears to be resistant to *C. difficile* toxins until 12–24 months of life [71]. While the reason for this asymptomatic colonisation with toxigenic strains remains unknown, potential explanations are the absence of toxin receptors, poorly developed cellular signalling pathways in the immature gut mucosa, or the presence of protective factors in the infantile gut [71,91]. Delmée et al. [68] showed that strains isolated from neonates and premature infants admitted in a neonatal care unit were different from those usually found in adults suffering CDI. In addition, most of the strains isolated from infants were non-toxigenic, and the toxigenic isolates belonged to serogroups never isolated from adults suffering from colitis at that time in the healthcare setting. Furthermore, in only 68 out of 105 stools harbouring toxigenic strains were faecal cytotoxins detected, which suggests that the neonatal isolates were poor toxin producers.

At this early stage of life, the presence of *C. difficile* has been reported to be associated with several bacterial species that are able to discriminate between colonised and non-colonised infants. Rousseau et al. [92] described *Ruminococcus gnavus* and *Klebsiella pneumoniae* as being more frequently presented in colonised

infants, while *Bifidobacterium longum* was associated with faecal microbiota of non-colonised individuals. High counts of lactobacilli in the intestines of infants, including *Lactobacillus paracasei* and *Lactobacillus plantarum*, have been suggested to have a protective effect against colonisation by *C. difficile* [93,94].

A further study compared the gut microbiome of infants suffering sudden infant death syndrome (SIDS) with that of normal babies. Surprisingly, detection of *C. difficile* was significantly associated with SIDS. Furthermore, SIDS babies had dual colonisation with both *C. perfringens* and *C. difficile* and triple colonisation *by C. perfringens*, *C. difficile* and *C. innocuum* more frequently than normal babies. The authors concluded that these associations may play a critical role in the final events of SIDS pathogenesis, including hypoxemia, fever, intrathoracic petechial, bradycardia and other signs of bacterial sepsis [95] (Table 1).

6. *C. difficile* in the elderly

While mortality associated with CDI is estimated at around 17%, it seems that this percentage could be higher among older people [96]. The presence of *C. difficile* PCR-ribotype 027 in patients between 60 and 90 years of age has been related to an increased likelihood of CDI-related death [97,98]. Some of the risk factors for CDI in the elderly are age-related immune senescence, comorbidities, surgical interventions, vitamin D deficiencies, Crohn's disease, irritable bowel disorders and immunosuppressive medications, including chemotherapy [20]. The constant movement of patients from nursing homes to hospitals and vice-versa may facilitate *C. difficile* transmission among elderly patients [99]. Changes in microbial composition with age will alter the metabolic capacity of the gut microbiota and the resistance against *C. difficile* colonisation. Hopkins et al. [100] associated advancing age with decreased bifidobacteria and increased *Bacteroides*, enterobacteria, clostridia and eubacteria species number and diversity [52,100,101]. Rea et al. [67] examined the carriage rate of *C. difficile* and the associated changes in the intestinal microbiota of elderly subjects recruited from the community, including out-patient, short-term respite and long-term hospital patients. A decrease in taxa assigned to patients with CDI compared with asymptomatic carriers was noted. Elderly patients suffering CDI presented a microbiota dominated by members of *Bacteroidetes* phylum and the *Lactobacillaceae* family. These patients also showed a decrease in number of *Bacteroides*, *Prevotella* and *Bifidobacterium*, and an increase of *Clostridium* spp. According to these findings, Hopkins et al. [52] also reported total numbers and diversity of lactobacilli and clostridia increased in CDI patients, including high numbers of *C. difficile*, *Clostridium ramosum* and *Clostridium sporosphaeroides*. *C. sporosphaeroides* is able to utilise pyruvate and lactate and its presence in CDI patients may be related to increased lactobacilli populations and lactate production. Other findings found in the same study were a reduction of anaerobic facultative species, and higher eubacteria, *Bacteroides*, *Enterobacteriaceae* and enterococci counts compared with healthy elderly subjects. The authors suggested that these species could contribute to profound changes in the biochemical capacity of the gut microbiota with age.

Modification of the gut microbiota by functional foods and beverages has been proposed as a good method of prophylaxis and treatment of CDI, especially in elderly people [52]. Several carbohydrates can affect the growth of *C. difficile*. Lactulose supplementation suppressed the growth of *C. difficile* and Bacteroides *spp.* [102] and fructo-oligosaccharides have been shown to modulate large intestinal inflammatory responses to *C. difficile* in antibiotic-compromised mice [103]. The reduced number of bifidobacteria species found in elderly patients suggests that probiotic therapy would be likely to succeed as a treatment for CDI in this population [52]. However, to date there is only moderate evidence for the effectiveness of probiotics in preventing primary CDI, and few data to support their use in secondary prevention of recurrent infection [104] (Table 1).

7. Microbiota of healthy donors and CDI patients before and after faecal microbiota transplantation

It has been suggested that after an episode of CDI the remaining microbiome is deficient in the ability to restore its barriers against *C. difficile* colonisation and, therefore, an ideal treatment would be the manipulation of the microbiome to increase colonisation resistance [105]. Faecal microbiota transplantation is now considered one of the most effective methods of choice to treat recurrent CDI. In this process, donor screening remains once of the most important factors for a successful treatment, as patients will adopt certain elements of the donor microbiota. In the last decade, several metagenomic analyses have been focused on the study of faecal microbiota of patients with CDI before and after faecal transplantation, and of healthy donors.

Comparing donors with individuals suffering CDI, Shahinas et al. [57] reported that at phylum level the microbiota of healthy individuals consisted predominantly of *Bacteroidetes* whereas CDI patients presented an over-abundance of fewer taxa, specifically a great abundance of *Proteobacteria*. It must be noted that CDI patients had been treated with vancomycin, which could have altered the stool microbiota and decreased diversity and richness. However, other studies have also observed a decreased overall diversity [105], high percentages of *Proteobacteria* and a paucity of *Bacteroidetes* and *Firmicutes* in CDI patients not receiving vancomycin therapy [55,105,106]. Vicent et al. [61] also reported that sequences corresponding to the phylum *Bacteroidetes* and to the families *Bacteroidaceae* and *Clostridiales* incertae sedis IX were depleted in CDI patients, while these subjects showed a higher percentage of *Enterococcaceae* compared to controls. In addition, authors observed reduced levels of the *Bacteroides-Porphyromonas-Prevotella* group and increased levels of facultative anaerobes in patients with CDI [61]. CDI stools also exhibited a depletion of *Ruminococcaceae* and *Lachnospiraceae*, which constitute the majority of butyrate-producing bacteria in the intestinal tract [55,57,107]. Zhang et al. [55] noted a relative paucity of *Bacteroides* and butyrate-producing bacteria *Coprococcus* and *Roseburia* (*Lachnospiraceae* family) as well as Faecalibacterium and Ruminococcus (*Ruminococcaceae* family) in CDI patients and *C. difficile* asymptomatic carriers. In the same study, other genera that appeared more abundantly in both CDI patients and in asymptomatic carriers were *Enterococcus* and *Lactobacillus*, but their relationship to bacterial colonisation or disease states is still unknown. However, a further study found no association between CDI and members of the enterobacteria, bifidobacteria or lactobacilli groups [61]. Shankar et al. [39] reported that faecal samples of CDI patients were abundant in *Gammaproteobacteria* and *Bacilli* and, at genus level, they detected high numbers of *Lactobacillus*, *Streptococcus* and *Enterobacter*. They also found that the patients' distal gut communities were completely restored within three days following faecal transplantation, being stable in each patient for at least four months and indistinguishable from that of the donor [39].

Successful faecal transplantation has been associated with a replenishment of *Roseburia* and *Bacteroides*, which are also involved in butyrate production [32]. After faecal transplantation, other successful markers that have been reported are an increase of richness and diversity, an eradication of *Proteobacteria* species and a restoration of *Firmicutes* and *Bacteroidetes* species [57,26] (Table 1).

8. Conclusions

Alterations to the intestinal microbiota of patients with an initial or recurrent episode of CDI are becoming more widely studied for diagnosis, treatment and prevention purposes. However, most of the available data has been obtained under different study conditions, including the influence of different antibiotics, diet, age of the individuals studied, underlying diseases or other medications that complicate the comparison of results among studies. Despite these limitations, it seems that there are specific and important differences in the abundance of key bacterial taxa between *C. difficile*-colonised patients and healthy individuals. Gut microbiota of CDI patients is severely compromised with a significant reduction in diversity and richness, due to antibiotic use in most cases. However, how the altered microbiome facilitates the colonisation of *C. difficile* is still not well understood. Further detailed information about the species composition of the gut microbiota is essential for prophylaxis and treatment of CDI and other pathogens for different age and health status groups within the population. Results of new omics techniques, such as metagenomics, will be critical in the future for further understanding the pathogenesis of *C. difficile* and other infectious agents, and to contribute towards the development of new more successful treatments and prevention measures, or fine-tuning existent measures such as diet, use of antibiotics and probiotics or faecal transplantation.

Acknowledgements

References

[1] M. Warny, J. Pepin, A. Fang, G. Killgore, A. Thompson, J. Brazier, E. Frost, L.C. McDonald, Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe, Lancet 366 (2005) 1079–1084.

[2] C. Simango, S. Uladi, Detection of Clostridium difficile diarrhoea in Harare, Zimbabwe, Trans. R. Soc. Trop. Med. Hyg. 108 (2014) 354–357.

[3] U. Deniz, N. Ulger, B. Aksu, M. Karavuş, G. Söyletir, Investigation of toxin genes of Clostridium difficile strains isolated from hospitalized patients with diarrhoea at Marmara University Hospital, Mikrobiyol. Bülteni 45 (2011) 1–10.

[4] I.T. Balassiano, E.A. Yates, R.M.C.P. Domingues, E.O. Ferreira, Clostridium difficile: a problem of concern in developed countries and still a mystery in Latin America, J. Med. Microbiol. 61 (Pt 2) (2012) 169–179.

[5] J.S. Weese, R. Finley, R.R. Reid-Smith, N. Janecko, J. Rousseau, Evaluation of Clostridium difficile in dogs and the household environment, Epidemiol. Infect. 138 (2010) 1100–1104.

[6] A. Schneeberg, M. Rupnik, H. Neubauer, C. Seyboldt, Prevalence and distribution of Clostridium difficile PCR ribotypes in cats and dogs from animal shelters in Thuringia, Germany, Anaerobe 18 (2012) 484–488.

[7] V. Båverud, A. Gustafsson, A. Franklin, A. Aspán, A. Gunnarsson, Clostridium difficile: prevalence in horses and environment, and antimicrobial susceptibility, Equine Veterinary J. 35 (2003) 465–471.

[8] C. Rodriguez, V. Avesani, J. Van Broeck, B. Taminiau, M. Delmée, G. Daube, Presence of Clostridium difficile in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium, Int. J. Food Microbiol. 166 (2013) 256–262.

[9] C.M. Jardine, R.J. Reid-Smith, J. Rousseau, J.S. Weese, Detection of Clostridium difficile in small and medium-sisized wild Mammals in Southern Ontario, Canada, J. Wildl. Dis. 49 (2013) 418–421.

[10] N. Al Saif, Brazier JS: the distribution of Clostridium difficile in the environment of South Wales, J. Med. Microbiol. 45 (1996) 133–137.

[11] V. Zidaric, S. Beigot, S. Lapajne, M. Rupnik, The occurrence and high diversity of Clostridium difficile genotypes in rivers, Anaerobe 16 (2010) 371–375.

[12] V. Pasquale, V. Romano, M. Rupnik, F. Capuano, D. Bove, F. Aliberti, K. Krovacek, S. Dumontet, Occurrence of toxigenic Clostridium difficile in edible bivalve molluscs, Food Microbiol. 31 (2012) 309–312.

[13] J.G. Songer, H.T. Trinh, G.E. Killgore, A.D. Thompson, L.C. McDonald, B.M. Limbago, Clostridium difficile in retail meat products, USA, 2007, Emerg. Infect. Dis. (15) (2009) 819–821.

[14] C. Eckert, B. Burghoffer, F. Barbut, Contamination of ready-to-eat raw vegetables with Clostridium difficile in France, J. Med. Microbiol. 62 (Pt 9) (2013) 1435–1438.

[15] M.M. Squire, T.V. Riley, One Health: the Human-animal-environment Interfaces in Emerging Infectious Diseases, Volume 365, Springer Berlin Heidelberg, Berlin, Heidelberg, 2013 (Current Topics in Microbiology and Immunology).

[16] A. Goorhuis, S.B. Debast, L.A.M.G. van Leengoed, C. Harmanus, D.W. Notermans, A.A. Bergwerff, E.J. Kuijper, Clostridium difficile PCR ribotype 078: an emerging strain in humans and in pigs? J. Clin. Microbiol. 46 (2008) 1157 author reply 1158.

[17] M.P.M. Hensgens, E.C. Keessen, M.M. Squire, T.V. Riley, M.G.J. Koene, E. de Boer, L.J.A. Lipman, E.J. Kuijper, Clostridium difficile infection in the community: a zoonotic disease? Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 18 (2012) 635–645.

[18] Stuart H. Cohen, Dale N. Gerding, Stuart Johnson, Ciaran P. Kelly, Vivian G. Loo, L. Clifford McDonald, Jacques Pepin, Mark H. Wilcox, Clinical Practice Guidelines for Clostridium difficile Infection in Adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), 2010.

[19] A.A. Venugopal, D.N. Gerding, S. Johnson, Clostridium difficile infection rates and spectrum of disease among peripartum women at one hospital from 2003 to 2007 with molecular typing analysis of recovered Clostridium difficile isolates, Am. J. Infect. Control 39 (2011) 206–211.

[20] C. Ghose, Clostridium difficile infection in the twenty-first century, Emerg. Microbes Infect. 2 (2013) e62.

[21] N. Banaei, V. Anikst, L.F. Schroeder, Burden of Clostridium difficile infection in the United States, N. Engl. J. Med. 372 (2015) 2368–2370.

[22] M.P.M. Hensgens, O.M. Dekkers, A. Demeulemeester, A.G.M. Buiting, P. Bloembergen, B.H.B. van Benthem, S. Le Cessie, E.J. Kuijper, Diarrhoea in general practice: when should a Clostridium difficile infection be considered? Results of a nested case-control study, Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 20 (2014) O1067–O1074.

[23] M. Salazar, L. Baskin, K.W. Garey, H.L. DuPont, Clostridium difficile-related death rates in Texas 1999-2005, J. Infect. 59 (2009) 303–307.

[24] E. Dubberke, Clostridium difficile infection: the scope of the problem, J. Hosp. Med. 7 (Suppl. 3) (2012) S1–S4.

[25] A. Asensio, S. Di Bella, A.L. Vecchio, S. Grau, W.M. Hart, B. Isidoro, R. Scotto, N. Petrosillo, M. Watt, J. Nazir, The impact of Clostridium difficile infection on resource use and costs in hospitals in Spain and Italy: a matched cohort study, Int. J. Infect. Dis. IJID Off. Publ. Int. Soc. Infect. Dis. (2015).

[26] A.M. Seekatz, J. Aas, C.E. Gessert, T.A. Rubin, D.M. Saman, J.S. Bakken, V.B. Young, Recovery of the gut microbiome following fecal microbiota transplantation, mBio 5 (2014) e00893–14.

[27] S.P. Borriello, The influence of the normal flora on Clostridium difficile colonisation of the gut, Ann. Med. 22 (1990) 61–67.

[28] A. Kramer, I. Schwebke, G. Kampf, How long do nosocomial pathogens persist on inanimate surfaces? A systematic review, BMC Infect. Dis. 6 (2006) 130.

[29] D. Heeg, D.A. Burns, S.T. Cartman, N.P. Minton, Spores of Clostridium difficile clinical isolates display a diverse germination response to bile salts, PloS One 7 (2012) e32381.

[30] S.P. Borriello, 12th C. L. Oakley lecture. Pathogenesis of Clostridium difficile infection of the gut, J. Med. Microbiol. 33 (1990) 207–215.

[31] D. Drudy, S. Fanning, L. Kyne, Toxin A-negative, toxin B-positive Clostridium difficile, Int. J. Infect. Dis. IJID Off. Publ. Int. Soc. Infect. Dis. 11 (2007) 5–10.

[32] D.N. Gerding, S. Johnson, M. Rupnik, K. Aktories, Clostridium difficile binary toxin CDT: mechanism, epidemiology, and potential clinical importance, Gut Microbes 5 (2014) 15–27.

[33] C. Eckert, A. Emirian, A. Le Monnier, L. Cathala, H. De Montclos, J. Goret, P. Berger, A. Petit, A. De Chevigny, H. Jean-Pierre, B. Nebbad, S. Camiade, R. Meckenstock, V. Lalande, H. Marchandin, F. Barbut, Prevalence and pathogenicity of binary toxin-positive Clostridium difficile strains that do not produce toxins A and B, New Microbes New Infect. 3 (2015) 12–17.

[34] R.L. Gebhard, D.N. Gerding, M.M. Olson, L.R. Peterson, C.J. McClain, H.J. Ansel, M.J. Shaw, M.L. Schwartz, Clinical and endoscopic findings in patients early in the course of clostridium difficile-associated pseudomembranous colitis, Am. J. Med. 78 (1985) 45–48.

[35] A.M. Jones, E.J. Kuijper, M.H. Wilcox, Clostridium difficile: a European perspective, J. Infect. 66 (2013) 115–128.

[36] M.A. Olsen, Y. Yan, K.A. Reske, M.D. Zilberberg, E.R. Dubberke, Recurrent Clostridium difficile infection is associated with increased mortality, Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 21 (2015) 164–170.

[37] C.P. Kelly, J.T. LaMont, Clostridium difficile infection, Annu. Rev. Med. 49 (1998) 375–390.

[38] D.N. Gerding, M.M. Olson, L.R. Peterson, D.G. Teasley, R.L. Gebhard, M.L. Schwartz, J.T. Lee, Clostridium difficile-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study, Arch. Intern. Med. 146 (1986) 95–100.

[39] V. Shankar, M.J. Hamilton, A. Khoruts, A. Kilburn, T. Unno, O. Paliy, M.J. Sadowsky, Species and genus level resolution analysis of gut microbiota in Clostridium difficile patients following fecal microbiota transplantation, Microbiome 2 (2014) 13.

[40] K.W. Altman, V. Chhaya, N.D. Hammer, S. Pavlova, B.J. Vesper, L. Tao, J.A. Radosevich, Effect of proton pump inhibitor pantoprazole on growth and

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morphology of oral Lactobacillus strains, Laryngoscope 118 (2008) 599—604.

[41] S. Dial, K. Alrasadi, C. Manoukian, A. Huang, D. Menzies, Risk of Clostridium difficile diarrheadiarrhoea among hospital inpatients prescribed proton pump inhibitors: cohort and case-control studies, CMAJ 171 (2004) 33—38.

[42] E.J. Woodmansey, Intestinal bacteria and ageing, J. Appl. Microbiol. 102 (2007) 1178—1186.

[43] L. Kyne, C. Merry, B. O'Connell, A. Kelly, C. Keane, D. O'Neill, Factors associated with prolonged symptoms and severe disease due to Clostridium difficile, Age Ageing 28 (1999) 107—113.

[44] E. Avershina, K. Rudi, Confusion about the species richness of human gut microbiota, Benef. Microbes (2015) 1—4.

[45] C.M. Theriot, V.B. Young, Microbial and metabolic interactions between the gastrointestinal tract and Clostridium difficile infection, Gut Microbes 5 (2014) 86—95.

[46] S.R. Gill, M. Pop, R.T. Deboy, P.B. Eckburg, P.J. Turnbaugh, B.S. Samuel, J.I. Gordon, D.A. Relman, C.M. Fraser-Liggett, K.E. Nelson, Metagenomic analysis of the human distal gut microbiome, Sci. (New York, NY) 312 (2006) 1355—1359.

[47] P.B. Eckburg, E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, D.A. Relman, Diversity of the human intestinal microbial flora, Sci. (New York, NY) 308 (2005) 1635—1638.

[48] H. Hayashi, M. Sakamoto, Y. Benno, Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods, Microbiol. Immunol. 46 (2002) 535—548.

[49] Y. Wang, P.-Y. Qian, Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies, PloS One 4 (2009) e7401.

[50] R.B.R. Ferreira, N. Gill, B.P. Willing, L.C.M. Antunes, S.L. Russell, M.A. Croxen, B.B. Finlay, The intestinal microbiota plays a role in Salmonella-induced colitis independent of pathogen colonization, PloS One 6 (2011) e20338.

[51] G.T. Macfarlane, S. Macfarlane, Bacteria, colonic fermentation, and gastro-intestinal health, J. AOAC Int. 95 (2012) 50—60.

[52] M.J. Hopkins, G.T. Macfarlane, Changes in predominant bacterial populations in human faeces with age and with Clostridium difficile infection, J. Med. Microbiol. 51 (2002) 448—454.

[53] A. Hague, B. Singh, C. Paraskeva, Butyrate acts as a survival factor for colonic epithelial cells: further fuel for the in vivo versus in vitro debate, Gastro-enterology 112 (1997) 1036—1040.

[54] P. Guilloteau, L. Martin, V. Eeckhaut, R. Ducatelle, R. Zabielski, F. Van Immerseel, From the gut to the peripheral tissues: the multiple effects of butyrate, Nutr. Res. Rev. 23 (2010) 366—384.

[55] L. Zhang, D. Dong, C. Jiang, Z. Li, X. Wang, Y. Peng, Insight into alteration of gut microbiota in Clostridium difficile infection and asymptomatic C. difficile colonizisation, Anaerobe 34 (2015) 1—7.

[56] S.H. Duncan, P. Louis, H.J. Flint, Lactate-utilizising bacteria, isolated from human feces, that produce butyrate as a major fermentation product, Appl. Environ. Microbiol. 70 (2004) 5810—5817.

[57] D. Shahinas, M. Silverman, T. Sittler, C. Chiu, P. Kim, E. Allen-Vercoe, S. Weese, A. Wong, D.E. Low, D.R. Pillai, Toward an understanding of changes in diversity associated with fecal microbiome transplantation based on 16S rRNA gene deep sequencing, mBio (2012) 3.

[58] D.A. Antonopoulos, S.M. Huse, H.G. Morrison, T.M. Schmidt, M.L. Sogin, V.B. Young, Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation, Infect. Immun. 77 (2009) 2367—2375.

[59] A. Vrieze, C. Out, S. Fuentes, L. Jonker, I. Reuling, R.S. Kootte, E. van Nood, F. Holleman, M. Knaapen, J.A. Romijn, M.R. Soeters, E.E. Blaak, G.M. Dallinga-Thie, D. Reijnders, M.T. Ackermans, M.J. Serlie, F.K. Knop, J.J. Holst, C. van der Ley, I.P. Kema, E.G. Zoetendal, W.M. de Vos, J.B.L. Hoekstra, E.S. Stroes, A.K. Groen, M. Nieuwdorp, Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity, J. Hepatology 60 (2014) 824—831.

[60] D.F. Newton, S. Macfarlane, G.T. Macfarlane, Effects of antibiotics on bacterial species composition and metabolic activities in chemostats containing defined populations of human gut microorganisms, Antimicrob. Agents Chemother. 57 (2013) 2016—2025.

[61] C. Vincent, D.A. Stephens, V.G. Loo, T.J. Edens, M.A. Behr, K. Dewar, A.R. Manges, Reductions in intestinal Clostridiales precede the development of nosocomial Clostridium difficile infection, Microbiome 1 (2013) 18.

[62] E. Ozaki, H. Kato, H. Kita, T. Karasawa, T. Maegawa, Y. Koino, K. Matsumoto, T. Takada, K. Nomoto, R. Tanaka, S. Nakamura, Clostridium difficile colonizisation in healthy adults: transient colonizisation and correlation with enterococcal colonizisation, J. Med. Microbiol. 53 (Pt 2) (2004) 167—172.

[63] A. Rodriguez-Palacios, C. Pickworth, S. Loerch, J.T. LeJeune, Transient fecal shedding and limited animal-to-animal transmission of Clostridium difficile by naturally infected finishing feedlot cattle, Appl. Environ. Microbiol. 77 (2011) 3391—3397.

[64] H. Kato, H. Kita, T. Karasawa, T. Maegawa, Y. Koino, H. Takakuwa, T. Saikai, K. Kobayashi, T. Yamagishi, S. Nakamura, Colonisation and transmission of Clostridium difficile in healthy individuals examined by PCR ribotyping and pulsed-field gel electrophoresis, J. Med. Microbiol. 50 (2001) 720—727.

[65] T. Mitsuoka, K. Hayakawa, N. Kimura, [The faecal flora of man. II. The composition of bifidobacterium flora of different age groups (author's transl)]. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrank-heiten und Hygiene Erste Abteilung Originale Reihe A, Med. Mikrobiol. Parasitol. 226 (1974) 469—478.

[66] I.J. Al-Jumaili, M. Shibley, A.H. Lishman, C.O. Record, Incidence and origin of Clostridium difficile in neonates, J. Clin. Microbiol. 19 (1984) 77—78.

[67] M.C. Rea, O. O'Sullivan, F. Shanahan, P.W. O'Toole, C. Stanton, R.P. Ross, C. Hill, Clostridium difficile carriage in elderly subjects and associated changes in the intestinal microbiota, J. Clin. Microbiol. 50 (2012) 867—875.

[68] M. Delmée, G. Verellen, V. Avesani, G. Francois, Clostridium difficile in neonates: serogrouping and epidemiology, Eur. J. Pediatr. 147 (1988) 36—40.

[69] M.M. Riggs, A.K. Sethi, T.F. Zabarsky, E.C. Eckstein, R.L.P. Jump, C.J. Donskey, Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic Clostridium difficile strains among long-term care facility residents, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 45 (2007) 992—998.

[70] P.D. Ziakas, I.M. Zacharioudakis, F.N. Zervou, C. Grigoras, E.E. Pliakos, E. Mylonakis, Asymptomatic carriers of toxigenic C. difficile in long-term care facilities: a meta-analysis of prevalence and risk factors, PloS One 10 (2015) e0117195.

[71] S. Jangi, J.T. Lamont, Asymptomatic colonization by Clostridium difficile in infants: implications for disease in later life, J. Pediatr. Gastroenterol. Nutr. 51 (2010) 2—7.

[72] C. Rousseau, I. Poilane, L. De Pontual, A.-C. Maherault, A. Le Monnier, A. Collignon, Clostridium difficile carriage in healthy infants in the community: a potential reservoir for pathogenic strains, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 55 (2012) 1209—1215.

[73] H. O'Toole, Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, Bacillus difficilis, Am. J. Dis. Child. 49 (1935).

[74] H.E. Larson, F.E. Barclay, P. Honour, I.D. Hill, Epidemiology of Clostridium difficile in infants, J. Infect. Dis. 146 (1982) 727—733.

[75] R.P. Bolton, S.K. Tait, P.R. Dear, M.S. Losowsky, Asymptomatic neonatal colonisation by Clostridium difficile, Arch. Dis. Child. 59 (1984) 466—472.

[76] J. Penders, C. Thijs, C. Vink, F.F. Stelma, B. Snijders, I. Kummeling, P.A. van den Brandt, E.E. Stobberingh, Factors influencing the composition of the intestinal microbiota in early infancy, Pediatrics 118 (2006) 511—521.

[77] H.S. Faden, D. Dryja, Importance of asymptomatic shedding of Clostridium difficile in environmental contamination of a neonatal intensive care unit, Am. J. Infect. Control (2015).

[78] G. Martirosian, S. Kuipers, H. Verbrugh, A. van Belkum, F. Meisel-Mikolajczyk, PCR ribotyping and arbitrarily primed PCR for typing strains of Clostridium difficile from a Polish maternity hospital, J. Clin. Microbiol. 33 (1995) 2016—2021.

[79] R.J. Sherertz, F.A. Sarubbi, The prevalence of Clostridium difficile and toxin in a nursery population: a comparison between patients with necrotizising enterocolitis and an asymptomatic group, J. Pediatr. 100 (1982) 435—439.

[80] M.B. Azad, T. Konya, H. Maughan, D.S. Guttman, C.J. Field, R.S. Chari, M.R. Sears, A.B. Becker, J.A. Scott, A.L. Kozyrskyj, Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months, CMAJ 185 (2013) 385—394.

[81] F. Fouhy, R.P. Ross, G.F. Fitzgerald, C. Stanton, P.D. Cotter, Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps, Gut Microbes 3 (2012) 203—220.

[82] G. Biasucci, M. Rubini, S. Riboni, L. Morelli, E. Bessi, C. Retetangos, Mode of delivery affects the bacterial community in the newborn gut, Early Hum. Dev. 86 (Suppl. 1) (2010) 13—15.

[83] M. Furuichi, E. Imajo, Y. Sato, S. Tanno, M. Kawada, S. Sato, Characteristics of Clostridium difficile colonizisation in Japanese children, J. Infect. Chemother. Off. J. Jpn. Soc. Chemother. 20 (2014) 307—311.

[84] P.L. Stark, A. Lee, The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life, J. Med. Microbiol. 15 (1982) 189—203.

[85] R.D. Rolfe, W. Song, Immunoglobulin and non-immunoglobulin components of human milk inhibit Clostridium difficile toxin A-receptor binding, J. Med. Microbiol. 42 (1995) 10—19.

[86] B. Kleessen, H. Bunke, K. Tovar, J. Noack, G. Sawatzki, Influence of two infant formulas and human milk on the development of the faecal flora in newborn infants, Acta Paediatr. (Oslo, Nor. 1992) 84 (1995) 1347—1356.

[87] F.M. Trejo, J. Minnaard, P.F. Perez, G.L. De Antoni, Inhibition of Clostridium difficile growth and adhesion to enterocytes by Bifidobacterium superna-tants, Anaerobe 12 (2006) 186—193.

[88] H. Kato, N. Kato, K. Watanabe, N. Ueno, H. Ushijima, S. Hashira, T. Abe, Application of typing by pulsed-field gel electrophoresis to the study of Clostridium difficile in a neonatal intensive care unit, J. Clin. Microbiol. 32 (1994) 2067—2070.

[89] C. Rousseau, L. Lemée, A. Le Monnier, I. Poilane, J.-L. Pons, A. Collignon, Prevalence and diversity of Clostridium difficile strains in infants, J. Med. Microbiol. 60 (Pt 8) (2011) 1112—1118.

[90] M. Camorlinga-Ponce, M. Gamboa, J.J. Barragan, O. Munoz, F.R. Fekety, J.F. Torres, Epidemiological aspects of Clostridium difficile in a pediatric hospital and its role in diarrheal disease, Eur. J. Clin. Microbiol. 6 (1987) 542—546.

[91] I. Adlerberth, H. Huang, E. Lindberg, N. Åberg, B. Hesselmar, R. Saalman, C.E. Nord, A.E. Wold, A. Weintraub, Toxin-producing Clostridium difficile strains as long-term gut colonizisers in healthy infants, J. Clin. Microbiol. 52 (2014) 173—179.

[92] C. Rousseau, F. Levenez, C. Fouqueray, J. Doré, A. Collignon, P. Lepage, Clostridium difficile colonizisation in early infancy is accompanied by changes in intestinal microbiota composition, J. Clin. Microbiol. 49 (2011) 858—865.

74

[93] P. Naaber, K. Klaus, E. Sepp, B. Björksten, M. Mikelsaar, Colonizisation of infants and hospitalizised patients with Clostridium difficile and lactobacilli, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 25 (Suppl. 2) (1997) S189—S190.

[94] P. Naaber, I. Smidt, J. Stsepetova, T. Brilene, H. Annuk, M. Mikelsaar, Inhibition of Clostridium difficile strains by intestinal Lactobacillus species, J. Med. Microbiol. 53 (Pt 6) (2004) 551—554.

[95] A.R. Highet, A.M. Berry, K.A. Bettelheim, P.N. Goldwater, Gut microbiome in sudden infant death syndrome (SIDS) differs from that in healthy comparison babies and offers an explanation for the risk factor of prone position, Int. J. Med. Microbiol. IJMM 304 (2014) 735—741.

[96] N.L. Crogan, B.C. Evans, Clostridium difficile: an emerging epidemic in nursing homes, Geriatr. Nurs. (New York, NY) 28 (2007) 161—164.

[97] M. Miller, D. Gravel, M. Mulvey, G. Taylor, D. Boyd, A. Simor, M. Gardam, A. McGeer, J. Hutchinson, D. Moore, S. Kelly, Health care-associated Clostridium difficile infection in Canada: patient age and infecting strain type are highly predictive of severe outcome and mortality, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 50 (2010) 194—201.

[98] J.H. Boone, L.R. Archbald-Pannone, K.N. Wickham, R.J. Carman, R.L. Guerrant, C.T. Franck, D.M. Lyerly, Ribotype 027 Clostridium difficile infections with measurable stool toxin have increased lactoferrin and are associated with a higher mortality, Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol. 33 (2014) 1045—1051.

[99] J.M. Mylotte, S. Russell, B. Sackett, M. Vallone, M. Antalek, Surveillance for Clostridium difficile infection in nursing homes, J. Am. Geriatr. Soc. 61 (2013) 122—125.

[100] M.J. Hopkins, R. Sharp, G.T. Macfarlane, Variation in human intestinal microbiota with age, Dig. Liver Dis. Off. J. Ital. Soc. Gastroenterol. Ital. Assoc. Study Liver 34 (Suppl. 2) (2002) S12—S18.

[101] S.L. Gorbach, L. Nahas, P.I. Lerner, L. Weinstein, Studies of intestinal microflora. I. Effects of diet, age, and periodic sampling on numbers of fecal microorganisms in man, Gastroenterology 53 (1967) 845—855.

[102] Y. Ito, H. Moriwaki, Y. Muto, N. Kato, K. Watanabe, K. Ueno, Effect of lactulose on short-chain fatty acids and lactate production and on the growth of faecal flora, with special reference to Clostridium difficile, J. Med. Microbiol. 46 (1997) 80—84.

[103] H.R. Gaskins, R.I. Mackie, T. May, K.A. Garleb, Dietary fructo-oligosaccharide modulates large intestinal inflammatory responses to Clostridium difficile in antibiotic-compromised mice, Microb. Ecol. Health & Dis. (1996) 9.

[104] C.T. Evans, S. Johnson, Prevention of Clostridium difficile infection with probiotics, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 60 (Suppl. 2) (2015) S122—S128.

[105] J.Y. Chang, D.A. Antonopoulos, A. Kalra, A. Tonelli, W.T. Khalife, T.M. Schmidt, V.B. Young, Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-associated diarrhea, J. Infect. Dis. 197 (2008) 435—438.

[106] A.R. Manges, A. Labbe, V.G. Loo, J.K. Atherton, M.A. Behr, L. Masson, P.A. Tellis, R. Brousseau, Comparative metagenomic study of alterations to the intestinal microbiota and risk of nosocomial Clostridum difficile-associated disease, J. Infect. Dis. 202 (2010) 1877—1884.

[107] V.C. Antharam, E.C. Li, A. Ishmael, A. Sharma, V. Mai, K.H. Rand, G.P. Wang, Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea, J. Clin. Microbiol. 51 (2013) 2884—2892.

1.4 Advanced age and *C. difficile* colonisation

Age-related changes in intestinal flora and host defences cause a decrease in species richness and diversity in the microbiota and therefore, it is possible that *C. difficile* can colonise and grow. Along with antibiotic treatment, immunosuppression or prolonged hospitalisation, advanced age has classically been considered to be a risk factor for *C. difficile* colonisation, and it has been related to an increase in mortality rate. The deteriorating health status of nursing home residents and the usual proximity between them promote bacterial colonisation, infection and the spread of spores. Therefore, retirement care facilities for elderly people have been pinpointed as frequent focus of *C. difficile* contamination.

While a reduced variability in the types of isolates from residential care facilities for elderly people was reported, the hypervirulent PCR-ribotype 027 remains the most common identified type in nursing homes regardless of their location. The presence of this PCR-ribotype in elderly patients increased likelihood of *C. difficile* infection related death. Contaminated areas of the environment can contribute towards *C. difficile* dissemination in healthcare settings. Elderly patients are often transferred to hospitals when they require special medical care, which may results in transmission of *C. difficile* strains between healthcare establishments (hospitals and nursing homes) and in the community.

This last part of the introduction reviews the current literature data on the occurrence of *C. difficile* colonisation in nursing home residents, as well as the main factors associated with the infection, the mortality rate and the genetic diversity of the isolates between different geographic areas.

C. difficile infection in elderly nursing home residents

Cristina Rodriguez, Nicolas Korsak, Bernard Taminiau, Véronique Avesani, Johan Van Broeck, Michel Delmée, Georges Daube

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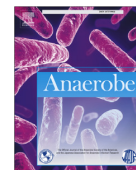
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Clostridium difficile infection in elderly nursing home residents

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ABSTRACT

Age-related changes in intestinal flora and host defences, the receipt of antibiotic treatment, and the presence of underlying diseases are some of the most common risk factors associated with *Clostridium difficile* infection. Therefore, retirement care facilities for elderly people have been pinpointed as frequent sources of contamination. There is only limited data regarding the presence and epidemiology of *C. difficile* in nursing homes, and this gap in the current literature emphasises the need to gain a better understanding of the situation in order to prevent the emergence of new outbreaks among this population group.

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1. Introduction

Clostridium difficile is a well-known anaerobic Gram-positive spore forming bacterium responsible for significant antibiotic-associated diarrhoea and pseudomembranous enterocolitis. Although reporting is not mandatory, the incidence of *C. difficile* infection (CDI) in hospitals has been established at both regional and national levels, ranging from 0 to 19.1 per 10, 000 patient days with an annual European economic burden estimated around €3000 million [1]. *C. difficile* related diarrhoea is frequently diagnosed among elderly residents in nursing homes and other long-term care facilities for older people [2—4]. Along with antibiotic treatment [4,5], advanced age has classically been considered to be a risk factor for *C. difficile* colonisation, and related to an increase in mortality rate [6—8].

Recent studies describe colonisation by toxigenic *C. difficile* strains as ten-times higher in nursing home residents than in the general population living outside long-term care facilities [9,10]. The deteriorating health status of nursing home residents and the typically close contact between them (including cohabitation in the same contaminated environment) promote bacterial colonisation, the development of infection and the spread of bacterial spores. In addition, the risk of *C. difficile* acquisition by nursing home residents during a hospital stay is significant [11]. Residents can also be

asymptomatic carriers while still representing a potential source of contamination among other patients [9].

Here, we review the current literature data on the occurrence of *C. difficile* colonisation in nursing homes. The main factors associated with infection are also analysed, as well as the mortality rate and the genetic diversity of the isolates between different geographic areas.

2. Methods

Publications analysed were searched on PudMed (http://www.ncbi.nlm.nih.gov/pubmed) with the terms "*C. difficile* nursing homes" and "*C. difficile* elderly". Additionally, further articles were included by reviewing the references of the articles identified.

3. Occurrence of *C. difficile* in nursing homes across different countries

A relatively low number of studies have estimated the prevalence of *C. difficile* in nursing homes and other long-term care facilities for the elderly. High isolation frequencies have been described in most of the studies conducted in USA, with up to 46% of residents testing positive for *C. difficile*. In contrast, in Canada, Europe, UK, Ireland or Australia the reported rates are much lower, varying between 0.8% and 10% (Table 1). However, it is necessary to note that sample size, age, or methodologies are not standardised among the available studies, making meaningful comparison of the results difficult. Seasonal differences should be also considered: a

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Table 1
Summary data for the presence of *C. difficile* in nursing homes and other long-term care facilities for elderly people across different geographic areas.

Country	Residence of the patients enrolled in the study	*C. difficile* colonization (%)	CDI case	Asymptomatic carriers of toxigenic *C. difficile* (T) or (NT) non-toxigenic carriers (%)	Main PCR-ribotypes identified	Mean aged of colonised residents	Study period	Reference
Germany	Nursing home[a]	11/240 (4.6) (0–10)[b]	1	T 9 (81.8) NT 1 (9.1)	014 001	83	2010–2011	[9]
France **UK**	Nursing home[a]	2.39/10,000 resident-days[d]	25[c]	–	027	79.8	2006–2009	[12]
Scotland **Ireland**	Care home residence[a]	19/2385 (0.80)[d]	19	–	–	≥65	2008–2009	[2]
Cork	Continuing care institution for the elderly	10/100 (10)	0	T 7 (7) NT (3)	–	82	–	[13]
Australia Melbourne	Residential aged care facility[a]	1/119 (0.84)	0	T/NT 1 (0.84)	–	79.2	2010	[14]
USA Maryland	Long-term care facility for the elderly	119/258 (46.1)[e]	119	–	–	78.3	2005–2010	[15]
New York	Nursing home[a]	0.52–0.67/10,000 [f]resident-days	102	–	–	83	2009–2011	[11]
Virginia	Nursing home[a]	235/489 (48.1)[g]	225	NT 10 (2.04)	027	–	2009	[10]
Pennsylvania	Long-term care veterans affairs	0.04–0.028/1000 resident-days	66	–	–	77	2004–2009	[16]
Ohio	Nursing home[a]	1.7–2.9/10,000 resident-days[d]	11,200	–	–	–	2006	[3]
Ohio	Long-term care facility[a]	40/73 (54.8)	5	A 35 (47.9)	027	70	2006	[17]
Rhode Island **Canada**	Nursing home[a]	11/172 (6.4)[h]	11	–	–	81.4–85.8	2008	[18]
Ontario	Nursing home	(2.1–8.1)	–	–	–	–	–	[4]

[a] More than one setting enrolled in study.
[b] Variation in *C. difficile* colonisation rate among 25 nursing homes.
[c] Only confirmed CDI 027 cases were taken into account.
[d] Results obtained from a national or regional level survey.
[e] Data from a survey conducted in a hospital reflecting the total of patients with CDI acquired in a long-term care facility in relation to the total number of hospitalised patients developing CDI.
[f] Incidence of CDI developed more than 30 days after admission.
[g] Data from nursing home residents obtained in a laboratory for *C. difficile* testing.
[h] Only patients in nursing homes with results of a urinalysis were studied.

higher number of *C. difficile* patients were observed during the winter months in a previous study conducted in Germany [19].

A reduced variability in the isolates from residential care facilities for elderly people was reported between different countries, with PCR ribotype 027 remaining the dominant type in nursing homes regardless of their location [10,17,20]. In an investigation of a large outbreak of *C. difficile* PCR-ribotype 027 infections in France from 2008 to 2009, elderly patients over 80 years old were found to be the main population affected. Some of these patients were probably transferred from hospitals to nursing homes (and vice versa) contributing to the spread of the strain [12]. In contrast, in a study conducted across 25 nursing homes in Germany, none of the isolates obtained were identified as PCR-ribotype 027, although this type had been largely isolated from hospitalised patients in this region. The authors hypothesised that this PCR-ribotype may be more related to CDI rather than asymptomatic carriage as in only one case did a resident develop the infection during the course of the study [9]. Other PCR-ribotypes most frequently found in nursing homes are 014 (accounting for between 8% and 30% of the isolates) and 001 (accounting for between 7% and 20% of the isolates) [9,10].

4. Factors associated with *C. difficile* colonisation in elderly people

Previous studies have highlighted certain factors that make people over 65 years old more susceptible to being colonised by *C. difficile* [4,5]. Antibiotic treatment and age-related changes in intestinal flora and host defences, as well as the presence of other underlying illness may promote *C. difficile* colonisation, the developing of the infection and (in some cases) further recurrences [21,22]. One previous study evaluating factors associated with *C. difficile* acquisition in residents of a nursing home found no apparent relationship between infection and dementia, incontinence, contact with other residents with diarrhoea or age over 82 years. However, the authors observed that previous CDI, hospital admission or antibiotic therapy seemed to be related to toxigenic *C. difficile* presence [9]. A further report identified no association between *C. difficile* carriage and gender, age over 65 years, the length of the hospital stay, previous infection with the bacterium or the use of proton pump inhibitors [13]. In another study of the epidemiology of CDI among elderly care home residents, the presence of a nasogastric or gastronomy feeding tube, incontinence, underlying diseases or an antibiotic treatment were identified as significant independent variables associated with the infection [35]. Antibiotic treatment has been shown to alter gut microbiota and to decrease the colonisation resistance for pathogens such as *C. difficile*, increasing the risk of developing the infection. Nevertheless, a study conducted in hospitalised patients aged 65 years or more reported 50% of the positive culture samples to be found in asymptomatic subjects with a history of an antibiotic usage (except clindamycin). Moreover, most of the strains obtained were toxigenic [23]. In the same study, a reduction in faecal microbial diversity was observed in patients with CDI but not in asymptomatic subjects from whom *C. difficile* had been isolated. Another study reported similar results with reduced numbers of *Bacteroides*, *Prevotella*, *Bifidobacteria* and an increase of facultative species such as *Clostridium* or *Lactobacillus* sp in the presence of

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CDI. Although this study found a decrease in the diversity of bifidobacterial species in favour of an increase in Bacteroides species in the faeces of healthy elderly people, the authors also found the microbiota of elderly patients with CDI markedly different from those without colonisation [24].

In a literature review of *C. difficile* associated small bowel enteritis involving analysis of 56 cases published from 1980 to 2010, the authors came to support the contention that immunosenescence and severe underlying disease could play a critical role in this infection [16].

On the other hand, a recent report defines prognostic markers for a complicated course of CDI, studying hospitalised patients with diarrhoea and with a positive result for the *C. difficile* toxin test. The mean age of these patients was 65 years. The study concludes that age (≥85 years), admission due to diarrhoea, diagnosis at the ICU department, recent abdominal surgery and hypotension were independent predictors of a complicated course of *C. difficile* infection [21].

5. *C. difficile* spores in room environments and contamination of nursing home residents in hospital

Several studies have reported the capacity of *C. difficile* to persist on the skin and in the room environment for between one and four weeks after therapy, and on inanimate surfaces for as long as five months [25,26]. There are few studies that refer to the presence of *C. difficile* in the environment of elderly patient hospital wards [4,27,28]. Contaminated areas of the environment such as floors, electronic thermometers and even the air can contribute towards *C. difficile* transmission in healthcare settings [4,29]. In patient rooms, the most commonly contaminated areas have been identified as bedside tables, bedrails and toilet floors [17,26,27].

Nursing home residents are often transferred to hospitals when they suffer an acute clinical problem or when they require special medical care. These situations can result in transmission of *C. difficile* strains between hospitals and nursing homes. A previous study found that approximately two-thirds of CDI cases occurred within 30 days of nursing home admission after hospitalisation [11]. An additional study states that the mean duration of hospital stay in elderly patients without *C. difficile* diarrhoea is 20 days while 75% of *C. difficile* infection cases in aged people occur by day 21 of a hospital stay. The authors conclude that CDI is, for many patients, the cause of their prolonged stay in hospital [30].

6. Mortality associated with *C. difficile* among elderly and nursing home residents

Although mortality associated with CDI is estimated at around 17%, it seems that this percentage could be higher among older people [31]. In a pooled analysis of *C. difficile* enteritis [16], authors found that age was significantly higher in the 18 (32%) non-survivors from a group of patients with a mean age of 66 years (subjects between 60 and 76 years old). The median time between the onset of *C. difficile* infection symptoms and death was 4 days. Similar finds were reported in an epidemiology survey conducted in Ohio where, within the total number of patients' deaths from CDI, mortality was consistently higher in the oldest age population [3]. Another recent study conducted in four different nursing homes in New York reported three deaths among 23 residents who develop *C. difficile* infection after more than 30 days following admission [11].

Further studies have attributed the presence of *C. difficile* PCR ribotype 027 in patients between 60 and 90 years of age with an increased likelihood of CDI related death [16,32,33]; however, studies concerning the incidence of this strain in nursing homes are limited. Besides the hypervirulent PCR-ribotype 027, other *C. difficile* types have been linked with the death of elderly patients living in long-term care facilities, such as PCR-ribotype 078. In a *C. difficile* outbreak which occurred in Irish hospitals and nursing homes, eight out of 15 subjects with PCR-ribotype 078 colonisation died, and in five of the cases the bacterium directly contributed to the death of the patients [34].

Despite these findings, other reports about CDI and related mortality in older people have not definitively established *C. difficile* as the causative agent of death [4,35]. Furthermore, in a cohort study of community-associated CDI infection among older people and the relationship between infection, antibiotic exposure, and care home residence, authors reported an increased mortality among subjects that whose infections were healthcare-onset, but not among CDI cases in the community [2]. Similarly, in the study of Garg et al. [15], the highest mortality was found among *C. difficile* infection cases in hospital (9.4%) while the percentage of deaths was lower in long-term care facility CDI cases (7.6%) and in community acquired infections (2.3%).

7. Conclusions

There seems to be clear evidence that *C. difficile* colonisation and infection is more likely in elderly patients, as many factors associated with ageing influence susceptibility. Despite the currently limited data on the age-related changes in gut microbiota, this may play a critical role in *C. difficile* colonisation. Antibiotics, as well as specific treatments or interventions, and other individual conditions that decrease immune defences appear to promote the development of infection.

Hospitals are traditionally considered to be the main focus of *C. difficile* contamination, but some studies have also highlighted long-term care facilities as an environment predisposed for transmission. The constant movement of patients from nursing homes to hospitals and vice versa may facilitate transmission of epidemic and non-epidemic *C. difficile* strains between both of the healthcare establishments.

The severity and mortality rate of CDI appears more elevated among nursing home residents than older people living in the community. In addition, the hypervirulent PCR-ribotype 027 have been described as the most prevalent strain in long-term care facilities for elderly people.

Although it is difficult to separate the increased CDI susceptibility of nursing home residents from that induced by other factors (e.g. exposure to antibiotics, hospitalisation), further studies are required to better understand the epidemiology of *C. difficile* in long-term care facilities, in both the presence and absence of an epidemic situation.

References

[1] Jones AM, Kuijper EJ, Wilcox MH. Clostridium difficile: a European perspective. J Infect 2013;66:115–28.
[2] Marwick CA, Yu N, Lockhart MC, McGuigan CC, Wiuff C, Davey PG, et al. Community-associated *Clostridium difficile* infection among older people in Tayside, Scotland, is associated with antibiotic exposure and care home residence: cohort study with nested case-control. J Antimicrob Chemother 2013;68:2927–33.
[3] Campbell RJ, Giljahn L, Machesky K, Cibulskas-White K, Lane LM, Poter K, et al. *Clostridium difficile* infection in Ohio hospitals and nursing homes during 2006. Infect Control Hosp Epidemiol 2009;30:526–33.
[4] Simor AE, Yake SL, Tsimidis K. Infection due to *Clostridium difficile* among elderly residents of a long-term-care facility. Clin Infect Dis 1993;17:672–8.
[5] Taslim H. *C. difficile* infection in the elderly. Acta Med Indones 2009;41: 148–51.
[6] Castrillon BO, Harbarth S, Prendki V. *Clostridium difficile* infections, specific issues in the elderly. Rev Med Suisse 2013;6:2044–8.
[7] Keller JM, Surawicz CM. *Clostridium difficile* infection in the elderly. Clin Geriatr Med 2014;30:79–93.

[8] Kee VR. *Clostridium difficile* infection in older adults: a review and update on its management. Am J Geriatr Pharmacother 2012;10:14—24.

[9] Arvand M, Moser V, Schwehn C, Bettge-Weller G, Hensgens MP, Kuijper EJ. High prevalence of *Clostridium difficile* colonisation among nursing home residents in Hesse, Germany. PlosOne 2012;7:e30183.

[10] Boone JH, Goodykoontz M, Rhodes SJ, Price K, Smith J, Gearhart KN, et al. *Clostridium difficile* prevalence rates in a large healthcare system stratified according to patient population, age, gender, and specimen consistency. Eur J Clin Microbiol Infect Dis 2012;31:1551—9.

[11] Mylotte JM, Russell S, Sackett B, Vllone M, Antalek M. Surveillance for *Clostridium difficile* infection in nursing homes. J Am Geriatr Soc 2013;61:122—5.

[12] Birgand G, Blanckaert K, Carbonne A, Coignard B, Barbut F, Eckert C, et al. Investigation of a large outbreak of *Clostridium difficile* PCR-ribotype 027 infections in northern France, 2006-2007 and associated clusters in 2008-2009. Euro Surveill 2010;24:19597.

[13] Ryan J, Murphy C, Twomey C, Ross RP, Rea MC, MacSharry J, et al. Asymptomatic carriage of *Clostridium difficile* in an Irish continuing care institution for the elderly: prevalence and characteristics. Ir J Med Sci 2010;179: 245—50.

[14] Stuart RL, Kotsanas D, Webb B, Vandergraaf S, Gillespie EE, Hoog GG, et al. Prevalence of antimicrobial-resistant organisms in residential aged care facilities. Med J Aust 2011;7:530—3.

[15] Garg S, Mirza YR, Girotra M, Kumar V, Yoselevitz S, Segon A, et al. Epidemiology of *Clostridium difficile*-associated disease (CDAD): a shift from hospital acquired infection to long-term care facility-based infection. Dig Dis Sci 2013;58:3407—12.

[16] Kim JH, Muder RR. *Clostridium difficile* enteritis: a review and pooled analysis of the cases. Anaerobe 2011;17:52—5.

[17] Riggs MM, Sheti AK, Zabarsky TF, Eckstein EC, Jump RLP, Donskey CJ. Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic *Clostridium difficile* strains among long term care facility residents. Clin Infect Dis 2007;45:992—8.

[18] Rotjanapan P, Dosa D, Thomas KS. Potentially inappropriate treatment of urinary tract infections in two Rhode Island nursing homes. Arch Intern Med 2011;14:438—43.

[19] Reil M, Hensgens MP, Kuijper EJ, Jakobiak T, Gruber H, Kist M, et al. Seasonality of *Clostridium difficile* infections in southern Germany. Epidemiol Infect 2012;140:1787—93.

[20] Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, et al. The changing epidemiology of *Clostridium difficile* infections. Clin Microbiol Rev 2010;23:529—49.

[21] Hensgens M, Dekkers OM, Goorhuis A, Lecessie S, Kuijper EJ. Predicting a complicated course of *Clostridium difficile* infection at the bedside. Clin Microbiol Infect 2014;20:0301—8.

[22] Kelly CP. Can we identified patients at high risk of recurrent *Clostridium difficile* infection? Clin Microbiol Infect 2012;18:21—7.

[23] Rea MC, O'Sullivan O, Shanahan F, O'Toole PW, Stanton C, Ross RP, et al. *Clostridium difficile* carriage in elderly subjects and associated changes in the intestinal microbiota. J Clin Microbiol 2012;50:867.

[24] Hopkins MJ, Macfarlane GT. Changes in predominant bacterial populations in human faeces with age and with Clostridium difficile infection. J Med Microbiol 2002;51:448—54.

[25] Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis 2006;6:130.

[26] Sjörberg M, Eriksson M, Andersson J, Noren T. Transmission of *Clostridium difficile* spores in isolation room environments and through hospital beds. APMIS 2014;30. http://dx.doi.org/10.1111/apm.12218.

[27] Wilcox MH, Fawley WN, Wigglesworth P, Parnell P, Verity P, Freeman J. Comparison of the effect of detergent versus hypochlorite cleaning on environmental contamination and incidence of *Clostridium difficile* infection. J Hosp Infect 2003;54:109—14.

[28] Fawley WN, Wilcox MH. Molecular epidemiology of endemic *Clostridium difficile* infection. Epidemiol Infect 2001;126:343—50.

[29] Brooks SE, Veal RO, Kramer M, Dore L, Schupf N, Adachi M. Reduction in the incidence of *Clostridium difficile* associated diarrhea in an acute care hospital and skilled nursing home care facility following replacement of electronic thermometers with single-use disposables. Infect Control Hosp Epidemiol 1992;13:98—103.

[30] MacGowan AP, Feeney R, Brown I, McCulloch SY, Reeves DS, Lovering AM. Health care resource utilization and antimicrobial use in elderly patients with community-acquired lower respiratory tract infection who develop *Clostridium difficile*-associated diarrhoea. J Antimicrob Chemother 1997;39: 537—41.

[31] Crogan NL, Evans BC. *Clostridium difficile*: an emerging epidemic in nursing homes. Geriatr Nurs 2007;28:161—4.

[32] Miller M, Gravel D, Mulvey M, Taylor G, Boyd D, Simor A, et al. Health care-associated *Clostridium difficile* infection in Canada: patient age and infecting strain type are highly predictive of severe outcome and mortality. Clin Infect Dis 2010;50:194—201.

[33] Boone JH, Archbald-Pannone LR, WickhaM KN, Carman RJ, Guerrant RL, Franck CT, et al. Ribotype 027 *Clostridium difficile* infections with measurable stool toxin have increased lactoferrin and are associated with a higher mortality. Eur J Clin Microbiol Infect Dis 2014;33:1045—51.

[34] Burns K, Morris-Downes M, Fawley WN, Smyth E, Wilcox MH, Fitzpatrick F. Infection due to *Clostridium difficile* ribotype 078: first report of cases in the Republic of Ireland. J Hosp Infect 2010;75:287—91.

[35] Canovas C, Merino FJ. Nosocomial *Clostridium difficile*-associated diarrhoea in patients older than 80 years. Rev Esp Geriatr Gerontol 2005;40:70—7.

CHAPTER 2: Objectives

C. difficile remains the leading cause of healthcare-associated diarrhea and outbreaks continue to occur worldwide. In Belgium, CDI is recognised as a major cause of diarrhea and pseudomembranous colitis in both acute and chronic healthcare institutions. But between 2008 and 2014, an increase in the proportion of community associated *C. difficile* cases and a decrease in the hospital-associated infection have been reported. The emerging detection of *C. difficile* in animals and foods raised questions on zoonotic, foodborne transmissions or the existence of an animal reservoir and its role in the changing epidemiology of CDI in humans.

The main hypothesis of the present dissertation was that companion animals, food animals, their environment and foods can drive epidemic *C. difficile* strains to exposed humans, and consequently expand the infection in the community.

To start the survey, *C. difficile* was first investigated in companion animals. The main objective was to assess the carriage of *C. difficile* in hospitalised horses and to investigate the possible influence of some risk factors in colonisation. The study was completed with the investigation of the gut microbiota of hospitalised horses by high-throughput sequencing analysis in order to attempt to determine whether the presence of diarrhea and/or the isolation of *C. difficile* were related to changes in the composition of the faecal microbiota.

The second objective was to assess *C. difficile* shedding in food animals (pigs and cattle) in farms and at slaughter, and to determine how the intestinal carriage of *C. difficile* represents a risk for carcass contamination in the slaughter line. In addition, to investigate the risk of food contamination, the presence of *C. difficile* spores in retail meat was also evaluated.

To complete the investigation about the role of livestock animals as sources of *C. difficile* contamination for humans, the third objective was to compare all the isolates obtained (from animals, carcasses, meats and human patients) by two different typing methods, multi-locus sequence typing (MLST) and multi-locus variable-number tandem-repeat analysis (MLVA) and to establish possible relationships between them.

The fourth objective was to determine the risk of ingestion of *C. difficile* spores on freshly prepared foods in the kitchen area of a nursing home, after cooking with recommended minimum temperatures. The study was performed in a nursing home as elderly are considered as a community at high risk for acquisition of CDI.

Besides foods, different areas of the environment such as floors, toilets or beds can contribute towards *C. difficile* transmission in healthcare settings. Spores of *C. difficile* are resistant to many antiseptic cleaners, and survive for long periods of time in the environment. The fifth objective was to evaluate the presence of *C. difficile* in patient rooms and other common areas of the same nursing home, in order to elucidate all potential sources of contamination.

Once the food and environmental contamination was evaluated in the nursing home, the sixth objective was to determine and follow the prevalence of *C. difficile* in the elderly care home subjects. In addition, high throughput sequencing analysis was used to characterise the faecal microbiota of the residents to evaluate the global evolutions of the total microbiota and to identify possible relationships between certain bacteria populations and *C. difficile* colonisation.

The final objective of this study was to survey the presence of *C. difficile* in another European country (Spain) and to compare the PCR-ribotype distribution with those observed in a second hospital in Belgium, during the same study period, in order to investigate if there was a regional or ward spread of some *C. difficile* strains implicated in human infections.

Overall, this study was conducted to assess if epidemic strains present in foods, animals and their environment are commonly found in humans, and to determine if some strains are moving from animals to humans, either directly or indirectly, causing infections in susceptible people in the community.

CHAPTER 3: Experimental section

3.1 Study of *C. difficile* in hospitalised horses: carriage rate and faecal microbiota characterisation

C. difficile is commonly associated with diarrhea and enterocolitis in horses. As in humans, the major risk factors for the development of CDI include hospitalisation or antibiotic therapy. A preliminary study was conducted in hospitalised horses at an equine medical teaching clinic to assess the presence of *C. difficile* at admission and also during hospitalisation. Results revealed an estimated *C. difficile* colonisation rate of 13.7% during a total of seven months of study. However, only two horses presented clinical signs of diarrhea associated with CDI. This finding suggests that while horses can harbour toxigenic and non-toxigenic *C. difficile,* the development of *C. difficile* associated diarrhea is more unusual. Molecular typing of the isolates revealed a wide variety of PCR-ribotypes, most of them toxigenic, including PCR-ribotype 014, UCL16L, UCL16a, UCL228, UCL9, UCL261 and UCL5a. This great variety of PCR-ribotypes detected could suggest that, for most of the horses, there was not clinic ward contamination, at least with a particular circulating strain. Similarly, the combination of clinical history and MLST analysis did not allow us to establish any relationship between the acquisition of *C. difficile* in the clinic and a particular circulating strain or between the presence of diarrhea and one particular type. In relation with antibiotic resistance, most of the isolates were resistance to ceftiofur but susceptible to metronidazole, moxifloxacin and vancomycin. Regarding gentamicin, it should not be tested. It is well described in the literature that aminoglycosides are less active against anaerobes.

Once the presence of *C. difficile* in hospitalised horses was assessed, a second study was conducted to investigate the nature of the bacterial communities present in horses developing diarrhoea, through comparison with faeces from horses without diarrhea. The study also examined the carriage of *C. difficile* at admission and attempted to determine the faecal microbiota of horses suffering CDI. High-throughput amplicon sequencing analysis revealed that a great part of the sequences (60%) were not identical (less than 1% mismatch) to sequence entries present in SILVA database. These finding underline the lack of knowledge regarding the horse gut microbiota. Bacterial diversity of the faecal microbiota in diarrhoeic horses was lower than in non-diarrhoeic horses in terms of species richness and in population evenness. Some taxa like *Fusobacteria, Actinobacillus* and *Porphyromonas* were detected more abundantly in horses with diarrhea, while *Akkermansia* was found in all of the horses studied. This bacterium is an appealing candidate to become a human probiotic, selected based on established mechanisms of preventive treatment of obesity and diabetes. The relevance of its presence in the equine intestinal microbiota deserves further investigation. The overall prevalence found for *C. difficile* colonization was lower than in the previous study (3.7%). However, animals were only sampled at the time of admission but not tracked during their hospitalisation as in the previous study,

in which animals were also sampled during their hospitalisation. In addition, it must be considered that the sampling size between studies is not identical. Five different PCR-ribotypes were identified: 014, UCL237, UCL49, UCL23f and. Only one of these types (UCL36) was non-toxigenic. None of these positive animals suffered an episode of diarrhea, which corroborates the hypothesis that *C. difficile* was transient in horses studied without overgrowth to trigger infection. Due to this lack of horses with infection during the study period, no association between CDI in horses and a specific modification of the microbiota could be demonstrated.

3.1.1 Carriage and acquisition rates of *C. difficile* in hospitalized horses, including molecular characterization, multilocus sequence typing and antimicrobial susceptibility of bacterial isolates

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Carriage and acquisition rates of *Clostridium difficile* in hospitalized horses, including molecular characterization, multilocus sequence typing and antimicrobial susceptibility of bacterial isolates

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ABSTRACT

Clostridium difficile has been identified as a significant agent of diarrhoea and enterocolitis in both foals and adult horses. Hospitalization, antibiotic therapy or changes in diet may contribute to the development of *C. difficile* infection. Horses admitted to a care unit are therefore at greater risk of being colonized. The aim of this study was to investigate the carriage of *C. difficile* in hospitalized horses and the possible influence of some risk factors in colonization. During a seven-month period, faecal samples and data relating the clinical history of horses admitted to a veterinary teaching hospital were collected. *C. difficile* isolates were characterized through toxin profiles, cytotoxicity activity, PCR-ribotyping, antimicrobial resistance and multilocus sequence typing (MLST). Ten isolates were obtained with a total of seven different PCR-ribotypes, including PCR-ribotype 014. Five of them were identified as toxinogenic. A high resistance to gentamicin, clindamycin and ceftiofur was found. MLST revealed four different sequencing types (ST), which included ST11, ST26, ST2 and ST15, and phylogenetic analysis showed that most of the isolates clustered in the same lineage. Clinical history suggests that horses frequently harbour toxigenic and non-toxigenic *C. difficile* and that in most cases they are colonized regardless of the reason for hospitalization; the development of diarrhoea is more unusual.

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1. Introduction

Most human *Clostridium difficile* infections (CDI) are acquired in hospitals and nursing homes following antibiotic therapy. It seems that infected patients and contaminated environments play an important role in the transmission of this pathogen (Bengualid et al., 2011). Moreover, colonized new admissions also contribute to *C. difficile* transmission in hospitals (Clabots et al., 1992). Reported prevalence rates of *C. difficile* range from 5.9% up to 11% in asymptomatic carriers at admission and from 4% up to 21% for hospital acquisition. More than 63% of infected patients remain asymptomatic (Barbut and Petit, 2001).

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C. difficile is an important agent of diarrhoea and enterocolitis in foals (Uzal et al., 2012) and horses (Arroyo et al., 2006; Weese et al., 2006). Newborn foals can suffer spontaneous *C. difficile* infection with watery or bloody diarrhoea several days before death (Diab et al., 2013). Additionally, in foals, a possible synergism of *C. perfringens* type C and *C. difficile* has been suggested, characterized by the presence of a necrotic mucosa with a superficial pseudomembrane, haemorrhage and vascular thrombosis in the small intestine and colon (Uzal et al., 2012). *C. difficile* has also been hypothesized to be the aetiology of duodenitis-proximal jejunitis disease in adult horses (Arroyo et al., 2006). As in humans, the major risks factors for the development of nosocomial *C. difficile* associated disease (CDAD) in horses include hospitalization, antibiotic therapy, changes in diet and pre- or post-surgical feed withdrawal. Transmission by oral–faecal route includes contact with other infected horses or a contaminated environment (Diab et al., 2013). An interspecies transmission, including one involving human beings, has also been speculated (Rodriguez-Palacios et al., 2013).

Carriage of multiple strains of *C. difficile* in the gastrointestinal tract of healthy horses has been reported (Schoster et al., 2012a). As in other young animals (particularly piglets and calves (Rodriguez et al., 2012)), neonatal foals are more likely than adult horses to be carriers of this bacterium (Rodriguez-Palacios et al., 2013). Mare–foal pairs can harbour *C. difficile* subclinically and therefore potentially serve as reservoirs for cross-colonization (Magdesian and Leutenegger, 2011).

Many articles have reported the prevalence of *C. difficile* in horses, mares and foals from different ranches or breeding farms over a specific period of time, but screening for *C. difficile* in an equine hospital has rarely been addressed. Only one previous study has examined the prevalence of *C. difficile* in horses with normal faeces admitted on Sundays and Mondays in a large animal clinic (Medina-Torres et al., 2011).

The main objective of this study was to assess the presence of *C. difficile* in hospitalized horses at an equine medical teaching clinic. Faeces of horses with soft or liquid bowel movements were analyzed at the moment of the episode of diarrhoea. In addition, horses with an extended clinical stay were tracked during their hospitalization. All the isolates were characterized by genotyping, PCR-ribotyping, toxigenic activity and antibiotic resistance. Further characterization was performed by multi-locus sequencing typing (MLST) analysis in order to study clonal relationships of the isolates.

2. Materials and methods

2.1. Sample collection

A prospective study was conducted over seven months at the Equine Clinic, Department of Companion Animals and Equids, Faculty of Veterinary Medicine, University of Liege, between January–April 2011 and October–December 2011. During this period, a total of 580 horses were admitted to the clinic (emergencies or consultations), with about 250 hospitalizations.

Between one and three samples were collected from each horse enrolled in the study. Eligible horses were animals with a hospital stay of at least one day. In addition, faeces of horses with soft or liquid bowel movements were analyzed at the moment of the episode of diarrhoea. Whenever possible, horses enrolled in the study whose hospital admission was prolonged over nine days were monitored every week for the presence of *C. difficile*. The exclusion criteria were horses with dysphoric mood, intolerable stress or other disease for which sampling by rectal manipulations was not recommended. Horses with a highly contagious infectious disease and isolated in quarantine boxes were also excluded from the study. All horses were documented for data relating to clinical history, diagnostic findings and treatment received during hospitalization. In addition, all predisposing factors for developing *C. difficile* infection (CDI), such as the prescription of antimicrobials, were carefully recorded. Faecal sampling was performed directly via the rectum. Samples obtained were scored as normal, diarrhoea or bloody diarrhoea faeces. All samples were processed on the same day immediately after transport to the laboratory (with travel at room temperature and for a maximum of 45 min). In the case of a diarrhoea episode during the night or on a non-working day, faecal samples were stored at 4 °C in the hospital for a maximum of 4 days before analysis.

2.2. C. difficile culture and characterization

Fresh faeces (0.1 g) were spread directly on home-made cycloserine cefoxitin fructose taurocholate (CCFT) plates (Delmée et al., 1987) and incubated in an anaerobic workstation (Led Techno, Heusden-Zolder, Belgium) at 37 °C for three days. At the same time, one gram of faeces was inoculated into 9 ml of CCFT broth as previously described (Rodriguez et al., 2012) and incubated anaerobically for 72 h at 37 °C. After the enrichment phase, approximately 10 μl of the broth was spread on CCFT plates and incubated anaerobically at 37 °C for two days. Initial identification of *C. difficile* colonies was based on morphological criteria such as yellowish colonies with an appearance of ground glass and a characteristic horse manure odour. One morphologically suspected colony per plate was subcultured onto blood agar (5% Sheep Blood; Biorad, Nazareth, Belgium) and checked using a *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid, Dardilly, France). Identification was confirmed by detection of a species-specific internal fragment of *tpi* and detection of genes for toxin A, B and binary toxin (*cdtA*) as described previously (Rodriguez et al., 2012). A cytotoxicity assay using confluent monolayer MRC-5 cells was carried out as previously described (Rodriguez et al., 2012).

2.3. Molecular typing of C. difficile isolates

PCR-ribotyping was performed using the primers and protocol of Bidet et al. (1999). International numbers were used for *C. difficile* strains that presented a PCR-ribotype profile matching the Cardiff ribotypes from the strain collection available in our laboratory. Otherwise, isolates were identified with internal nomenclature.

All isolates were additionally retested by the Genotype Cdiff system (Hain Lifescience, Nehren, De), according to the manufacturer's instructions, for the presence of the *tpi*, all toxin genes (*tcdA*, *tcdB*, *cdtA* and *cdtB*) and deletions in the regulator gene *tcdC* and *gyrA* mutation.

2.4. Antimicrobial susceptibility testing

All of the horse isolates were tested for susceptibilities to a total of 10 antimicrobial agents by disc diffusion ($n = 8$) and E-test ($n = 2$).

Disc diffusion was performed with standard disc (Becton-Dickinson, Erembodegem, Belgium) of rifampin (25 µg), erythromycin (15 µg), oxytetracycline (30 µg), vancomycin (30 µg), penicillin (10 µg), clindamycin (2 µg), ceftiofur (30 µg) and gentamicin (10 µg) on Brucella Blood Agar with hemin and vitamin K1 (Becton-Dickinson) according to the French Society of Microbiology (SFM) (www.sfm-microbiologie.org) protocols. These antimicrobials were chosen because they are widely used in the equine teaching hospital featured in the study, or because they have been associated with *C. difficile*-associated disease or its treatment. The zone diameters were read after 24 h of anaerobic incubation at 37 °C. The resistant and susceptible zone diameters were defined as reported by Delmée and Avesani (1988): rifampin no zone and >23 mm, erythromycin <13 mm and >20 mm, oxytetracycline <14 mm and >23 mm, clindamycin no zone and >12 mm. The zone diameter breakpoint for vancomycin was ≥19 mm as proposed by Erikstrup et al. (2012). Susceptibility and resistance to penicillin was defined at the limits of <8 mm and ≥29 mm established by Cattoir et al. (2008). The remaining diameters were based on the only values available for other Gram + bacteria reported by Marie et al. (2000) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) as follows: ceftiofur <21 mm and ≥21 mm and gentamicin <20 mm and ≥20 mm.

Susceptibility to metronidazole and moxifloxacin was determined by the Etest method (Lucron ELITechGroup St-Martens-Latem, Belgium) on Schaedler with Vit K1 and 5% sheep blood (Becton-Dickinson) according to the manufacturer's instructions. Metronidazole was tested because it is the first choice antibiotic for adult horses with diarrhoea in the equine hospital studied (if they are excluded from the human food chain). Resistance to moxifloxacin was evaluated because it is quite common in human *C. difficile* isolates (Barbut et al., 2007). Plates were incubated anaerobically at 37 °C for 48 h. The susceptibility and resistance breakpoints for metronidazole ($s \leq 8$ µg/ml; $r \geq 32$ µg/ml) and moxifloxacin ($s \leq 2$ µg/ml; $r \geq 8$ µg/ml) used for interpretation were those recommended by the Clinical and Laboratory Standard Institute (CLSI, 2010). *Bacteroides fragilis* ATCL 25285 was tested as a quality control.

2.5. C. difficile multilocus sequencing

Seven housekeeping loci (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA* and *tpi*) were used for the analysis of *C. difficile* isolates by MLST according to the protocol described previously by Griffiths et al. (2010). PCR products were purified with a Wizard SV Gel and PCR Clean-Up System kit (Promega, Leiden, The Netherlands). The Sanger sequencing reaction was carried out with the BigDye terminator kit version 3.1 (Applied Biosystems, Life technologies Europe BD, Gent, Belgium) and resolved with a 3730 ABI capillary sequencer (Applied Biosystems) (48 capillaries). Results were analyzed using the Geneious program (http://www.geneious.com). The allele number, clade and sequence type (ST) were assigned according to the *C. difficile* MLST reference database (http://pubmlst.org/cdifficile). A dendrogram was constructed using the Geneious program (Drummond et al., 2013).

3. Results

3.1. C. difficile prevalence in hospitalized horses

During the course of the seven-month study period, 102 faecal samples were collected from a total of 73 hospitalized horses. Eighteen horses were sampled on more than one occasion either because they had a long hospital stay or because they suffered an additional diarrhoea episode after the first collection. Ten out of a total of 73 horses (13.7%) tested positive for *C. difficile* but only two presented clinical signs of diarrhoea associated with CDI. Most of the positive samples (8/10) were detected after three days of enrichment, but two tested positive in direct culture without enrichment of the faeces. Because these two samples also tested positive after three enrichment days, a total of 12 strains were obtained from 10 horses. None of the horses tested positive for *C. difficile* on more than one occasion.

Of the total of 73 animals tested, 41 horses (56.2%) presented gastrointestinal disorders (diarrhoea or colic). *C. difficile* was detected in five (12.2%) of these 41 horses with gastrointestinal problems. Two had diarrhoea and were suspected to suffer from CDI (one new born foal with bloody diarrhoea and septicaemia and one adult horse with diarrhoea and anorexia). Three other adult horses tested positive for *C. difficile* had colic with a diagnosis of impaction and volvulus of the jejunum, nephrosplenic entrapment and incarceration of the jejunum, respectively. The remaining 36 horses (87.8%) tested negative for *C. difficile*, with intestinal disorders diagnosed as the following: 18 had diarrhoea (50%) and another 18 (50%) suffered an episode of colic. The most common causes of colic for these 18 *C. difficile* negative horses included: colon impaction (5/18; 27.8%), nephrosplenic entrapment (4/18; 22.2%) and colonic displacement (3/18; 16.7%).

Of the remaining 32 animals (43.8%) not affected by gastrointestinal problems, *C. difficile* was isolated from five horses (15.6%). The clinical diagnosis of each positive animal was hip fracture, sinusitis, wound on leg, chorioptic mange and paraphimosis, respectively. In contrast, 84.4% (27/32) of the horses without gastrointestinal problems tested negative for *C. difficile*. Their clinical diagnoses were varied; for example mange and other parasites ($n = 7$), wounds ($n = 6$), dislocation and bone fracture ($n = 2$), among others.

Table 1
Clinical characteristics of the 73 horses enrolled in the study and comparison of of *C. difficile* colonized versus non-colonized horses.

	C. difficile positive horses (%)	*C. difficile* negative horses (%)
Overall totals (%)	10 of 73 (13.7)	63 of 73 (86.3)
Mean age in years[a]	9.1	11.8
Sort by gender		
Mare	4 (40)	29 (46)
Stallion	1 (10)	8 (12.7)
Gelding	5 (50)	26 (41.3)
Sort by size		
Light horses	8 (80)	53 (84.1)
Heavy horses	2 (20)	4 (6.3)
Ponies	0 (0)	6 (9.5)
Horses with gastrointestinal disorders 41/73 (56.2)	5 of 41 (12.2)	36 of 41 (87.8)
Diarrhoea	2 (40)	18 (50)
Colic	3 (60)	18 (50)
Horses without gastrointestinal disorders 32/73 (43.8)	5 of 32 (15.6)	27 of 32 (84.4)
Dislocation/bone fracture	1 (20)	2 (7.4)
Chorioptic mange/other parasites	1 (20)	7 (25.9)
Wounds	1 (20)	6 (22.2)
Others	2 (40)	12 (44.4)
Horses with an antibiotic treatment 44/73 (60.3)	9 of 44 (20.5)	35 of 44 (79.5)
Penicillin	1 (11.1)	2 (5.7)
Penicillin[b]–Gentamicin	1 (11.1)	7 (20)
Cefquinome	6 (66.7)	9 (25.7)
Cefquinome[c] combined treatment	1 (11.1)	6 (17.1)
Others[d]	0 (0)	11 (31.4)
Horses with a surgery intervention 27/73 (37)	1 of 27 (3.7)	26 of 27 (96.3)
Intestinal surgery	1 (100)	11 (42.3)
Other type of surgery	0 (0)	15 (57.7)

[a] Mean age of the hospitalized horses.
[b] Antimicrobial treatment of penicillin combined with gentamicin.
[c] Association of cefquinome with other antibiotics such as gentamicin, metronidazole, penicillin or marbofloxacin.
[d] Including single or combined antimicrobial therapy.

In relation to clinical interventions and treatments, previous gastrointestinal surgery had been carried out on 11 of the negative horses, but in only one of the 10 *C. difficile* positive animals. Regarding the antimicrobial therapy, a total of nine horses tested positive for *C. difficile* had previously received an antibiotic medication. Cefquinome and penicillin were prescribed for six and one positively tested horses, respectively. A combined antibiotic treatment composed of two or more different antibiotics was administrated to the two other positively tested horses. Among the animals that tested negative for *C. difficile*, 35 had received antimicrobial therapy. Cefquinome and penicillin was administrated to nine and two negatively tested horses, respectively, while 37.1% (13/35) of *C. difficile* negative horses had received a combined antibiotic treatment (Table 1).

Of the 10 horses carrying *C. difficile* in their faeces, two animals had been assessed positive less than two days after admission (H7252 and H3113). Another two horses were suspected of being colonized during their hospitalization (H4850 and H0521), as they were both tested negative for *C. difficile* during initial sampling on admission. For the remaining six positively tested horses, initial sampling could not be performed until five days after admission. Monitoring of the hospitalized horses reveals that two *C. difficile* positive animals tested negative after 10 and 20 days of clinical stay (H6647 and H3113). Three horses were euthanized during the study. One of them (H7516) was

identified as positive for *C. difficile* only in a second sampling after euthanasia (Table 2).

3.2. Toxin gene profiles, toxin activity, PCR-ribotyping and genotype Cdiff test

Seven different PCR-ribotypes were identified. Only one strain has a ribotype profile corresponding to an international collection number (014). The remaining isolates were not associated with any reference Cardiff ribotypes (UCL16L″, UCL5a, UCL228, UCL9, UCL261, UCL16a). Five out of these seven different PCR ribotypes had toxic activity (75% of all isolates). All toxigenic isolates encoded toxin A and B, while only PCR-ribotype UCL5a also contained the binary toxin (Table 2).

The two animals assessed as *C. difficile* positive after less than two days of admission carried toxigenic *C. difficile* PCR ribotypes UCL16L″ and UCL5a. Isolates from the horses suspected to have been colonized during the hospitalization were also toxigenic and identified as PCR ribotypes UCL16a and UCL228. Gastrointestinal surgery or diarrhoea was not associated with the presence of a specific PCR-ribotype.

3.3. Antibiotic resistance

Isolates were tested for resistance to a total of 10 antibiotics. All strains were susceptible to vancomycin,

Table 2
Detailed information of hospitalized horses positive for *C. difficile* and molecular type of the isolates.

Date of sampling	Positive animal identification	Age (years)	Hospital stay before positive culture (days)	Diagnostic	Diarrhoea	Intestinal surgery	Antibiotic treatment	Positive culture Direct[a]	3 Days[b]	PCR-ribotype	Toxin profile
17/01/11	H7252	11	1	Colic (nephrosplenic entrapment)	–	–	–	–	+	UCL16L″	A⁺B⁺CDT⁻
24/01/11	H5017	11	5	Colic (impaction and volvulus of the jejunum)	–	–	CEF	–	+	UCL16a	A⁺B⁺CDT⁻
31/01/11	H4850	3	22	Paraphimosis	–	–	PEN	+	+	UCL228	A⁺B⁺CDT⁻
03/03/11	H5197	16	35	Chorioptic mange	–	–	CEF	–	+	UCL9	A⁻B⁻CDT⁻
31/03/11	H6695	4	8	Colic (incarceration of the jejunum)	–	+	PEN-GEN	+	+	UCL261	A⁻B⁻CDT⁻
08/04/11	H6647	4	17	Wound on leg	–	–	CEF	–	+	UCL9	A⁻B⁻CDT⁻
29/04/11	H7516	8 days	7	Septicaemia	+[c]	–	LZ-MAR-CEF	–	+	014	A⁺B⁺CDT⁻
14/11/11	H3113	11	2	Anorexia	+	–	CEF	–	+	UCL5a	A⁺B⁺CDT⁺
24/11/11	H2867	6	21	Sinusitis	–	–	CEF	–	+	UCL5a	A⁺B⁺CDT⁺
16/12/11	H0521	2	16	Hip fracture	–	–	CEF	–	+	UCL16a	A⁺B⁺CDT⁻

[a] Positive results detected after direct culture of the faeces.
[b] Positive results detected after 3 days of enrichment.
[c] Bloody diarrhoea.
CEF: cefquinome; PEN: penicillin; GEN: gentamicin; ENR: enrofloxacin; LZ: metronidazole; MAR: marbloxacin.

metronidazole and rifampicin. In addition, all of the isolates showed full sensibility to moxifloxacin, with the exception of one non-toxigenic isolate (H5197) that had intermediate resistance to this drug. These results were correlated with the absence of a mutation in the *gyrA* gene. Moreover, all the strains were resistant to clindamycin, gentamicin and ceftiofur. For penicillin, only one isolate (H3113) was resistant while all others showed intermediate resistance. Resistance to erythromycin was detected in one non-toxigenic isolate (H5197) and also in one toxigenic strain (H2867) that was also intermediately resistant to tetracycline (Fig. 1). The only isolate from a horse without an antibiotic treatment (H7252) showed a very similar antimicrobial susceptibility to the other strains with coresistance to clindamycin, gentamicin and ceftiofur and intermediate resistance to penicillin.

3.4. C. difficile MLST analysis

In order to determine the allelic diversity between *C. difficile* strains from the hospitalized horses, each isolate was characterized by MLST. The analyses revealed four different sequencing types (ST), which included ST11, ST26, ST2 (two isolates) and ST15 (one isolate). Only the two strains included in ST11 and identified as PCR-ribotype UCL5a were binary toxin positive. The two nontoxigenic PCR-ribotypes UCL9 and UCL261 were related with ST26 and ST15, respectively. All the strains with the same PCR-ribotype (UCL9 and UCL16a) clustered in the same lineage. For three strains (H7252, H7516 and H4850) ST assignment was not possible as no loci sequence combination matched the allelic profile of the isolates.

Two different clades were assigned. Clade 1 was correlated with PCR ribotypes UCL261 (non-toxigenic isolate) and UCL16a. No clade assignation was available for the types UCL9, UCL16L″, UCL228 and 014. The two

isolates with PCR ribotype UCL5a were included in clade 5, which appear in a different cluster from all the remaining strains (Fig. 1).

4. Discussion

Few studies describing the carriage of *C. difficile* in hospitalized horses exist, and the majority of these focus their investigation on animals with a particular health problem, such as duodenitis–proximal jejunitis (Arroyo et al., 2006), post-operative diarrhoea or colic (Niwa et al., 2013), acute haemorrhagic diarrhoea (Uzal et al., 2012), antibiotic associated diarrhoea (Barr et al., 2013), mare–foal pairs infection (Magdesian and Leutenegger, 2011) or the prevalence variation in different intestinal compartments after euthanasia (Schoster et al., 2012b). Overall, we reported an estimated *C. difficile* colonization rate of 13.7% (10/73) in an equine hospital setting during a total of seven months of study. Only two horses out of 73 (incidence of 2.7%) presented clinical sign of diarrhoea associated with CDI. From currently available data, it seems that foals are more likely to suffer the infection, and that antimicrobials increase the risk of developing the disease. Thean et al. (2011) observed a *C. difficile* prevalence of 23% (14/62) in horses with diarrhoea tested in Australia (10 of them were foals). Baverud et al. (2003) reported a prevalence of 42% (18/48) in horses that developed acute colitis during antibiotic treatment, and 6% (4/72) in mature horses with no history of antibiotic administration. The results of our study suggest a low endemic CDI incidence in horses admitted to the veterinary hospital studied.

Limited information is available regarding the presence of *C. difficile* in hospitalized horses in the absence of clinical signs of *C. difficile* disease. Medina-Torres et al. (2011) observed a prevalence of 4.8% (4/82) from patients at a
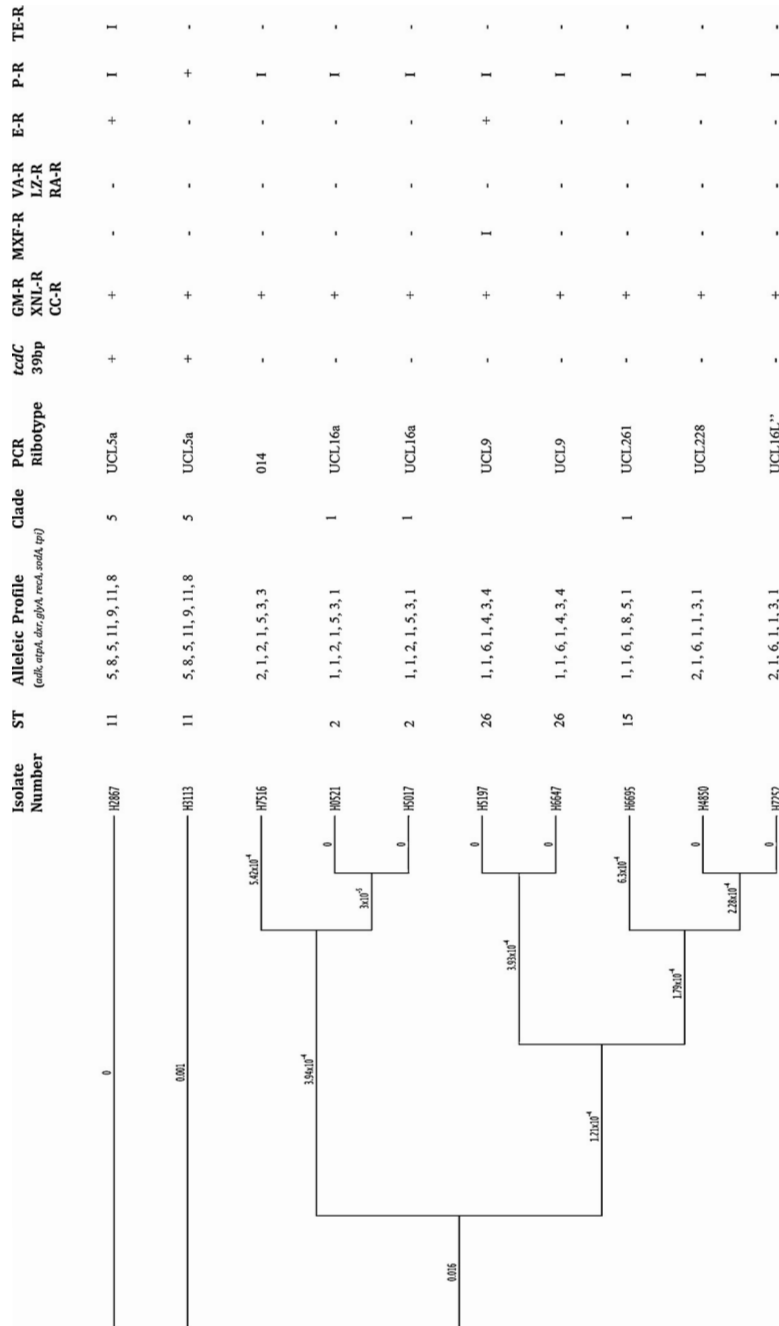
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Fig. 1. Neighbour-joining phylogenetic tree constructed with the MLST results showing the relationships between horse *C. difficile* strains. ST: sequence type; *tcdC* 39 bp: presence of deletions in the regulator gene *tcdC*; *gyrA* mut: presence of mutation in the *gyrA* gene; GM-R: gentamicin resistance; XNL-R: ceftiofur resistance; CC-R: clindamycin resistance; MXF-R: moxifloxacin resistance; VA-R: vancomycin resistance; LZ-R: metronidazole resistance; RA-R: rifampicin resistance; E-R: erythromycin resistance; P-R: penicillin resistance; TE-R: oxytetracycline resistance; I: intermediate antimicrobial resistance.

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veterinary teaching hospital with normal faeces at the time of admission. In the present study, the colonization rate detected in horses without CDI was 11% (8/73) over the seven months. Remarkably, none of the horses tested positive for *C. difficile* on more than one occasion. These findings suggest that colonization is transient in most cases. This colonization may be influenced by many stress situations that alter the intestinal flora, often difficult to quantify (change of diet, transportation, hospitalization and surgical or medical treatment among others) (Baverud, 2004). Transit shedding of *C. difficile* has been previously reported in healthy adult horses (Schoster et al., 2012b) but also in cattle (Rodriguez-Palacios et al., 2011) and humans (Ozaqui et al., 2004).

The mean age of horses enrolled in this study was ten years, with the great majority older than two years; nevertheless an association between an age of more than 24 months and the carriage of *C. difficile* could not be established. Three foals under 17 months old participated in the study and of these only an eight day-old filly was colonized with *C. difficile*. As previously reported, foals may develop *C. difficile* infection in the first days of life (Diab et al., 2013). In this case, the animal presented the classic signs of spontaneous *C. difficile* infection including depression and bloody diarrhoea (Diab et al., 2013). Forty eight hours after the hospital admission, the foal was treated with metronidazole, cefquinome and marbofloxacin. An initial sampling of faeces was carried out four days after the start of antibiotic treatment with a negative result for the presence of *C. difficile*. A second sample was collected 24 h later, directly from the large intestine after euthanasia. The second analysis revealed the carriage of toxigenic *C. difficile* in the intestinal contents. It has been suggested that even though faecal samples can demonstrate the presence of *C. difficile*, rectal samples might not absolutely reflect the status of proximal compartments (Schoster et al., 2012b).

Intestinal flora perturbations and colic may facilitate the proliferation of *C. difficile* in horses (Donaldson and Palmer, 1999). In this study two *C. difficile* positive horses (H5017 and H6695) presented signs of colic and had received an antibiotic therapy, but only one harboured a toxigenic *C. difficile* strain (the other was colonized with a non-toxigenic type). Two horses with health problems other than enteric disease and treated with antibiotics tested positive for toxigenic *C. difficile* after nine and 18 days of hospitalization. They had tested negative at the time of admission, and it was therefore suspected they were colonized during the period of hospitalization. However, neither of the horses showed signs of *C. difficile* infection. Two other positive horses became negative eight days after stopping cefquinome antibiotic treatment. This observation correlates with the results of numerous studies reporting antibiotic exposure as the most important risk factor for *C. difficile* colonization in humans, including clindamycin, cephalosporins or fluoroquinolones (Deshpande et al., 2013; Slimings and Riley, 2014). Nevertheless, in the present study no relation between *C. difficile* infections and clinical signs or medication could be obtained, since the group of positive horses was too small.

A wide variety of types were observed without the predominance of a particular PCR-ribotype. In most instances (six horses out 10), animals did not present any of the clinical signs classically associated with *C. difficile* infection, such as diarrhoea, abdominal pain, colic, nausea, depression or dehydration (Keel and Songer, 2006; Diab et al., 2013). This correlates with the findings of previous studies describing horses infected by *C. difficile* subclinically (Magdesian and Leutenegger, 2011; Schoster et al., 2012a; Schoster et al., 2012b). Moreover, it has been suggested that healthy or sick carriers without any signs of the *C. difficile* infection may harbour strains that do not produce toxins (Uzal et al., 2012). In the present study, three positive-tested horses were colonized with non-toxigenic *C. difficile* strains (PCR-ribotypes UCL9 and UCL261). Two of these horses showed no sign of enteric disease. Non-toxigenic *C. difficile* strains have been described in horses (Thean et al., 2011) and other farm animals including pigs and cattle (Pelaez et al., 2013; Rodriguez et al., 2013; Zidaric et al., 2012). Furthermore, the non-toxigenic PCR-ribotype UCL9 has been previously isolated from suckling piglets in Belgium (Rodriguez et al., 2012).

Resistance to more than one antimicrobial tested was found among the isolates obtained. Resistance of *C. difficile* to multiple antimicrobials has been previously described in several studies conducted in both humans and animals (Pelaez et al., 2013; Pirs et al., 2013; Zidaric et al., 2012; Weber et al., 2013). Furthermore, all of the isolates were resistant to ceftiofur and gentamicin, which is not surprising as these antibiotics are the most commonly used in the equine clinic. Even though gentamicin has been associated with *C. difficile* diarrhoea in horses (Diab et al., 2013), no relationship between treatment of animals with this medication and the appearance of soft or liquid bowel movements could be established. Furthermore, no association between antimicrobial resistance and toxigenic strains was observed, which correlates with the findings of a previous study (Pituch et al., 2005). Data observed suggested that antibiotics might have an important role in *C. difficile* colonization, but that the distribution of other enteric bacteria may be involved in the occurrence of diarrhoea. As previously suggested by Diab et al. (2013), metagenomic analysis of the gut microbiome is useful in improving understanding not only of *C. difficile* associated diarrhoea but also other etiologies of diarrhoea in horses.

Some studies have used MLST to discriminate human *C. difficile* strains, but only a few have applied this method to animal isolates (Lemée et al., 2004; Lemée and Pons, 2010; Stabler et al., 2012). To date, this study is the first to address genetic analysis of *C. difficile* by MLST in an equine hospital. We found a clear concordance between some PCR-ribotypes and ST as described in human isolates from hospitals (Dingle et al., 2011; Weber et al., 2013). Two horse isolates (both of them PCR-ribotype UCL5a) were included in ST 11. In the literature, ST 11 is correlated with PCR-ribotypes 078, 126 or 033 among others (Knetsch et al., 2012). Remarkably, all of them are binary toxin positive, as are our two strains PCR-ribotype UCL5a. For three strains, the allelic profile over the seven loci did not

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match with any number assigned to the unique allelic profiles available on the PubMLST database. Two of these isolates are closely related in the constructed neighbour-joining phylogenetic tree, and the allelic profile is the same. Nonetheless, results obtained do not allow us to establish any relationship between the acquisition of *C. difficile* in the hospital and a particular circulating strain, or between the presence of diarrhoea and one particular sequence type. The same observation has previously been reported in an epidemiological surveillance of *C. difficile* infection in a tertiary care hospital (Weber et al., 2013).

Our study has several limitations. Firstly, only 18 of 73 horses were monitored for the presence of *C. difficile* throughout their stay in the clinic because only animals whose admission was prolonged (over nine days) were monitored. In other cases, the animal's health status prevented monitoring. Consequently, some horses colonized during hospitalization may have been missed, and it was not possible to determine the duration of carriage for some other positive animals. Additionally, the small number of *C. difficile* positive horses made it impossible to conduct an epidemiological analysis. For this reason, care has to be taken when interpreting the results. For six positive horses, initial sampling could not be performed until five days after admission; we were therefore not able to determine if they were colonized during or before admission. Only two positive samples were detected directly without enrichment media, while in eight other cases detection required an enrichment step. This finding indicates that in most cases the spore load was low and the colonization may have been transient. Another limitation of the antibiotic resistance test is the need to use different criteria breakpoints when SFM criteria were not available, and with a different culture media than usually recommended. Additionally, the lack of sufficient reference strains in our laboratory only allowed us to identified one ribotype profile corresponding to an international collection number while the remaining 6 PCR-ribotypes were identified with an internal nomenclature.

In conclusion, the results of this study show that CDI is very rare in the studied equine clinic and that *C. difficile* colonization is transient by different toxigenic and non-toxigenic types, suggesting that an appropriate infection prevention strategy may reduce the associated disease.

Acknowledgements

References

Arroyo, L.G., Henry, R., Stampfli, H.R., Weese, J.S., 2006. Potential role of *Clostridium difficile* as a cause of duodenitis-proximal jejunitis in horses. J. Med. Microbiol. 55, 605–608.

Barbut, F., Petit, J.C., 2001. Epidemiology of *Clostridium difficile*-associated infections. Clin. Microbiol. Infect. 7, 405–410.

Barbut, F., Mastrantonio, P., Delmée, M., Brazier, J., Kuijper, E., Poxton, I., European Study Group of *Clostridium difficile* (ESGCD), 2007. Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. Clin. Microbiol. Infect. 13, 1048–1057.

Barr, B.S., Waldrige, B.M., Morresey, P.R., Reed, S.M., Clark, C., Belgrave, R., Donecker, J.M., Weigel, D.J., 2013. Antimicrobial-associated diarrhoea in three equine referral practices. Equine Vet. J. 45, 154–158.

Baverud, V., 2004. Clostridium difficile diarrhea: infection control in horses. Vet. Clin. Equine 20, 615–630.

Baverud, V., Gustafsson, A., Franklin, A., Aspan, A., Gunnarsson, A., 2003. *Clostridium difficile*: prevalence in horses and environment and antimicrobial susceptibility. Equine Vet. J. 35, 465–471.

Bengualid, V., Umesh, K.C., Alapati, J., Berger, J., 2011. *Clostridium difficile* at a community hospital in the Bronx, New York: incidence, prevalence and risk factors from 2006 to 2008. Am. J. Infect. Control 39, 183–187.

Bidet, P., Barbut, F., Lalande, V., Burghoffer, B., Petit, J.C., 1999. Development of a new PCR-ribotyping method based on ribosomal RNA gene sequencing. FEMS Microbiol. Lett. 175, 261–266.

Cattoir, V., Ould-Hocine, Z.F., Legrand, P., 2008. Antimicrobial susceptibility of *Clostridium difficile* clinical isolates collected from 2001 to 2007 in a French university hospital. Pathol. Biol. 56, 7–8.

Clabots, C.R., Johnson, S., Olson, M.M., Peterson, L.R., Gerding, D.N., 1992. Acquisition of *Clostridium difficile* by hospitalized patients: evidence for colonized new admissions as a source of infection. J. Infect. Dis. 166, 561–567.

CLSI, 2010. 20th Informational Supplement, vol. 30 No. 1. Clinical and Laboratory Standards Institute, Wayne, PA. USA, M100-S20.

Delmée, M., Vandercam, B., Avesani, V., Michaux, J.L., 1987. Epidemiology and prevention of *Clostridium difficile* in a leukaemia unit. Eur. J. Clin. Microbiol. 6, 623–627.

Delmée, M., Avesani, V., 1988. Correlation between serogroup and susceptibility to chloramphenicol clindamycin, erythromycin, rifampicin and tetracycline among 308 isolates of *Clostridium difficile*. J. Antimicrob. Chemother. 22, 325–331.

Deshpande, A., Pasupuleti, V., Thota, P., Pant, C., Rolston, D.D., Sferra, T.J., Hernandez, A.V., Donskey, C.J., 2013. Community-associated *Clostridium difficile* infection and antibiotics: a meta-analysis. J. Antimicrob. Chemother. 68, 1951–1961.

Diab, S.S., Songer, G., Uzal, F.A., 2013. *Clostridium difficile* infection in horses: a review. Vet. Microbiol. 167, 42–49.

Dingle, K.E., Griffiths, D., Didelot, X., Evans, J., Vaughan, A., Kachrimaidau, M., Stoesser, N., Jolley, K.T., Harding, R.M., Peto, T.E., Fawley, W., Walker, A.S., Wilcox, M., Crook, D.W., 2011. Clinical *Clostridium difficile*: clonality and pathogenicity locus diversity. Plos One 6, e19993.

Donaldson, M.M., Palmer, J.E., 1999. Prevalence of *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxin A in feces of horses with diarrhea and colic. J. Am. Vet. Med. Assoc. 215, 358–361.

Drummond, A.J., Ahston, B., Buxton, S., Cheung, M., Heled, J., Kearse, M., Moir, R., Stones-Avas, S., Thierer, T., Wilson, A., 2013. Geneious v4.8, Available from ⟨http://www.geneious.com⟩

Erikstrup, L.T., Danielsen, T.K.L., Hall, V., Olsen, K.E.P., Kristensen, B., Kahlmeter, G., Fuursted, K., Justesen, U.S., 2012. Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disk diffusion correlates. Clin. Microbiol. Infect. 18, 266–272.

European Committee on Antimicrobial Susceptibility testing clinical break-points (EUCAST). http://www.eucast.org/clinical_breakpoints.

Griffiths, D., Fawley, W., Kachrimanidou, M., Bowden, R., Crook, D.W., Fung, R., Golubchik, T., Harding, R.M., Jeffery, M., Jolley, K.A., Kirton, R., Peto, T.E., Rees, G., Stoesser, N., Vaughan, A., Walker, A.S., Young, B.C., Wilcox, M., Dingle, K.E., 2010. Multilocus sequence typing of *Clostridium difficile*. J. Clin. Microbiol. 48, 370–378.

Keel, M.K., Songer, J.G., 2006. The comparative pathology of *Clostridium difficile*-associated disease. Vet. Pathol. 43, 225–240.

Knetsch, C.W., Terveer, E.M., Lauber, C., Gorbalenya, A.E., Harmanus, C., Kuijper Ed, J., Corver, J., van Leeuwen, H.C., 2012. Comparative analysis of an expanded *Clostridium difficile* reference strain collection. Infect. Genet. Evol. 12, 1577–1585.

Magdesian, K.G., Leutenegger, C.M., 2011. Real-time PCR and typing of *Clostridium difficile* isolates colonizing mare–foal pairs. Vet. J. 190, 119–123.

Marie, J., Morvan, H., Berthelot-Hérault, F., Sanders, P., Kempf, I., Gautier-Bouchardon, A.V., Jouy, E., Kobisch, M., 2000. Antimicrobial susceptibility of *Streptococcus suis* isolated from swine in France and from humans in different countries between 1996 and 2000. J. Antimicrob. Chemother. 50, 201–209.

Medina-Torres, C.E., Weese, J.S., Staempfli, H.R., 2011. Prevalence of *Clostridium difficile* in horses. Vet. Microbiol. 152, 212–215.

Niwa, H., Kato, H., Hobo, S., Kinoshita, Y., Ueno, T., Katayama, Y., Hariu, K., Oku, K., Senoh, M., Kuroda, T., Nakai, K., 2013. Postoperative *Clostridium difficile* infection with PCR ribotype 078 strain identified at necropsy in five thoroughbred racehorses. Vet. Rec. 173, 607.

Lemée, L., Dhalluin, A., Pestel-Caron, M., Lemeland, J.F., Pons, J.L., 2004. Multilocus sequence typing of humans and animal *Clostridium difficile* isolates of various toxigenic types. J. Clin. Microbiol. 42, 2609–2617.

Lemée, L., Pons, J.L., 2010. Multilocus sequence typing for *Clostridium difficile*. Methods Mol. Biol. 646, 77–90.

Ozaqui, E., Kato, H., Kita, H., Karasawa, T., Maegawa, T., Koino, Y., Matsumoto, K., Takada, T., Nomoto, K., Tanaka, R., Nakamura, S., 2004. *Clostridium difficile* colonization in healthy adults: transient colonization and correlation with enterococcal colonization. J. Med. Microbiol. 53, 167–172.

Pituch, H., Obuch-Woszczatyriski, P., Wultanska, D., Meisel-Mikolajczyk, F., Luczak, M., 2005. A survey of metronidazole and vancomycin resistance in strains of Clostridium difficile isolated in Warsaw, Poland. Anaerobe 11, 197–199.

Pelaez, T., Alcala, L., Blanco, J.L., Alvarez-Perez, S., Marin, M., Martin-Lopez, A., Catalan, P., Reigadas, E., Garcia, M.E., Bouza, E., 2013. Characterization of swine isolates of *Clostridium difficile* in Spain: a potential source of epidemic multidrug resistant strains? Anaerobe 22, 45–49.

Pirs, T., Avbersek, J., Zdovc, I., Krt, B., Andlovic, A., Lejko-Zupanc, T., Rupnik, M., Ocepek, M., 2013. Antimicrobial susceptibility of animal and human isolates of *Clostridium difficile* by broth microdilution. J. Med. Microbiol. 62, 1478–1485.

Rodriguez, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., Daube, G., 2012. *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 18, 621–625.

Rodriguez, C., Avesani, V., Van Broeck, J., Taminiau, B., Delmée, M., Daube, G., 2013. Presence of *Clostridium difficile* pigs and cattle intestinal contents and carcass contamination at slaughterhouse in Belgium. Int. J. Food Microbiol. 166, 256–262.

Rodriguez-Palacios, A., Pickworth, C., Loerch, S., Lejeune, J.T., 2011. Transient fecal sheeding and limited animal-to-animal transmission of *Clostridium difficile* by naturally infected finishing feedlot cattle. Appl. Environ. Microbiol. 77, 3391–3397.

Rodriguez-Palacios, A., Borgmann, S., Kline, T.R., Lejeune, J.T., 2013. *Clostridium difficile* in foods and animals: history and measures to reduce exposure. Anim. Health Res. Rev. 14, 11–29.

Schoster, A., Staempfli, H.R., Arroyo, L.G., Reid-Smith, R.J., Janecko, N., Shewen, P.E., Weese, J.S., 2012a. Longitudinal study of *Clostridium difficile* and antimicrobial susceptibility of *Escherichia coli* in healthy horses in a community setting. Vet. Microbiol. 159, 364–370.

Schoster, A., Arroyo, L.G., Staempfli, H.R., Shewen, P.E., Weese, J.S., 2012b. Presence and molecular characterization of *Clostridium difficile* and *Clostridium perfringens* in intestinal compartments of healthy horses. BMC Vet. Res. 8, 94.

Slimings, C., Riley, T.V., 2014. Antibiotics and hospital aquired *Clostridium difficile* infection: update of systematic review and meta-analysis. J. Antimicrob. Chemother. 69, 881–891.

Stabler, R.A., Dawson, L.F., Valiente, E., Cairns, M.D., Martin, M.J., Donahue, E.H., Riley, T.V., Songer, J.G., Kuijper Ed, J., Dingle, K.E., Wren, B.W., 2012. Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations. PLoS One 7, e31559.

Thean, S., Elliott, B., Riley, T.V., 2011. *Clostridium difficile* in horses in Australia—a preliminary study. J. Med. Microbiol. 60, 1188–1192.

Uzal, F.A., Diab, S.S., Blanchard, P., Moore, J., Anthenill, L., Shahriar, F., Garcia, J.P., Songer, J.G., 2012. *Clostridium perfringens* type C and *Clostridium difficile* co-infection in foals. Vet. Microbiol. 156, 395–402.

Weber, I., Riera, E., Déniz, C., Pérez, J.L., Oliver, A., Mena, A., 2013. Molecular epidemiology and resistance profiles of *Clostridium difficile* in a tertiary care hospital in Spain. Int. J. Med. Microbiol. 303, 128–133.

Weese, J.S., Toxopeux, L., Arroyo, L., 2006. *Clostridium difficile* associated diarrhoea in horses within the community: predictors clinical presentation and outcome. Equine Vet. J. 38, 185–188.

Zidaric, V., Pardon, B., Dos Vultos, T., Deprez, P., Brouwer, M.S.M., Roberts, A.P., Henriques, A.O., Rupnik, M., 2012. Different antibiotic resistance and sporulation properties within multiclonal *Clostridium difficile* PCR Ribotypes 078 126 and 033 in a single calf farm. Appl. Environ. Microbiol. 78, 8515–8522.

3.1.2 Faecal microbiota characterisation of horses using 16 rDNA barcoded pyrosequencing, and carriage rate of *C. difficile* at hospital admission

Cristina Rodriguez, Bernard Taminiau, Bastien Brévers, Véronique Avesani, Johan Van Broeck, Aurélie Leroux, Marjorie Gallot, Antoine Bruwier, Hélène Amory, Michel Delmée, Georges Daube

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Faecal microbiota characterisation of horses using 16 rdna barcoded pyrosequencing, and carriage rate of clostridium difficile at hospital admission

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Abstract

Background: The equine faecal microbiota is very complex and remains largely unknown, while interspecies interactions have an important contribution to animal health. *Clostridium difficile* has been identified as an important cause of diarrhoea in horses. This study provides further information on the nature of the bacterial communities present in horses developing an episode of diarrhoea. The prevalence of *C. difficile* in hospitalised horses at the time of admission is also reported.

Results: Bacterial diversity of the gut microbiota in diarrhoea is lower than that in non-diarrhoeic horses in terms of species richness (p-value <0.002) and in population evenness (p-value: 0.02). Statistical differences for *Actinobacillus*, *Porphyromonas,* RC9 group*, Roseburia* and *Ruminococcaceae* were revealed. *Fusobacteria* was found in horses with diarrhoea but not in any of the horses with non-diarrheic faeces. In contrast, *Akkermansia* was among the three predominant taxa in all of the horses studied. The overall prevalence of *C. difficile* in the total samples of hospitalised horses at admission was 3.7 % (5/134), with five different PCR-ribotypes identified, including PCR-ribotype 014. Two colonised horses displayed a decreased bacterial species richness compared to the remaining subjects studied, which shared the same *Bacteroides* genus. However, none of the positive animals had diarrhoea at the moment of sampling.

Conclusions: The abundance of some taxa in the faecal microbiota of diarrhoeic horses can be a result of microbiome dysbiosis, and therefore a cause of intestinal disease, or some of these taxa may act as equine enteric pathogens. *Clostridium difficile* colonisation seems to be transient in all of the horses studied, without overgrowth to trigger infection. A large proportion of the sequences were unclassified, showing the complexity of horses' faecal microbiota.

Background

Equine gut microbiota is poorly characterised and studies are currently underway to increase understanding of how defined microbial communities are able to interfere with different bacterial aetiologies of diarrhoea in horses. Recently, high-throughput amplicon sequencing analysis has been introduced to investigate the intestinal microbiota of healthy horses and horses with colitis [1–3]. However, the equine faecal microbiome is still largely unknown, as

are interspecies interactions and their contribution to animal health [1, 3].

In horses, diarrhoea and colitis have been associated with a number of different pathogenic agents, including *Clostridium difficile, Clostridium perfringens, Salmonella* spp and *Escherichia coli* [4–7]. *Clostridium difficile* is amongst the most important agents of diarrhoea and serious colitis in horses [8]. While both adult horses and foals can suffer *C. difficile* enteric disease, it seems that foals are more likely to be colonised by the bacterium [9]. A diagnosis of *C. difficile* infection (CDI) requires clinical suspicion as well as detection of pre-formed *C. difficile* toxins TcdA and/or TcdB (or transcripts) in non-enriched specimens. Other possible causes of acute colitis must also be ruled out [8].

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However, in most cases of diarrhoea, the aetiology remains unclear and the prevalence of *C. difficile* colonisation in hospitalised horses has rarely been addressed [10, 11].

As in humans, the major risk factors for the development of CDI are antibiotic treatment and hospitalisation [8]. However, some cases of infection have been also reported in horses without previous exposure to these risk factors, including in foals at 2 to 5 days of age [12]. Furthermore, it has been reported that up to 7 % of horses carry spores of *C. difficile* without showing any signs of diarrhoea [10, 13].

The first objective of this study was to provide further information on the nature of the bacterial community present in horses developing diarrhoea, including possible alterations in the microbiota profiles as a result of antibiotic treatment, through comparison with faeces from horses without diarrhoea. This study also aimed to examine, by culture of horse faeces at admission to an Equine Clinic, the carriage rate of *C. difficile*. Isolates obtained were characterised in terms of PCR-ribotype, toxigenic activity and antibiotic resistance. Further metagenetic analyses were performed to compare the faecal microbiota of *C. difficile* in colonised and non-colonised horses.

Results

Bacterial community present in horses with and without diarrhoea

A group of 10 horses with diarrhoea at the moment of sampling were compared with 10 non-diarrhoeic horses via metagenetic analysis. All of the animals ($n = 20$) tested negative for *C. difficile* by faeces culture (Table 1). Pyrosequencing yielded between 4,000 and 5,000 reads per sample (Additional file 1). The microbiota composition for each horse is presented at phylum level (Fig. 1),

Table 1 Detailed information on twenty *C. difficile* negative horses studied via high-throughput amplicons sequencing analysis with and without diarrhoea

Clinical history of horses							
Date of sampling	Animal number	Age (years)	Diagnostic	Diarrhea	Hospital stay (days)	Antibiotic treatment (days) [1]	NSAIDs treatment
9/10/13	13	12	Colic	+	5	Pen-Gen (1)[2]	F-M
21/10/13	14	3	Diarrhoea	+	3	-	-
28/10/13	15	21	Diarrhoea and colic	+	2	SXT (2)	F-M
5/11/13	17	13	Equine atypical myopathy	+	1	-	-
18/11/13	23	9	Diarrhoea and colic	+	1	-	Dipyrone
18/11/13	25	2	Haemorrhagic enterocolitis Hyperlipemia	+	1	-	-
22/11/13	26	5	Diarrhoea and weight loss	+	1	Xnl (1)	F-M Dipyrone
26/11/13	27	6	Colic	+	2	-	F-M
26/11/13	28	11	Colic	+	4	Pen-Gen-LZ (1)[2]	F-M Dipyrone Firocoxib
19/12/13	29	2	Diarrhoea	+	2	-	-
8/10/13	11	11	Oesophageal obstruction	-	1	Pen-Gen-LZ (1)[2]	F-M
9/10/13	12	8	Horse fall	-	10	Xnl (7)[2]	Dipyrone
28/10/13	16	21	Wound	-	3	SXT (4)[2]	Dipyrone
7/11/13	18	8	Equine atypical myopathy	-	1	-	-
8/11/13	19	6	Colic	-	1	-	-
12/11/13	20	6	Colic	-	1	-	F-M
13/11/13	21	5	Equine atypical myopathy	-	1	-	-
18/11/13	22	3	Osteochondritis dissecans screening	-	1	-	-
18/11/13	24	9	Colic	-	2	Pen-Gen (2)[2]	F-M
19/12/13	30	2	Arthroscopy	-	3	-	-

NSAIDs nonsteroidal anti-inflamatory dugs
Pen penicillin, *Gen* gentamicin, *Xnl* ceftiofur, *SXT* trimethoprim/sulfamethoxazole, *LZ* metronidazole
F-M Flunixin meglumine
[1]Time before antibiotic administration in days [2]Antibiotic treatment in progress at the time of sampling

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Fig. 1 Microbiota faecal composition at phylum level for horses with and without diarrhoea. Bar chart detailing the relative abundance of the 17 core phylotypes common to the two groups of horses (with and without diarrhea)
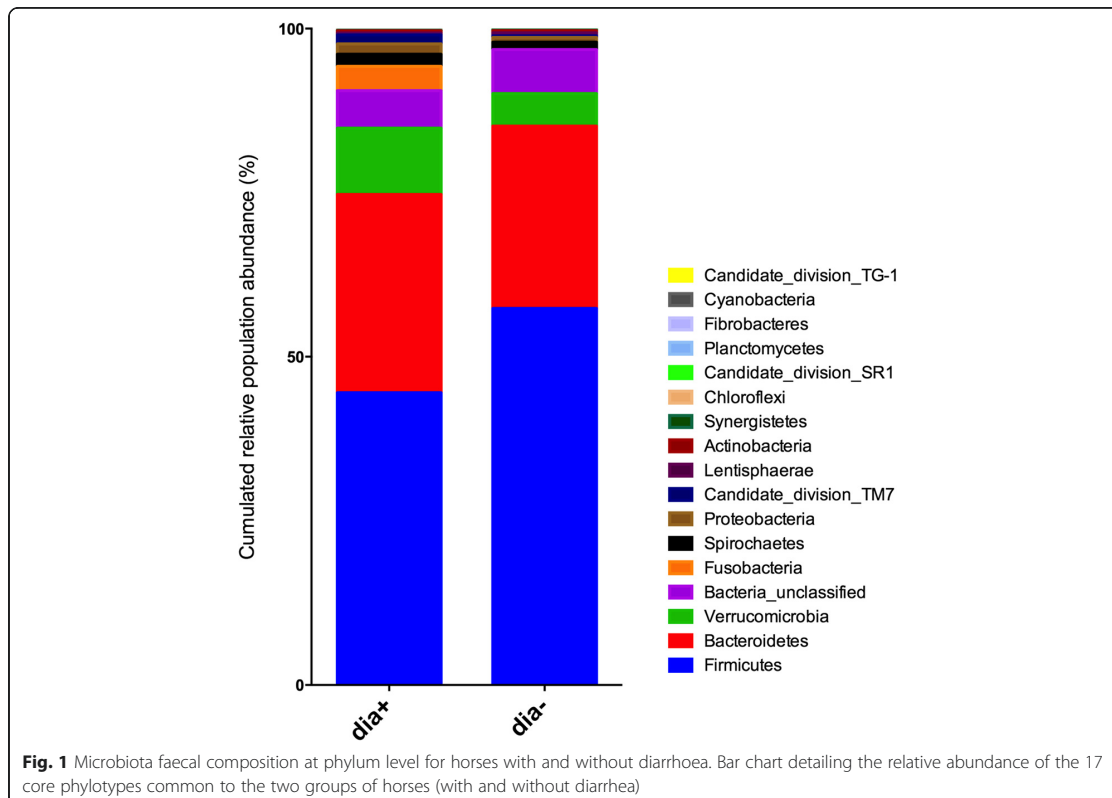
genus level (Fig. 2) and species level (Additional file 2). The more abundant bacterial families found for both groups were *Lachnospiraceae* (between 7 % and 39 %), *Ruminococcaceae* (between 2.7 % and 28.7 %), *Verrucomicrobiaceae* (between 0 % and 43.1 %), and *Prevotellaceae* (between 0.6 % and 17.5 %). Bacterial diversity of the gut microbiota in diarrhoea was lower than in non-diarrhoeic horses (p-value: 0.0105). This effect was observed both in terms of species richness and in the population evenness (Fig. 3). Principal coordinate analysis (PCoA) of the diarrhoeic and non-diarrhoeic horses did show the sample distribution along the 3 main axes (PC1-PC2-PC3) (Additional file 3). Analysis of Molecular Variance (AMOVA) revealed a significant difference between the variance of both groups taken as a single group and the variance of each group (sum of squares (ss): 0.53 and 11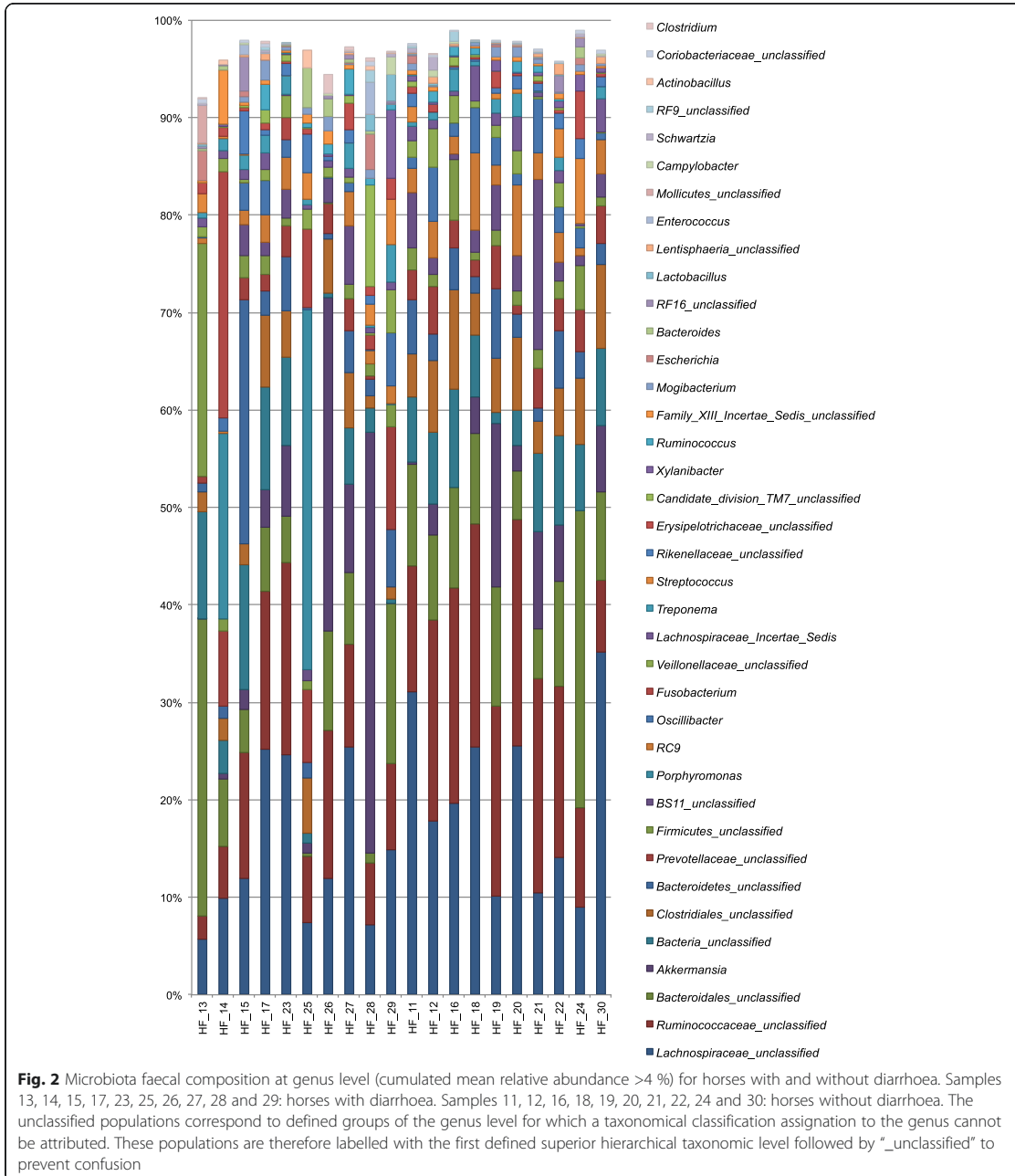.66 among and within groups respectively; AMOVA test statistic Fs: 1.57; p-value: 0.006). Unifrac weighted analysis further showed that both groups share different population structure (Wscore: 0.88 and p-value: <0.001). Thus, the microbiota structure of each group was showed statistically different from each other. The relative abundance of each genus in both groups were compared in order to identify the populations responsible for this difference

(White *t*-test), leading to statistical differences for *Actinobacillus*, *Porphyromonas,* RC9 gut group, *Roseburia* and a taxonomically undefined population belonging to the *Ruminococcaceae* (*Ruminococcaceae* unclassified) (Fig. 4).

Clostridium difficile prevalence in horses at admission and strain characterisation

During the three-month study period, the total number of horses admitted at the clinic for either emergencies or consultation was 302, with 141 hospitalisations. A total of 136 samples were collected from the 134 hospitalised horses enrolled in the study. Two horses were sampled on two different times because they suffered a diarrhoeal episode during hospitalisation. However, these horses did not test positive for *C. difficile* on any of the sample days. The overall prevalence of *C. difficile* in the faecal microbiota of hospitalised horses at admission was 3.7 % (5/134). All horses testing positive were adult animals aged between four and 16 years old.

Altogether, 52 of the total of 134 horses studied (38.8 %) presented gastrointestinal problems at admission, but *C. difficile* was isolated from only three of these animals (two with colic and one with proximal enteritis). In these three horses a nasogastric tube had been passed before the faecal

Fig. 2 Microbiota faecal composition at genus level (cumulated mean relative abundance >4 %) for horses with and without diarrhoea. Samples 13, 14, 15, 17, 23, 25, 26, 27, 28 and 29: horses with diarrhoea. Samples 11, 12, 16, 18, 19, 20, 21, 22, 24 and 30: horses without diarrhoea. The unclassified populations correspond to defined groups of the genus level for which a taxonomical classification assignation to the genus cannot be attributed. These populations are therefore labelled with the first defined superior hierarchical taxonomic level followed by "_unclassified" to prevent confusion

collection. However, none of them had previously received an antibiotic treatment. Nineteen horses (14.2 %) had diarrhoea at admission but all tested negative for the bacterium (Additional File 4).

The remaining 82 horses sampled (61.2 %) were not affected by gastrointestinal disorders. In this group, *C. difficile* was detected in two horses. The clinical diagnoses in these two infected horses were multiple wounds and wound with tendon injury, respectively. Both had received a similar antibiotic treatment (gentamicin and penicillin with or without ceftiofur) and non-steroidal anti-inflammatory drugs (dypirone). Age (9 years) and the gender (female)

Fig. 3 Bacterial biodiversity, bacterial richness and bacterial evenness in *C. difficile* negative horses with and without diarrhoea. Box plot of richness, evenness and diversity values showed that the microbiota structure of each group (diarrhoeic and non diarrhoeic horses) is statistically different from each other. Whiskers represent minimum and maximum value. Bottom and top of the box are the first and the third quartile. The median is shown as a band inside the box

Fig. 4 Bacterial genus whose relative abundance was statistically different between the 2 groups. Result of a White test (p value <0.05). Box plot showing mean relative sequence abundance of *Actinobacillus, Porphyromonas*, RC9, *Roseburia* and *Ruminococcaceae_unclassified* in horses with and without diarrhea. The error bar indicates the diversity between samples in terms of proportions of sequences

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of the two horses were also the same (Table 2). In terms of antimicrobial therapy, 54 out of 134 horses studied (40.3 %) had previously received antimicrobial therapy (prior to the study period), but most ($n = 52$) tested negative for *C. difficile*. Among them, the most common drug used was ceftiofur, which was administered to 16 (11.9 %) horses.

Four of the equine isolates were positive for *tcdA, tcdB* and binary toxin CDT genes while only one was non-toxinogenic. The presence of TcdB was confirmed by cytotoxicity assay using confluent monolayer MRC-5 cells. None of the isolates presented an 18, 39-base pair deletion or a deletion at 117 of the *tcdC* gene. Five different PCR-ribotypes were detected. Only one strain had a ribotype profile associated with the reference Cardiff collection number (014). The remaining isolates were identified as UCL237, UCL49, UCL23f and UCL36 (non-toxigenic PCR-ribotype). PCR-ribotypes UCL49, 014 and UCL23f were isolated from three animals with gastrointestinal problems while PCR-ribotypes UCL237 and UCL36 were recovered from the two horses with wounds (Table 2).

Only the non-toxigenic strain PCR-ribotype UCL36 showed resistance to metronidazole (minimum inhibitory concentration (MIC) = 40 μg/ml, average of two essays) and erythromycin. For clindamycin, only one isolate (PCR-ribotype 014) was susceptible, while all others were fully resistant. Intermediate resistance for penicillin was observed in all of the isolates tested. The isolate PCR-ribotype UCL237 also exhibited intermediate resistance to tetracycline, while all the rest were susceptible. There was no vancomycin, moxifloxacin or rifampicin resistance detected, but all the strains were resistant to ceftiofur (Table 2).

Microbiota composition for *C. difficile* positive and negative horses

Stool samples from all horses testing positive for *C. difficile* ($n = 5$) were studied in order to obtain further information about the microbiota composition of the colonised subjects. As during the entire study period (3 months) only five animals were positive for the bacterium, we could only use five *C. difficile*-negative horses as control group, but with similar clinical history (Additional file 5). In both *C. difficile* colonised and non-colonised horses, the dominant taxa were *Lachnospiraceae* (ranging from 3.2 to 20.8 %), *Bacteroidales* (ranging from 5 to 29.0 %) and *Ruminococcaceae* (ranging from 7 to 17.9 %). In the group of *C. difficile* positive horses, only one animal (10) presented a predominance of *Bacteroides* (36.8 %) and *Akkermansia* (19.5 %). The same *Bacteroides* genus was found in another *C. difficile* positive sample (01) at a level of 7.2 % (Fig. 1). Only 45 distinct OTUs (with a mean abundance greater than 4 %) were identified, representing 25-40 % of the relative abundance of the microbial taxa in the faecal samples (Fig. 5 and Additional file 6).

However, bacterial biodiversity, bacterial richness and bacterial evenness were not statistically different between *C. difficile* colonised and non-colonised horses (p-value > 0.05). Ordination analysis with PCoA supported the grouping of most of the individuals into one group (Additional file 7), which confirmed by non significant results from AMOVA (sum of squares (ss) 0.41 and 3.18 among and within groups respectively; test statistic for AMOVA (Fs) 1.03; p-value: 0.389) and Unifrac analysis of sample clustering (Wscore: 0.97 and p-value: 0.92). Indeed, White test abundance population comparison between groups performed at the different taxonomical levels identified two taxa (RF16 group and *Clostridiales*) for which the relative abundance was statistically different (Additional file 8).

Microbiota composition relation with gastro-intestinal disorder diagnostics

We grouped the microbiota profiling data from the 20 samples from diarrhoeic and non diarrhoeic samples with the 10 *C. difficile* positive and negative samples together and assigned them to diagnosis categories: Colic ($n = 12$), Enteritis ($n = 1$), Enterocolitis ($n = 1$), diarrhoea ($n = 3$) and others (13). We used this clustering in order to identify genus whose abundance could be related to one particular category. Statistical abundance comparison with ANOVA2 between colic, diarrhoea and other categories highlight a higher abundance of *Escherichia* and *Streptococcus* genera in colic group compared to the others and higher abundance of *Akkermansia*, *Fusobacterium*, *Porphyromonas* and *Xylanibacter* genera in the Diarrhoea group (Additional file 9). Enteritis and Enterocolitis group, having only one sample could not be included in statistical abundance comparison but each sample was dominated by 2 defined genus: *Bacteroides* and *Parabacteroides* in the Enteritis sample and *Porphyromonas* and *Fusobacterium* Enterocolitis sample (Additional file 10).

Discussion

High-throughput amplicon sequencing analysis is one of the methods of choice in the study of complex gut microbiota ecosystems [14]. However, most of the studies reported bacteria populations at the phylum and class level while the genus and species level were explored only in a few recent studies [15, 16]. Higher taxonomic resolutions (genus or species level) may reveal more differences in population structure than phylum or class level [17] and provide the degree of precision necessary for clinical diagnosis [18]. As previously demonstrated, selecting the accurate region of 16S ribosomal DNA (rDNA) gene to sequence is essential in determining the utility of microbial genomics for species-level assignments [19]. In the present study we report genus and species labelling based on V1-V3 region.

Table 2 Detailed information on _C. difficile_ positive horses, including molecular characterization and antibiotic resistance of the isolates

C. difficile positive horses

Date of sampling	Animal identification	Age (years)	Diagnostic	Diarrhoea	Hospital stay (days)	Antibiotic treatment (days)[e]	AINS treatment	Isolates characterisation												
								PCR-ribotype	CE	tcdA tcdB	cdtA cdtB	tcdC[a]	gyrA[b]	MUT	PEN	CC	TE	VA MXF RA	LZE	XNL
15/10/2013	01	9	Wound and tendon injury	-	1	Pen-Gen-Xnl (1)[f]	Dipyrone	UCL237	+	+	-	-		I		R	I	S	S	R
05/11/2013	03	16	Colic[c]	-	1	-	Flunixin meglumine	UCL49	+	+	-	-		I		R	S	S	S	R
12/11/2013	04	4	Colic[d]	-	2	-	-	014	+	+	-	-		I		S	S	S	S	R
03/12/2013	09	9	Multiple wounds	-	4	Pen-Gen (5)[f]	Dipyrone	UCL36	-	-	-	-		I		R	S	S	R	R
09/12/2013	10	4	Proximal enteritis	-	4	-	Firocoxib Meloxidyl Dipyrone	UCL23f	+	+	-	-		I		R	S	S	S	R

MUT mutation
_CE_ cytotoxicity assay using confluent monolayer MRC-5 cells
Gen gentamicin, _Pen_ penicillin, _CC_ clindamycin, _TE_ tetracycline, _VA_ vancomycin, _MXF_ moxifloxacin, _RA_ rifampicin, _LZ_ metronidazole, _E_ erythromycin, _XNL_ ceftiofur
_I_ intermediate antimicrobial resistance
[a]Presence of deletions in the regulator gene _tcdC_ (118 bp-39 bp-17 bp)
[b]Presence of mutation in the _gyrA_ gene associated with moxifloxacin resistance
[c]Colic secondary to a gaseous distension of the caecum and a retraction of the colon
[d]Colic secondary to a pelvic flexure impaction (suspicion of a digestive tract verminosis causing weight loss)
[e]Time before antibiotic administration in days [f]Antibiotic treatment in progress at the time of sampling

Fig. 5 Microbiota faecal composition at genus level (cumulated mean relative abundance >4 %) of *C. difficile* culture-positive and -negative horses. Samples HF_01, 03, 04, 09 and 10: *C. difficile* positive horses detected by faeces culture. Samples HF_02, 05, 06, 07 and 08: *C. difficile* negative horses detected by faeces culture
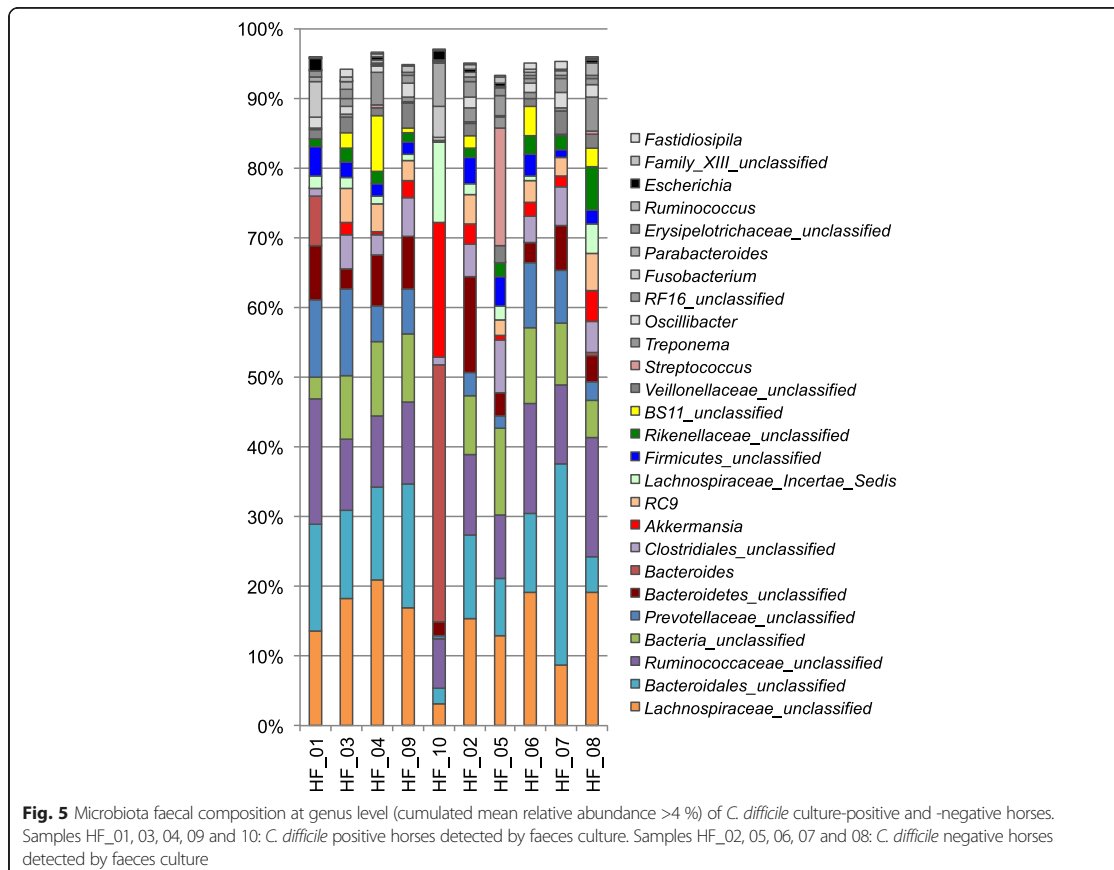
To determine whether the presence of diarrhoea was related to changes in the composition of the faecal microbiota of horses, a strict screening process was carried out among all the samples obtained over three months, in order that the two groups (with and without diarrhoea) were as similar as possible and therefore comparable. As observed in a previous study investigating the microbiota in the equine large intestine via 16S ribosomal DNA sequencing [1], a great part of the sequences (60 %) were not identical (less than 1 % mismatch) to sequence entries present in SILVA database (v1.15). Even among the sequences identical to known entries, the species name was seldom taxonomically defined. These findings underline the lack of knowledge regarding a good part of the horse gut microbiota stressing the need for further research on fundamental microbiology either on taxonomic as well on the functional level.

Interestingly, *Akkermansia* was found in 90 % of horses studied, with a relative abundance ranging between 0.03 % and 43.1 %. This bacterium is an appealing candidate to become a human probiotic, selected based on established mechanisms of preventative treatment of obesity and diabetes [20–22]. Only one previous study [23] describes the genus *Akkermansia* in the equine intestinal microbiota In this study we reported the presence of *Akkermansia muciniphila* and *Akkermansia EU779370* in the faecal microbiota of horses with and without diarrhoea. *Akkermansia EU779370* population identified in this study is 100 % identical to the *Akkermansia EU779370* GenBank entry (for the sequenced V1-V3 section). It represents a potential new *Akkermansia* species as it shares only 90 % of nucleotide identity with *Akkermansia muciniphila.* The relevance of this finding deserves further investigation.

Overall, the composition of the microbiota of all horses studied was dominated by the same taxa as previously described [4]. However, the differences observed in the cumulative mean relative abundance among individuals in these dominant bacterial groups may be linked to recent dietary history [17] or to antimicrobial therapy [23]. Diarrhoea has been associated with changes in the faecal microbiota composition of humans but alterations in the equine gut microbiota has been rarely addressed [24]. In the present study,

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Actinobacillus (0.3 % mean abundance in diarrhoeic group versus 0.004 in non-diarrhoeic group) and *Porphyromonas* taxa (5.6 % mean abundance in diarrheic group versus 0.002 in non-diarrhoeic group) were detected more abundantly in horses with diarrhoea. These results contrast with a previous study of commensal bacteria in acute diarrhoea in children, where *Porphyromonas* species were in the lowest proportions during acute diarrhoea compared with levels during periods of normal gastrointestinal health [25]. *Fusobacteria* were found in horses with diarrhoea but not in any of the horses with normal faeces. In a previous study, *Fusobacterium* spp. was also found in higher percentages in horses with colitis, which could be a consequence of overgrowth due to bacterial dysbiosis or an aetiological agent of disease [4]. In human beings, *Fusobacteria* have been associated with colorectal carcinomas and adenomas [26]. However, there are no previous studies describing this bacterium as an equine enteropathogen [4].

After clustering of the different feces samples into more defined diagnosis categories, genus *Escherichia* and *Streptococcus* were more abundant in horses with diarrhoea without any other symptoms. A more refined analysis revealed that the species involved were *Escherichia coli* and *Streptococcus equinus*. If the first is long known to be associated with diarrhoea, the second is the most common *Streptococcus* found in horse feces. In the colic group, *Fusobacterium* and *Porphyromonas* genera were found in higher amount compared to other groups but were composed of yet unknown species. As mentioned above, little is known regarding the involvement of these bacteria in gastro-intestinal disorders. Finally, two horses suffered from enteritis and enterocolitis and their 16S profiling revealed a domination (above 30 % of the sample sequences) of genus *Bacteroides* in the enteritis case and genus *Porphyromonas* in the enterocolitis case. The *Bacteroides* population was mainly represented by *Bacteroides heparinolyticus*. This species, originally characterised as an agent of periodontitis in human [27], is phylogenetically related to *Bacteroides fragilis* which is well-known enterotoxinogenic bacteria involved in human infections [28]. However, there is still no evidence of *B. heparinolyticus* involvement in gut disorder in horse or in human and it is thus unclear whether its abundance is related to the symptomatology.

We are aware that the limited size of the analysed cohort reduces the strength and the scope of our results. Larger cohort studies will be needed to improve our knowledge on diarrhoea impact on horse microbiota.

In the present study, the carriage of *C. difficile* at the time of admission was examined. The overall prevalence found was 3.7 % (5/134). None of the positive animals had diarrhoea at the moment of sampling which may suggest that *C. difficile* colonisation in these horses was transient in most cases. In the literature, there is only one previous study that investigated the presence of *C. difficile* in horses

at admission to a veterinary teaching hospital [11]. Our results correlate with the findings of this study, which reported a prevalence of 4.8 % (4/82). In another previous study conducted at the same Belgian Equine Clinic, we observed a *C. difficile* colonisation rate of 13.7 % (10/73). However, in that study, animals were not only sampled at the time of admission but also tracked during their hospitalisation, which could explain the higher prevalence found [10].

From the five *C. difficile* positive horses found, three of them presented gastrointestinal problems (colic and proximal enteritis) with a nasogastric tube passed before sampling. Nasogastric tube placement has been previously identified as a risk factor for *C. difficile* infection [11]. Two other horses without enteric perturbations were also colonised by *C. difficile*. Both had suffered wounds and were treated with antibiotics. Previous studies reported intestinal flora perturbations and antibiotic exposure as the most significant risk factors for *C. difficile* proliferation in horses [5].

There were five different PCR-ribotypes detected among the five *C. difficile* positive animals, which suggests that a wide variety of *C. difficile* strains circulate in horses, as previously reported [11, 29], including PCR-ribotype 014. All the isolates were resistant to ceftiofur and four out of five were also resistant to clindamycin, which agrees with the findings of previous studies [10, 30]. Only one isolate was resistant to metronidazole and erythromycin. Surprisingly, this strain (PCR-ribotype UCL36) was the only non-toxigenic isolate. A high degree of resistance to antimicrobials (including erythromycin and clindamycin) in non-toxigenic strains has been reported previously [30] but the role in disease development or prevention is still unknown [31].

We were unable to identify *C. difficile* by pyrosequencing analysis in the stool samples with positive cultures. In humans, it is considered that feces harbour up to 10^{12} bacteria per gram [32]. Thus, our sampling of thousands of sequences limits our detection ability to populations above 10^9 bacteria per gram. The horses testing positive by culture did not have any clinical signs of *C. difficile* disease and the isolate was obtained only after three days of enrichment. While the results of high-throughput amplicon sequencing analysis are limited by the small number of animals positive for *C. difficile*, and by the fact that none of the animals suffered CDI, the findings for each colonised horse should not be dismissed as they provide a first insight, albeit limited, about the impact of *C. difficile* colonisation in the horses' gut microbiota, which merits further investigation.

Conclusions

Metagenomic analysis is a promising tool to identify correlations between changes in the gut microbiota and intestinal

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diseases. The abundance of *Actinobacillus, Porphyromonas* and *Fusobacteria* in the faecal microbiota of diarrhoeic horses deserves special attention, as it can be a result of microbiome dysbiosis, and therefore a cause of intestinal disease, or in the case of *Fusobacteria*, may act as equine enteric pathogen. Furthermore, the high proportion of *Akkermansia* in all of the horses studied and its role in the intestine merits further investigation. *Clostridium difficile* colonisation seems to be transient in all of the horses studied without overgrowth to trigger infection. For a great variety of bacterial species the currently available systems are not able to confidently assign taxonomy, which shows how complex and still unknown the equine microbiome is.

Methods
Inclusion criteria and sampling
Samples were collected over a three month period (October to December 2013) at the Equine Clinic, Department of Companion Animals and Equids, Faculty of Veterinary Medicine, University of Liege.

All hospitalised horses during this period with a clinic stay of at least one day were eligible. Subjects were all selected without regard to their diagnosis or the possible duration of hospitalisation. The exclusion criteria were horses exhibiting dysphoric mood, intolerable stress, or any other disease for which sampling by rectal manipulations was not recommended. Samples were collected between day one and day two following admission. In addition, all horses developing an episode of diarrhoea during their hospital stay were sampled for a second time. Horses were documented for data relating to clinical history, diagnostic findings and treatment received, including the prescription of antimicrobial agents. Faecal sampling was performed directly via rectal. Samples obtained were scored as normal faeces, diarrhoea or bloody diarrhoea faeces. All samples were processed on the same day immediately after transport (at room temperature) to the laboratory (approximately 15 min after sampling). In cases of emergency admission (i.e., during the night or on a non-working day), two samples per horse were collected. The first sample was collected in an individual identified sterile 50 ml tube for further culture to detect *C. difficile*; the second was collected using the Stool DNA stabiliser (PSP^R Spin Stool DNA Plus Kit 00310, Invitek) and stored at 4 °C in the hospital for a maximum of three days before processing. After culture of faeces, all samples were frozen immediately at –80 °C before DNA extraction.

16S rDNA pyrosequencing and data analysis
Among all the faecal samples collected, clinical history of each subject was investigated in order to select two homogenous groups of horses (with and without diarrhoea) with the same number of individuals in each group. Both groups were matched for age, pathologies, previous hospital stay and medical treatment. A total of 20 faecal samples (ten with diarrhoea and ten without diarrhoea) were further studied. A second selection was done on the pool of 140 horses to gather 2 homogenous groups of horses, either or not positive for *C. difficile* by classical microbiology ($n = 5$), which were matched for age, pathologies, previous hospital stay and medical treatment. Finally, the 30 samples were grouped together in a third analysis and clustered into defined diagnostic category based upon diagnostic (Table 1; Table 2; Additional file 5). The resulting diagnostic categories are: colic—horses suffering from abdominal pain ($n = 12$); diarrhoea—horses with 3 or more loose or liquid stools per day, without other symptom ($n = 3$); enteritis—horse with ileon inflammation ($n = 1$); enterocolitis—horse with ileon and colon inflammation ($n = 1$) and others—horses with non Gastro-intestinal disorders ($n = 13$).

Total DNA was extracted from the stool samples with the PSP^R Spin Stool DNA Plus Kit 00310 (Invitek), following the manufacturer's recommendations. The DNA was eluted into DNase/RNase-free water and its concentration and purity were evaluated by absorbance measurement using the NanoDrop ND-1000 spectrophotometer (NanoDrop ND-1000, Isogen). PCR-amplification of the V1-V3 region of the 16S rDNA was performed as previously described [33]. Primers E9-29 and E514-530 [33–35] were selected for their theoretical ability to generate the lowest possible amplification capability bias among the various bacterial phyla [36]. The oligonucleotide design included 454 Life Sciences' A or B sequencing titanium adapters (Roche Diagnostics) and multiplex identifiers (MIDs) fused to the 5′ end of each primer. The master mix composition consisted of 5 units of FastStart high fidelity polymerase (Roche Diagnostics), 1x enzyme reaction buffer, 200 μM dNTPs (Eurogentec), 0.2 μM of each primer and 100 ng of genomic DNA in a volume of 100 μl. The amplification was carried out in a gradient thermocycler (Eppendorf) as follows: denaturation at 94 °C for 15 min followed by 25 cycles of 94 °C for 40 s, 56 °C for 40 s; 72 °C for 1 min; and a final elongation step at 72 °C for 7 min. PCR products were run on 1 % agarose gel electrophoresis and purified using the SV PCR purification kit (Promega Benelux). Picogreen dsDNA quantitation assay (Isogen) was performed in order to assess the quality and quantity of the products. All libraries were run in the same titanium pyrosequencing reaction using Roche multiplex identifiers, and amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche).

Sequence reads were processed using MOTHUR software package v1.32 [37] and denoised using the Pyronoise algorithm [38]. Trimming criteria of the reads was applied as follows: read lengths no shorter than 425 bp, an exact match to the barcode, and 1 mismatch allowed to the proximal primer [33]. Sequences were checked for

the presence of chimeric amplifications using the UCHIME algorithm [39].

The read sets obtained were compared with a reference data set of aligned sequences of the corresponding region derived from the SILVA database (v1.15) of full-length rDNA sequences [40] implemented in MOTHUR [41]. The final reads were clustered into operational taxonomic units (OTUs) using the nearest MOTHUR neighbour algorithm with a 0.03 distance unit cut-off. Taxonomic identity was attributed to each OTU by comparison with the SILVA v1.15 database [37] (80 % homogeneity cut-off) [33]. When taxonomic identification fell below the 80 % treshold, the taxonomic level was labelled with the first defined level from higher level followed by the term "_unclassified".

All unique sequences for each OTU were further compared with the SILVA data set version v1.15 using the BLASTN algorithm [42, 43], as MOTHUR is not suitable to taxonomic assignment beyond the genus level. For each OTU, a consensus detailed taxonomic identification was given based upon the identity (less than 1 % of mismatch with the aligned sequence) and the metadata associated with the most frequent hits leading to 3 kind of labelling : (i) the population is identical to a taxonomically defined species and is labelled "genus_species"; (ii) the population is identical to a reference sequence belonging to a still undefined species and is labelled "genus_NCBI Accession Number"; (iii) the sequence is not identical to any known sequence and is arbitrarily labelled with its OTU number [33].

Subsample datasets were obtained and used to evaluate ecological indicators (the richness and microbial diversity of the samples) using MOTHUR. Population structure and community membership were assessed with MOTHUR using distance matrices based on the Jaccard index (a measure of community membership; which considers the number of shared OTUs but not their abundance) and the Yue and Clayton measure of dissimilarity (a measure of community structure which considers shared OTUs and their relative abundances) [44]. Richness estimation (Chao1 estimator) [45], microbial biodiversity (non-parametric (NP) Shannon diversity index) [46], and the population evenness (Shannon evenness) [47] were calculated using MOTHUR. Chao 1 estimator was used to estimate the richness of the detected species (OTUs) in a sample [33].

Ordination and statistical analysis and biosample accession numbers

Ordination analysis were performed with Vegan package in R [48].

Principal coordinate analysis (PCoA) was applied to visualise the biodiversity between the two groups [49]. Statistical analysis regarding community structure and composition were performed with AMOVA and UNIFRAC

implemented in MOTHUR v1.32. Analysis of molecular variance (AMOVA) was used for estimating population differentiation [50]. Unifrac unweighted analysis, which accounts for the relative abundance of each of the taxa within communities, was used to evaluate differences in population structure between pairs of sample categories [51]. The differences were considered significant for a p-value of less than 0.05; all the results given are the means ± the standard deviations of the results between the samples of each category [33]. Statistical differences between bacterial biodiversity, richness and evenness were assessed using two-sided unpaired *t*-test using PRISM 6 (Graphpad Software). In order to highlight statistical differences in the bacterial population abundance between categories, a White tests (paired comparisons) and ANOVA with Tukey post-hoc test were performed using STAMP software [52]. All the biosample sequences have been deposited at the National Center for Biotechnology Information (NCBI) [53] and are available under de Bioproject ID PRJNA279335.

Clostridium difficile culture, identification and characterisation

For isolation of *C. difficile*, one gram of faeces was inoculated into 9 ml of CCFT (cycloserine cefoxitin fructose taurocholate) broth as previously described [54] and incubated anaerobically for 72 h at 37 °C. A 10 μl aliquot of the enriched broth was spread on CCFT plates and incubated anaerobically at 37 °C for three days. One presumptive colony per plate was subcultured onto blood agar 5 % Sheep Blood (Biorad) and checked using a *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid). Identification, toxin gene profile, deletions in the regulator gene *tcdC*, and *gyrA* mutation (gene associated with moxifloxacin resistance) were determined using the Genotype Cdiff system (Hain Lifescience) according to the manufacturer's instructions. The supernatant from each pure culture was tested for cytotoxicity assay (TcdB) using confluent monolayer MRC-5 cells, as previously described [54]. A PCR ribotyping method based on capillary gel was performed using the primers recommended by Bidet et al. [55]. The isolates with a PCR-ribotype profile from which reference strains were available in our laboratory were designated with the relevant Cardiff international number. Otherwise, isolates were identified with an internal nomenclature beginning with UCL.

Antibiotic resistance

Clostridium difficile isolates (*n* = 5) were tested for susceptibilities to a panel of nine antimicrobial agents by disc diffusion (*n* = 7) and E-test (*n* = 2). The antimicrobials studied were chosen because they have been associated with *C. difficile* infection or its treatment, or

because they were widely used in the equine teaching hospital used in the study.

Resistance to rifampin (25 µg), erythromycin (15 µg), oxitetracycline (30 µg), vancomycin (30 µg), penicillin (10 µg), clindamycin (2 µg), and ceftiofur (30 µg) (Becton-Dickinson) was tested by disc diffusion assay on Brucella Blood Agar with hemin and vitamin K1 (Becton-Dickinson) according to the French Society of Microbiology [56] protocols. Zone diameters were read after 24 h of anaerobic incubation at 37 °C and interpreted as previously described [10].

Susceptibility to metronidazole and moxifloxacin was determined using the Etest method (Lucron ELITechGroup) on Schaedler with Vit K1 and 5 % sheep blood (Becton-Dickinson) according to the manufacturer's instructions. Plates were incubated anaerobically at 37 °C for 48 h. The susceptibility and resistance breakpoints for metronidazole (s ≤8 µg/ml; r ≥32 µg/ml) and moxifloxacin (s ≤ 2 µg/ml; r ≥8 µg/ml) used for interpretation were those recommended by the Clinical and Laboratory Standard Institute [57]. *Bacteroides fragilis* ATCC 25285 was tested as a quality control.

Metagenetic analysis

Rectal faecal samples of all animals positive for *C. difficile* ($n = 5$) were analysed by 16S rDNA pyrosequencing (as described above) in order to recover further information about the microbiota composition of horses colonised by the bacterium. A further group of non-colonised horses ($n = 5$) was used as a control. Control subjects were selected on basis of their similarity to colonised horses, including same clinical history (pathologies and antimicrobial treatment), age and previous hospital stay, to obtain two groups as similar as possible.

Ethics

This study required no experimental research on animals, only the use of collected feces. Therefore, this study did not require approval from Animal Ethics Committee following Belgian (royal decree M.B.10.07.2013) and European legislation (2010/63/UE).

Additional files

Additional file 1: Quality analysis of the metagenetic libraries created for the horse faecal samples analysed. (DOCX 35 kb)

Additional file 2: Microbiota faecal composition at species level (cumulative mean relative abundance >4 %) of horses with and without diarrhoea. Samples 13, 14, 15, 17, 23, 25, 26, 27, 28 and 29: horses with diarrhoea. Samples 11, 12, 16, 18, 19, 20, 21, 22, 24 and 30: horses without diarrhoea. (DOCX 146 kb)

Additional file 3: Principal coordinate analysis of the diarrhoeic and non-diarrhoeic horses. The 3 graphs show the sample distribution along the 3 main axes (PC1-PC2-PC3). (DOCX 83 kb)

Additional file 4: Clinical history comparison between *C. difficile* colonised and non-colonised horses. [a]Antimicrobial treatment of two or more antibiotics. [b]Including single or combined antimicrobial therapy (DOCX 63 kb)

Additional file 5: Detailed information on five *C. difficile* negative horses studied via high-throughput amplicon sequencing analysis and compared with *C. difficile* colonised horses. NSAIDs: nonsteroidal anti-inflammatory drugs. Pen: penicillin; Gen: gentamicin; SXT: trimethoprim/sulfamethoxazole (DOCX 46 kb)

Additional file 6: Microbiota faecal composition at species level (cumulative mean relative abundance >4 %) of *C. difficile* culture-positive and -negative horses. Samples 01, 03, 04, 09 and 10: *C. difficile* positive horses detected by faeces culture. Samples 02, 05, 06, 07 and 08: *C. difficile* negative horses detected by faeces culture. (DOCX 161 kb)

Additional file 7: Principal coordinate analysis of *C. difficile* positive and negative horses. The 3 graphs show the sample distribution along the 3 main axes (PC1-PC2-PC3). (DOCX 73 kb)

Additional file 8: Bacterial genus whose relative abundance was statistically different between *C. difficile* positive and negative horses. Result of a White test (p value <0.05) pairwise comparison. (DOCX 95 kb)

Additional file 9: Bacterial genus whose relative abundance was statistically different between Colic, diarrhoea and Others diagnosis groups. The graph illustrates the mean relative abundance for the selected genera. Groups were compared by ANOVA 2 analysis with post-hoc Tukey-kramer multiple comparison. The inside table shows the pairwise difference (p-value < 0.05) represented by different letters. (DOCX 159 kb)

Additional file 10: Abundance of the dominant genera found in the enteritis and enterocolitis samples. The graph shows the relative abundance of the 2 dominant genera found in enteritis and enterocolitis samples as well as their mean relative abundance in the other diagnosis categories. (DOCX 132 kb)

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References

1. Daly K, Stewart CS, Flint HJ, Shirazi-Beechey SP. Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. FEMS Microbiology Ecology. 2001;38:141–51.
2. Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Newbold CJ. Identification of a core bacterial community within the large intestine of the horse. PloS one. 2013;8:e77660.
3. O' Donnell MM, Harris HMB, Jeffery IB, Claesson MJ, Younge B, O' Toole PW, et al. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. Letters in applied microbiology. 2013;57:492–501.

4. Niwa C, Kato H, Hobo S, Kinoshita Y, Veno T, Katayama Y, et al. Postoperative Clostridium difficile infection with PCR ribotype 078 strain identified at necropsy in five throughbred racehorses. Veterinary Research. 2013;173:607.

5. Donaldson MT, Palmer JE. Prevalence of Clostridium perfringens enterotoxin and Clostridium difficile toxin A in feces of horses with diarrhea and colic. Journal of the American Veterinary Medical Association. 1999;215:358–61.

6. Hartnack AK, Van Metre DC, Morley PS. Salmonella enterica shedding in hospitalized horses and associations with diarrhea occurrence among their stablemates and gastrointestinal-related illness or death following discharge. Journal of the American Veterinary Medical Association. 2012;240:726–33.

7. Van Duijkeren E, van Asten AJ, Gaastra W. Characterization of Escherichia coli isolated from adult horses with and without enteritis. The Veterinary quarterly. 2000;22:162–6.

8. Diab SS, Songer G, Uzal FA. Clostridium difficile infection in horses: a review. Veterinary microbiology. 2013;167:42–9.

9. Rodriguez-Palacios A, Borgmann S, Kline TR, LeJeune JT. Clostridium difficile in foods and animals: history and measures to reduce exposure. Animal health research reviews / Conference of Research Workers in Animal Diseases. 2013;14:11–29.

10. Rodriguez C, Taminiau B, Brévers B, Avesani V, Van Broeck J, Leroux AA, et al. Carriage and acquisition rates of Clostridium difficile in hospitalized horses, including molecular characterization, multilocus sequence typing and antimicrobial susceptibility of bacterial isolates. Veterinary microbiology. 2014;172:309–17.

11. Medina-Torres CE, Weese JS, Staempfli HR. Prevalence of Clostridium difficile in horses. Veterinary microbiology. 2011;152:212–5.

12. Jones RL, Adney WS, Shideler RK. Isolation of Clostridium difficile and detection of cytotoxin in the feces of diarrheic foals in the absence of antimicrobial treatment. Journal of clinical microbiology. 1987;25:1225–7.

13. Arroyo LG, Stämpfli HR, Weese JS. Potential role of Clostridium difficile as a cause of duodenitis-proximal jejunitis in horses. Journal of medical microbiology. 2006;55(Pt 5):605–8.

14. Zhou Y, Burnham C-AD, Hink T, Chen L, Shaikh N, Wollam A, et al. Phenotypic and genotypic analysis of Clostridium difficile isolates: a single-center study. Journal of clinical microbiology. 2014;52:4260–6.

15. Shankar V, Hamilton MJ, Khoruts A, Kilburn A, Unno T, Paliy O, et al. Species and genus level resolution analysis of gut microbiota in Clostridium difficile patients following fecal microbiota transplantation. Microbiome. 2014;2:13.

16. Shahinas D, Silverman M, Sittler T, Chiu C, Kim P, Allen-Vercoe E, Weese S, Wong A, Low DE, Pillai DR: Toward an understanding of changes in diversity associated with fecal microbiome transplantation based on 16S rRNA gene deep sequencing. mBio 2012. 3 doi: 10.1128/mBio.00338-12.

17. Fernandes KA, Kittelmann S, Rogers CW, Gee EK, Bolwell CF, Bermingham EN, et al. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PloS one. 2014;9:e112846.

18. Salipante SJ, Sengupta DJ, Rosenthal C, Costa G, Spangler J, Sims EH, et al. Rapid 16S rRNA next-generation sequencing of polymicrobial clinical samples for diagnosis of complex bacterial infections. PloS one. 2013;8:e65226.

19. Conlan S, Kong HH, Segre JA. Species-level analysis of DNA sequence data from the NIH Human Microbiome Project. PloS one. 2012;7:e47075.

20. Cani PD, Everard A. [Akkermansia muciniphila: a novel target controlling obesity, type 2 diabetes and inflammation?]. Médecine Sciences. 2014;30:125–7.

21. Shin N-R, Lee J-C, Lee H-Y, Kim M-S, Whon TW, Lee M-S, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut. 2014;63:727–35.

22. Underwood MA. Intestinal dysbiosis: novel mechanisms by which gut microbes trigger and prevent disease. Preventive medicine. 2014;65:133–7.

23. Costa MC, Stämpfli HR, Arroyo LG, Allen-Vercoe E, Gomes RG, Weese J. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. BMC veterinary research. 2015;11:19.

24. Costa MC, Arroyo LG, Allen-Vercoe E, Stämpfli HR, Kim PT, Sturgeon A, et al. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. PloS one. 2012;7:e41484.

25. Balamurugan R, Janardhan HP, George S, Raghava MV, Muliyil J, Ramakrishna BS. Molecular studies of fecal anaerobic commensal bacteria in acute diarrhea in children. Journal of pediatric gastroenterology and nutrition. 2008;46:514–9.

26. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome research. 2012;22:299–306.

27. Okuda K, Kato T, Shiozu J, Takazoe I, Nakamura T. Bacteroides heparinolyticus sp. nov. Isolated from Humans with Periodontitis. International Journal of Systematic Bacteriology. 1985;35:438–42.

28. Sears CL. The toxins of Bacteroides fragilis. Toxicon : official journal of the International Society on Toxinology. 2001;39:1737–46.

29. Thean S, Elliott B, Riley TV. Clostridium difficile in horses in Australia - a preliminary study. Journal of Medical Microbiology. 2011;60:1188–92.

30. Martirossian G, Sokół-Leszczyńska B, Mierzejewski J, Meisel-Mikołajczyk F. [Occurrence of Clostridium difficile in the digestive system of dogs]. Medycyna doświadczalna i mikrobiologia. 1992;44:49–54.

31. Natarajan M, Walk ST, Young VB, Aronoff DM. A clinical and epidemiological review of non-toxigenic Clostridium difficile. Anaerobe. 2013;22:1–5.

32. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, et al. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Applied and environmental microbiology. 1999;65:4799–807.

33. Delcenserie V, Taminiau B, Delhalle L, Nezer C, Doyen P, Crevecoeur S, et al. Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. Journal of dairy science. 2014;97:6046–56.

34. Bindels LB, Neyrinck AM, Salazar N, Taminiau B, Druart C, Muccioli GG, et al. Non Digestible Oligosaccharides Modulate the Gut Microbiota to Control the Development of Leukemia and Associated Cachexia in Mice. PloS one. 2015;10:e0131009.

35. De Maesschalck C, Eeckhaut V, Maertens L, De Lange L, Marchal L, Nezer C, et al. The effects of xyloccpaxoligosaccharides on performance and microbiota in broiler chickens. Applied and environmental microbiology. 2015;81(17):5880–8.

36. Wang Y, Qian P-Y. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PloS one. 2009;4:e7401.

37. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology. 2009;75:7537–41.

38. Quince C, Lanzén A, Curtis TP, Davenport RJ, Hall N, Head IM, et al. Accurate determination of microbial diversity from 454 pyrosequencing data. Nature methods. 2009;6:639–41.

39. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics (Oxford, England). 2011;27:2194–200.

40. Silva. http://www.arb-silva.de/. Accessed 1 May 2015.

41. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic acids research. 2007;35:7188–96.

42. Nucleotide Blast. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. Accessed 1 May 2015.

43. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990;215:403–10.

44. Eshar D, Weese JS. Molecular analysis of the microbiota in hard feces from healthy rabbits (Oryctolagus cuniculus) medicated with long term oral meloxicam. BMC veterinary research. 2014;10:62.

45. Chao A, Bunge J. Estimating the number of species in a stochastic abundance model. Biometrics. 2002;58:531–9.

46. Chao A, Shen T-J: Nonparametric estimation of Shannon's index of diversity when there are unseen species in sample. Environmental and Ecological Statistics, 10:429–443

47. Mulder CPH, Bazeley-White E, Dimitrakopoulos PG, Hector A, Scherer-Lorenzen M, Schmid B. Species evenness and productivity in experimental plant communities. Oikos. 2004;107:50–63.

48. Dixon P. VEGAN, a package of R functions for community ecology. Journal of Vegetation Science. 2003;14:927–30.

49. Gower J. Wiley StatsRef: Statistics Reference Online. Chichester: John Wiley & Sons, Ltd; 2014.

50. Excoffier L, Smouse PE, Quattro JM. Analysis of Molecular Variance Inferred From Metric Distances Among DNA Haplotypes: Application to Human Mitochondrial DNA Restriction Data. Genetics. 1992;131:479–91.

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51. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and environmental microbiology. 2005;71:8228–35.

52. Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. Bioinformatics (Oxford, England). 2010;26:715–21.

53. National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov/. Accessed 1 May 2015.

54. Rodriguez C, Taminiau B, Van Broeck J, Avesani V, Delmée M, Daube G. Clostridium difficile in young farm animals and slaughter animals in Belgium. Anaerobe. 2012;18:621–5.

55. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC. Development of a new PCR-ribotyping method for Clostridium difficile based on ribosomal RNA gene sequencing. FEMS microbiology letters. 1999;175:261–6.

56. Société Française de Microbiologie. http://www.sfm-microbiologie.org/. Accessed 1 May 2015.

57. Standards - CLSI :CLSI. http://clsi.org/standards/. Accessed 1 May 2015.

Additional file 1: **Quality analysis of the metagenetic libraries created for the horse faecal samples analysed.**

Item	Total number (or %)	Mean read length, nucleotide
Raw reads	137,319	510
Postdenoising/filtering	118,429	452
Loss in denoising (%)	13.8	
Postchimeric detection	104,608	450
Chimeric reads (%)	11.7	
OTU 0.03[a]	8,086	

Microbiota faecal composition at species level (cumulative mean relative abundance >4 %) of horses with and without diarrhoea. Samples 13, 14, 15, 17, 23, 25, 26, 27, 28 and 29: horses with diarrhoea. Samples 11, 12, 16, 18, 19, 20, 21, 22, 24 and 30: horses without diarrhoea.
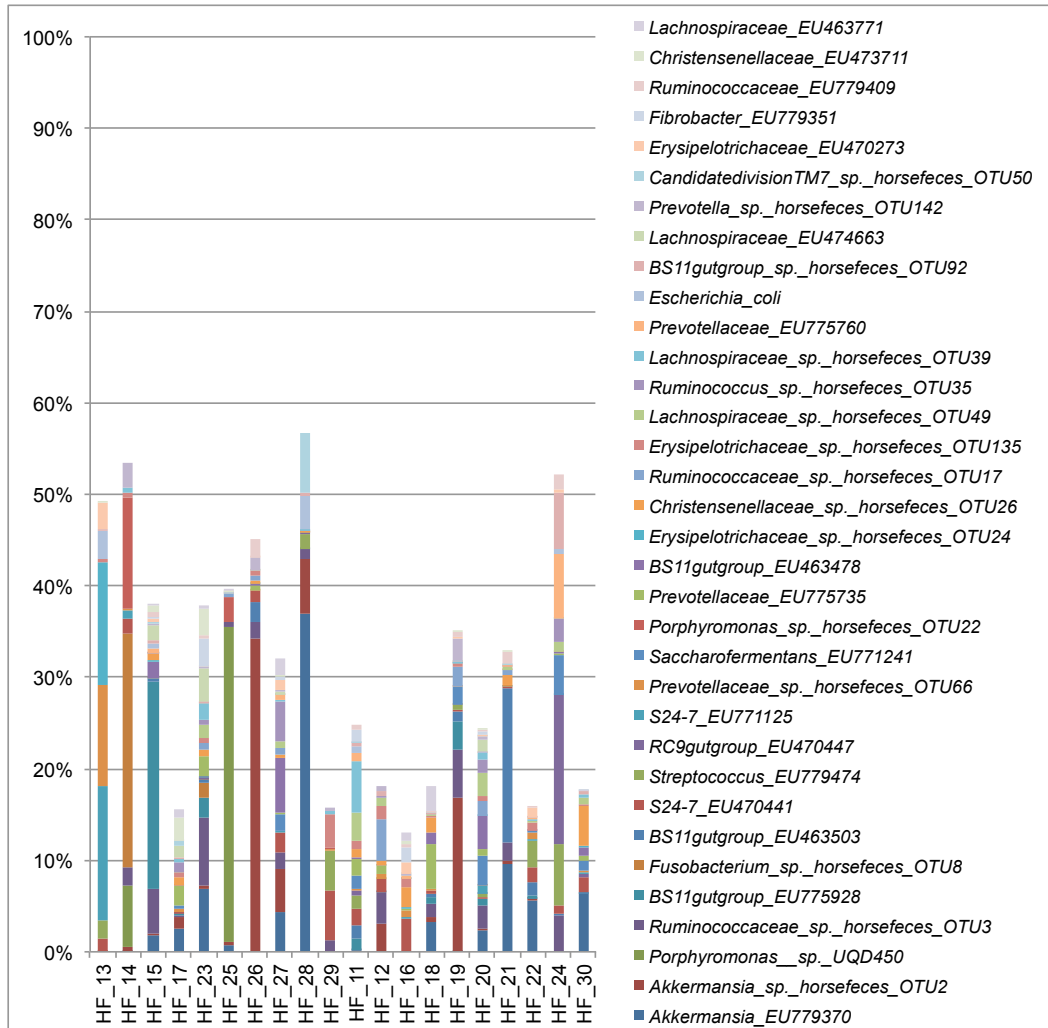
Additional file 3: **Principal coordinate analysis of the diarrhoeic and non-diarrhoeic horses.** The 3 graphs show the sample distribution along the 3 main axes (PC1-PC2-PC3).
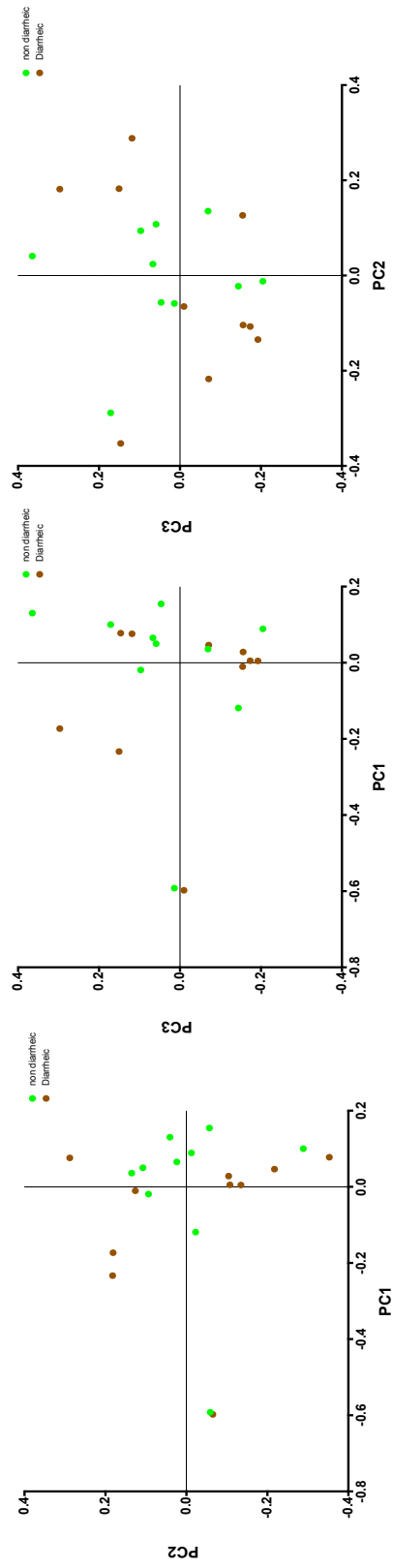
Additional file 4: **Clinical history comparison between C. difficile colonised and non-colonised horses.** [a]Antimicrobial treatment of two or more antibiotics. [b]Including single or combined antimicrobial therapy

	C. difficile negative horses (%)	*C. difficile* positive horses (%)
Overall totals (%)	129 of 134 (96.3%)	5 of 134 (3.7%)
Mean age in years	10.2	8.4
Sort by size		
Heavy horses (>650kg)	16 (12.4)	0 (0)
Light horses (<650kg)	94 (72.8)	4 (80)
Ponies/donkeys	19 (14.7)	1 (20)
Sort by gender		
Mare	62 (48.9)	3 (60)
Stallion	24 (18.6)	0 (0)
Gelding	43 (33.3)	2 (40)
Horses with gastrointestinal disorders 52/134 (38.8)	49 (94.2)	3 (5.8)
Colic	30 (61.2)	2 (66.6)
Diarrhoea	18 (36.7)	0 (0)
Colic and diarrhoea	1 (2.0)	0 (0)
Others	0 (0)	1 (33.3)
Horses without gastrointestinal disorders 82/134 (61.2)	79 (96.3)	3 (3.7)
Orthopaedics/bone fracture	23 (29.1)	0 (0)
Wounds	14 (17.7)	2 (66.6)
Muscular dystrophy	8 (10.1)	0 (0)
Ophthalmology	8 (10.1)	0 (0)
Weight loss	3 (3.8)	0 (0)
Others	23 (29.1)	1 (33.3)
Horses with an antibiotic treatment 54/134 (40.3)	52 (96.3)	2 (3.7)
Penicillin	4 (7.7)	0 (0)
Penicillin-Gentamicin[a]	9 (17.3)	1 (50)
Penicillin-Gentamicin-Metronidazole[a]	3 (5.8)	0 (0)
Ceftiofur	16 (30.8)	0 (0)
Trimethoprim/sulfamethoxazole	6 (11.5)	0 (0)
Others[v]	14 (27)	1 (50)
Placement of a nasogastric tube 43/134 (32.1)	40 (93)	3 (7)

Additional file 5: Detailed information on five C. difficile **negative horses studied via high-throughput amplicon sequencing analysis and compared with** C. difficile **colonised horses.** NSAIDs: nonsteroidal anti-inflammatory drugs. Pen: penicillin; Gen: gentamicin; SXT: trimethoprim/sulfamethoxazole

			Clinical history of *C. difficile* negative horses				
Date of sampling	Animal identification	Age (years)	Diagnostic	Diarrhoea	Hospital stay (days)	Antibiotic treatment	NSAIDS treatment
05/10/2013	02	17	Colic (displacement of the colon to an abnormal location)	-	1	-	Flunixin meglumine Dipyrone
13/11/2013	05	3	Colic (recurrent colic due to gastric ulcers)	-	4	-	-
19/11/2013	06	5	Oesophageal obstruction	-	1	-	-
22/11/2013	07	7	Wound	-	2	Pen-Gen-SXT	Flunixin meglumine Dipyrone
26/11/2013	08	7	Jaw wound	-	4	Cefquinome	Dipyrone

Additional file 6: **Microbiota faecal composition at species level (cumulative mean relative abundance >4 %) of** C. difficile **culture-positive and -negative horses.**
Samples 01, 03, 04, 09 and 10: *C. difficile* positive horses detected by faeces culture.
Samples 02, 05, 06, 07 and 08: *C. difficile* negative horses detected by faeces culture.
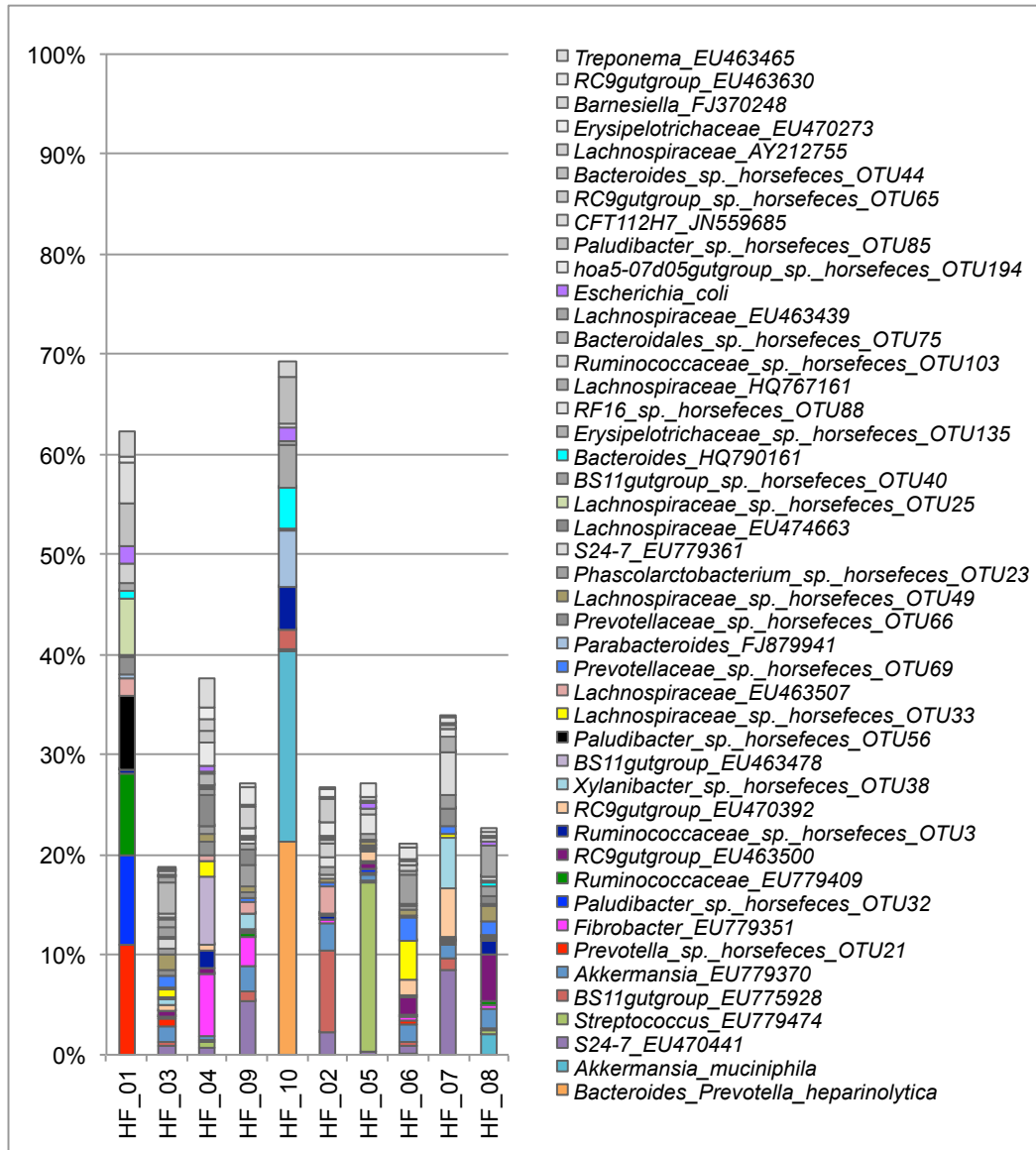
Additional file 7: **Principal coordinate analysis of** C. difficile **positive and negative horses.** The 3 graphs show the sample distribution along the 3 main axes (PC1-PC2-PC3).
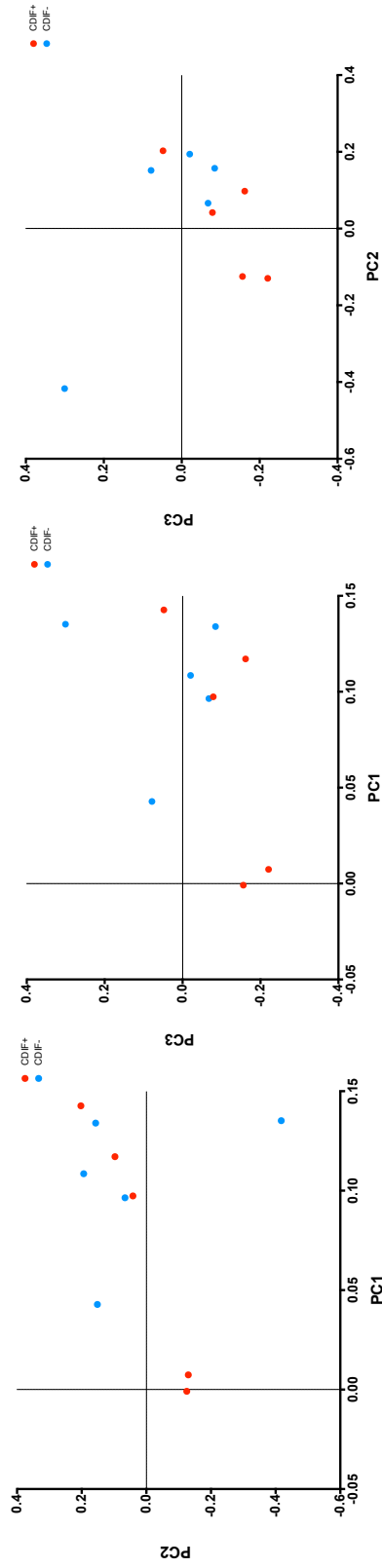
[Additional file 8:](#) **Bacterial genus whose relative abundance was statistically different between** C. difficile **positive and negative horses.** Result of a White test (p value <0.05) pairwise comparison.

Statistical differences - genus level - CDIF
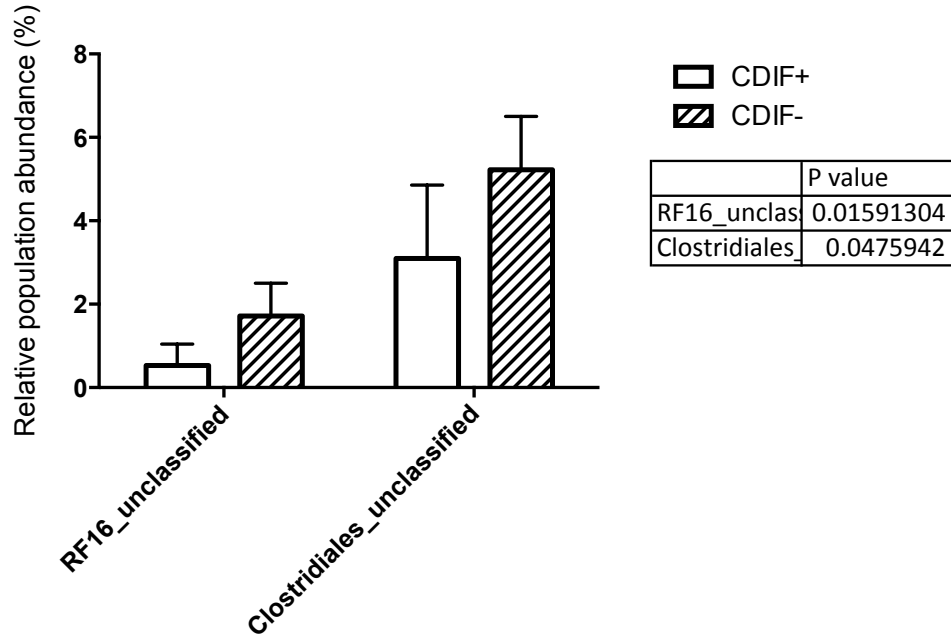
	P value
RF16_unclas	0.01591304
Clostridiales	0.0475942

Additional file 9: Bacterial genus whose relative abundance was statistically different between Colic, diarrhoea and Others diagnosis groups. The graph illustrates the mean relative abundance for the selected genera. Groups were compared by ANOVA 2 analysis with post-hoc Tukey-kramer multiple comparison. The inside table shows the pairwise difference (p-value < 0.05) represented by different letters.

Statistical differences - genus level - diagnosis groups
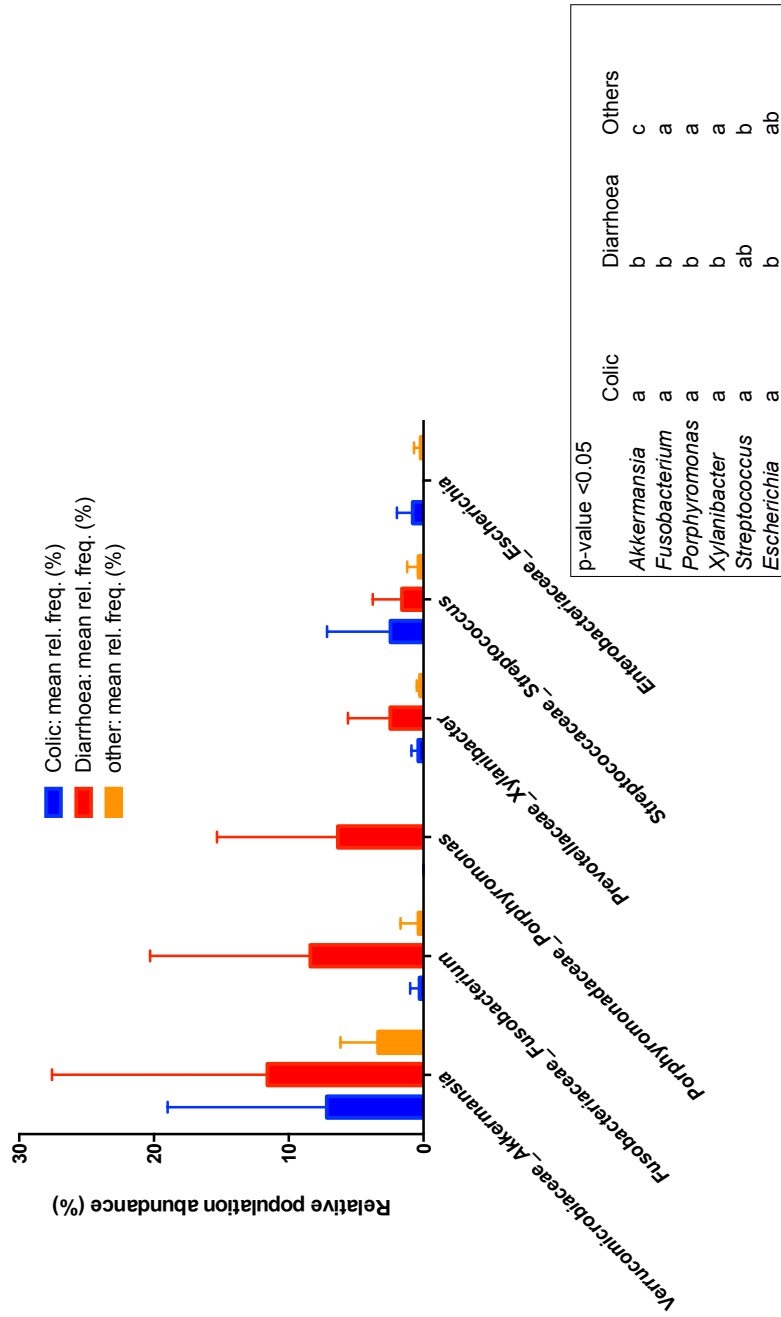
p-value <0.05	Colic	Diarrhoea	Others
Akkermansia	a	b	c
Fusobacterium	a	b	a
Porphyromonas	a	b	a
Xylanibacter	a	b	a
Streptococcus	a	ab	b
Escherichia	a	b	ab

<u>Additional file 10</u>: **Abundance of the dominant genera found in the enteritis and enterocolitis samples.** The graph shows the relative abundance of the 2 dominant genera found in enteritis and enterocolitis samples as well as their mean relative abundance in the other diagnosis categories
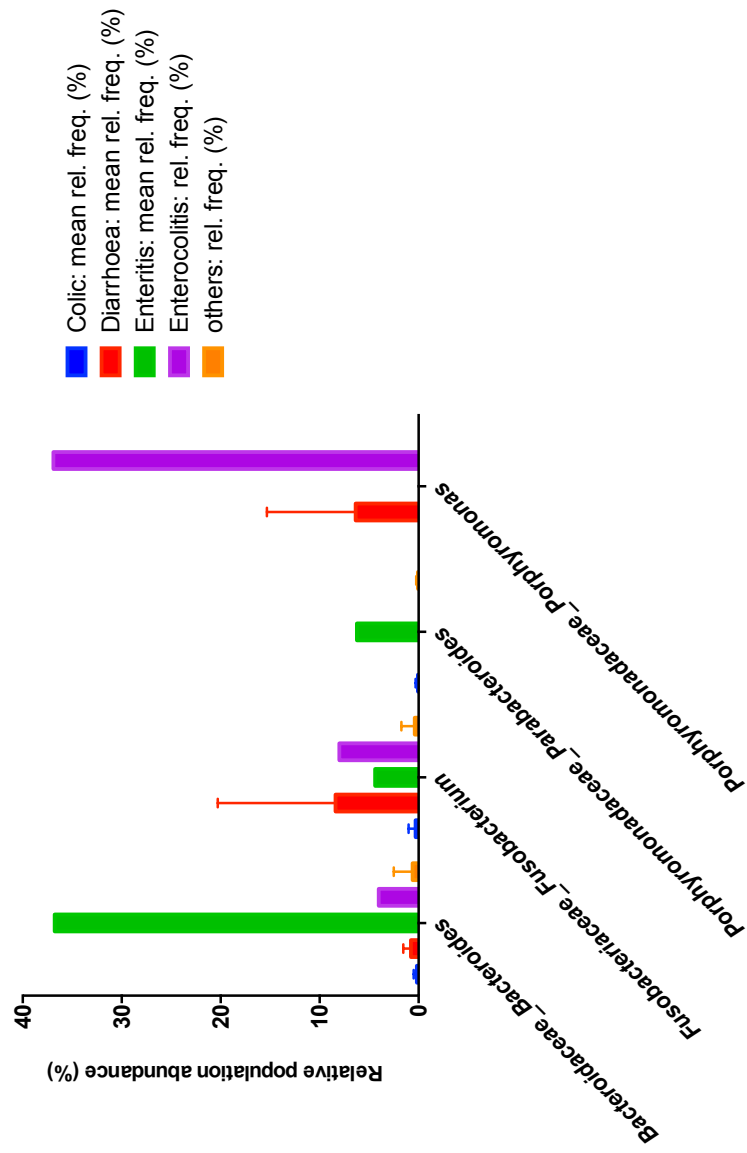
3.2 *C. difficile* in food animals: potential sources for zoonotic and foodborne contamination

Once the presence of the enteropathogen *C. difficile* was evidenced in both horses with and without any signs of disease, the next concern was to determine if food animals on farms and at slaughter could also be sources of toxigenic *C. difficile* and therefore, reservoirs for human infection. In order to address that concern, a first study was conducted to evaluate the carriage of *C. difficile* in healthy newborn piglets and calves on farms, and in full-grown cattle and pigs at slaughter line. Results obtained revealed a high colonisation in clinically healthy suckling piglets (78.3%) and in calves (22.2%) less than three months of age. In contrast, a reduction in colonisation rate with age was observed. At slaughter, *C. difficile* was recovered with a prevalence of 6.9% from cattle aged between 11 and 52 months, but from none of the intestinal samples collected from pigs between 5 and 6 months of age. The increased susceptibility to colonisation in young animals may be due to an immature endogenous microbiota. However, there is little evidence to explain why some newborn animals do not develop the disease despite being colonised with *C. difficile* strains. Some hypotheses are the absence of toxin receptors, which have been largely studied, or poorly developed cellular signalling pathways in the immature gut mucosa. In addition, the colostrum intake and colostrum antibody concentration could be crucial in the development (or not) of the disease. Among the different PCR-ribotypes identified (078, 002, 015, 014, 081 and 087) the predominance of the type 078 in calves and piglets from breeding farms has not been explained. In contrast, isolates from cattle presented the widest range in PCR-ribotype variety, most of them being toxigenic.

The results obtained in the previous study presented evidence that healthy food animals are carriers of toxigenic *C. difficile* strains on farms and at the time of harvest. Therefore, it was important to determine if such carriage represents a risk for carcass contamination at slaughter line. Intestinal contents and carcass samples from pigs and cattle were collected from a single slaughterhouse. Intestinal samples were collected from the large intestine of each animal, directly from the viscera processing area. In the same day, carcasses were sampled just after fast chilling in the chilling room. *C. difficile* was isolated in 1% and 9.9% of the pig and cattle intestinal contents and in 7.9% and 7% of cattle and pig carcass samples respectively. Despite the relative high prevalence of *C. difficile* in intestinal contents and on carcasses, only one animal was positive for both samples but PCR-ribotypes were not identical. This finding suggests that cross contaminations during processing might occur. Moreover, while only 1% of pig intestinal contents were detected positive for *C. difficile*, the prevalence of the bacterium on pig carcasses was much higher (7%), which, in agree with the previous observation, may reflects a faecal contamination through at slaughter line. A total of 19 different PCR-ribotypes were identified, with predominance of PCR-ribotypes 078 and 014. Furthermore, some of

the PCR-ribotypes most prevalent in hospitals in Belgium in the same year that the study was conducted were detected in pigs and cattle samples (PCR-ribotypes 014, 078, 023, 015, UCL16L, UCL5a and UCL46).

It was revealed that slaughter animals are carriers of toxigenic *C. difficile* associated with infections in humans, and carcass contamination occurs inside the slaughter line. To investigate genetic relationships between *C. difficile* isolates from human faecal samples, pig and cattle intestinal and carcasses samples, MLST and MLVA were performed. Six clonal groups of strains were obtained from the same animal species, irrespective of the isolation date. Three of these six clonal groups of strains (detected in hospitalised patients and in cattle and pigs intestinal contents at slaughter) were identified as PCR-ribotype 078. MLST analysis revealed that all of the other human and animal isolates with a given PCR-ribotype clustered in the same lineage. Identical *C. difficile* strains were also detected in the same animal species for PCR-ribotypes 014, UCL16U and UCL36, although interspecies transmission was no evident. The fact that isolates were obtained from subjects (human beings and animals) localised in different geographical regions and in different environments should be also considered, which would explain the divergence of strains.

3.2.1 *C. difficile* in young farm animals and slaughter animals in Belgium

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Pathogenesis and toxins

Clostridium difficile in young farm animals and slaughter animals in Belgium

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ABSTRACT

Faecal carriage of *Clostridium difficile* in healthy animals has been reported recently, especially in piglets and calves. However there is limited data about carriage in animals just prior to slaughter in Europe. The main objective of this study was to determine the presence of *C. difficile* in pigs and cattle at the slaughterhouse. *C. difficile* was isolated in 6.9% of the cattle at the slaughterhouse. None of the pig slaughter samples were positive for *C. difficile* after an enrichment time of 72 h. For complementary data, a short study was conducted in piglets and calves at farms. *C. difficile* was more prevalent in piglets (78.3%) than in calves (22.2%) on the farms. Regarding the piglet samples, 27.8% of the positive samples were detected without enrichment of stools. The PCR ribotype 078 was predominant in farm animals. Samples isolated from slaughter cattle presented the widest range in PCR-ribotype variety, and the most prevalent PCR ribotype was 118a UCL. The results of this study confirm that *C. difficile* is present in slaughter animals in Belgium with a large percentage of toxigenic strains also commonly found in humans.

1. Introduction

Clostridium difficile is an anaerobic, Gram-positive, spore-forming, rod-shaped bacterium that has been widely characterized as a serious pathogen agent in humans and animals. In humans it may be considered as the most commonly isolated bacterium in patients developing nosocomial diarrhoea and, in the most serious cases, pseudomembranous colitis after use of antibiotics.

C. difficile has also been isolated from stools of different types of animals suffering from severe enteric diseases [1–3]. Recent isolation of this bacterium in healthy young carrier animals demonstrates the possible existence of an animal reservoir [4,5]. Some of those animals, like piglets and calves, are destined to enter the human food supply chain. In addition, a significant number of animal isolates are correlated with PCR ribotypes found in humans, establishing a possible relationship between them [6,7]. Similar studies in food, including retail meat [8,9], revealed the presence of *C. difficile* ribotypes in relation with human *C. difficile* infections (CDI) [5]. This recent observation suggests a potential risk of foodborne infections linked to *C. difficile* but, at the moment, there is not much data describing *C. difficile* in animals at the slaughterhouse in Europe.

The aim of this study was to determine the presence of *C. difficile* in full-grown animals at the slaughterhouse and young animals on farms and to compare the main PCR-ribotypes in the animals with those found in humans.

2. Materials and methods

2.1. Samples

A total of 437 intestinal and faecal samples were collected from pigs and cattle on farms and at a single slaughterhouse during a sample collection campaign performed in Belgium from January to July in 2011.

During this period, 202 intestinal samples from cattle and 194 from pigs were collected over 9 different visits to a local slaughterhouse. The yearly production of this slaughterhouse is approximately 32,000 cattle and 205,000 pigs with a work schedule of four days a week. Cattle intestinal samples were recovered from animals coming from 55 different herds on seven different sampling occasions. All samples were from animals aged between 11 and 52 months. The information regarding the specific age and the herd of 24 cattle intestinal samples is not available. Pig intestinal samples were collected from animals between 5 and 6 months of age and collected on 4 different occasions. All intestinal contents were collected from the slaughter line, directly from the large intestine in the viscera processing area. The samples were collected weekly on

rotating days. Each sample (approximately 50 g of intestinal contents) was kept in individual, identified, sterile 50 ml tubes. They were kept at room temperature for a maximum of 1 h until their arrival in the laboratory where they were stored at 4 °C. All of the intestinal samples were analysed within 2 h of sample collection.

Samples from 23 newborn pigs were collected from 3 different breeding farms. On each farm, faecal samples were collected from individual piglets from at least two different litters. The farms used an all in/all out system. Piglets were stimulated to make them defecate in individual sterile 50 ml tubes. The piglets did not have diarrhoea and were still suckling (<15 days old).

Faecal samples of 18 non-diarrhoeic clinically healthy calves were collected from 5 local farms. The rectal faecal samples were obtained using sterile gloves and kept in sterile 50 ml tubes. Calves were less than 3 months of age at the time of sampling.

All freshly collected faecal samples were processed on the same day after transport to the laboratory at room temperature for a maximum of 2 h. In those cases when there was a delay in analysis, samples were stored for a maximum of 3 days at 4 °C before processing.

2.2. Isolation of C. difficile

Culture of all samples was carried out as described by Delmée et al. [10] with minor modifications. Briefly, approximately 0.1 g of faeces were spread directly on cycloserine cefoxitin fructose agar taurocholate medium (CCFAT), freshly prepared in the laboratory as previously described [11]. Plates were incubated anaerobically for 72 h at 37 °C. The anaerobic atmosphere in the jar was created using AnaeroGen™ sachet (Oxoid, Dardilly, FR) and checked using an anaerobic indicator BR0055B (Oxoid).

At the same time an enrichment step was performed. One gram of sample was inoculated into 9 ml of home-made cycloserine cefoxitin fructose taurocholate enrichment broth (CCFBT), as previously described [11], but without agar, and incubated anaerobically for 72 h at 37 °C. Subsequently, 10 μl of the broth was spread on CCFAT [11] and incubated at 37 °C for two days. Colonies of *C. difficile* were identified from culture plates by morphological criteria as greyish colonies with an appearance of ground glass and a characteristic horse manure odour. Between one and four presumptive colonies per plate were subcultured onto blood agar (5% Sheep Blood; BioRad, Nazareth, BE). Multiple colonies were taken when morphologies suggested more than one type of ribotype or when the presumptive colonies were too small to ensure isolation on the blood agar.

2.3. Identification of C. difficile by latex agglutination assay, polymerase chain reaction and GenoType Cdiff test

2.3.1. Latex agglutination assay

All non-haemolytic and morphologically suspect colonies were checked using a *C. difficile* latex agglutination rapid test (*C. difficile* Test Kit DR 1107A; Oxoid) according to the manufacturer's instructions. Briefly, using an inoculating loop, the suspect colony was mixed in a drop *C. difficile* latex reagent (Oxoid). The agglutination was examined after 1–2 min. In the event of uncertain agglutination, the procedure was to classify it as unconfirmed agglutination and to continue with the same process as would have been done with a positive sample.

2.3.2. C. difficile identification and toxin genes detection

Total DNA was harvested from a single *C. difficile* colony picked up from a group of colonies cultured onto blood agar (5% Sheep Blood; BioRad) using a sterile loop and suspended in 150 μl of a chelex 100 solution 5% (BioRad). The suspension was shaken in a thermomix (Eppendorf, Hamburg, DE) (950 rpm, 15 min, 56 °C and 10 min, 95 °C) and then centrifuged (1400 rpm, 60 s). The resulting supernatant was recovered, and the DNA quantity was measured by absorbance spectrophotometry (Nanodrop-2000; Thermo-Scientific, NanoDrop products, Wilmington, USA). Next, detection of a species-specific internal fragment of the *tpi* gene, toxin B gene [12] and detection of CDT (*cdtA*) [13] were performed according to the multiplex PCR protocol of Lemée et al. [12]. Another PCR was performed for the detection of the toxin A encoding gene according to the primers and protocol from Lemée et al. [12]. PCR products were separated by electrophoresis in 2% agarose gel (Eurogentec, Seraing, Be) stained with 0.1 μl GelGreen™ ml^{-1} (VWR, Leuven, Be) in 1× TAE buffer (VWR) for 30 min at 100 V and analysed under UV light.

2.3.3. GenoType CDiff test

All strains with discrepant results in cytotoxicity and amplification of toxin genes were additionally tested using the molecular genetic GenoType CDiff test system (Hain Lifescience, Nehren, DE).

The test allowed the detection of *tpi* and all the toxins' genes (*tcdA*, *tcdB*, *cdtA* and *cdtB*), and made possible the detection of deletion in the regulator gene *tcdC* (18 bp and 39 bp deletion or single base deletion at position 117).

Procedures and reagents for DNA extraction, DNA amplification, hybridization and hybridization wash were accomplished following the manufacturer's instructions. DNA amplification and hybridization were performed respectively in a commercial thermocycler (PerkinElmer, Massachusetts, USA) and a TwinCubator (Hain Lifescience).

2.4. Cytotoxicity assay using cell lines

Toxic activity of the isolated strains was confirmed by a cytotoxic assay on cells. A suspension of colonies, from a 48 h subculture on Columbia agar supplemented with 5% Sheep Blood (Becton Dickinson, Erembodegem, BE), was prepared with 1 ml of physiological solution. After centrifugation of the suspension (13,500 rpm for 10 min), 75 μl of supernatant from each sample was placed into 1 well containing 0.75 ml of confluent monolayer MRC-5 cells prepared in the laboratory. The concentration of the cells was 15 million cells per 100 ml of a solution MEM REGA (Life technologies, Darmstadt, DE). Plates were incubated at 37 °C under 5% CO$_2$ atmosphere and examined at 24 and 48 h. In addition, 75 μl of specific *C. difficile* antitoxin (TechLab, Virginia, USA) was used in order to confirm the specificity of the cytotoxic activity by neutralization.

2.5. 16S–23S intergenic spacer region PCR-ribotyping

The PCR-ribotyping was based on the patterns comparison of PCR products from the 16S–23S rDNA intergenic spacer regions using primers described by Barbut et al. [14]: primer 16S (5′-GTG–CGG–CTG–GAT–CAC–CTC–CT-3′) (forward primer) and 23S primer labelled at the 5′ end with carboxyfluorescein (FAM) (5′-FAM–CCC–TGC–ACC–CTT–AAT–AAC–TTG–ACC-3′) (reverse primer). The DNA extraction was performed using a chelex 100 solution 5% (Biorad) as previously described [15].

For PCR amplification, 3 μl of DNA (diluted 1:10) was added to a 22 μl mixture containing 2 mM of final concentration of MgCl$_2$, 2.5 μl of Buffer II 10× (Applied Biosystems, California, USA), 200 μM of final concentration of DNTP (Eurogentec), 1.25 Units of final concentration of AmpliTaq (Applied Biosystems) and 0.2 μM of final concentration of each PCR primer. The reactions were performed in a commercial thermocycler (PerkinElmer) under the following

conditions: 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C with a final extension at 72 °C for 30 min.

Amplicon size was analysed by capillary electrophoresis using a 3100 automatic sequencer (ABI 3100 Automated Capillary DNA Sequencer and GeneScan Analysis) (Applied Biosystems). A 35–500 bp ROX ladder (Applied Biosystems) was used as an internal marker for each sample.

Strains with an international number have a ribotype profile corresponding to one of the 23 profiles of the Brazier collection, which are available to every reference laboratory in Europe. If a strain has a profile that does not correspond to any of the 23, an internal number beginning with UCL was given.

3. Results

3.1. Isolation of C. difficile

A total of 220 cattle samples were analysed. *C. difficile* was isolated from 4/18 samples from calves (22.2%) and from 14/202 samples from slaughter cattle (6.9%). In slaughterhouse, three positive samples were found in January during the same sampling day. In February 6 positive samples were found also during the same sampling day, and in March 1 positive sample was found in one sampling day and 4 positive samples in another sampling day.

Samples from 217 pigs were also analysed. *C. difficile* was isolated from 18/23 piglets (78.3%). None of the 194 samples collected from pigs on the slaughter line was tested positive for *C. difficile*.

Isolates from cattle and calves were obtained only with enrichment culture. In piglets, 5 out of 18 positives were also obtained by direct culture (27.8%). Thus, only 5 of all study isolates obtained were recovered by direct culture and only from piglets.

3.2. Cytotoxicity assay using cell lines, toxin gene detection and GenoType CDiff test

Thirty-one (86.1%) of the total isolates ($n = 36$) were positive on cytotoxicity assay using cell lines. All calf isolates ($n = 4$) were positive for the cytotoxicity assay, while only one piglet was

negative. Among isolates recovered from cattle intestinal samples, 10 were positive and 4 were negative. These results were compared with the PCR results targeting *tpi*, *tcdA*, *tcdB* and *cdtA* and with results of the GenoType CDiff test system. Simplex PCR for *tcdA* showed only 14 positive isolates and 22 negative isolates. Subsequently, GenoType CDiff method was used to retest the 22 *tcdA*− in previously test and indicated a total of 17 *tcdA*+ isolates. Using GenoType CDiff test and multiplex PCR results, 31 strains contained *tcdA* and *tcdB* genes, and 17 strains contained *cdt*A gene coding for the binary toxin. Only 5 strains (9, 238, 103, 36, and 273 UCL) were negative for all toxin genes. Only the 239, 172 UCL and all of the 078 ribotype strains had a 39 bp deletion in the regulator gene *tcdC* (Table 1).

3.3. PCR-ribotyping

Eighteen different PCR ribotypes were identified with a predominance of type 078 in calves and piglets from breeding farms (75% (3/4) and 66.7% (12/18) respectively). Other identified ribotypes were 002, 015, 014, 081 and 087 while the rest of the strains were not associated with any international Brazier types. Isolates from cattle presented the widest range in PCR-ribotype variety (Table 1). None of the positive animals were identified as a bearer of more than one PCR-ribotype strain.

4. Discussion

C. difficile has been isolated in a significant proportion of calves from breeding farms (22.2%), which is in accordance with the prevalence obtained in analogous studies [16,17]. This study was the first to target *C. difficile* isolated from cattle (between 11 and 52 months old) at a slaughterhouse in Belgium, and the observed prevalence (6.9%) was higher than expected. Two isolates were from cattle of 11 and 52 months old. The twelve remaining positives were from animals between 16 and 24 months. One of the most remarkable aspect of these findings is the high prevalence given the age of the animals. *C. difficile* has not been extensively described in older food animals, especially in Europe. A small proportion of culture positives were reported from cattle just prior to slaughter [17–19]. In Europe, Hoffer et al. [18] reported only one positive stool

Table 1
PCR-ribotypes and toxin gene profiles of *Clostridium difficile* isolated from food animals.

Animal group	PCR-ribotype	No. isolates	Cytotoxicity assay	Detection of toxin genes by PCR			GenoType CDiff test system						
				tcdA	tcdB	cdtA	tcdA	tcdB	cdtA	cdtB	tcdC 18 bp	tcdC 39 bp	tcdC 117 bp
Piglets	078	12	+	−	+	+	+	+	+	+	−	+	−
	002	3	+	+	+	−	*	*	*	*	*	*	*
	172 UCL	1	+	−	+	+	+	+	+	+	−	+	−
	239 UCL	1	+	−	+	+	+	+	+	+	−	+	−
	9 UCL	1	−	−	−	−	−	−	−	−	−	−	−
Calves	078	3	+	−	+	+	+	+	+	+	−	+	−
	015	1	+	+	+	−	*	*	*	*	*	*	*
Cattle	002	1	+	+	+	−	*	*	*	*	*	*	*
	014	1	+	+	+	−	*	*	*	*	*	*	*
	081	1	+	+	+	−	*	*	*	*	*	*	*
	087	1	+	+	+	−	*	*	*	*	*	*	*
	118 UCL	1	+	+	+	−	*	*	*	*	*	*	*
	16l UCL	1	+	+	+	−	*	*	*	*	*	*	*
	16r UCL	1	+	+	+	−	*	*	*	*	*	*	*
	118a UCL	2	+	+	+	−	*	*	*	*	*	*	*
	20a UCL	1	+	+	+	−	*	*	*	*	*	*	*
	238 UCL	1	−	−	−	−	−	−	−	−	−	−	−
	103 UCL	1	−	−	−	−	−	−	−	−	−	−	−
	36 UCL	1	−	−	−	−	−	−	−	−	−	−	−
	273 UCL	1	−	−	−	−	−	−	−	−	−	−	−

*Isolates were not tested using Genotype CDiff test system.

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sample collected on a slaughter line, in a total of 204 calves aged between four and six months. Only one recent study (in Pennsylvania) [20] pointed to a prevalence of 12% in five and a half month-old calves just before slaughter.

C. difficile was recovered from a high prevalence of piglets (78.3%) from breeding farms. This finding is in accordance with analogous studies [21] and indicates a significant colonization rate of *C. difficile* in suckling piglets. Interestingly, piglets in our study were clinically healthy, even though a very high percentage tested positive. More important, *C. difficile* was detected by direct plating, indicating high spore counts. However, in our study none of the intestinal samples collected from pigs on the slaughter line tested positive for *C. difficile* after an enrichment of three days. This finding is lower than the prevalence in slaughter pigs found in analogous studies [6,18,22,23], but indicates the significant reduction in colonization rate with age that has been reported by others [1,17,21].

In most cases, isolation of *C. difficile* from animals requires an enrichment stage, but the current literature does not conclusively establish a standard method for isolating *C. difficile* from animal faecal samples. Due to this lack of a standard and the negative results of the first 179 slaughter pig samples, the enrichment step was increased 10 times to a maximum duration of 30 days. This experimental modification was applied to the last 15 samples, and 3 positives resulted (two PCR ribotypes 078 and one PCR ribotype 081). However, thirty days of enrichment is an appreciably long technique for laboratory purposes, and the sample size for the experimental study was small for drawing conclusions. It might be desirable for further investigations to focus to a greater degree on how the enrichment duration influences results, in order to work towards an accepted standard procedure for detection of *C. difficile* in animals.

Six PCR ribotypes out of a total of eighteen found could be assigned to international Brazier types. Even though the PCR ribotype 078 was predominant in piglets and calves, it was not identified in any isolates from older cattle. This result was in accordance with previous studies [17,22,24]. The PCR ribotype 002 was the second most identified ribotype in piglets on breeding farms and was identified in one sample from the cattle. The greatest variety of PCR ribotypes was found in slaughter cattle (13 different PCR ribotypes among 14 isolates), including types 014, 087 and 081. Of the six PCR-ribotypes assigned to international Brazier types identified in the present study, four (014, 015, 078 and 002) are among the seventeen most frequent PCR ribotypes identified in humans in Europe [25].

In Belgium, the predominant PCR ribotypes identified in patients are currently 027, 014, 020, 002, 46 UCL, 078, 015 and 001, sorted by decreasing values in number of isolates (unpublished data). Four of these ribotypes were isolated in the present study in the following proportions 2.7% (014), 11.1% (002), 41.7% (078) and 2.7% (015).

Most of the isolates in this study possessed gene encoding for both A and B toxins. In addition, some of them were also $cdtA^+$. However, all PCR ribotypes 078, 239 and 172 UCL were $tcdA^-$ by simplex PCR and $tcdA^+$ by GenoType CDiff test systems. The simplex PCR primers used appear to be very insensitive compared to both cytotoxicity assay and GenoType Cdiff method. The discrepancies between GenoType CDiff test and simplex PCR may be due to the choice of the *tcdA* primers [26]. Variability in the genes *tcdA* and *tcdB* has been previously studied [27], and is the basis of a novel *C. difficile* toxinotyping scheme [28].

The results obtained in this study present evidence of the presence of toxigenic *C. difficile* isolates in piglets and calves on farms and in cattle stools on the slaughter line in Belgium. This suggests a potential risk of retail meat contamination. Nevertheless,

there is a need for more studies focussing on straight carcass and meat tests to demonstrate a true animal-to-human transmission through the food chain.

References

[1] Rodriguez-Palacios A, Staempfli HR, Duffield T, Peregrine AS, Trotz-Williams L, Arroyo L, et al. Clostridium difficile PCR ribotypes in calves, Canada. Emerg Infect Dis 2006;13:485–7.
[2] Songer GJ, Anderson MA. Clostridium difficile: an important pathogen of food animals. Anaerobe 2006;12:1–4.
[3] Thean S, Elliott B, Riley TV. Clostridium difficile in horses in Australia – a preliminary study. J Med Microbiol 2011;60:1188–92.
[4] Rupnik M. Clostridium difficile: (re)emergence of zoonotic potential. Clin Infect Dis 2010;583–4.
[5] Weese JS. Clostridium difficile in food – innocent bystander or serious threat? Clin Microbiol Infect 2010;16:3–10.
[6] Indra A, Lassnig H, Baliko N, Much P, Fiedler A, Huhulescu S, et al. Clostridium difficile: a new zoonotic agent? Wien Klin Wochenschr 2009;121: 91–5.
[7] Rupnik M, Widmer A, Zimmermann O, Eckert C, Barbut F. Clostridium difficile toxinotype V, ribotype 078, in animals and humans. J Clin Microbiol 2008;46: 2146.
[8] Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. Clostridium difficile in retail ground meat, Canada. Emerg Infect Dis 2007;13:485–7.
[9] Songer GJ, Trinh HT, Killgore GE, Thompson AD, McDonald LC, Limbago BM. Clostridium difficile in retail meat products, USA. Emerg Infect Dis 2007; 2009(15):819–21.
[10] Delmée M, Van Broeck J, Simon A, Janssens M, Avesani V. Laboratory diagnosis of Clostridium difficile-associated diarrhoea: a plea for culture. J Med Microbiol 2005;54:187–91.
[11] Delmée M, Vandercam B, Avesani V, Michaux JL. Epidemiology and prevention of Clostridium difficile infections in a leukaemia unit. Eur J Clin Microbiol 1987; 6:623–7.
[12] Lemée L, Dhalluin A, Testelin S, Mattrat M, Maillard K, Lemeland J, et al. Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (toxin A), and tcdB (toxin B) genes for toxigenic culture of Clostridium difficile. J Clin Microbiol 2004;42:5710–4.
[13] Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of Clostridium difficile. FEMS Microbiol Lett 2000;186:307–12.
[14] Barbut F, Richard A, Hamadi K, Chomette V, Burghoffer B, Petit JC. Epidemiology of recurrence or reinfections of Clostridium difficile-associated diarrhea. J Clin Microbiol 2000;38:2386–8.
[15] O'Neill GL, Ogunsola FT, Brazier JS, Duerden BI. Modification of a PCR ribotyping method for application as a routine typing scheme for Clostridium difficile. Anaerobe 1996;2:205–9.
[16] Avbersek J, Janezic S, Pate M, Rupnik M, Zidaric V, Logar K, et al. Diversity of Clostridium difficile and other animals in Slovenia. Anaerobe 2009;15: 252–5.
[17] Costa MC, Stämpfli HR, Arroyo LG, Pearl DL, Weese JS. Clostridium difficile on a veal farm: prevalence, molecular characterization and tetracycline resistance. Vet Microbiol 2011;152:379–84.
[18] Hoffer E, Haechler H, Frei R, Stephan R. Low occurrence of Clostridium difficile in faecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. J Food Prot 2010;73:973–5.
[19] Thitaram SN, Frank JF, Lyon SA, Siragusa GR, Bailey JS, Lombard JE, et al. Clostridium difficile from healthy food animals: optimized isolation and prevalence. J Food Prot 2011;74:130–3.
[20] Houser AB, Soehnlen KM, Wolfgang RD, Lysczek RH, Burns MC, Jayarao MB. Prevalence of Clostridium difficile toxin genes in the faeces of veal calves and incidence of ground veal contamination. Foodborne Pathog Dis 2012;9: 32–6.
[21] Alvarez-Perez S, Blanco J, Bouza E, Alba P, Gibert X, Maldonado J, et al. Prevalence of Clostridium difficile in diarrhoeic and non-diarrhoeic piglets. Vet Microbiol 2009;137:302–5.
[22] Keessen EC, van den Berkt AJ, Haasjes NH, Harmanus C, Kuijper EJ, Lipman LJ. The relation between farm specific factors and prevalence of Clostridium difficile in slaughter pigs. Vet Microbiol 2011;154:130–4.
[23] Baker AA, Davis E, Rehberger T, Rosener D. Prevalence and diversity of toxigenic Clostridium perfringens and Clostridium difficile among swine herds in the Midwest. Appl Environ Microbiol 2010;76(9):2961–6.

[24] Hopman NE, Keesen EC, Harmanus C, Sanders IM, van Leengoed LA, Kuijper EJ, et al. Acquisition of *Clostridium difficile* by piglets. Vet Microbiol 2011;21:186–92.

[25] Bauer MP, Notermans DW, Monnet DL, van Dissel JT, Kuijper EJ, ECDIS Study Group. *Clostridium difficile* infection in Europe: a hospital based survey. Lancet 2011;377:63–73.

[26] Rodriguez-Palacios A, Pickworth C, Loerch S, LeJeune JT. Transient faecal shedding and limited animal-to-animal transmission of *Clostridium difficile* by naturally infected finishing feedlot cattle. Appl Environ Microbiol 2011;77: 3391–7.

[27] Rupnik M, Avesani V, Janc M, von Eichel-Streiber C, Delmée M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J Clin Microbiol 1998;36:2240–7.

[28] Rupnik M. *Clostridium difficile* toxinotyping. Methods Mol Biol 2010;646: 67–76.

3.2.2 Presence of *C. difficile* in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium

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Presence of *Clostridium difficile* in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium

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ABSTRACT

The objective of this study was to evaluate the presence of *Clostridium difficile* in intestinal and carcass samples collected from pigs and cattle at a single slaughterhouse. *C. difficile* was isolated in 1% and 9.9% of the pig and cattle intestinal contents and in 7.9% and 7% of cattle and pig carcass samples respectively. A total of 19 different PCR-ribotypes were identified, among them types 078 and 014. Seven of 19 ribotypes correlated with the PCR-ribotypes involved in human *C. difficile* infections in Belgium. This study confirms that animals are carriers of *C. difficile* at slaughter and ribotypes are identical than those in humans, and that carcass contamination occurs inside the slaughterhouse.

1. Introduction

Clostridium difficile is an anaerobic, spore-forming bacterium that remains the main cause of nosocomial diarrhoea in humans after use of antibiotics. *C. difficile* has also been described in other environments outside of hospitals, such as soil, river and seawater samples (Al Saif and Brazier, 1996; Pasquale et al., 2011; Zidaric et al., 2010) and in animals, in which it can also cause enteric disease (Rodriguez-Palacios et al., 2006; Songer and Anderson, 2006). The possibility of transmission of *C. difficile* pathogenic isolates between animals, environments and humans has been suggested (Janezic et al., 2012).

In recent years, the interest in *C. difficile* in food and in food animals has increased, leading to studying animals as a possible reservoir and a potential risk for food borne infections linked to *C. difficile*. Studies in various countries have determined differences in the prevalence of *C. difficile* in animals just before slaughter (Baker et al., 2010; Hoffer et al., 2010; Houser et al., 2012; Keeseen et al., 2011; Rodriguez et al., 2012). In addition, many types, including PCR-ribotype 078, are present in humans, animals (Debast et al., 2009; Janezic et al., 2012) and meat (Boer et al., 2011; Curry et al., 2012; Weese et al., 2009). The PCR-ribotype 078 was among the three most prevalent ribotypes of *C. difficile* isolated from humans in Europe in 2009 (Bauer et al., 2011), and it also appears to be associated with increased virulence (Goorhuis et al., 2008) as the highly virulent epidemic strain *C. difficile* 027 (Kuijper et al., 2006). However, there is not much data describing *C. difficile* on carcasses at the slaughterhouse, and studies have failed to establish

the importance of the faecal contamination of the carcass on the slaughter line.

Differences in prevalence have been observed between studies. These differences between continents may be due to geographical differences in occurrence, seasonality or methodological variations (Hensgens et al., 2012; Rodriguez-Palacios et al., 2009; Weese, 2010). In most cases, isolation of *C. difficile* from animals requires an enrichment stage, and the methods recently used to detect *C. difficile* in animal samples have varied greatly. The influence of different factors such as enrichment time can affect the recovery rates of *C. difficile* in faecal samples or carcasses (Limbago et al., 2012).

The primary objective of this study was to determine the presence of *C. difficile* in intestinal contents and on carcasses in full-grown animals at the slaughterhouse. Additionally, the influence of the enrichment duration was evaluated with a method of 3 enrichment days and an increased enrichment step to a maximum of 30 days. *C. difficile* isolates were characterized and compared to the main PCR-ribotypes found in humans in Belgium.

2. Materials and methods

2.1. Sampling

Sampling was carried out between September 2011 and May 2012, and a total of 402 cattle and pig samples, including intestinal contents and carcass samples were collected. Intestinal contents and carcass samples were collected from a single slaughterhouse. This local slaughterhouse has a mean daily production of 154 cattle and 985 pigs with a work schedule of four days a week. Intestinal and carcass samples were collected between September and November 2011 on nine different

rotatory days at 1 to 2 week intervals. Between 4 and 20 consecutively slaughtered animals were sampled. Cattle samples were recovered from animals coming from 57 different herds. Most of the samples were from animals between 15 and 56 months old. Only two animals sampled were younger than 12 months, and 6 were older than 7 years. Pig samples were collected from animals between 5 and 6 months of age coming from 14 different herds with an average weight of 96 kg per carcass.

2.1.1. Intestinal contents

A total of 101 intestinal samples from cattle and 100 from pigs were recovered from the large intestine of each animal at the slaughter line, directly from the viscera processing area. Approximately 50 g of intestinal contents were collected by making an incision of approximately 3 cm on the top of the cecum. The sample content was extracted by applying pressure on the surface and after discarding the first outgoing content. All collected samples were kept in individual, identified, sterile 50 ml tubes at room temperature for a maximum of 5 h. They were processed the same day, immediately upon arrival at the laboratory.

2.1.2. Carcass samples

In total, 101 carcasses from cattle and 100 from pigs were sampled 2 h after slaughter, just after fast chilling in the chilling room. For cattle, 80.1% of carcass samples (81 animals) were taken from the same animal from which the intestinal content had been collected. In those cases when it was not possible to take the intestinal content and the carcass swabs from the same animal, another carcass in the chilling room was randomly selected for swabbing. In the case of pig samples, intestinal contents and carcass swabs were always taken from different animals.

Samples were taken from half carcasses according to the Belgian Royal Decree of 20 August 2002 from four different places. Briefly, cotton cosmetic pads were first moistened in sterile buffered peptone water with cysteine 0.5% (Oxoid, Dardilly, France). A 400 cm² area of rump, flank, brisket and posterior face of the anterior limb were swabbed with the wet side of the cotton, representing in total an area of 1600 cm² on each cattle carcass. On pig carcasses, swabs were taken from ham (100 cm²), basin (100 cm²), sternum (300 cm²) and forelimb (100 cm²), covering a total area of 600 cm². After using the wet side of the cotton, the same procedure was repeated in the same areas with the dry cotton face. For each carcass swab, an effort was made to exert the maximum pressure possible. Swabs from each half carcass were placed together in sterile, identified 100 ml tubes. They were kept at room temperature for a maximum of 2 h until arrival at the laboratory, where they were immediately processed.

2.2. C. difficile isolation and characterization

Culture of all samples was performed with an enrichment step. The enrichment broth used, cycloserine cefoxitin fructose taurocholate (CCFT), was freshly prepared in the laboratory as described by Delmée et al. (1987) but without agar. One gram of intestinal sample was inoculated into 9 ml of CCFT as previously described (Rodriguez et al., 2012). Carcass swabs were put into 50 ml of CCFT. Subsequently, the enrichment broth of each sample was incubated in an anaerobic workstation (Led Techno, Heusden-Zolder, Belgium) at 37 °C for 3 and 30 days. After each phase of enrichment, approximately 10 μl of the broth was spread on home-made cycloserine cefoxitin fructose agar taurocholate agar plates (CCFAT) (Delmée et al., 1987) and incubated anaerobically for 48 h at 37 °C. Colonies of *C. difficile* were identified from culture plates by morphological criteria as yellowish colonies with an appearance of ground glass and a characteristic horse manure odour. One morphological suspected colony per plate was subcultured onto blood agar (5% Sheep Blood; Biorad, Nazareth, Belgium) and checked using a *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid, Dardilly, France). Multiple colonies where taken only when presumptive colonies were too small to ensure isolation on the blood agar or when

morphologies suggested more than one type of colony (4 samples). Confirmation of *C. difficile* by detection of a species-specific internal fragment of *tpi* and detection of genes for toxin B and binary toxin (*cdtA*) were performed according to a specific multiplex PCR as described previously (Rodriguez et al., 2012). A second simplex PCR for the detection of the toxin A encoding gene was performed according to the primers of Antikainen et al. (2009) and the protocol of Lemée et al. (2004). Monolayer MRC-5 cells were used in order to confirm the cytotoxic activity as described previously (Rodriguez et al., 2012).

2.3. GenoType CDiff test and PCR-ribotyping

In addition, all of the isolates were tested using the Genotype CDiff test system (HainLifescience, Nehren, DE). The test detects specific internal fragments of *tpi* and all the toxin genes (*tcdA*, *tcdB*, *cdtA* and *cdtB*) and also deletion in the regulator gene *tcdC* (18 bp and 39 bp deletions or single base deletion at position 117). Procedures and reagents were accomplished following the manufacturer's instructions. PCR-ribotyping was performed with primers used for amplification of 16S–23S intergenic spacer regions previously described by Bidet et al. (1999). DNA was extracted using a chelex 100 solution 5% (Biorad, Nazareth, Belgium) as described by O'Neill et al. (1996). PCR amplification was performed following a previously described protocol (Rodriguez et al., 2012). Amplicon size was analysed by capillary electrophoresis using the ABI 3100 Automated Capillary DNA Sequencer and GeneScan Analysis (Applied Biosystems, California, USA). As an internal marker, 35–500 bp ROX ladder (Applied Biosystems, California, USA) was used for each sample. The isolates with an international number had presented a PCR-ribotype profile corresponding to one of the 23 reference Cardiff ribotypes from the strain collection available in our laboratory. If a strain had a profile that did not correspond to any of the 23, an arbitrary internal number beginning with UCL was given.

3. Results

3.1. Prevalence of C. difficile in intestinal contents and carcass samples

A total of 202 cattle samples were analysed. *C. difficile* was isolated from 10/101 (9.9%) intestinal samples from slaughter cattle. Most of the positive samples (8/10) were already isolated after 3 days of enrichment, but 2 positive samples were only isolated after 30 enrichment days. In cattle carcass samples, *C. difficile* was isolated from 8/101 samples (7.9%). Seven positive samples were detected after 3 days of enrichment, and only 1 sample was negative after 3 enrichment days but positive after 30 enrichment days. Positive samples were from animals aged between 11 months and 6 years. Two animals with positive carcasses came from the same herd, while the remaining positive samples (intestinal contents and carcasses) were from animals coming from different herds. Only in one case was *C. difficile* detected in the intestinal content and on the carcass sample of the same animal (Table 1).

From pigs, a total of 200 samples were analysed. *C. difficile* was isolated from 1/100 (1%) intestinal samples from slaughter pigs. This sample was already detected after 3 days of enrichment, and it was also positive after 30 enrichment days. On pig carcass samples *C. difficile* was isolated from 7/100 samples (7%) on the same sampling day. Six positives were detected after 3 and 30 days of enrichment, while 1 sample was only positive after 30 days of enrichment (Table 2).

All of the positive samples (intestinal and carcass samples from pigs and cattle) detected after 3 days of enrichment were also positive after 30 enrichment days.

3.2. PCR-ribotyping, toxin activity, toxin genes detection and Genotype CDiff

From the total of 26 positive samples, 19 different PCR-ribotypes were identified. Six of these PCR-ribotypes have a ribotype profile

Table 1
Clostridium difficile in intestinal and carcass samples obtained from slaughter cattle per sampling day and herd after 3 and 30 days of enrichment.

Date of sampling	Positive animals (total animals)	Positive herds (total herds)	Positive herd identification (animal sampled by herd)	Positive animals in each herd	Age of positive animals (months)	Positive Intestinal contents		PCR-ribotypes isolated from Intestinal contents		Positive Carcasses		PCR-ribotypes isolated from Carcasses	
						3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]
15/09/11	0 (7)	0 (7)											
23/09/11	3 (11)	3 (4)	A (7)	1[c]	26	+	+	UCL118	UCL118				
			B (1)	1[c]	17	+	+	UCL16R	UCL16L				
			C (3)	1	76	+	+	UCL270	UCL5a				
30/09/11	2 (12)	2 (8)	D (3)	1	24	+	+	UCL273/029[d]	029				
			E (1)	1	30	+	+	UCL254/078[d]	UCL254				
07/10/11	2 (7)	1 (6)	F (5)	2[c]	19					+	+	UCL5a	UCL5a
					18					+	+	015	015
21/10/11	1 (12)	1 (8)	G (1)	1	11	−	+		014				
04/11/11	2 (12)	2 (7)	H (1)	1	17	+	+	078	078				
			I (1)	1	18	−	+		078				
18/11/11	3 (11)	3 (8)	J (1)	1	22	+	+	UCL103	UCL103				
			K (1)	1	20					+	+	UCL16u	UCL16u
			L (1)	1	23					+	+	UCL16u	UCL16u
22/11/11	3 (6)	3 (5)	M (1)	1	20					+	+	023	023
			N (1)	1	20	+	+	078	078	+	+	UCL11	UCL11
			O (1)	1	18						+		UCL23d
29/11/11	1 (4)	1 (4)	P (1)	1	22					+	+	UCL5a	UCL5a

Shadowed parts of the table mean that no positives were found in the samples.
[a] Positive results detected after 3 days of enrichment.
[b] Positive results detected after 30 days of enrichment.
[c] Intestinal and carcass samples were not taken from the same animal.
[d] Two different PCR-ribotypes isolated from the same sample.

corresponding to the international collection (078, 014, 029, 023, 015, 081), while the rest of the strains were not associated with any reference Cardiff ribotypes (Table 3). The same PCR-ribotype was isolated from cattle intestinal contents and cattle carcass samples (PCR-ribotype UCL5a) (Table 3). PCR-ribotype 078 was isolated from pig and cattle intestinal contents. PCR-ribotype 014 was isolated from cattle intestinal contents and from pig carcasses (Table 3).

From cattle, in two intestinal contents more than one PCR-ribotype (n = 2) was isolated from each sample after 3 days of enrichment, while after 30 enrichment days only one PCR-ribotype was detected (Table 1). For the other positive samples, the same PCR-ribotype was detected after 3 and 30 days of enrichment except in two intestinal samples (positive herd B: 3 days with PCR-ribotype UCL16R and 30 days with PCR-ribotype UCL16L; positive herd C: 3 days with PCR-ribotype UCL270 and 30 days with PCR-ribotype UCL5a). Two animals with positive carcasses came from the same herd (herd F), but different

PCR-ribotypes were detected on each carcass (Table 1). Regarding the only animal testing positive in both intestinal content and carcass samples, different PCR-ribotypes were detected from each sample (intestinal content with PCR-ribotype 078; carcass with PCR-ribotype UCL11) (Table 1). Isolates from intestinal contents presented the widest variety in PCR-ribotypes (11 different PCR-ribotypes among the 19 identified) followed by the isolates from carcasses (6 different PCR-ribotypes identified) (Table 3).

From pigs, the same PCR-ribotype was detected after 3 and 30 days of enrichment, except in the positive intestinal content where two different PCR-ribotypes were isolated (3 days with PCR-ribotype 078; 30 days with PCR-ribotype UCL46) (Table 2). All positive carcasses (n = 7) came from the same sampling day, and 3 different PCR-ribotypes were identified (014, 081 and UCL36) (Table 2).

Forty-two of the total isolates (n = 50) had a toxic activity confirmed by the cytotoxicity assay. In cattle intestinal contents a total of 8

Table 2
Clostridium difficile in intestinal and carcass samples obtained from slaughter pigs per sampling day after 3 and 30 days of enrichment.

Date of sampling	Positive animals (total animals)		Positive Intestinal contents		PCR-ribotypes isolated from Intestinal contents		Positive Carcasses		PCR-ribotypes isolated from Carcasses	
	Intestinal contents	Carcass samples	3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]
23/09/11	0 (20)	0 (20)								
30/09/11	0 (12)	0 (12)								
07/10/11	0 (12)	0 (12)								
21/10/11	0 (12)	0 (12)								
04/11/11	1 (12)	0 (12)	+	+	078	UCL46				
22/11/11	0 (12)	0 (12)								
29/11/11	0 (20)	7 (20)					+	+	UCL36	UCL36
							+	+	014	014
							+	+	014	014
							+	+	081	081
							+	+	UCL36	UCL36
								+		014
							+	+	014	014

Shadowed parts of the table mean that no positives were found in the samples.
[a] Positive results detected after 3 days of enrichment.
[b] Positive results detected after 30 days of enrichment.

Table 3
PCR-ribotypes, *tcdC* and toxin gene profiles of *C. difficile* isolated from cattle and pigs intestinal contents, carcass and meat samples.

Animal group	Sample type	PCR-ribotype	No. isolates	Toxin activity	Detection of toxin genes by PCR			Genotype CDiff test system						
					tcdA	*tcdB*	*cdtA*	*tcdA*	*tcdB*	*cdtA*	*cdtB*	*tcdC*18 bp	*tcdC*39 bp	*tcdC*117 bp
Cattle	Intestinal contents	078	6	+	+	+	+	+	+	+	+	−	+	−
		UCL5a	1	+	+	+	+	+	+	+	+	−	+	−
		014	1	+	+	+	−	+	+	−	−	−	−	−
		UCL16L	1	+	+	+	−	+	+	−	−	−	−	−
		029	2	+	+	+	−	+	+	−	−	−	−	−
		UCL118	2	+	+	+	−	+	+	−	−	−	−	−
		UCL16R	1	+	+	+	−	+	+	−	−	−	−	−
		UCL254	2	+	+	+	−	+	+	−	−	−	−	−
		UCL270	1	−	−	−	−	−	−	−	−	−	−	−
		UCL273	1	−	−	−	−	−	−	−	−	−	−	−
		UCL103	2	−	−	−	−	−	−	−	−	−	−	−
	Carcass samples	023	2	+	+	+	+	+	+	+	+	+	+	−
		UCL5a	4	+	+	+	+	+	+	+	+	−	+	−
		UCL11	2	+	+	+	+	+	+	+	+	−	+	−
		015	2	+	+	+	−	+	+	−	−	+	−	−
		UCL16u	4	+	+	+	−	+	+	−	−	−	−	−
		UCL23d	1	+	+	+	−	+	+	−	−	−	−	−
Pork	Intestinal contents	078	1	+	+	+	+	+	+	+	+	−	+	−
		UCL46	1	+	+	+	−	+	+	−	−	−	−	−
	Carcass samples	014	7	+	+	+	−	+	+	−	−	−	−	−
		081	2	+	+	+	−	+	+	−	−	−	−	−
		UCL36	4	−	−	−	−	−	−	−	−	−	−	−

PCR-ribotypes had toxic activity, and only three were identified as non-toxigenic, while all cattle carcass PCR-ribotypes identified were toxigenic. Among PCR-ribotypes recovered from pigs, only one strain from carcass was non-toxigenic. These results are obtained at the same time by the PCR results targeting *tpi, tcdA, tcdB* and *cdtA* and by the results of the GenoType CDiff test system. All the toxigenic identified isolates contained *tcdA* and *tcdB* genes. All isolates of PCR-ribotypes 078, 023, UCL5a and UCL11 also contained *cdtA* and *cdtB* gene coding for the binary toxin and had a 39 bp deletion in the regulator gene *tcdC*. An 18 bp deletion in *tcdC* was only detected in all the isolates of PCR-ribotypes 015 and 023. All the isolates of PCR-ribotypes UCL 270, UCL 273, UCL 103 and UCL 36 were negative for all toxin genes (Table 3).

4. Discussion

The present study determined the prevalence of *C. difficile* in intestinal contents and carcasses in slaughter pigs and cattle in Belgium and also includes toxin activity, toxin gene detection and detection of deletion in *tcdC* gene of all isolates.

C. difficile was isolated most frequently from intestinal contents (9.9%) of cattle at the slaughterhouse. In our previous study in Belgium (Rodriguez et al., 2012) and in two other studies in The United States (Houser et al., 2012; Thitaram et al., 2011) the prevalence reported ranges between 6.3% and 12% in cattle just before slaughter. This prevalence is much lower in other studies conducted in slaughter cattle in Europe (Hoffer et al., 2010; Koene et al., 2011). The difference in prevalence among studies may be due to geographical, seasonal or methodological variations as previously described (Hensgens et al., 2012; Weese, 2010). The condition that the sampling size between studies is not identical should also be considered. Limited information is available for the prevalence of *C. difficile* on cattle carcasses at the slaughterhouse. Rodriguez-Palacios et al. (2011) reported 0 positive carcasses from a total of 168 samples analysed. Houser et al. (2012) detected the *tpi* housekeeping gene by PCR in 4 of 100 carcass swabs. One of these carcasses was also positive for the *tcdA* gene, but *C. difficile* was not isolated using culture techniques. This present study is the first to target *C. difficile* isolated from cattle carcasses at the slaughterhouse with an observed prevalence of 7.9% (8/101). Positive samples were

detected on three different sampling days. Despite the high prevalence of *C. difficile* in intestinal contents and on carcasses, only one animal was positive for both samples but ribotypes were not identical. These results suggest that carcass contamination during processing might occur.

C. difficile was recovered from only one intestinal content (1%) from pigs at the slaughterhouse. Reported prevalence rates of *C. difficile* vary widely among other studies conducted in different countries. A prevalence ranging between 3.3 and 8.6% was reported in The Netherlands (Keeseen et al., 2011), Austria (Indra et al., 2009), The United States (Norman et al., 2009) and Canada (Weese et al., 2011). In other studies the prevalence described is much lower: in Switzerland (0%) (Hoffer et al., 2010), in The United States (0.3%) (Susick et al., 2012) and in our previous study in Belgium (0%) (Rodriguez et al., 2012). However, as in the case of intestinal contents from cattle, the condition that the sampling size or methodologies between studies are not identical must be considered. Carcasses from 7% (7/100) of the slaughter pigs were positive for *C. difficile*. There are only a few studies describing *C. difficile* on pig carcasses at the slaughterhouse. In The United States Susick et al. (2012) reported a prevalence of 2.2% (4/182) and 2.5% on post-evisceration and post-chill swabs respectively in antimicrobial free pigs. Harvey et al. (2011) detected 3 positive *C. difficile* samples from a total of 10 sponge swabs collected from carcass hide, post-excision hide and ears in a processing plant in Texas. Another recent study conducted in Canada (Hawken et al., 2013) reported a total of 3 out of 20 positive carcasses (15%) sampled at post-bleed and a further 3 out of 20 (15%) at post-evisceration. To the authors' knowledge, this current study is the first study to isolate *C. difficile* from pig carcasses at the slaughterhouse in Europe. However, the 80 negative carcasses previously collected in 6 different sampling days and the sole positive sample of intestinal content detected corroborate that the prevalence of *C. difficile* in slaughter pigs is very low. The higher prevalence of *C. difficile* in pig carcasses reported in the present study (7%) could be explained by an unusually high contamination in the slaughtered herd or a previous faecal contamination through at the slaughter line. In the sampling plan developed for pigs, first, intestinal contents were collected from consecutively slaughtered animals coming from the same herd. Next, carcasses from another herd were sampled in the chilling room. These carcasses were from animals

Table 4
C. difficile PCR-ribotypes isolated from pig at cattle at slaughter age and comparison with the most frequent PCR-ribotypes isolated from humans.

Cardiff PCR-Ribotypes	Cattle Faecal samples No. Isolates (%)	Country	Reference	Cattle Carcass samples No. Isolates (%)	Country	Reference	Pigs Faecal samples No. Isolates (%)	Country	Reference	Pigs Carcass samples No. Isolates (%)	Country	Reference	Humans Belgium 2011[2]	Europe 2008[3]
014	1 (7.1)	Belgium	Present study[1]	–	–	–	9 (15.5)	Netherlands	Keeseen et al., 2011	7 (50)	Belgium	Present study[1]	56 (12.1)	61 (16)
	1 (7.1)	Belgium	Rodriguez et al., 2012											
	1 (33.3)	Austria	Indra et al., 2009											
002	1 (7.1)	Belgium	Rodriguez et al., 2012	–	–	–	1 (1.7)	Netherlands	Keeseen et al., 2011	–	–	–	37 (8)	18 (5)
027	–	–	–	–	–	–	–	–	–	–	–	–	36 (7.8)	19 (5)
078	4 (28.6)	Belgium	Present study[1]	–	–	–	1 (50)	Belgium	Present study[1]	6 (100)	Canada	Hawken et al., 2013	35 (7.6)	31 (8)
	1 (100)	Switzerland	Hoffer et al., 2010				20 (67)	Canada	Weese et al., 2011	3 (100)	USA	Harvey et al., 2011		
	4 (100)	Canada	Costa et al., 2011				18 (31)	Netherlands	Keeseen et al., 2011	–	–	–		
020	–	–	–	–	–	–	–	–	–	–	–	–	31 (6.7)	61 (16)
001	–	–	–	–	–	–	2 (3.4)	Netherlands	Keeseen et al., 2011	–	–	–	10 (2.1)	37 (10)
023	–	–	–	1 (12.5)	Belgium	Present study[1]	2 (3.4)	Netherlands	Keeseen et al., 2011	–	–	–	10 (2.1)	10 (3)
012	6 (85.7)	Netherlands	Koene et al., 2011	–	–	–	–	–	–	–	–	–	9 (2)	17 (4)
015	–	–	–	1 (12.5)	Belgium	Present study[1]	2 (3.4)	Netherlands	Keeseen et al., 2011	–	–	–	4 (0.87)	13 (3)
081	1 (7.1)	Belgium	Rodriguez et al., 2012	–	–	–	–	–	–	1 (16.7)	Belgium	Present study[1]	–	
087	1 (7.1)	Belgium	Rodriguez et al., 2012	–	–	–	–	–	–	–	–	–	–	
033	1 (14.3)	Netherlands	Koene et al., 2011	–	–	–	–	–	–	–	–	–	–	
013	–	–	–	–	–	–	5 (8.6)	Netherlands	Keeseen et al., 2011	–	–	–	–	
018	–	–	–	–	–	–	–	–	–	–	–	–	–	23 (6)
106	–	–	–	–	–	–	–	–	–	–	–	–	–	20 (5)
017	–	–	–	–	–	–	–	–	–	–	–	–	–	14 (4)
126	–	–	–	–	–	–	–	–	–	–	–	–	–	12 (3)

(1) PCR-ribotypes of isolates after 30 days of enrichment were only taken into account in the case of being different of the previously found after 3 days of enrichment.
(2) Unpublished data from the National *Reference Laboratory* for *Clostridium difficile* in Belgium.
(3) Data from the study of Bauer et al. (2011).

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slaughtered before the animals from which the intestinal contents had been taken. Therefore no conclusion about the focus of the contamination can be stated.

The detection method that was used in this study was performed without ethanol-shock treatment. A previously pilot study in our laboratory demonstrated that the detection of the *C. difficile* colonies improved without pre-treatment of the samples (unpublished data). Furthermore, the medium used (CCFT) is an excellent selective and differential medium for *C. difficile*, as described previously (Delmée et al., 1987; George et al., 1979). This detection method has two enrichment phases. In our first study the enrichment step was prolonged to a maximum duration of 30 days in 15 pig intestinal samples that tested negative after 3 days of enrichment. A total of three new positive samples resulted (Rodriguez et al., 2012). As the sample size (n = 15) was small for drawing conclusions, in the present study the enrichment step of 30 days was applied to all samples. Additional positives were found in 20% of cattle intestinal samples (n = 2), 12.5% of cattle carcasses (n = 1) and 14.3% of pig carcasses (n = 1). It seems that the increase in the time of enrichment improves the sensitivity of the method. However 30 days of enrichment is a long technique for laboratory purposes for the slight increase of the sensitivity observed. Moreover, a bacterial competition or a low level of *C. difficile* in the enrichment broth could explain the differences between 3 and 30 days of enrichment, which can have a direct impact on the results (Weese et al., 2009). After 30 days of enrichment, rarely other colonies than *C. difficile* were found. The presence of other bacteria in the plate was more relevant after 3 days of enrichment. The finding of different PCR-ribotypes in some of the samples after 3 and 30 enrichment days reinforced this hypothesis.

Six PCR-ribotypes out of the total of 19 found could be assigned to international Brazier types. Intestinal contents and carcass samples from slaughter cattle showed the greatest variety of PCR-ribotypes. Intestinal contents also showed a considerable percentage of non-toxigenic PCR-ribotypes. Some of these strains, like PCR-ribotype UCL273 had not been isolated before in humans in Belgium. Moreover, several different PCR-ribotypes were obtained in some single intestinal samples from cattle. This finding is in accordance with a previous study describing the presence of more than one different type of isolate in rectal samples of calves (Zidaric et al., 2012).

Other studies in various countries have also identified *C. difficile* PCR-ribotypes from slaughter animals closely related to human PCR-ribotypes (Table 4). In 2011 in Belgium the most prevalent PCR-ribotypes in hospitals were 014, 002, 027, 078, 020, UCL46, UCL16l, UCL26, 001, 023, UCL23f, 012, UCL16b, 015, UCL5a, UCL20a and UCL49 sorted by decreasing values in number of isolates (unpublished data). A total of 7 of these 17 ribotypes were isolated in the present study.

In conclusion, this study shows that toxigenic *C. difficile* is present in the slaughterhouse in Belgium, among them PCR-ribotypes 078, 029 and 014. Carcasses were contaminated with a variety of PCR-ribotypes that were not found in the intestinal samples for the same animals, suggesting a slaughterhouse environmental contamination. This study further documented that animals are carriers of *C. difficile* at slaughter, and carcass contamination occurs inside the slaughterhouse.

Acknowledgements

References

Al Saif, N., Brazier, J.S., 1996. The distribution of *Clostridium difficile* in the environment of South Wales. Journal of Medical Microbiology 45, 133–137.

Antikainen, J., Pasanen, T., Mero, S., Tarkka, E., Kirveskari, J., Kotila, S., Mentula, S., Könönen, E., Virolainen-Julkunen, A.R., Vaara, M., Tissari, P., 2009. Detection of virulence genes of *Clostridium difficile* by multiplex PCR. Acta Phatologica, Microbiologica et InmunologicaScandinavica 117, 607–613.

Baker, A.A., Davis, E., Rehberger, T., Rosener, D., 2010. Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the Midwest. Applied and Environmental Microbiology 76, 2961–2966.

Bauer, M.P., Notermans, D.W., van Benthem, B.H.B., Brazier, J.S., Wilcox, M.H., Rupnik, M., Monnet, D.L., van Dissel, J.T., Kuijper, E.J., 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. The Lancet 377, 63–73.

Bidet, P., Barbut, F., Lalande, V., Burghoffer, B., Petit, J.C., 1999. Development of a new PCR-ribotyping method based on ribosomal RNA gene sequencing. FEMS Microbiology Letters 175, 261–266.

Boer, E., Zwartkruis-Nahuis, A., Heuvelink, A.E., Harmanus, C., Kuijper, E.J., 2011. Prevalence of *Clostridium difficile* in retailed meat in The Netherlands. International Journal of Food Microbiology 144, 561–564.

Costa, M.C., Stämpfli, H.R., Arroyo, L.G., Pearl, D.L., Weese, J.S., 2011. Epidemiology of *Clostridium difficile* on a veal farm: prevalence, molecular characterization and tetracycline resistance. Veterinary Microbiology 152, 379–384.

Curry, S.R., Marsh, J.W., Schlackman, J.L., Harrison, L.H., 2012. Prevalence of *Clostridium difficile* in uncooked ground meat products from Pittsburgh, Pennsylvania. Applied and Environmental Microbiology 78, 4183–4186.

Debast, S.B., van Leengoed, L.A., Goorhuis, A., Harmanus, C., Kuijper, E.J., Bergwerff, A.A., 2009. *Clostridium difficile* PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. Environmental Microbiology 11, 505–511.

Delmée, M., Vandercam, B., Avesani, V., Michaux, J.L., 1987. Epidemiology and prevention of *Clostridium difficile* in a leukaemia unit. European Journal of Clinical Microbiology 6, 623–627.

George, W.L., Sutter, V.L., Citron, D., Finegold, S.M., 1979. Selective and differential medium for isolation of *Clostridium difficile*. Journal of Clinical Microbiology 9, 214–219.

Goorhuis, A., Bakker, D., Corver, J., Debast, S.B., Harmanus, C., Notermans, D.W., Bergwerff, A.A., Dekker, F.W., Kuijper, E.J., 2008. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotypes 078. Clinical Infection Infectious? Disease 47, 1162–1170.

Harvey, R.B., Norman, K.N., Andrews, K., Norby, B., Hume, M.E., Scanlan, C.M., Hardin, M.D., Scott, H.M., 2011. *Clostridium difficile* in retail meat and processing plants in Texas. Journal of Veterinary Diagnostic Investigation 23, 807–811.

Hawken, P., Weese, J.S., Friendship, R., Warriner, K., 2013. Longitudinal study of *Clostridium difficile* and Methicillin-resistant *Staphylococcus aureus* associated with pigs from weaning through to the end processing. Journal of Food Protection 76, 624–630.

Hensgens, M.P.M., Keessen, E.C., Squire, M.M., Riley, T.V., Koene, M.G.J., De Boer, E., Lipman, L.J.A., Kuijper, E.J., European Society of Clinical Microbiology and Infectious Diseases Study Group for Clostridium difficile (ESGCD), 2012. *Clostridium difficile* infection in the community: a zoonotic disease? Clinical Microbiology and Infectious Disease 18, 635–645.

Hoffer, E., Haechler, H., Frei, R., Stephan, R., 2010. Low occurrence of *Clostridium difficile* in faecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. Journal of Food Protection 73, 973–975.

Houser, A.B., Soehnlen, K.M., Wolfgang, R.D., Lysczek, R.H., Burns, M.C., Jayarao, M.B., 2012. Prevalence of *Clostridium difficile* toxin genes in the faeces of veal calves and incidence of ground veal contamination. Foodborne Pathogens and Disease 9, 32–36.

Indra, A., Lassnig, H., Baliko, N., Much, P., Fiedler, A., Huhulescu, S., Allerberger, F., 2009. *Clostridium difficile*: a new zoonotic agent? Wiener KlinischeWochenschrift 121, 91–95.

Janezic, S., Ocepek, M., Zidaric, V., Rupnik, M., 2012. *Clostridium difficile* genotypes other than ribotype 078 that are prevalent among human, animal and environmental isolates. Biomedical Central Microbiology 12, 48.

Keeseen, E.C., van den Berkt, A.J., Haasjes, N.H., Harmanus, C., Kuijper, E.J., Lipman, L.J., 2011. The relation between farm specific factors and prevalence of *Clostridium difficile* in slaughter pigs. Veterinary Microbiology 154, 130–134.

Koene, M.G.J., Mevious, D., Wagenaar, J.A., Harmanus, C., Hensgens, M.P.M., Meetsma, A.M., Putirulan, F.F., Van Bergen, M.A.P., Kuijper, E.J., 2011. *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. Clinical Microbiology and Infection 18, 778–784.

Kuijper, E.J., Coignard, B., Tull, P., ESCMID Study Group Clostridium difficile, EU Member States, European Centre for Disease Prevention and Control, 2006. Emergence of Clostridium difficile-associated disease in Nord America and Europa. Clinical Microbiology and Infection 12, 2–18.

Lemée, L., Dhalluin, A., Testelin, S., Mattrat, M., Maillard, K., Lemeland, J., Pons, J.L., 2004. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxin B) genes for toxigenic culture of *Clostridium difficile*. Journal of Clinical Microbiology 42, 5710–5714.

Limbago, B., Thompson, A.D., Greene, S.A., MacCannell, D., MacGowan, C.E., Jolbitado, E., Hardin, H.D., Estes, S.R., Weese, J.S., Songer, J.G., Gould, L.H., 2012. Development of a consensus method for culture of *Clostridium difficile* from meat and its use in a survey of U.S. retail meats. Food Microbiology 32, 448–451.

Norman, K.N., Harvey, R.B., Scott, H.M., Hume, M.E., Andrews, K., Brawley, A.D., 2009. Varied prevalence of *Clostridium difficile* in an integrated swine operation. Anaerobe 15, 256–260.

O'Neill, G.L., Ogunsola, F.T., Brazier, J.S., Duerden, B.I., 1996. Modification of PCR ribotyping method for application as a routine typing scheme for *Clostridium difficile*. Anaerobe 2, 205–209.

Pasquale, V., Romano, V.J., Rupnik, M., Dumontet, S., Ciznar, I., Aliberti, F., Mauri, F., Saggiomo, V., Krovacek, K., 2011. Isolation and characterization of *Clostridium difficile* from shellfish and marine environments. Folia Microbiologica 56, 431–437.

Rodriguez, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., Daube, G., 2012. *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 18, 621–625.

Rodriguez-Palacios, A., Staempfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., Weese, J.S., 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerging Infectious Diseases 12, 1730–1736.

Rodriguez-Palacios, A., Reid-Smith, R.J., Staempfli, H.R., Daignault, D., Janecko, N., Avery, B.P., Martin, H., Thompson, A.D., McDonald, L.C., Limbago, B., Weese, J.S., 2009. Possibility of seasonality of *Clostridium difficile* in retail meat, Canada. Emerging Infectious Diseases 15, 802–805.

Rodriguez-Palacios, A., Pickworth, C., Loerch, S., LeJeune, T., 2011. Transient faecal shedding and limited animal-to-animal transmission of *Clostridium difficile* by naturally infected finishing feedlot cattle. Applied and Environmental Microbiology 77, 3391–3397.

Songer, J.G., Anderson, M.A., 2006. *Clostridium difficile*: an important pathogen of food animals. Anaerobe 12, 1–4.

Susick, E.K., Putnam, M., Bermudez, D.M., Thakur, S., 2012. Longitudinal study comparing the dynamics of *Clostridium difficile* in conventional and antimicrobial free pigs at farm and slaughter. Veterinary Microbiology 25, 172–178.

Thitaram, S.N., Frank, J.F., Lyon, S.A., Siragusa, G.R., Bailey, J.S., Lombard, J.E., Haley, C.A., Wagner, B.A., Dargatz, D.A., Fedorka-Cray, P.J., 2011. *Clostridium difficile* from healthy food animals: optimized isolation and prevalence. Journal of Food Protection 74, 130–133.

Weese, J.S., 2010. *Clostridium difficile* in food - innocent bystander or serious threat? Microbiology and Infectious Disease 16, 3–10.

Weese, J.S., Avery, B.P., Rousseau, J., Reid-Smith, R.J., 2009. Detection and enumeration of *Clostridium difficile* spores in retail beef and pork. Applied and Environmental Microbiology 75, 5009–5011.

Weese, J.S., Rousseau, J., Deckert, A., Gow, S., Reid-Smith, R.J., 2011. *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus* shedding by slaughter-age pigs. Biomedical Central Veterinary Research 7, 41.

Zidaric, V., Beigot, S., Lapajne, S., Rupnik, M., 2010. The occurrence and high diversity of *Clostridium difficile* genotypes in rivers. Anaerobe 16, 371–375.

Zidaric, V., Pardon, B., Dos Vultos, T., Deprez, P., Brouwer, M.S.M., Roberts, A.P., Henriques, A.O., Rupnik, M., 2012. Multiclonal presence of *Clostridium difficile* PCR ribotypes 078, 126 and 033 within a single calf farm is associated with differences in antibiotic resistance and sporulation properties. Applied and Environmental Microbiology 78, 8515–8522.

3.2.3 Investigation of *C. difficile* interspecies relatedness using multilocus sequence typing, multilocus variable-number tandem-repeat analysis and antimicrobial susceptibility testing

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Investigation of *Clostridium difficile* interspecies relatedness using multilocus sequence typing, multilocus variable-number tandem-repeat analysis and antimicrobial susceptibility testing

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ABSTRACT

Multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA) and antimicrobial susceptibility were performed on 37 animal and human *C. difficile* isolates belonging to 15 different PCR-ribotypes in order to investigate the relatedness of human and animal isolates and to identify possible transmission routes.

MLVA identified a total of 21 different types while MLST only distinguished 12 types. Identical *C. difficile* strains were detected in the same animal species for PCR-ribotypes 014, 078, UCL 16U and UCL 36, irrespective of their origin or the isolation date. Non clonal strains were found among different hosts; however, a high genetic association between pig and cattle isolates belonging to PCR-ribotype 078 was revealed. MLVA also showed genetic differences that clearly distinguished human from animal strains. For a given PCR-ribotype, human and animal strains presented a similar susceptibility to the antimicrobials tested. All strains were susceptible to vancomycin, metronidazole, chloramphenicol and rifampicin, while PCR-ribotypes 078, UCL 5a, UCL 36 and UCL 103 were associated with erythromycin resistance.

The data suggest a wide dissemination of clones at hospitals and breeding-farms or a contamination at the slaughterhouse, but less probability of interspecies transmission. However, further highly discriminatory genotyping methods are necessary to elucidate interspecies and zoonotic transmission of *C. difficile*.

Introduction

Clostridium difficile is an anaerobic spore-forming bacterium responsible for serious enterocolitis in humans and animals. Several outbreaks have been reported in recent years, particularly in hospitals, making *C. difficile* a primarily nosocomial pathogen in humans (Jones et al., 2013). However, the increasing number of *C. difficile* infections (CDI) acquired in the community (Hensgens et al., 2012) and the large number of reports describing food (Rodriguez-Palacios et al., 2013), animal (Susick et al., 2012) and environmental (Zidaric et al., 2010; Hargreaves et al., 2013) reservoirs of the bacterium suggest possible transmission outside healthcare settings. For this reason, several studies have considered food and animals as potential sources for human community-acquired CDI (Rodriguez-Palacios et al., 2013).

Previous studies have focused on comparing *C. difficile* isolates from diverse sources and hosts (Lemée et al., 2005; Bakker et al., 2010; Griffiths et al., 2010; Stabler et al., 2012). Multilocus variable-number tandem-repeat analysis (MLVA) and multilocus sequence typing (MLST) have been recently developed to bring out phylogenetic relationships among the *C. difficile* population. MLVA shows a high level of discrimination and is considered useful for tracking outbreak strains geographically and for identifying cross-infection clusters between patients (Killgore et al., 2008; Manzoor et al., 2011). Data obtained by MLST can be used to determine the molecular phylogeny of *C. difficile* isolates and are highly transportable interlaboratory (Killgore et al., 2008).

Few studies using MLVA and MLST methods have focused on comparing human and animal isolates. Three previous studies (Lemée et al., 2004, 2005; Stabler et al., 2012) analysed isolates from various hosts by MLST, and the MLVA technique has been applied to determine the relatedness of *C. difficile* PCR ribotype 078 and 027 isolates from different hosts (Debast et al., 2009; Bakker et al., 2010).

In this study MLVA and MLST were both used to investigate genetic relationships between *C. difficile* isolates from human faecal

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Table 1
PCR-ribotypes, toxin activity, gen profile and in vitro antibiotic resistance of the human and animal strains characterised by MLST.

Strain identification	Origin	Source	PCR-ribotype	Toxin activity[a]	Toxin genes[b]	tcdC deletion[b]			GyrA mutation[b]	
						tcdC 18bp	tcdC 36bp	tcdC 117	gyrA Mut 1A	gyrA Mut 1B
5828	Human	NRCCD[d]	078	+	A$^+$ B$^+$ CDT$^+$	−	+	−	−	−
5063	Human	NRCCD[d]	078	+	A$^+$ B$^+$ CDT$^+$	−	+	−	+	−
4667	Human	NRCCD[d]	078	+	A$^+$ B$^+$ CDT$^+$	−	+	−	−	−
6136	Pig	Intestinal contents[e]	078	+	A$^+$ B$^+$ CDT$^+$	+	−	+	−	−
6135	Pig	Intestinal contents[e]	078	+	A$^+$ B$^+$ CDT$^+$	+	−	+	−	−
7485	Cow	Intestinal contents[f]	078	+	A$^+$ B$^+$ CDT$^+$	−	+	−	−	−
6423	Cow	Intestinal contents[f]	078	+	A$^+$ B$^+$ CDT$^+$	−	+	−	+	−
1101	Human	NRCCD[d]	UCL 11	+	A$^+$ B$^+$ CDT$^+$	NT	NT	NT	NT	NT
6412	Cow	Carcass[f]	UCL 11	+	A$^+$ B$^+$ CDT$^+$	−	+	−	−	−
5001	Human	NRCCD[d]	UCL 5a	+	A$^+$ B$^+$ CDT$^+$	NT	NT	NT	NT	NT
6408	Cow	Carcass[f]	UCL 5a	+	A$^+$ B$^+$ CDT$^+$	−	+	−	+	−
4592	Human	NRCCD[d]	014	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
4455	Human	NRCCD[d]	014	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6420	Cow	Intestinal contents[f]	014	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
7071	Pig	Carcass[f]	014	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6414	Pig	Carcass[f]	014	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6415	Pig	Carcass[f]	014	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6427	Cow	Intestinal contents[f]	UCL 16L	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
1601	Human	NRCCD[d]	UCL 16U	+	A$^+$ B$^+$ CDT$^-$	NT	NT	NT	NT	NT
6410	Cow	Carcass[f]	UCL 16U	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6413	Cow	Carcass[f]	UCL 16U	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
4597	Human	NRCCD[d]	015	+	A$^+$ B$^+$ CDT$^-$	NT	NT	NT	NT	NT
6409	Cow	Carcass[f]	015	+	A$^+$ B$^+$ CDT$^-$	+	−	−	−	−
8387	Human	NH[c]	UCL 36	−	A$^-$ B$^-$ CDT$^-$	−	−	−	−	−
8378	Human	NH[c]	UCL 36	−	A$^-$ B$^-$ CDT$^-$	−	−	−	−	−
7083	Pig	Carcass[f]	UCL 36	−	A$^-$ B$^-$ CDT$^-$	−	−	−	−	−
7078	Pig	Carcass[f]	UCL 36	−	A$^-$ B$^-$ CDT$^-$	−	−	−	−	−
8101	Human	NRCCD[d]	081	+	A$^+$ B$^+$ CDT$^-$	NT	NT	NT	NT	NT
7077	Pig	Carcass[f]	081	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6140	Pig	Intestinal contents[e]	081	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
4181	Human	NRCCD[d]	UCL 46	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
7488	Pig	Intestinal contents[f]	UCL 46	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6421	Cow	Intestinal contents[f]	UCL 118	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6425	Cow	Intestinal contents[f]	UCL 270	−	A$^-$ B$^-$ CDT$^-$	−	−	−	−	−
7487	Cow	Intestinal contents[f]	UCL 103	−	A$^-$ B$^-$ CDT$^-$	−	−	−	−	−
6430	Cow	Intestinal contents[f]	UCL 254	+	A$^+$ B$^+$ CDT$^-$	−	−	−	−	−
6411	Cow	Carcass[f]	023	+	A$^+$ B$^+$ CDT$^+$	+	+	−	−	−

NT, not tested.
[a] Toxin activity was determined using confluent monolayer MRC-5 cells.
[b] Results obtained by the Genotype CDiff test system and/or multiplex PCR.
[c] *C. difficile* isolates from patients of a Belgian nursing home (unpublished data).
[d] *C. difficile* strains isolated from hospitalised patients at the Belgium National Reference Centre for *Clostridium difficile*.
[e] Rodriguez et al. (2012).
[f] Rodriguez et al. (2013).

samples, pig and cattle intestinal and carcasses samples isolated in the same geographic region and during the same time period. Additionally, all of the isolates were characterised and compared with respect to their antibiotic resistance.

Materials and methods

Isolates

A total of 37 isolates were investigated. Eleven human isolates were obtained from the *C. difficile* collection at the Microbiology Unit, Catholic University of Louvain (the human *C. difficile* reference laboratory in Belgium). Another two human isolates were obtained from aged residents at a local Belgian nursing home. In animals, a total of 24 isolates were analysed; 12 were obtained from animal intestinal samples at slaughter (four from pigs and eight from cattle). The remaining 12 isolates were recovered from carcasses at the slaughterhouse (six from pigs and six from cattle). Isolates were first characterised using PCR-ribotype and toxin gene profiles using the multiplex PCR and Genotype CDiff systems (Rodriguez et al., 2012, 2013) (Table 1).

MLVA

All of the *C. difficile* intestinal and carcass isolates from pigs and cattle were analysed by MLVA. In addition, three isolates from hospital patients (PCR-ribotype 078) and two isolates from nursing home residents (PCR-ribotype UCL 36) were further typed by MLVA in order to compare them with the same PCR-ribotypes found in animals. These human isolates were selected on the basis of their prevalence in Belgian healthcare settings.

DNA extraction was performed using a Chelex 100 Solution 5% (BioRad) as described previously (O'Neill et al., 1996). A6, B7, C6, E7, G8, CDR5 and CDR60 variable-number-tandem repeat (VNTR) loci were amplified as previously described with minor modifications (Manzoor et al., 2011). Briefly, three separate PCR duplexes (A6-B7; C6-E7; CDR5-CDR60) and one single PCR (G8) were performed. Forward PCR primers for loci CDR60, E7 and B7 were labelled with hexa-chlorofluorescein (HEX) while the remaining loci (A6, C6, CDR5 and G8) were labelled with 6-carboxyfluorescein (FAM). PCR fragments were analysed using multi-coloured capillary electrophoresis on an ABI3130 automatic sequencer (Applied Biosystems). Electropherograms were analysed using Genemapper V4.0 software (Life Technologies) and copy numbers were determined. The summed absolute difference between two MLVA-typed isolates was the calculated summed tandem repeat difference (STRD) at all seven loci using the Manhattan coefficient (Marsh et al., 2006). Isolates with MLVA STRD ≤ 2 were indicative of a high degree of genetic relatedness and the value was used to define MLVA clusters (Best et al., 2014). For each VNTR, the Simpson and Hunter–Gaston's diversity indices were calculated using the VNTR diversity and confidence extractor software (V-DICE).[1]

MLST

C. difficile animal (*n* = 24) and human (*n* = 13) isolates were further characterised by MLST. Isolates were sequence typed using seven housekeeping loci (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA* and *tpi*) according to the scheme described by Griffiths et al.

[1] See: http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl (accessed 20 February 2014).

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(2010). PCR products were purified with a Wizard SV Gel and PCR Clean-Up System Kit (Promega) and sequenced (10 ng of DNA) with PCR forward or reverse primers using the Sanger sequencing reaction BigDye Terminator Kit version 3.1 (Applied Biosystems). Results were analysed using the Geneious program.[2] The allele number, clade and sequence type (ST) were assigned according to the *C. difficile* MLST reference database.[3] A dendrogram was constructed using the Geneious program. Prim's algorithm was applied to draw a minimum-spanning tree from allelic profile data.[4]

Concordance and discriminatory ability of MLVA and MLST methods

Simpson's index of diversity was used to compare the discriminatory ability of MLVA and MLST by measuring the probability that two unrelated strains will be differentiated by the two typing methods (Hunter and Gaston, 1988). Adjusted Wallace coefficient and corresponding confidence intervals (CI) were used to determine the concordance between MLST and MLVA typing methods (Severiano et al., 2011). Simpson's diversity index and concordance calculations were performed with an accessible online tool for quantitative assessment of classification agreement.[5]

Antibiotic resistance Etest testing

Susceptibility to metronidazole, moxifloxacin and vancomycin was determined by Etest strips (Lucron ELITechGroup) on Brucella Blood Agar with hemin and vitamin K1 (Becton-Dickinson) according to the manufacturer's instructions. Plates were anaerobically incubated at 37 °C for 48 h. The susceptibility (s) and resistance (r) breakpoints for metronidazole (s ≤ 8 μg/mL; r ≥ 32 μg/mL) and moxifloxacin (s ≤ 2 μg/mL; r ≥ 8 μg/mL) used for interpretation were those recommended by the Clinical and Laboratory Standard Institute (CLSI, 2010). Vancomycin MIC breakpoints (s ≤ 2 μg/mL; r ≥ 2 μg/mL) were established following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) rules.[6] *Bacteroides fragilis* ATCL 25285 was included as a quality control.

Antibiotic resistance disc susceptibility testing

Disc diffusion was performed with standard discs (Becton-Dickinson) of rifampin (25 μg), erythromycin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg) and clindamycin (2 μg) on Brucella Blood Agar with hemin and vitamin K1 (Becton-Dickinson) in accordance with the French Society of Microbiology (SFM) guidelines.[7] The zone diameters were read after 48 h of anaerobic incubation at 37 °C.

The zone diameters were established as previously reported by Delmée and Avesani (1988): rifampin no zone and >23 mm; erythromycin <13 mm and >20 mm; tetracycline <14 mm and >23 mm; chloramphenicol <10 mm and >20 mm; clindamycin no zone and >12 mm. *Bacteroides fragilis* ATCL 25285 was also tested as a quality control.

Results

C. difficile *MLVA analysis*

A total of 26 *C. difficile* isolates were typed with MLVA. For PCR-ribotypes 078 and UCL 36, the animal and carcass isolates were compared with the human isolates circulating in Belgian hospitals and nursing homes. No amplification was obtained in loci A6, CDR5 and B7 for strains belonging to PCR-ribotypes UCL5a, UCL11 and 078 isolated from pig and cattle intestinal contents and carcasses. In contrast, the three human PCR-ribotypes 078 were positive for both loci A6 and B7 with invariable results in locus A6 and two amplifications in locus B7. For the PCR-ribotype UCL 036, although the two human isolates were different from the pig carcass strains they were similar to each other with a STRD < 2. All animal isolates belonging to the same PCR-ribotype (078, 014, UCL 16U or UCL 36) obtained from the same animal species (pig or cow) and from the same type of sample (intestinal contents or carcasses) were clonal by MLVA (STRD ≤ 2). Furthermore, clonal animal isolates were detected not only within the same sampling day but also among strains collected in different dates (Table 2).

The large number of MLVA clusters identified in this study is probably related with the allelic diversity observed among the MLVA loci. The highest number of different repeats in the sample set was found for loci C6 (n = 24) and B6 (n = 15). In contrast, loci CDR5 and CDR60 presented the lowest allelic diversity. However, these loci were valuable to discriminate isolates PCR-ribotype 023, UCL 16u and 081 (CDR5), and isolates PCR-ribotype 015, 081, 023, UCL 118 and UCL 254 (CDR60) (Table 3).

C. difficile *MLST analysis*

A total of 12 different STs were found by MLST. ST assignment was not possible for two isolates (PCR-ribotypes UCL 254 and 014) as no loci sequence combination matched the allelic profile of the isolates. A clear concordance was found between most of the PCR-ribotypes studied and ST. However, PCR-ribotype 014 correlated with two different STs (49 and 2) while ST 11 and ST 49 were assigned to more than one different PCR-ribotype (Fig. 1). Most of the isolates belonged to clade 1, which cluster a great variety of PCR-ribotypes, including the six non-toxigenic types. Clade 5 was attributed to PCR-ribotypes 078, UCL 5a and UCL 11 whereas only one isolate (PCR-ribotype 023) was related to clade 3.

A phylogenetic tree shows a correlation between isolates with the same PCR-ribotype regardless of their origin (carcass, intestinal contents or human faeces) (Fig. 1). According to the minimum spanning tree, the nearest neighbour strains belonged to ST 49, ST 2, ST 14, ST 45 and ST 6, which corresponded with PCR-ribotypes 014, 015, UCL 16U, UCL 16L, UCL 118 and UCL 46 respectively (Fig. 2).

Discriminatory power and concordance among genotyping methods

Results of Simpson's diversity index showed that MLVA had the greatest discriminatory power, with and index of diversity value of 0.979 (Table 4). MLVA identified a total of 21 different types, of which six grouped more than one isolate. However, most of these types (n = 15) were defined by a single isolate. MLST only distinguished 12 different STs (sequence types) generating an index of diversity value of 0.868. Wallace coefficient of concordance among genotyping methods reveals that the probability of predicting the correct ST based on knowledge of MLVA type was 71%. There were four STs that represented multiple MLVA types (comprising between two and five different MLVA types for one ST). For example, MLST could not discriminate between L, M and N MLVA groups, classified as ST15 (Fig. 1).

C. difficile *antimicrobial susceptibility*

All human and animal isolates were susceptible to vancomycin, metronidazole, chloramphenicol and rifampicin. In addition, all of the isolates showed full sensibility to tetracycline except for PCR-ribotypes UCL 5a and UCL 11 which both showed intermediate resistance to this drug. Only two isolates (one animal and one human strain) belonging to PCR-ribotype 078 and the two isolates belonging to PCR-ribotype UCL 5a showed in vitro resistance to moxifloxacin by Etest. These results were correlated with the presence of a mutation in the *gyrA* gene by Genotype CDiff system. Moreover, four isolates assigned to PCR-ribotypes UCL 36 (n = 1), UCL 11 (n = 2) and UCL 078 (n = 1) were resistant to clindamycin, while resistance to erythromycin was detected in 13/37 (35.1%) isolates belonging to PCR-ribotypes UCL 36, UCL 103, 078 and UCL 5a (Fig. 1).

Discussion

In studies of *C. difficile* isolates, MLVA and MLST have shown limited application to date (Griffiths et al., 2010) and few studies have investigated the phylogenic relatedness of isolates from humans

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Table 2
Characterisation of the 26 main *C. difficile* types by MLVA.

PCR-ribotype	MLVA profile								Strain identification	MLVA type	Sample type	Date of the isolation
	A6	B7	C6	E7	G8	CDR5	CDR60	Total				
078	10.6	9.1 25.1[a]	37 38[a]	8.1	9.8	NA	2.2 8.7 4.2[b]	76.8[c]	**5828**	A	Human	2011
	10.6	9.1 38.3[a]	37	8.1 14.3[a]	10.7	NA	2.2 8.7 4.3[b]	77.7[c]	**5063**	A	Human	2010
	10.6	9.2[a] 12	26.5	9.1 10.9[a]	12.7	NA	2.2	70.2[c]	**4667**	B	Human	2010
	NA	NA	30.5	8.2	11.7	NA	2.2 1.6[a]	52.6[c]	**6136**	C	Pig IC	22/06/2011
	NA	NA	30.5	8.1	11.7	NA	2.2 1.6[a]	52.5[c]	**6135**	C	Pig IC	22/06/2011
	NA	NA	33.7	8	10.8	NA	2.2 1.6[a]	57.8[c]	**7485**	D	Cow IC	22/11/2011
	NA	NA	34.8	8	11.7	NA	2.2 1.6[a]	56.7[c]	**6423**	D	Cow IC	04/11/2011
UCL 11	NA	NA	41.9	8	10.8	NA	2.2	63.8	**6412**	E	Beef C	22/11/2011
UCL 5a	NA	NA	28.5	8	11.7	NA	2.2 1.6[a]	50.4[c]	**6408**	F	Beef C	07/10/2011
014	27.9	22.2	25.5 24.4[a]	6	7	6.8	7.2	102.6[c]	**6420**	G	Cow IC	18/11/2011
	19.2	13.1	27.5	7	9.9	6.8	7.2	90.7	**7071**	H	Pork C	29/11/2011
	20.2	13.1	27.5 26.5[a]	7	9.9	6.8	7.2	91.7[c]	**6414**	H	Pork C	29/11/2011
	19.3	13.1	27.5 26.5[a]	7	9.9	6.8	7.2	91.8[c]	**6415**	H	Pork C	29/11/2011
UCL 16L	25	12.1	40.9	3.9	8.9	6.8	10.2	107.8	**6427**	I	Cow IC	23/09/2011
UCL 16U	34.9	9	24.4	6	8.9	7.8	7.2	98.2	**6410**	J	Beef C	18/11/2011
	35.9	9	25.4	6	8.9	7.8	7.2	100.2	**6413**	J	Beef C	18/11/2011
015	14.5	14	48.7	2.8	10.8	4.2	6.2	101.2	**6409**	K	Beef C	07/10/2011
UCL36	31.8	18.1	35.8	8	10.8	4.9	11.2	118.5	**8387**	L	Human	19/04/2013
	19.2	17	42.8	8	9.9	4.9	10.2	112	**8378**	M	Human	08/03/2013
	17.3	15.1	20.4	8	10.8	4.9	12.2	88.7	**7083**	N	Pork C	29/11/2011
	17.3	18.1	19.4	8	10.8	4.8	12.2	90.6	**7078**	N	Pork C	29/11/2011
081	27.9	21	51.8	2.9 26[a]	7	4.9	27.3	142.8	**7077**	O	Pork C	29/11/2011
	26	14.9	40	2.7	8.9	5.9	26.2	124.6	**6140**	P	Pig IC	23/09/2011
UCL 118	27.9	19	18.4	7	8.9	4.2	8.2	93.6	**6421**	Q	Cow IC	23/09/2011
UCL 270	37.9	14.1	15.4	6	13.7	4.8	11.2	103.1	**6425**	R	Cow IC	23/09/2011
UCL 103	34.9	12.1	38	8	11.8	4.8	12.2	121.8	**7487**	S	Cow IC	30/09/2011
UCL 254	25	13.1	29.5	5	11.8	4.2	3.3	91.9	**6430**	T	Cow IC	22/11/2011
023	NA	18.1	34.7	8.1	14.6	8.8	2.3	86.6	**6411**	U	Pork C	22/11/2011

NA, no amplification; C, carcass; IC, intestinal contents.
[a] Two fragments amplified for the same locus.
[b] Three fragments amplified for the same locus.
[c] When there were two or more amplifications for the same fragment, only the first value indicated in the table has been taken into account to make the sum (total).

and from different animal species isolated in a restricted geographical region. Lemée et al. (2005) used a set of 74 *C. difficile* isolates from various hosts, geographic sources and PCR-ribotypes to investigate the allelic diversity and population structure of the isolates by MLST. Bakker et al. (2010) studied the relatedness of human and porcine *C. difficile* PCR-ribotype 078 isolates from four European countries using MLVA. In the present study, characterisation of *C. difficile* isolates was achieved using both MLST and MLVA methods. Six clonal groups of strains were obtained from the same animal species, irrespective of the isolation date. Furthermore, MLST revealed that all of the other human and animal isolates with a given PCR-ribotype clustered in the same lineage.

Table 3
Simpson and Hunter–Gaston diversity indices of MLVA VNTRs studied.

Simpson diversity index					Hunter-Gaston diversity index				
Locus	Diversity index	Confidence interval	K	Max (pi)	Locus	Diversity index	Confidence interval	K	Max (pi)
C6	0.952	0.934–0.969	24	0.107	C6	0.987	0.969–1.000	24	0.107
B7	0.895	0.845–0.945	15	0.214	B7	0.929	0.879–0.979	15	0.214
A6	0.885	0.825–0.946	14	0.250	A6	0.918	0.857–0.979	14	0.250
G8	0.865	0.818–0.912	11	0.214	G8	0.897	0.850–0.944	11	0.214
E7	0.827	0.745–0.908	11	0.321	E7	0.857	0.775–0.939	11	0.321
CDR60	0.821	0.736–0.907	11	0.321	CDR60	0.852	0.766–0.938	11	0.321
CDR5	0.814	0.739–0.888	8	0.321	CDR5	0.844	0.770–0.918	8	0.321

Diversity index for VNTR data is a measure of the variation of the number of repeats at each locus (ranges from 0.0 [no diversity] to 1.0 [complete diversity]). Confidence interval: precision of the diversity index; expressed as 95% upper and lower boundaries. K, number of different repeats present at this locus in this sample set. Max (pi) is the fraction of samples that have the most frequent repeat number in this locus (range 0.0–1.0).

Isolate Number	ST	Alleieic Profile (*atp, adk, dxr, glyA, recA, sodA, tpi*)	Clade	PCR Ribotype	CC-R	TE-R	E-R	MXF-R	Origin
8387	15	1, 1, 6, 1, 8, 5, 8	1	UCL 36	–	–	+	–	Human
8378	15	1, 1, 6, 1, 8, 5, 8	1	UCL 36	–	–	+	–	Human
7083	15	1, 1, 6, 1, 8, 5, 8	1	UCL 36	–	–	+	–	Pig carcass
7078	15	1, 1, 6, 1, 8, 5, 8	1	UCL 36	+	–	+	–	Pig carcass
8101	9	1, 1, 6, 1, 1, 6, 1	1	081	–	–	–	–	Human
7077	9	1, 1, 6, 1, 1, 6, 1	1	081	–	–	–	–	Pig carcass
6140	9	1, 1, 6, 1, 1, 6, 1	1	081	–	–	–	–	Pig
6421	45	1, 4, 6, 1, 1, 5, 1	1	UCL 118	–	–	–	–	Cow
7488	6	1, 2, 6, 1, 1, 5, 1	1	UCL 46	–	–	–	–	Pig
4181	6	1, 2, 6, 1, 1, 5, 1	1	UCL 46	–	–	–	–	Human
6425	48	1, 1, 2, 1, 1, 5, 1	1	UCL 270	–	–	–	–	Cow
6409	10	1, 2, 2, 1, 1, 3, 1	1	015	–	–	–	–	Cow carcass
7487	125	1, 4, 6, 1, 4, 26, 1	1	UCL 103	–	–	+	–	Cow
4592	2	1, 1, 2, 1, 5, 3, 1	1	014	–	–	–	–	Human
4455	2	1, 1, 2, 1, 5, 3, 1	1	014	–	–	–	–	Human
4597	2	1, 1, 2, 1, 5, 3, 1	1	015	–	–	–	–	Human
6420	2	1, 1, 2, 1, 5, 3, 1	1	014	–	–	–	–	Cow
6427	14	1, 1, 2, 1, 5, 5, 3	1	UCL 16L	–	–	–	–	Cow
1601	49	1, 1, 2, 1, 5, 3, 3	1	UCL 16U	–	–	–	–	Human
7071	49	1, 1, 2, 1, 5, 3, 3	1	014	–	–	–	–	Pig carcass
6414	49	1, 1, 2, 1, 5, 3, 3	1	014	–	–	–	–	Pig carcass
6413	49	1, 1, 2, 1, 5, 3, 3	1	UCL 16U	–	–	–	–	Cow carcass
6410	49	1, 1, 2, 1, 5, 3, 3	1	UCL 16U	–	–	–	–	Cow carcass
6430	NA	1, 1, 2, 11, 1, 3, 1	NA	UCL 254	–	–	–	–	Cow
6415	NA	1, 1, 2, 1, 9, 3, 3	NA	014	–	–	–	–	Pig carcass
6411	5	6, 1, 4, 7, 2, 8, 7	3	023	–	–	–	–	Cow carcass
5828	11	8, 5, 5, 11, 9, 11, 8	5	078	–	–	+	–	Human
5063	11	8, 5, 5, 11, 9, 11, 8	5	078	–	–	+	+	Human
5001	11	8, 5, 5, 11, 9, 11, 8	5	UCL 5a	–	I	+	+	Human
4667	11	8, 5, 5, 11, 9, 11, 8	5	078	–	–	+	–	Human
1101	11	8, 5, 5, 11, 9, 11, 8	5	UCL 11	+	I	–	–	Human
7485	11	8, 5, 5, 11, 9, 11, 8	5	078	–	–	+	–	Cow
6423	11	8, 5, 5, 11, 9, 11, 8	5	078	–	–	+	+	Cow
6412	11	8, 5, 5, 11, 9, 11, 8	5	UCL 11	+	I	–	–	Cow carcass
6408	11	8, 5, 5, 11, 9, 11, 8	5	UCL 5a	–	I	+	+	Cow carcass
6136	11	8, 5, 5, 11, 9, 11, 8	5	078	+	–	+	–	Pig
6135	11	8, 5, 5, 11, 9, 11, 8	5	078	–	–	+	–	Pig

Fig. 1. Neighbour-joining phylogenetic tree constructed with the MLST results showing the relationships between animal and human *C. difficile* strains. Allelic Profile: atp, adk, dxr, glyA, recA, sodA, tpi; ST, sequence type; NA, no available data; MXF-R, moxifloxacin resistance; E-R, erythromycin resistance; CC-R, clindamycin resistance; TE-R, tetracycline resistance; I, intermediate antimicrobial resistance.

Three of the six clonal groups of strains were identified as PCR-ribotype 078. They were detected in two hospitalised patients, in two cattle intestinal contents at slaughter and in two pigs' intestinal contents also obtained at the slaughterhouse (specifically, in the viscera processing area). The human PCR-ribotype 078 clonal strains were isolated from two different Belgian provinces in two different years (unpublished data). The porcine strains were obtained on the same sampling day in one slaughterhouse; this could suggest a widespread dissemination of *C. difficile* at the slaughterhouse (Rodriguez et al., 2012) or even in the pig farm, as previously described (Keessen et al., 2011). The same conditions were observed for the two PCR-ribotype 078 isolates from cattle intestinal contents. These were considered clonal by MLVA, but in this case the isolates were obtained on two different sampling days and the animals proceeded from different herds. As previously suggested, this finding may indicate either an epidemiological connection between farms (Scheeberg et al., 2013) or slaughterhouse contamination. However, clonal strains were not detected among *C. difficile* isolates from pig and cattle origins, which indicates that cross-contamination between the two slaughter lines (bovine and porcine) during processing is unlikely.

Identical *C. difficile* strains were also detected in the same animal species for PCR-ribotypes 014, UCL 16U and UCL 36. These results indicate that clonal *C. difficile* strains are circulating among the same animal species (including humans), although interspecies transmission was not evident. In a previous study, Kenetsch et al. (2014) reported transmission between farmers and pigs but the authors did not exclude the possibility of a common environmental source of *C. difficile* for both populations. In addition, more than half of the sequenced farmer/pig pairs were not clonal. As in our study, isolates were obtained from subjects localised in different geographical regions and in different environments. It was consequently very unlikely that identical *C. difficile* isolates would be found among the different hosts although MLST and MLVA revealed a close relation between them. Furthermore, for PCR-ribotype 078 MLVA showed a higher genetic association between pig and cattle *C. difficile* isolates (STRD ≤ 6) than between animal and human isolates (STRD ≥ 20).

Varshney et al. (2014) observed significant genotypic and phenotypic differences between meat and human isolates for a variety of PCR-ribotypes, while a few meat isolates (including PCR-ribotype 078) were very similar to human *C. difficile* strains. It has been suggested that relatedness between human and animal isolates of PCR-ribotype 078 is a consequence of less natural variability in this type than in other types (Bakker et al., 2010). However, our results indicate genetic differences that clearly distinguish between human and animal isolates.

As has been previously reported, MLST shows less discriminatory power than MLVA but does establish the *C. difficile* genetic lineage (Marsh et al., 2010). In our study some limitations of the MLVA technique were observed. Three loci, including A6, B7 and CDR5, did not seem to be stable and disappeared from some isolates, recording a null result. Variations in loci total size were also observed for some of the isolates, possibly due to the weak stability of the loci in vitro after several passages which may cause isolates with closely related MLVA profiles to appear non-clonal (Wuyts et al.,

Fig. 2. Minimum spanning tree of 37 *C. difficile* human, animal and carcass isolates based on MLST data. Each circle represents a unique sequence type. The number of strains that share an identical MLST type are shown in parenthesis: ST-15 (8), ST-5 (2), ST-14 (2), ST-49 (10), ST-2 (8), ST-48 (2), ST-10 (2), ST-Null (2), ST-11 (22), ST-9 (6), ST-125 (2), ST-45 (2), ST-6 (4).

2013). Further studies to investigate the stability of these loci are therefore required. In addition, MLVA provides little insight regarding genetic relatedness. In consequence, it may be useful to combine both methods (MLVA and MLST) in order to resolve phylogenetic diversity (Zaiss et al., 2009) although the best alternative could be whole genome sequencing, which is generally considered to be the next generation tool to type bacterial strains.

Resistance of *C. difficile* to multiple antimicrobials has been described in several previous studies in both humans and animals (Pelaez et al., 2013; Pirs et al., 2013). Even though some isolates were resistant to both moxifloxacin and erythromycin or to clindamycin and erythromycin, no association between antimicrobial resistance and toxigenic isolates was observed in our study, echoing the results of previous work by Pituch et al. (2005). Furthermore, for a given PCR-ribotype, human and animal strains presented a similar susceptibility to the antimicrobials we tested.

Our study has some limitations. Firstly, only two different human PCR-ribotypes were analysed by MLVA because the other types found in animals were either not available in the human collections in our laboratory or have not been frequently isolated in previous studies

Table 4
Simpson's index of diversity and adjusted Wallace coefficient of concordance among genotyping methods.

Method	Simpson's diversity index			Adjusted Wallace coefficient of concordance (95% CI)	
	Number of units	ID	95% CI	Sequence typing MLST	MLVA type
MLVA	21	0.979	0.961–0.997	0.712 (0.440–0.984)	
MLST	12	0.868	0.780–0.955		0.101 (0.000–0.234)

ID, index of diversity; CI, confidence interval.

conducted in Belgian hospitals or nursing homes. Additionally, the lack of sufficient reference strains in our laboratory only allowed us to identify five ribotype profiles corresponding to an international collection number while the remaining PCR-ribotypes were identified using an internal nomenclature.

Conclusions

This study shows that clonal *C. difficile* strains circulate among the same animal species or among human patients, irrespective of the geographic area and the isolation date. The typing methods used also reveal close relationships between isolates of different species, but less genetic similarity among human and animal strains. However, animal and human strains cluster in the same lineage. Our data evidence the need for highly discriminatory genotyping methods, not only to elucidate the possible transmission routes between humans and animals but also to investigate animal-to-animal transmission in herds or cross-contamination at slaughterhouses.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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References

Bakker, D., Corver, J., Harmanus, C., Goorhuis, A., Keessen, E.C., Fawley, W.N., Wilcox, M.H., Kuijper, E.J., 2010. Relatedness of human and animal *Clostridium difficile* PCR ribotype 078 isolates determined on the basis of multilocus variable-number tandem-repeat analysis and tetracycline resistance. Journal of Clinical Microbiology 48, 3744–3749.

Best, E.L., Parnell, P., Thirkell, G., Verity, P., Copland, M., Elsa, P., Denton, M., Hobson, R.P., Wilcox, M.H., 2014. Effectiveness of deep cleaning followed by hydrogen peroxide decontamination during high *Clostridium difficile* infection incidence. Journal of Hospital Infection 87, 25–33.

CLSI, 2010. Clinical and Laboratory Standard Institute. http://clsi.org/ (accessed 20 January 2015).

Debast, S.B., van Leengoed, L.A., Goorhuis, A., Harmanus, C., Kuijper, E.J., Bergwerff, A.A., 2009. *Clostridium difficile* PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. Environmental Microbiology 11, 505–511.

Delmée, M., Avesani, V., 1988. Correlation between serogroup and susceptibility to chloramphenicol, clindamycin, erythromycin, rifampicin and tetracycline among 308 isolates of *Clostridium difficile*. Journal of Antimicrobial Chemotherapy 22, 325–331.

Griffiths, D., Fawley, W., Kachrimanidou, M., Bowden, R., Crook, D.W., Fung, R., Golubchik, T., Harding, R.M., Jeffery, M., Jolley, K.A., et al., 2010. Multilocus sequence typing of *Clostridium difficile*. Journal of Clinical Microbiology 48, 770–778.

Hargreaves, K.R., Coluin, H.V., Patel, K.V., Clokie, J.J., Clokie, M.R., 2013. Genetically diverse *Clostridium difficile* strains harbouring abundant prophages in an estuarine environment. Applied Environmental Microbiology 79, 6236–6243.

Hensgens, M.P.M., Keessen, E.C., Squire, M.M., Riley, T.V., Koene, M.G.J., de Boer, E., Lipman, L.J.A., Kuijper, E.J., 2012. *Clostridium difficile* infection in the community: A zoonotic disease? Clinical Microbiology Infection 18, 635–645.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. Journal of Clinical Microbiology 26, 2465–2466.

Jones, A.M., Kuijper, E.J., Wilcox, M.H., 2013. *Clostridium difficile*: A European perspective. Journal of Infection 66, 115–128.

Keessen, E.C., Donswijk, C.J., Hol, S.P., Hermanus, C., Kuijper, E.J., Lipman, L.J., 2011. Arial dissemination of *Clostridium difficile* on a pig farm and its environment. Environmental Research 111, 1027–1032.

Kenetsch, C.W., Connor, T.R., Mutreja, A., van Dorp, S.M., Sanders, I.M., Browne, H.P., Harris, D., Lipman, L., Keessen, E.C., Corver, J., 2014. Whole genome sequencing reveals potential spread of Clostridium difficile between humans and farm animals in the Netherlands, 2002 to 2011. Eurosurveillance 19, 20954.

Killgore, G., Thompson, A., Johnson, S., Brazier, J., Kuijper, E., Pepin, J., Frost, E.H., Svelkoul, P., Nicholson, B., van den Berg, R.J., et al., 2008. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: Restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. Journal of Clinical Microbiology 46, 431–437.

Lemée, L., Dhalluin, A., Pestel-Caron, M., Lemeland, J.F., Pons, J.L., 2004. Multilocus sequence typing of humans and animal *Clostridium difficile* isolates of various toxigenic types. Journal of Clinical Microbiology 42, 2609–2617.

Lemée, L., Bourgeois, I., Ruffin, E., Collignon, A., Lemeland, J.F., Pons, J.L., 2005. Multilocus sequence analysis and comparative evolution of virulence-associated genes and housekeeping genes of *Clostridium difficile*. Microbiology (Reading, England) 151, 3171–3180.

Manzoor, S.E., Tanner, H.E., Marriott, C.L., Brazier, K.S., Hardy, K.J., Platt, S., Hawkey, P.M., 2011. Extended multilocus variable-tandem-repeat analysis of *Clostridium difficile* correlates exactly with ribotype and enables identification of hospital transmission. Journal of Clinical Microbiology 49, 3523–3530.

Marsh, J.W., O'Leary, M.M., Shutt, K.A., Pasculle, A.W., Johnson, S., Gerding, D.N., Muto, C.A., Harrison, L.H., 2006. Multilocus variable-number tandem-repeat analysis for investigation of *C. difficile* transmission in hospitals. Journal of Clinical Microbiology 44, 2558–2566.

Marsh, J.W., O'Leary, M.M., Shutt, K.A., Sambol, S.P., Johnson, S., Gerding, D.N., Harrison, L.H., 2010. Multilocus variable-number tandem-repeat analysis and multilocus sequence typing reveal genetic relationships among *Clostridium difficile* isolates genotyped by restriction endonuclease analysis. Journal of Clinical Microbiology 48, 412–418.

O'Neill, G.L., Ogunsola, F.T., Brazier, J.S., Duerden, B.I., 1996. Modification of a PCR ribotyping method for application as a routine typing scheme for *Clostridium difficile*. Anaerobe 2, 205–209.

Pelaez, T., Alcala, L., Blanco, J.L., Alvarez-Perez, S., Marin, M., Martin-Lopez, A., Catalan, P., Reigadas, E., Garcia, M.E., Bouza, E., 2013. Characterization of swine isolates of *Clostridium difficile* in Spain: A potential source of epidemic multidrug resistant strains? Anaerobe 22, 45–49.

Pirs, T., Avbersek, J., Zdovc, I., Krt, B., Andlovic, A., Lejko-Zupanc, T., Rupnik, M., Ocepek, M., 2013. Antimicrobial susceptibility of animal and human isolates of *Clostridium difficile* by broth microdilution. Journal of Medical Microbiology 62, 1478–1485.

Pituch, H., Obuch-Woszczatyriski, P., Wultanska, D., Meisel-Mikolajczyk, F., Luczak, M., 2005. A survey of metronidazole and vancomycin resistance in strains of *Clostridium difficile* isolated in Warsaw, Poland. Anaerobe 11, 197–199.

Rodriguez, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., Daube, G., 2012. *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 18, 621–625.

Rodriguez, C., Avesani, V., Van Broeck, J., Taminiau, B., Delmée, M., Daube, G., 2013. Presence of *Clostridium difficile* in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium. International Journal of Food Microbiology 2, 256–262.

Rodriguez-Palacios, A., Borgmann, S., Kline, T.R., LeJeune, J.T., 2013. *Clostridium difficile* in foods and animals: History and measures to reduce exposure. Animal Health Research Review 14, 11–29.

Scheeberg, A., Neubauer, H., Schmoock, G., Baier, S., Harlizius, J., Nienhoff, H., Brase, K., Zimmermann, S., Seyboldt, C., 2013. *Clostridium difficile* genotypes in piglet populations in Germany. Journal of Clinical Microbiology 51, 3796.

Severiano, A., Pinto, F.R., Ramirez, M., Carriço, J.A., 2011. Adjusted Wallace coefficient as a measure of congruence between typing methods. Journal of Clinical Microbiology 49, 3997–4000.

Stabler, R.A., Dawson, L.F., Valiente, E., Cairns, M.D., Martin, M.J., Donahue, E.H., Riley, T.V., Songer, J.G., Kuijper, E.J., Dingle, K.E., et al., 2012. Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations. PLoS ONE 7, e31559.

Susick, E.K., Putnam, M., Bermudez, D.M., Thakur, S., 2012. Longitudinal study comparing the dynamics of *Clostridium difficile* in conventional and antimicrobial free pigs at farm and slaughter. Veterinary Microbiology 25, 172–178.

Varshney, J.B., Very, K.J., Williams, J.L., Hegarty, J.P., Stewart, D.B., Lumadue, J., Venkitanarayanan, K., Jayarao, B.M., 2014. Characterization of *Clostridium difficile* isolates from human faecal samples and retail meat from Pennsylvania. Foodborne Pathogens Diseases 11, 822–829.

Wuyts, V., Mattheus, W., De Lamine de Bex, G., Wildemauwe, C., Roosens, N.H.C., Marchal, K., De Keersmaecker, S.C.J., Bertrand, S., 2013. MLVA as a tool for public health surveillance of human *Salmonella typhimurium*: Prospective study in Belgium and evaluation of MLVA loci stability. PLoS ONE 8, e84055.

Zaiss, N.H., Rupnik, M., Kuijper, E.J., Harmanus, C., Michielsen, D., Janssens, K., Nübel, H., 2009. Typing *Clostridium difficile* strains based on tandem repeat sequences. BMC Microbiology 8, 6.

Zidaric, V., Beigot, S., Lapajne, S., Rupnik, M., 2010. The occurrence and high diversity of *Clostridium difficile* genotypes in rivers. Anaerobe 16, 371–375.

3.3 *C. difficile* in retail meats

In previous studies, *C. difficile* was isolated from livestock (pigs and cattle) at slaughter and it was revealed that carcass contamination occurs inside the slaughterhouse. Therefore data obtained suggest a potential risk of retail meat contamination. The human *C. difficile* infectious dose is not known. Among healthy people with normal intestinal microbiota, the ingestion of low quantities of spores may have no major repercussions. However, a small dose of spores ingested with a disruption in the gut microbiota equilibrium could lead to *C. difficile* colonisation and infection. The main objective of this study was to evaluate the presence of *C. difficile* in retail meat sold in market places in Belgium. Freshly minced meat, including pure pork or pure beef burgers and sausages, were sampled from 21 different retailers between January and June 2012. *C. difficile* was isolated from 3/133 (2.3%) retail beef samples and from 5/107 (4.7%) retail pork samples. Positive beef samples came from 3 different establishments on 3 different sampling days. For pork samples, one establishment was positive for 3 retail pork samples collected on three different sampling days. Most of the isolates were identified as PCR-ribotypes 078 or 014. Only one isolate was non-toxinogenic (UCL378). The relationships among the human and meat isolates were examined using MLST analysis. The most common sequence type (ST) was ST49 (corresponding with PCR-ribotype 014), followed by ST11 (corresponding with PCR-ribotype 078). ST11 has been frequently related with human disease. Furthermore, some isolates are indistinguishable from the Belgian human isolates by MLST.

Multilocus sequence typing analysis and antibiotic resistance of *C. difficile* strains isolated from retail meat and humans in Belgium

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Multilocus sequence typing analysis and antibiotic resistance of *Clostridium difficile* strains isolated from retail meat and humans in Belgium

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ABSTRACT

Clostridium difficile has been isolated from food animals and meat, specially ground pork and ground beef. The recovered isolates were closely related to *C. difficile* human strains, indicating that animals and food are possible transmission routes of human *C. difficile* infection. The main objective of this study was to characterize *C. difficile* isolates from retail meat and to compare with human isolates recovered from hospital patients in Belgium. Raw meat (beef and pork) was obtained from the retail trade. *C. difficile* was recovered from 2.3% of the beef samples and from 4.7% of the pork samples. A total of 4 different PCR-ribotypes were identified with a large percentage of types 078 and 014. Resistance to moxifloxacin and erythromycin was detected. The multi-locus sequence typing (MLST) analysis showed that meat and human isolates cluster in the same lineage. This study reveals the presence of toxigenic *C. difficile* in retail meat in Belgium with predominance PCR-ribotypes 078 and 014, which are among the four most prevalent ribotypes of *C. difficile* isolated from humans in Europe.

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1. Introduction

Clostridium difficile is an anaerobic spore-forming bacterium recognized as the major cause of nosocomial diarrhoea in humans after antimicrobial therapy. In animals, as pigs, calves, horses, ostriches, dogs or poultry, *C. difficile* also seems to be an important cause of enteric disease (Arruda et al., 2013; Cooper et al., 2013; Diab et al., 2013; Frazier et al., 1993; Rodriguez-Palacios et al., 2006a,b; Songer and Anderson, 2006; Weese and Armstrong, 2003). However, a carriage of toxigenic and non-toxigenic *C. difficile* has been also described in animals without diarrhoea like Iberian free-range pigs (Alvarez-Pérez et al., 2013), wild mammals (Jardine et al., 2013), sheep and lambs (Knight and Riley, 2013), cats and dogs (Schneeberg et al., 2012; Wetterwik et al., 2013), horses (Schoster et al., 2012), pigs and cattle (Baker et al., 2010; Houser et al., 2012; Rodriguez et al., 2012).

A few recent studies have focused on the study of *C. difficile* in intestinal contents and on the carcasses in pigs and cattle at slaughter (Harvey et al., 2011; Hawken et al., 2013; Rodriguez et al., 2013). These studies showed that full-grown animals are carriers of *C. difficile* and carcass contamination occurs inside the slaughterhouse, suggesting a potential risk of retail meat contamination.

Moreover recent isolation of *C. difficile* in a variety of meat products as chicken meat (De Boer et al., 2011; Indra et al., 2009), ground turkey meat (Harvey et al., 2011), pork sausages (Curry et al., 2012; Harvey et al., 2011; Songer et al., 2009), chorizo (Curry et al., 2012; Songer et al., 2009), retail beef or retail pork (Metcalf et al., 2010; Rodriguez-Palacios et al., 2009; Songer et al., 2009; Visser et al., 2012; Weese et al., 2009) reinforces the hypothesis about a potential risk of foodborne infections linked to this bacterium. In addition, some of these meat isolates were identified as PCR-ribotypes 078 (De Boer et al., 2011; Harvey et al., 2011; Songer et al., 2009; Weese et al., 2009), 027 (Rodriguez-Palacios et al., 2009; Metcalf et al., 2010; Songer et al., 2009; Weese et al., 2009), 014 (Rodriguez-Palacios et al., 2009) or 001 (De Boer et al., 2011) which are correlated with the types implicated in human disease (Bauer et al., 2011).

PCR-ribotypes 078 and 027 have been largely isolated from beef and pork retail meat in USA and Canada. Those strains are the most frequently recovered ribotypes (Harvey et al., 2011; Metcalf et al., 2010; Rodriguez-Palacios et al., 2007; Songer et al., 2009; Weese et al., 2009). On the contrary, these types have not been isolated from meat samples in Europe. Furthermore, no studies conducted

in Europe have recovered *C. difficile* from pure pork meat (Hensgens et al., 2012) in retail stores.

Multi-locus sequence typing analysis (MLST) for *C. difficile* has been developed to study clonal relationships of the bacterial populations (Griffiths et al., 2010; Lemée et al., 2004a). Recent MLST studies on *C. difficile* have focused on human isolates but have not been widely adopted in animal and food strains. Lemée et al. (2004a, 2005) investigated the allelic diversity and population structure of *C. difficile* strains including isolates from animal host while Stabler et al. (2012) used MLST analysis to study 16 *C. difficile* isolates from human, animal and food sources.

In a previous study, a *C. difficile* carcass contamination was reported at the slaughterhouse in Belgium with an observed prevalence of 7.9% and 7% in cattle and pig carcasses respectively (Rodriguez et al., 2013). The main objective of this study was to evaluate the presence of *C. difficile* in retail meat sold in market places in Belgium. Additionally, *C. difficile* isolates were characterized with respect to the presence of toxin genes, toxigenic activity and antibiotic resistance. MLST was used in order to determine genetic relationships between meat and human *C. difficile* isolates recovered from hospital patients in Belgium.

2. Materials and methods

2.1. Sampling

A total of 133 beef samples and 107 pork samples were collected from 21 different retailers between January and June 2012. Between 5 and 18 samples from pork and beef were collected weekly (one beef and one pork sample by establishment). Each establishment was visited at least three times in order to carry out the mentioned sample collection. For each sample, between 200 and 400 g of freshly minced meat (pure beef or pure pork) were purchased. Pure pork or pure beef burgers and sausages were included. The purchase date, analyse date, quantity and store name for each sample were registered. Samples were transferred at room temperature to the laboratory (about 3 h) and stored for a maximum of 3 days at 4 °C before processing.

2.2. Isolation and characterization of C. difficile

A total of 10 g of minced meat was added to 90 ml of home-made cycloserine cefoxitin fructose taurocholate (CCFT) as previously described (Delmée et al., 1987), but without agar, and both were homogenised for 2 min in the Pulsifier^R (Led Techno, Heusden-Zolder, Belgium) and then incubated in an anaerobic workstation (Led Techno, Heusden-Zolder, Belgium) at 37 °C for 3 days. After incubation, approximately 10 µl of the broth was spread on home-made cycloserine cefoxitin fructose agar taurocholate agar plates (CCFAT) (Delmée et al., 1987) and incubated anaerobically for 48 h at 37 °C. Presumptive cultures (irregular yellowish colonies with an appearance of ground glass and a characteristic horse manure odour) were checked using a *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid, Dardilly, France) and subcultured anaerobically onto blood agar (5% Sheep Blood; Biorad, Nazareth, Belgium). Identification as *C. difficile* was confirmed by detection of a species-specific internal fragment of *tpi* by PCR as described previously (Lemée et al., 2004b). Detection of genes for toxin B (Lemée et al., 2004b) and binary toxin (*cdtA*) (Antikainen et al., 2009) were also performed in the same multiplex PCR (Rodriguez et al., 2012). For the detection of the toxin A encoding gene, another PCR assay was performed using the primers of Antikainen et al. (2009) and protocol of Lemée et al. (2004b).

2.3. Toxin activity

To detect the presence of toxin activity of isolates, a cytotoxicity assay was carried out as previously described (Rodriguez et al., 2012). Briefly, the cytotoxicity testing was performed on a 48 h anaerobic broth culture contained confluent monolayer MRC-5 cells and 75 ml of a suspension of *C. difficile* colonies. In addition, 75 µl of specific *C. difficile* antitoxin-B kit (T500, TechLab, Virginia, USA) was used in order to confirm the specificity of the cytotoxic activity by neutralization, according to the manufacturer's instructions.

2.4. PCR-ribotyping and genotype CDiff test system

PCR-ribotyping was performed by amplification of 16S-23S intergenic spacer regions (Bidet et al., 1999). Total DNA was extracted according to O'Neill et al. (1996). PCR amplification and amplicon size analysis were performed as previously described (Rodriguez et al., 2012). International number was used for *C. difficile* strains that presented a PCR-ribotype profile which matched the Cardiff ribotypes from the strain collection available in our laboratory. Otherwise, isolates were coded as UCL followed by an internal number.

In addition, all of the isolates were analysed using the Genotype Cdiff test system (Hain Lifescience, Nehren, De). This method was used to retest the presence of the *tpi* and all toxin genes (*tcdA, tcdB, cdtA* and *cdtB*). This test detected the deletions in the regulator gene *tcdC* (18 bp and 39 bp deletions or single base deletion at position 117) and *gyrA* mutation associated with moxifloxacin resistance (Drudy et al., 2007).

2.5. Antimicrobial susceptibility testing

2.5.1. Etest testing

Susceptibility to metronidazole, moxifloxacin and vancomycin was determined by Etest strips (Lucron ELITechGroup St- Martens-Latem, Belgium) on Schaedler with Vit K1 and 5% sheep blood (Becton-Dickinson) according to the manufacturer's instructions. Plates were anaerobically incubated at 37 °C for 48 h. The susceptibility (*s*) and resistant (*r*) breakpoints for metronidazole ($s \leq 8$ µg/ml; $r \geq 32$ µg/ml) and moxifloxacin ($s \leq 2$ µg/ml; $r \geq 8$ µg/ml) used for interpretation were those recommended by the Clinical and Laboratory Standard Institute (CLSI, 2010). Vancomycin MIC breakpoints ($s \leq 2$ µg/ml; $r \geq 2$ µg/ml) were established following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). *Bacteroides fragilis* ATCL 25285 was included as a quality control.

2.5.2. Disc susceptibility testing

Disc diffusion was performed with standard discs (Becton-Dickinson, Erembodegem, Belgium) of rifampin (25 µg), erythromycin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg) and clindamycin (2 µg) on *Brucella* Blood Agar with hemin and vitamin K1 (Becton-Dickinson) in accordance with the French Society of Microbiology (SFM) (www.sfm-microbiologie.org). The zone diameters were read after 48 h of anaerobic incubation at 37 °C. The zone diameters were established as previously reported by Delmée and Avesani (1988): rifampin no zone and >23 mm, erythromycin <13 mm and >20 mm, tetracycline <14 mm and >23 mm, chloramphenicol <10 mm and >20 mm and clindamycin no zone and >12 mm. *B. fragilis* ATCL 25285 was also tested as quality control.

2.6. Multilocus sequence typing analysis

All of the *C. difficile* meat isolates were further characterized by MLST. In addition, 6 isolates from hospital patients (three PCR-ribotypes 078, two PCR-ribotypes 014 and one PCR-ribotype UCL57) were obtained from the human *C. difficile* collection at the Microbiology Unit, Catholic University of Louvain (reference human *C. difficile* laboratory in Belgium). These human strains were previously characterized by the same methods described in this work and selected on the basis of their PCR-ribotype, toxin activity, toxin genes presence, deletions in the regulator gene *tcdC*, *gyrA* gen mutations and antimicrobial susceptibility, to be as similar as possible to the isolates from meat. The six human PCR-ribotypes were further typed by MLST in order to compare them with the same PCR-ribotypes we found in ground meat. MLST analysis was performed using the housekeeping genes (*adk, atpA, dxr, glyA, recA, sodA* and *tpi*) and the amplification conditions previously described by Griffiths et al. (2010). Seven PCR amplicons were obtained for each isolate. PCR products were purified using the kit Wizard SV Gel and PCR Clean-Up System (Promega, Leiden, The Netherlands) according to the manufacturer's instructions and sequenced (10 ng of DNA) with PCR forward or reverse primers. Purification of sequencing products was performed using a Magnetic bead CLEANSEQ kit (Agencourt Bioscience Corporation, Massachusetts, USA). Capillary electrophoresis was carried out using 3730 Genetic Analyser (Applied Biosystems, California, USA), 48 capillaries. The analysis of the two sequences obtained for each gene fragment was conducted using the Geneious program (http://www.geneious.com). The assignment of the allele number, clade and sequence type (ST) was performed with *C. difficile* MLST website (http://pubmlst.org/cdifficile). Sequences were concatenated and a neighbour-joining phylogenetic tree was constructed using Geneious program (Drummond et al., 2013).

3. Results

3.1. Prevalence of C. difficile in meat samples

A total of 240 meat samples were analysed. *C. difficile* was isolated from 3/133 (2.3%) retail beef samples that corresponded to beef burger and ground beef. Positive beef samples came from 3 different establishments on 3 different sampling days. From retail pork samples, *C. difficile* was isolated from 5 (4.7%) of 107 samples collected from 3 different establishments after 3 days of enrichment, including ground pork, pork sausages and chipolata. One establishment was positive for 3 retail pork samples collected on three different sampling days.

3.2. PCR-ribotyping, toxin activity, toxin genes detection, and genotype CDiff test

A total of 4 different PCR-ribotypes were identified among the 8 positive samples. Two of these PCR-ribotypes have a ribotype profile corresponding to the international collection (078, 014), while the 2 other strains were not associated with any reference Cardiff ribotypes (UCL57 and UCL378). PCR-ribotypes 078 and 014 were isolated from beef and pork retail meat samples (Table 1).

Seven of the total isolates ($n = 8$) displayed cytotoxic activity. All the isolates ($n = 3$) found in retail beef were toxigenic. Among PCR-ribotypes recovered from retail pork ($n = 5$), only one isolate was non-toxigenic. These results were also corroborated by the PCR results targeting *tpi, tcdA, tcdB* and *cdtA* and by the results of the GenoType CDiff test system. All toxin positive isolates contained *tcdA* and *tcdB* genes. PCR-ribotypes 078 also contained *cdtA* and *cdtB* gene coding for the binary toxin and had a 39 bp deletion in the regulator gene *tcdC*. GyrA mutation, related with moxifloxacin resistance, was also detected in all of the PCR-ribotypes 078 identified. Neither 18 bp nor 117 bp deletion in *tcdC* was detected in any isolates. Isolate of PCR-ribotype UCL 378 were negative for all toxin genes (Table 1).

3.3. C. difficile antimicrobial susceptibility

A total of four *C. difficile* isolates (50%) were fully susceptible to all the antimicrobials tested. Only two isolates (25%) (strain 2404 and 2405) belonging to PCR-ribotype 078, showed intermediate resistance to tetracycline by disc diffusion test, in vitro resistance to moxifloxacin by Etest and a mutation in the *gyrA* gene by Genotype Cdiff test. In addition, one of these two isolates (strain 2404) also showed resistance to erythromycin by disc diffusion test.

Of the four isolates identified as PCR-ribotype 014, only one isolate recovered from ground pork (strain 2012) and one isolate recovered from ground beef (strain 3030) showed intermediate resistance to clindamycin by disc diffusion test as defined by Delmée and Avesani (1988). All the meat isolates were fully susceptible to metronidazole, vancomycin, chloramphenicol and rifampin.

3.4. C. difficile MLST analysis

Among the 14 isolates analysed, a total of 6 different STs were identified. The most common sequence type was ST49 (5 isolates) followed by ST11, which included 4 of the isolates. ST identification and PCR ribotyping are compared in Fig. 1. A concordance between PCR-ribotypes and STs was found. Most of the PCR-ribotypes 078

Table 1
PCR-ribotypes, toxin activity and gene profile of *Clostridium difficile* isolated from retail meat samples.

Sample type		Isolate number	PCR-ribotype	Toxin activity	Detection of toxin genes by PCR			Genotype CDiff test system							
					tcdA	*tcdB*	*cdtA*	*tcdA*	*tcdB*	*cdtA*	*cdtB*	*tcdC* 18 bp[a]	*tcdC* 39 bp[a]	*tcdC* 117 bp[a]	*gyrA* MUT[b]
Retail Beef	Beef burger	2404	078	+	+	+	+	+	+	+	+	−	+	−	+
	Beef burger	2001	014	+	+	+	−	+	+	−	−	−	−	−	−
	Ground beef	3030	014	+	+	+	−	+	+	−	−	−	−	−	−
Retail Pork	Chipolata	2405	078	+	+	+	+	+	+	+	+	−	+	−	+
	Ground pork	2012	014	+	+	+	−	+	+	−	−	−	−	−	−
	Ground pork	2403	014	+	+	+	−	+	+	−	−	−	−	−	−
	Pork sausage	1003	UCL57	+	+	+	−	+	+	−	−	−	−	−	−
	Pork sausage	1703	UCL378	−	−	−	−	−	−	−	−	−	−	−	−

[a] Presence of deletions in the regulator gene *tcdC*.
[b] Presence of mutation in the *gyrA* gene associated with moxifloxacin resistance.

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Isolate Number	ST	Allelic Profile (adk, atpA, dxr, glyA, recA, sodA, tpi)	Clade	PCR Ribotype	tcdC 39bp	GyrA mut	MXF-R	E-R	CC-R	Origin
1703	124	11, 7, 14, 14, 6, 1, 10	4	UCL378	-	-	-	-	-	Pork sausage
2404	11	5, 8, 5, 11, 9, 11, 8	5	078	+	+	+	+	-	Beef burger
4667	11	5, 8, 5, 11, 9, 11, 8	5	078	+	-	-	-	-	Human stool
5828	11	5, 8, 5, 11, 9, 11, 8	5	078	+	-	-	+	-	Human stool
5063	186	5, 8, 5, 11, 15, 11, 8	5	078	+	+	+	+	-	Human stool
2405	11	5, 8, 5, 11, 9, 11, 8	5	078	+	+	+	-	-	Pork Chipolata
1003	21	2, 2, 2, 1, 1, 1, 3	ND	UCL57	-	-	-	-	-	Pork sausage
1010	21	2, 2, 2, 1, 1, 1, 3	ND	UCL57	-	-	-	-	-	Human stool
4455	2	1, 1, 2, 1, 5, 3, 1	1	014	-	-	-	-	-	Human stool
2001	49	1, 1, 2, 1, 5, 3, 1	1	014	-	-	-	-	-	Beef burger
2012	49	1, 1, 2, 1, 5, 3, 3	1	014	-	-	-	-	I	Ground pork
2403	49	1, 1, 2, 1, 5, 3, 3	1	014	-	-	-	-	-	Ground pork
3030	49	1, 1, 2, 1, 5, 3, 3	1	014	-	-	-	-	I	Ground beef
3973	49	1, 1, 2, 1, 5, 3, 3	1	014	-	-	-	-	+	Human stool

Fig. 1. Neighbour-joining phylogenetic tree showing the relationships between meat and human *C. difficile* strains. ST: sequence type. *tcdC* 39 bp: Presence of deletions in the regulator gene *tcdC*. *gyrA* mut: Presence of mutation in the *gyrA* gene. MXF-R: moxifloxacin resistance. E-R: erythromycin resistance. CC-R: moxifloxacin resistance. I: intermediate antimicrobial resistance.

(meat and human *C. difficile* isolates) were associated with ST11, and only one human PCR-ribotype 078 was assigned to ST186. Similarly, all the isolates from meat identified as PCR-ribotype 014 shared the same ST (ST49) whereas the two PCR-ribotypes 014 from humans were associated with two different STs (ST2 and ST49). The single nontoxigenic isolate from meat (PCR-ribotype UCL 378) was related with ST124.

A total of 3 different clades were assigned. All the PCR-ribotypes 078 and 014 belonged to clade 5 and 1 respectively. Non-clade assignation was available for the meat and human PCR-ribotypes UCL57 (ST21). The sole nontoxigenic isolate (strain 1703) was included in clade 4.

The relationships among the human and meat isolates were examined using the concatenated sequences of the seven MLST loci (*adk, atpA, dxr, glyA, recA, sodA* and *tpi*) to construct a neighbour-joining phylogenetic tree. This neighbour-joining tree confirms the correlation between meat and human isolates with the same PCR-ribotype and the correlation between toxigenic type and ST. The only nontoxigenic isolate (ST124) appears in a different cluster from all of the other toxigenic meat and human isolates analysed (Fig. 1).

4. Discussion

C. difficile was isolated from 5 of 107 (4.7%) of pork and from 3 of 133 (2.3%) of beef meat samples, which is in agreement with the previously reported prevalence in other studies in Europe with a similar sampling size (Bouttier et al., 2010; Jöbstl et al., 2010; Von Abercron et al., 2009) but lower than the prevalence detected in North America, which ranges between 1.8 and 20% of positives (Rodriguez-Palacios et al., 2009; Weese et al., 2009).

C. difficile carcass contamination was reported at the slaughterhouse in Belgium with an observed prevalence of 7.9% and 7% in cattle and pig carcasses respectively (Rodriguez et al., 2013). The present study confirms that the detection of *C. difficile* in pig and cattle carcasses appears to carry through into Belgian retail meats. In our previous study, we investigated the contamination of carcasses by *C. difficile* spores in a single slaughterhouse. Here, we have sampled a wide selection of meat of many brands from various large and small supermarkets and butcher's shops. These meat samples were originated from different slaughterhouses and meat processing plants around the country. Therefore, this study provides a more comprehensive picture of the presence and prevalence of *C. difficile* in Belgian retail meats.

Most of the isolates in this study showed toxic activity and genes encoding for toxins A and B. The only non-toxigenic strain PCR-ribotype UCL378 obtained from a pork sausage had not been isolated before in humans in Belgium. *C. difficile* non-toxigenic types have also been isolated from cattle and pig intestinal contents (Rodriguez et al., 2013, 2012) with a percentage ranging between 14% and 16%. The presence of these non-toxigenic types in meat provides further evidence that the source of *C. difficile* in retail meats may involve faecal contamination of carcasses at slaughter.

The most common PCR-ribotypes recovered from pork and beef retail samples were PCR-ribotypes 014 (50%) and 078 (25%). With regard to the authors' knowledge, this study is the first one to isolate PCR-Ribotypes 078 and 014 in retail meats in Europe and the first one to recover from pure pork meat in retail stores in Europe. These two types have also been recently isolated from cattle and pork intestinal contents and carcass samples in a slaughterhouse in Belgium (Rodriguez et al., 2013), suggesting that food animals can be a possible reservoir and a potential risk for foodborne infections.

Furthermore, a hospital-based survey in Belgium (2011) (unpublished data) and in Europe (2009) (Bauer et al., 2011) identified the PCR-ribotypes 014 and 078 among the four most prevalent PCR ribotypes of *C. difficile* isolated from humans.

Zidaric et al., (2012) find that antibiotic resistance patterns may help to differentiate strains of *C. difficile* within a ribotype. The in vitro antimicrobial susceptibility testing in the present study has been performed by disc diffusion and E-test method. The disc diffusion technique has been suggested as an inexpensive option for a routine antimicrobial susceptible testing (Erikstrup et al., 2012) which permits a clear-cut discrimination between resistant and sensitive *C. difficile* strains (Delmée and Avesani, 1988). However, some minor errors have been reported in the correlation between results of gradient test and disc diffusion method for metronidazole, vancomycin (Wong et al., 1999) and moxifloxacin (Erikstrup et al., 2012). In the present study susceptibility to these three drugs has been tested by E-test method while resistance to the other four antimicrobial agents was determined by disc diffusion method. Data obtained reveals that the *C. difficile* meat isolates were sensitive to most of the antibiotic agents used in this study. All the strains identified as PCR-ribotype 078 were resistant to moxifloxacin and carried a mutation in *gyrA* gene. Amino acid substitutions in the *gyrA* or *gyrB* genes have been implicated in *C. difficile* resistance to some fluoroquinolones as ciprofloxacin, gatifloxacin, levofloxacin or moxifloxacin (Dridi et al., 2012; Drudy et al., 2006). Nonetheless, in the majority of the human *C. difficile* clinical strains, fluoroquinolone resistance is caused by alterations in the *gyrA* gene (Drudy et al., 2007; Spigaglia et al., 2009). Resistance to moxifloxacin and other fluoroquinolones is quite common in human and food isolates. A prospective study of *C. difficile* infections in Europe (Barbut et al., 2007) revealed that 37.5% of the clinical strains circulating in hospitals showed resistance to moxifloxacin. In meat, moxifloxacin resistance has been previously reported for *C. difficile* isolates belonging to PCR-ribotype 027 (Songer et al., 2009), 012 (Bouttier et al., 2010) and 053 (Jöbstl et al., 2010). Multidrug resistance has been detected in one isolate PCR-ribotype 078 which also showed resistance to erythromycin and fell within the intermediate zone for tetracycline. Any of the strains were resistant to both antibiotics erythromycin and clindamycin. This condition has been less common observed but previously described in human *C. difficile* isolates (Delmée and Avesani, 1988). In agreement with other studies in food (Harvey et al., 2011; Rodriguez-Palacios et al., 2007), low susceptibility to clindamycin in *C. difficile* retail meat isolates has been observed. In humans, 44.4%, 46.1% and 9.2% of the strains circulating in hospitals showed resistance to erythromycin, clindamycin, and tetracycline respectively (Barbut et al., 2007).

Few studies have used multilocus sequence typing (MLST) technique to established genetic relationships of human and animal *C. difficile* strains. In two previous studies (Lemée et al., 2004a; Lemée et Pons, 2010) a MLST scheme was developed to study the allelic diversity between *C. difficile* strains from different hosts. The results obtained showed that animal and human isolates do not cluster in distinct lineages. Another recent study analysed strains of animal and food origin. Results showed that both of them were associated with the two sequence types (ST1 and ST11) frequently related with human disease (Stabler et al., 2012). The present study is the first to apply MLST analysis to *C. difficile* isolates from meat and humans in the same geographical region within a given PCR ribotype and an antimicrobial susceptibility profile. As previously reported, a close relation between PCR-ribotypes and STs was observed as well as a congruent association between clade and PaLoc *tcdC* variants (Dingle et al., 2011). The more prevalent STs detected (ST11 and ST49) corresponds to PCR-ribotypes 014 and 078 and are included in clades 5 and 1 respectively. In humans, a high prevalence of isolates belonging to clade 1 and clade 5 (ST11) was also recently described in a clinical study in Spain (Weber et al., 2013). Furthermore, MLST analysis shows that meat and human strains isolated in this study cluster in the same lineage.

They are some limitations of this study. We are not able to assert that animals are the sole origin of *C. difficile* meat contamination. The source may involve faecal or environmental contamination of carcasses at slaughterhouse but also contamination during processing or in retail meat markets. In the current study, it was not possible to identify the facility of origin or slaughterhouse for all of the samples. Therefore we cannot define if one slaughterhouse or processing plant has a great risk of *C. difficile* meat contamination than another. Furthermore, as all of the analysis were performed by enrichment culture of the samples, it is not possible to determine the numbers of *C. difficile* cells presented in the positive samples.

In summary, this study shows that toxigenic *C. difficile* are present in retail meats in Belgium. Among the types identified, 078 and 014 were the most prevalent PCR-ribotypes. Furthermore, meat strains isolated are indistinguishable from the Belgian human isolates by PCR-ribotype, toxin activity, toxin genes, MLST, antimicrobial resistance and genes for moxifloxacin resistance, suggesting a potential risk of foodborne infections linked to *C. difficile*.

Acknowledgements

References

Alvarez-Pérez, S., Blanco, J.L., Pélaez, T., Astorga, R.J., Harmanus, C., Kuijper, E., Garcia, M.E., 2013. High prevalence of the epidemic *Clostridium difficile* PCR ribotype 078 in Iberian free-range pigs. Anaerobe 95, 358—361.

Antikainen, J., Pasanen, T., Mero, S., Tarkka, E., Kirveskari, J., Kotila, S., Mentula, S., Könönen, E., Virolainen-Julkunen, A.R., Vaara, M., Tissari, P., 2009. Detection of virulence genes of *Clostridium difficile* by multiplex PCR. Acta Phatol. Microbiol. Inmunol. Scand. 117, 607—613.

Arruda, P.H., Madson, D.M., Ramirez, A., Rowe, E., Lizer, J.T., Songer, J.G., 2013. Effect of age, dose and antibiotic therapy on the development of *Clostridium difficile* infection in neonatal piglets. Anaerobe 22, 104—110.

Baker, A.A., Davis, E., Rehberger, T., Rosener, D., 2010. Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the Midwest. Appl. Environ. Microbiol. 76, 2961—2966.

Barbut, F., Mastrantonio, P., Delmée, M., Brazier, J., Kuijper, E., Poxton, I., European Study Group of Clostridium difficile (ESGCD), 2007. Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. Clin. Microbiol. Infect. 13, 1048—1057.

Bauer, M.P., Notermans, D.W., van Benthem, B.H.B., Brazier, J.S., Wilcox, M.H., Rupnik, M., Monnet, D.L., van Dissel, J.T., Kuijper, E.J., 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377, 63—73.

Bidet, P., Barbut, F., Lalande, V., Burghoffer, B., Petit, J.C., 1999. Development of a new PCR-ribotyping method based on ribosomal RNA gene sequencing. FEMS Microbiol. Lett. 175, 261—266.

Bouttier, S., Barc, M.C., Felix, B., Lambert, S., Collignon, A., Barbut, F., 2010. *Clostridium difficile* in ground meat, France. Emerg. Infect. Dis. 16, 733—735.

CLSI, 2010. Clinical and Laboratory Standards Institute. In: 20th Informational Supplement, vol. 30. No. 1. M100—S20. Wayne, PA. USA.

Curry, S.R., Marsh, J.W., Schlackman, J.L., Harrison, L.H., 2012. Prevalence of *Clostridium difficile* in uncooked ground meat products from Pittsburgh, Pennsylvania. Appl. Environ. Microbiol. 78, 4183—4186.

Cooper, K.K., Songer, J.G., Uzal, F.A., 2013. Diagnosing clostridial enteric disease in poultry. J. Vet. Diagn. Investig. 25, 314—327.

De Boer, E., Zwartkruis-Nahuis, A., Heuvelink, A.E., Harmanus, C., Kuijper, E.J., 2011. Prevalence of *Clostridium difficile* in retailed meat in The Netherlands. Int. J. Food Microbiol. 144, 561—564.

Delmée, M., Vandercam, B., Avesani, V., Michaux, J.L., 1987. Epidemiology and prevention of *Clostridium difficile* in a leukaemia unit. Eur. J. Clin. Microbiol. 6, 623—627.

Delmée, M., Avesani, V., 1988. Correlation between serogroup and susceptibility to chloramphenicol, clindamycin, erythromycin, rifampicin and tetracycline among 308 isolates of *Clostridium difficile*. J. Antimicrob. Chemother. 22, 325—331.

Diab, S.S., Songer, G., Uzal, F.A., 2013. *Clostridium difficile* infection in horses: a review. Vet. Microbiol.. http://dx.doi.org/10.1016/j.vetmic.2013.03.03.

Dingle, K.E., Griffiths, D., Didelot, X., Evans, J., Vaughan, A., Kachrimaidou, M., Stoesser, N., Jolley, K.T., Harding, R.M., Peto, T.E., Fawley, W., Walker, A.S., Wilcox, M., Crook, D.W., 2011. Clinical *Clostridium difficile*: clonality and pathogenicity locus diversity. Plos One 6, e19993.

Dridi, L., Tankovic, J., Burghoffer, B., Barbut, F., Petit, J.C., 2012. *GyrA* and *gyrB* Mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. J. Antimicrob. Chemother. 46, 3418—3421.

Drudy, D., Quinn, T., O'Mahony, R., Kyne, L., O'Gaora, P., Fanning, S., 2006. High-level resistance to moxifloxacin and gatifloxacin associated with a novel mutation in *gyrB* in toxin-A-negative, toxin-B-positive *Clostridium difficile*. J. Antimicrob. Chemother. 58, 1264—1267.

Drudy, D., Kyne, L., O'Mahony, R., Fanning, S., 2007. *GyrA* mutations in fluoroquinolone-resistant *Clostridium difficile* PCR-027. Emerg. Infect. Dis. 13, 504—505.

Drummond, A.J., Ahston, B., Buxton, S., Cheung, M., Heled, J., Kearse, M., Moir, R., Stones-Avas, S., Thierer, T., Wilson, A., 2013. Geneious v4.8. Available from: http://www.geneious.com/.

Erikstrup, L.T., Danielsen, T.K.L., Hall, V., Olsen, K.E.P., Kristensen, B., Kahlmeter, G., Fuursted, K., Justesen, U.S., 2012. Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disk diffusion correlates. Clin. Microbiol. Infect. 18, 266—272.

Frazier, K.S., Herron, A.J., Hines 2nd, M.E., Gaskin, J.M., Altman, N.H., 1993. Diagnosis of enteritis and enterotoxemia due to *Clostridium difficile* in captive ostriches (Struthio camelus). J. Vet. Diagn. Investig. 5, 623—625.

Griffiths, D., Fawley, W., Kachrimanidou, M., Bowden, R., Crook, D.W., Fung, R., Golubchik, T., Harding, R.M., Jeffery, M., Jolley, K.A., Kirton, R., Peto, T.E., Rees, G., Stoesser, N., Vaughan, A., Walker, A.S., Young, B.C., Wilcox, M., Dingle, K.E., 2010. Multilocus sequence typing of *Clostridium difficile*. J. Clin. Microbiol. 48, 370—378.

Harvey, R.B., Norman, K.N., Andrews, K., Norby, B., Hume, M.E., Scanlan, C.M., Hardin, M.D., Scott, H.M., 2011. *Clostridium difficile* in retail meat and processing plants in Texas. J. Vet. Diagn. Investig. 23, 807—811.

Hawken, P., Weese, J.S., Friendship, R., Warriner, K., 2013. Longitudinal study of *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus* associated with pigs from weaning through to the end processing. J. Food Prot. 76, 624—630.

Hensgens, M.P.M., Keessen, E.C., Squire, M.M., Riley, T.V., Koene, M.G.J., De Boer, E., Lipman, L.J.A., Kuijper, E.J., 2012. European Society of Clinical Microbiology and Infectious Diseases Study Group forClostridium difficile (ESGCD), Clostridium difficile infection in the community: a zoonotic disease? Clin. Microbiol. Infect. Dis. 18, 635—645.

Houser, A.B., Soehnlen, K.M., Wolfgang, R.D., Lysczek, R.H., Burns, M.C., Jayarao, M.B., 2012. Prevalence of *Clostridium difficile* toxin genes in the faeces of veal calves and incidence of ground veal contamination. Foodborne Pathog. Dis. 9, 32—36.

Indra, A., Lassnig, H., Baliko, N., Much, P., Fiedler, A., Huhulescu, S., Allerberger, F., 2009. *Clostridium difficile*: a new zoonotic agent? Wien. Klin. Wochenschr. 121, 91—95.

Jardine, C.M., Reid-Smith, R.J., Rousseau, J., Weese, J.S., 2013. Detection of *Clostridium difficile* in small and medium-size wild mammals in southern Ontario, Canada. J. Wildl. Dis. 49, 418—421.

Jöbstl, M., Heuberger, S., Indra, A., Nepf, R., Kofer, J., Wagner, M., 2010. *Clostridium difficile* in raw products of animal origin. Int. J. Food Microbiol. 138, 172—175.

Knight, D.R., Riley, T.V., 2013. *Clostridium difficile* in Australian sheep and lambs. Appl. Environ. Microbiol. 79, 5689—5692.

Lemée, L., Dhalluin, A., Pestel-Caron, M., Lemeland, J.F., Pons, J.L., 2004a. Multilocus sequence typing of humans and animal *Clostridium difficile* isolates of various toxigenic types. J. Clin. Microbiol. 42, 2609—2617.

Lemée, L., Dhalluin, A., Testelin, S., Mattrat, M., Maillard, K., Lemeland, J., Pons, J.L., 2004b. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxin B) genes for toxigenic culture of *Clostridium difficile*. J. Clin. Microbiol. 42, 5710—5714.

Lemée, L., Bourgeois, I., Ruffin, E., Collignon, A., Lemeland, J.F., Pons, J.L., 2005. Multilocus sequence analysis and comparative evolution of virulence-associated genes and housekeeping genes of *Clostridium difficile*. Microbiology 151, 3171—3180.

Lemée, L., Pons, J.L., 2010. Multilocus sequence typing for *Clostridium difficile*. Methods Mol. Biol. 646, 77—90.

Metcalf, D., Reid-Smith, R.J., Avery, B.P., Weese, J.S., 2010. Prevalence of *Clostridium difficile* in retail pork. Can. Vet. J. 51, 873—876.

O'Neill, G.L., Ogunsola, F.T., Brazier, J.S., Duerden, B.I., 1996. Modification of PCR ribotyping method for application as a routine typing scheme for *Clostridium difficile*. Anaerobe 2, 205—209.

Rodriguez, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., Daube, G., 2012. *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 18, 621—625.

Rodriguez, C., Avesani, V., Van Broeck, J., Taminiau, B., Delmée, M., Daube, G., 2013. Presence of *Clostridium difficile* pigs and cattle intestinal contents and carcass contamination at slaughterhouse in Belgium. Int. J. Food Microbiol. 166 (2), 256—262.

Rodriguez-Palacios, A., Staempfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., Weese, J.S., 2006a. *Clostridium difficile* in retail ground meat, Canada. Emerg. Infect. Dis. 13, 485—487.

Rodriguez-Palacios, A., Staempfli, H.R., Duffield, T., Weese, J.S., 2006b. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12, 1730—1736.

Rodriguez-Palacios, A., Staempfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., Weese, J.S., 2007. Clostridium difficile in retail ground meat, Canada. Emerg. Infect. Dis. 13, 485—487.

Rodriguez-Palacios, A., Reid-Smith, R.J., Staempfli, H.R., Daignault, D., Janecko, N., Avery, B.P., Martin, H., Thompson, A.D., McDonald, L.C., Limbago, B., Weese, J.S., 2009. Possibility of seasonality of *Clostridium difficile* in retail meat, Canada. Emerg. Infect. Dis. 15, 802—805.

Stabler, R.A., Dawson, L.F., Valiente, E., Cairns, M.D., Martin, M.J., Donahue, E.H., Riley, T.V., Songer, J.G., Kuijper, Ed J., Dingle, K.E., Wren, B.W., 2012. Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations. Plos One 7, e31559.

Schneeberg, A., Rupnik, M., Neubauer, H., Seyboldt, C., 2012. Prevalence and distribution of *Clostridium difficile* PCR ribotypes in cats and dogs from animals shelters in Thuringia, Germany. Anaerobe 18, 484—488.

Schoster, A., Arroyo, L.G., Staempfli, H.R., Shewen, P.E., Weese, J.S., 2012. Presence an molecular characterization of *Clostridium difficile* and *Clostridium perfringens* in intestinal compartments of healthy horses. Biomed. Cent. Microbiol. 29, 94.

Songer, J.G., Anderson, M.A., 2006. *Clostridium difficile* : an important pathogen of food animals. Anaerobe 12, 1—4.

Songer, J.G., Trinh, H.T., Killgore, G.E., Thompson, A.D., McDonald, L.C., Limbago, B.M., 2009. *Clostridium difficile* in retail meat products, USA, 2007. Emerg. Infect. Dis. 15, 819—821.

Spigaglia, P., Barbanti, F., Louie, T., Barbut, F., Mastrantonio, P., 2009. Molecular analysis of the *gyrA* and *gyrB* quinolone resistance-determining regions of fluoroquinolone-resistant *Clostridium difficile* mutants selected in vitro. Antimicrob. Agents Chemother. 53, 2463—2468.

Visser, M., Sephri, S., Olson, N., Mulvey, M.R., Alfa, M.J., 2012. Detection of *Clostridium difficile* in retail ground meat products in Manitoba. Can. J. Infect. Dis. Med. Microbiol. 23, 28—30.

Von Abercron, S.M., Karlsson, F., Wigh, G.T., Wierup, M., Krovacek, K., 2009. Low occurrence of *Clostridium difficile* in retail ground meat in Sweden. J. Food Prot. 72, 1732—1734.

Weber, I., Riera, E., Déniz, C., Pérez, J.L., Oliver, A., Mena, A., 2013. Molecular epidemiology and resistance profiles of *Clostridium difficile* in a tertiary care hospital in Spain. Int. J. Med. Microbiol. 303, 128—133.

Weese, J.S., Armstrong, J., 2003. Outbreak of *Clostridium difficile*-associated disease in a small animal veterinary teaching hospital. J. Vet. Intern. Med. 17, 813—816.

Weese, J.S., Avery, B.P., Rousseau, J., Reid-Smith, R.J., 2009. Detection and enumeration of *Clostridium difficile* spores in retail beef and pork. Appl. Environ. Microbiol. 75, 5009—5011.

Wetterwik, K.J., Trowald-Wigh, G., Fernström, L.L., Krovacek, K., 2013. *Clostridium difficile* in faeces from healthy dogs and dogs with diarrhea. Acta Vet. Scand. 55, 23.

Wong, S.S., Woo, P.C., Luk, W.K., Yuen, K.Y., 1999. Susceptibility testing of *Clostridium difficile* against metronidazole and vancomycin by disk diffusion and Etest. Diagn. Microbiol. Infect. Dis. 34, 1—6.

Zidaric, V., Pardon, B., Dos Vultos, T., Deprez, P., Brouwer, M.S.M., Roberts, A.P., Henriques, A.O., Rupnik, M., 2012. Multiclonal presence of Clostridium difficile PCR ribotypes 078, 126 and 033 within a single calf farm is associated with differences in antibiotic resistance and sporulation properties. Appl. Environ. Microbiol. 78, 8515—8522.

3.4 *C. difficile* in long-term-care facilities for the elderly

In the previous study it was shown that toxigenic *C. difficile* strains are present in retail meats. Therefore, it's plausible to assume that people may be continuously exposed to a small numbers of the bacterium by food products. A low number of potentially infectious *C. difficile* spores under healthy intestinal conditions may have not effect. However, aged patients (which are frequently hospitalised, under antibiotic exposure and/or suffer from different underlying diseases) are more susceptible to endure perturbations in the gut microbial ecosystem. In this case, a small dose of spores ingested with an altered gut microbiota may be able to trigger infection.

The following three studies were conducted at a local Belgian nursing home at the same time period. The first aimed to assess the presence of *C. difficile* on freshly prepared food in the kitchen area of the retirement home and to examine the presence of the bacterium in the environment, including kitchen surfaces, patient rooms, toilets at common lounges. Results obtained showed that none of the tested surfaces were positive for the bacterium, indicating that the number of spores in the environment must have been low and not detectable with the methodology used, and the reason for this might be the efficacy of implemented cleaning protocols to control the spread of *C. difficile* spores on the nursing home. However, in this study we have not investigated the effectiveness of different cleaning protocols against *C. difficile* spore dissemination and thus, this statement is only a hypothesis. Regarding food samples, *C. difficile* was isolated from only one food sample composed by pork sausage and salad. This low recovery rate from foods could be explained by the fact that *C. difficile* spores have been observed to be inhibited after a heat shock at 85°C, with a sub-lethal effect at 71-75°C. The PCR-ribotype isolated was identified as PCR-ribotype 078, which has been isolated from retail pork in Belgium in a previous study, and which is among the most common PCR-ribotypes found in Belgian and other European hospitals. Nevertheless, contamination of the freshly prepared meal could have originated from any of the ingredients or by the food handlers.

In the second study performed in parallel, the main objective was to assess and follow the prevalence of *C. difficile* among a group of 23 residents of the nursing home during a total of seven weeks. The bacterium was detected in seven out 23 (30.4%) residents during the entire study period, but there was only one episode of CDI diagnosed by the medical service of the nursing home. Four different PCR-ribotypes were identified but the most common type isolated was the hypervirulent ribotype 027. MLVA showed clonal relatedness of the *C. difficile* isolates and cross-infection between patients. Additionally, barcoded pyrosequencing was used to characterise the faecal microbiota of the elderly residents, to evaluate the global evolutions of the total microbiota and to identify possible relationships between certain bacteria populations and *C. difficile* colonisation. Comparison between *C. difficile* positive and negative residents showed an increase in microbial diversity in *C. difficile* positive

individuals. A marked decrease in genus *Akkermansia* and a high abundance of *Lachnospiraceae* were detected in *C. difficile* positive individuals. The study of the microbial phylotype composition of the samples showed that almost all of the samples were clustered in a sub-tree corresponding to a single resident, underlining that each resident studied had his own bacterial imprint and that it was stable during the entire study. Full characterisation of the gut microbiota in a large number of patients suffering infection due to *C. difficile* is warranted and will provide a high valued insight for new therapeutic and prevention approaches.

3.4.1 *C. difficile* from food and surface samples in a Belgian nursing home: an unlikely source of contamination

Cristina Rodriguez, Nicolas Korsak, Bernard Taminiau, Véronique Avesani, Johan Van Broeck, Philip Brach, Michel Delmée, Georges Daube

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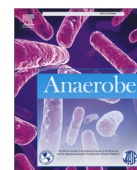
Note

Clostridium difficile from food and surface samples in a Belgian nursing home: An unlikely source of contamination

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ARTICLE INFO

ABSTRACT

This study investigates the contamination of foods and surfaces with *Clostridium difficile* in a single nursing home. *C. difficile* PCR-ribotype 078 was found in one food sample and in none of the tested surfaces. These results indicate that food and surfaces are an unlikely source of *C. difficile* infection in this setting.

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Clostridium difficile is a spore-forming anaerobic bacterium recognised as a major cause of nosocomial colitis and antibiotic associated diarrhoea. The main risk factors for *C. difficile* infection (CDI) are considered to be patient age over 65 years; gut microbiota changes; previous hospitalization; antibiotic exposure and certain underlying diseases [1,2]. Nursing homes can therefore be particularly susceptible to outbreaks [3], and the environment (e.g. contaminated surfaces in resident rooms) and foods might play a significant role in the nosocomial transmission of this bacterium [4,5].

The aim of this study was to assess the presence of *C. difficile* on freshly prepared food in the kitchen area of a nursing home and to examine the presence of this bacterium in the environment, including kitchen surfaces, patient rooms, toilets and common lounges of the retirement home.

The study was conducted over four months, from March to June 2013, at a local Belgian nursing home with a total capacity of 110 beds. This healthcare facility implemented cleaning protocols to assure an appropriate eradication of *C. difficile* spores. Residents'

rooms are cleaned and disinfected daily using bleach-based disinfectants (sodium hypochlorite 10%). Subsequent automated gaseous decontamination (stabilised hydrogen peroxide 6%) is also performed weekly. However, for residents suffering from CDI, the automated gaseous decontamination of the room is performed every day.

During the study period, one case of CDI was diagnosed by the nursing home medical services between weeks 11 and 14 while an additional case was detected 9 days before the study began. Furthermore, during the same study period another research work was conducted in the same nursing home. A group of 23 elderly-care home residents was followed weekly to evaluate the presence of *C. difficile*. Preliminary results revealed 7 out of 23 monitored residents positives for *C. difficile* at least once. Regarding positive residents to the bacterium, 57.1% (4 out of 7) suffered at least one diarrhoea episode at the time of sample collection. However, the nursing home did not experience a *C. difficile* outbreak during the study period. The main *C. difficile* strains found in the nursing home residents were identified as PCR-ribotypes 027, UCL36 and UCL16a (unpublished data). UCL followed by an international number was used only for *C. difficile* strains that presented a PCR-ribotype profile which not matched the Cardiff ribotypes from the strain collection available in our laboratory. None of the human strains isolated during the entire study were

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identified as PCR-ribotype 078.

In this nursing home, all meals for residents served daily in the canteen are prepared in-house by the kitchen staff. A second kitchen is available for residents who want to prepare their own meals. Every day (where possible), approximately 90 g of the main course of the midday meal prepared in the canteen and 60 g from a meal prepared in the resident's kitchen were sampled and stored at $-20\,°C$ in the nursing home's freezer for a maximum of 6 days. This meal quantity was the maximum provided by the two kitchen's services of the nursing home and included all food sorts of the meal in equal parts. Every Friday, samples were collected and transported to the laboratory for immediate analysis. The food preparation date, analysis date, quantity, and ingredients for each sample were recorded.

Samples from surfaces were taken on two different occasions with a 65-day interval between them (5th March and 8th May). A variety of areas in the canteen kitchen (including trays and meal delivery carts for the canteen and rooms) kitchen-staff locker room and bathroom, residents' rooms, private bathrooms, residence hall, lifts and staircase railings were tested. Samples from common areas, the kitchen and the staff bathroom and locker room were swabbed after routine cleaning. Resident rooms were sampled before (n = 4) or after (n = 4) being cleaned. A variety of residents' rooms were sampled, covering a range of occupancy and need requirements; private rooms were sampled from dependant, semi-dependant and independent residents. The dependant classification was used for residents who were confined to bed. Residents able to get out of bed but who need assistance with walking, toileting or eating in the canteen were classified as semi-dependant. Independent residents were identified as those who can perform all basic tasks necessary for a normal life without additional care support. On the first day of sampling, surface swabs were collected from randomly chosen rooms. During the second visit, sampling was performed in rooms from residents previously tested and identified as positive for *C. difficile*, based on the previous results of faeces culture (Table 1). Samples were collected using a sterile cotton swab first moistened in sterile buffered peptone water with cysteine 0.5% (Oxoid, Dardilly, France). A total area of approximately 100 cm^2 was swabbed for each sample. Swabs were placed in sterile and labelled 10 ml tubes. They were kept at room temperature for a maximum of 5 h until arrival at the laboratory, where they were immediately processed.

An enrichment culture was performed on all samples. A total of 50 g of meal was added to 150 ml of cycloserine cefoxitin fructose taurocholate (CCFT) broth (produced in-house), as previously described [6] but without agar, and both were homogenized 3 min in a stomacher device (Interscience, Saint-Nom-La Brechete, France). Surface swabs were placed into 10 ml of CCFT. The enrichment broth was incubated for 3 days in the case of food

samples and for 10 days in the case of surface samples, both at $37\,°C$ in an anaerobic workstation (Led Techno, Heusden-Zolder, Belgium). Subsequently, approximately 10 μl of the broth was spread on to CCFT plates [6] and incubated anaerobically for 48 h at $37\,°C$. Suspected colonies were identified by *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid) and by *C. difficile*-specific PCR, as previously described [7]. A cytotoxicity assay using MRC-5 cells was performed, as described elsewhere [8].

C. difficile isolates were characterised using the GenotypeCDiff test system (HainLifescience, Nehren, Germany) for detection of all toxin genes (*tcdA, tcdB, cdtA* and *cdtB*), the deletions in the regulator gene *tcdC* (18 bp and 39 bp deletions or single base deletion at position 117), and *gyrA* mutation, according to the manufacturer's instructions. PCR-ribotyping was performed as described previously [9].

A total of 246 environmental surfaces and 188 food samples were analysed. *C. difficile* was isolated from only one meal sample (0.53%) composed of pork sausage, mustard sauce and carrot salad. This meal had been prepared in the canteen kitchen. The isolate had a toxic activity confirmed by cytotoxicity assay and contained the genes coding for the A, B and binary toxin. A 39 bp deletion in the regulator gene *tcdC* was also detected. The isolate was identified as PCR-ribotype 078.

This finding suggest that the contamination level in prepared meals is lower than the contamination reported in other raw foods such as meat or vegetables where the prevalence ranged between 0.9% and 20% [10]. Regarding the prevalence of *C. difficile* in foods in Belgium, only one previous study reported the presence of the bacterium in retail meat with a percentage of positive samples ranging between 2.3% and 4.7% [11]. One possible explanation for this low recovery rate in prepared meals is that an inhibitory effect on *C. difficile* spores has been observed after a heat shock at $85\,°C$, with a sub-lethal effect at $71\,°C$ [12]. In this study, a total of 70 out of 188 (70.7%) samples analysed were composed entirely from cooked ingredients, while 55 out of 188 (29.3%) contained one or more raw ingredients such as lettuce, tomato or raw meat. The only positive sample for *C. difficile* was recovered from a meal composed of carrot salad, mustard sauce and pork sausage. However, contamination could have originated from any of the ingredients (as they were analysed together) or even from the kitchener's hands. The PCR-ribotype isolated (078) has been frequently reported in pork retail meat in USA and Canada [10] but has also been considered as an emergent hypervirulent strain in humans [13]. Nevertheless, in the present study none of the circulating clinical *C. difficile* isolates in the nursing home were identified as PCR-ribotype 078. These results indicate that food is an unlikely source of *C. difficile* infection in the nursing home evaluated.

Surprisingly, none of the surfaces sampled were positive for *C. difficile*, even after 10 enrichment days on selective medium.

Table 1
Characteristics of the resident's rooms sampled.

Room identification	Floor	Resident health condition	Sampling before or after cleaning room routine	Resident faeces analysis for *C. difficile*
0	1	Semi-independent resident	Sampling before being cleaned	Not tested
A	1	Independent resident	Sampling after being cleaned	Not tested
B	1	Dependent resident	Sampling after being cleaned	Not tested
C	2	Independent resident	Sampling before being cleaned	Not tested
D	2	Dependent resident	Sampling after being cleaned	Positive[a]
E	2	Independent resident	Sampling before being cleaned	Positive[a]
F	3	Independent resident	Sampling before being cleaned	Positive[c]
G	3	Dependent resident	Sampling after being cleaned	Positive[b]

[a] Resident positive for *C. difficile* at least one week before and at the time of sampling.
[b] Resident positive for *C. difficile* one week before sampling.
[c] Resident positive for *C. difficile* two weeks before sampling.

Previous studies have reported an environmental contamination of *C. difficile* spores in hospitals with a prevalence ranging between 2.4% and 47% [14–17]. A range of culture methods have been proposed to isolate environmental *C. difficile,* including swabbing [14], electrostatic cloths [15] contact CCFT plates [16] and rodac plates [17]. This diversity in the methodology can help to explain the variations in spore detection. For this reason, a standard method to estimate the environmental contamination of *C. difficile* spores would be desirable. However, care was taken to achieve robust results: a large number of samples were performed from a variety of surface types, including the zones commonly contaminated, e.g. bedside table, bedrail or toilet floor [14]; sampling was performed on two different days separated by a large time interval (65 days); and an effort was made to exert the maximum pressure possible for each swab. Because of these measures, our results indicate that the implemented clean program to control the spread of *C. difficile* on the nursing home is effective and if there was any surface contamination, the number of spores in the environment must have been very low and not easy detectable.

In conclusion, the results of this study indicate that the nursing home environment and meals studied are unlikely to serve as vector of *C. difficile* infection. As meals from two different kitchens with different hygiene procedures were tested over a period of four months, we report a low *C. difficile* contamination level in ready-to-eat foods prepared in the nursing home's kitchens. For the environment, it is probable that results vary significantly between retirement and healthcare establishments according to procedures for routine cleaning and disinfecting, or even depending on the procedure used to isolate *C. difficile.*

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References

[1] G. Dulny, M. Zalewska, G. Mlynarczyk, An analysis of risk factors of *Clostridium difficile* infection in patients hospitalized in the teaching hospital in 2008, Prz. Epidemiol. 63 (2013) 445–450, 547-51.

[2] J.M. Wenish, D. Schmid, H.W. Kuo, E. Simons, F. Allerberger, V. Michl, et al., Hospital-acquired *Clostridium difficile* infection: determinants for severe disease, Eur. J. Clin. Microbiol. Infect. Dis. 31 (2012) 1923–1930.

[3] M. Arvand, V. Moser, C. Schwehn, G. Bettge-Weller, M.P. Hensgens, EdJ. Kuijper, High prevalence of *Clostridium difficile* colonization among nursing home residents in Hesse, Germany, PLoS One 7 (2012) e30183.

[4] C.D. Faires, D.L. Pearl, O. Berke, R.J. Reid-Smith, J.S. Weese, The identification and epidemiology of methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile* in patient rooms and the ward environment, BMC Infect. Dis. 13 (2013) 342.

[5] D.G. Hoover, A. Rodriguez-Palacios, Transmission of *Clostridium difficile* in foods, Infect. Dis. Clin. N. Am. 27 (2013) 675–685.

[6] M. Delmée, B. Vandercam, V. Avesani, J.L. Michaux, Epidemiology and prevention of *Clostridium difficile* infections in a leukaemia unit, Eur. J. Clin. Microbiol. 6 (1987) 623–627.

[7] C. Rodriguez, V. Avesani, J. Van Broeck, B. Taminiau, M. Delmée, G. Daube, Presence of *Clostridium difficile* in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium, Int. J. Food Microbiol. 2 (2013) 256–262.

[8] C. Rodriguez, B. Taminiau, J. Van Broeck, V. Avesani, M. Delmée, G. Daube, *Clostridium difficile* in young farm animals ans slaughter animals in Belgium, Anaerobe 18 (2012) 621–625.

[9] P. Bidet, F. Barbut, V. Lalande, B. Burghoffer, J.C. Petit, Development of a new PCR-ribotyping method base on ribosomal RNA gene sequencing, FEMS Microbiol. Lett. 175 (1999) 261–266.

[10] M.P.M. Hensgens, E.C. Keessen, M.M. Squire, T.V. Riley, M.G.J. Koene, E. De Boer, L.J.A. Lipman, E.J. Kuijper, European Society of Clinical Microbiology and Infectious Diseases Study Group for *Clostridium difficile* (ESGCD). *Clostridium difficile* infection in the community: a zoonotic disease? Clin. Microbiol. Infect. Dis. 18 (2012) 635–645.

[11] C. Rodriguez, B. Taminiau, V. Avesani, J. Van Broeck, M. Delmée, G. Daube, Multilocus sequence typing analysis and antibiotic resistance of *Clostridium difficile* strains isolated from retail meat and humans in Belgium, Food Microbiol. 42 (2014) 166–171.

[12] A. Rodriguez-Palacios, J.T. LeJeune, Moist-heat resistance, spore aging, and superdormancy in *Clostridium difficile*, Appl. Environ. Microbiol. 77 (2011) 3085–3091.

[13] A. Goorhuis, D. Bakker, J. Corver, S.B. Debast, C. Haemanus, D.W. Notermans, et al., Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078, Clin. Infect. Dis. 47 (2008) 1162–1170.

[14] M. Sjörberg, M. Eriksson, J. Andersson, T. Noren, Transmission of *Clostridium difficile* spores in isolation room environments and through hospital beds, APMIS 122 (2013) 800–803.

[15] A. Kramer, I. Schwebke, G. Kampf, How long do nosocomial pathogens persist on inanimate surfaces? A systematic review, BMC Infect. Dis. 6 (2006) 130.

[16] J.S. Weese, H.R. Staempfli, J.F. Prescott, Isolation of environmental *Clostridium difficile* from a veterinary teaching hospital, J. Vet. Diagn Investig. 12 (2000) 449–452.

[17] K.H. Kim, R. Fekety, D.H. Batts, D. Brown, M. Cudmore, J. Silva Jr., D. Waters, Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-induced colitis, J. Infect. Dis. 143 (1981) 42–50.

3.4.2 Longitudinal survey of *Clostridium difficile* presence and gut microbiota composition in a Belgian nursing home

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Abstract

Background: Increasing age, several co-morbidities, environmental contamination, antibiotic exposure and other intestinal perturbations appear to be the greatest risk factors for *C. difficile* infection (CDI). Therefore, elderly care home residents are considered particularly vulnerable to the infection. The main objective of this study was to evaluate and follow the prevalence of *C. difficile* in 23 elderly care home residents weekly during a 4-month period. A *C. difficile* microbiological detection scheme was performed along with an overall microbial biodiversity study of the faeces content by 16S rRNA gene analysis.

Results: Seven out of 23 (30.4%) residents were (at least one week) positive for *C. difficile*. The most common PCR-ribotype identified was 027. 16S-profiling analyses revealed that each resident has his own bacterial imprint, which is stable during the entire study. Variations in the abundance of taxa have been observed in the faecal microbiota of colonised residents. Positive *C. difficile* status is not associated with microbiota richness or biodiversity reduction in this study. A decrease of *Akkermansia* in positive subjects to the bacterium has repeatedly found.

Conclusions: This association of classical microbiology protocol with pyrosequencing allowed to follow *C. difficile* in patients and to identify several other bacterial populations whose abundance is correlated with *C. difficile*. The link between *Akkermansia,* gut inflammation and *C. difficile* colonisation merits further investigations

Keywords: *C. difficile*, elderly care home residents, 16S rRNA gene analysis

Background

Clostridium difficile is a Gram-positive, anaerobic, spore-forming, rod-shaped bacterium that has been widely described in the intestinal tract of humans and animals. In 1978, *Clostridium difficile* was recognized as a cause of antibiotic associated diarrhoea and, in the most serious cases pseudomembranous colitis [1-3]. Since then, many outbreaks have been reported; most of them were associated with the emergence of a specific subtype, hyper-virulent PCR-ribotype 027 [4]. Nowadays, *C. difficile* is a worldwide public health concern as it is considered the major cause of antibiotic-associated infections in healthcare settings [5]. A recent report of *C. difficile* infection (CDI) cost-of-illness attributes a mean cost ranging from 8,911 to 30,049 USD for hospitalised patients (per patient/admission/episode/infection) in the USA [6] and annual economic burden estimated around 3,000 million euro in Europe [7].

CDI is more commonly diagnosed among older people in nursing homes. High isolation frequencies have been described in USA, with up to 46% of elderly residents testing positive for *C. difficile*, while in Europe or Canada the reported rates are much lower, varying between 0.8% and 10% [8]. This is partly because elderly people are more commonly in hospitals, have an antibiotic treatment and age-related changes in intestinal flora and host defences, as well as the presence or other underlying health problem [8-10]. These factors can have an impact on the intestinal microbiota, which may promote *C. difficile* colonisation and the development of the infection [11]. Therefore, a new concern of several studies has been the identification of the microbial communities implicated in the CDI through the use of new sequencing techniques, like metagenomics [12].

The aim of this study was to evaluate and follow the prevalence of *C. difficile* among the residents of a Belgian nursing home. Multilocus variable number of tandem repeats analysis (MLVA) was performed to determine the genetic diversity of the *C. difficile* isolates and possible cross-infection between patients. Additionally, 16S rRNA gene sequencing was used to characterize the faecal microbiota of the elderly residents, to evaluate the global evolutions of the total microbiota and to identify possible relationships between certain bacteria populations and *C. difficile* colonization, diarrhoea and antibiotic treatment.

Results

Prevalence of *C. difficile*

A total of 242 faecal samples were collected from 23 residents in seventeen consecutive weeks (resident number 11 was excluded from the study as he finally did not agree to participate in the survey). Two subjects passed away within the four-month study period. Seven of 23 monitored residents were positive for *C. difficile* at least once (Table 1).

There was only one case of CDI diagnosed during the study (subject 01). He was diagnosed in week eleven of the study after suffer more than three episodes consecutives of diarrhea *C. difficile* was detected in 14 out of these 30 diarrhoeal samples (43.7%). Regarding the antimicrobial therapy, a total of five residents tested positive for *C. difficile* had previously received an antibiotic medication. Probiotic treatment was noted in 4 residents, two of them were positive for *C. difficile* even 5 weeks after the first administration. Only one resident (number 08) was hospitalized during the study (Table 2).

Table 1. Detailed information on 23 nursing home residents enrolled in the study, including the detection of *C. difficile* with and without enrichment

Resident Identification	Week																
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
01	E	E	‡	-	-	‡	-	-	‡	E	E	E	‡	D	‡	D	-
02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
03	-	-	-	‡	-	-	-	-	-	‡	-	-	‡	-	‡	‡	‡
04	-	-	-	-	-	-	-	-	-	-	-	‡	-	-	-	-	-
05	-	-	-	‡	‡	-	‡	‡	-	-	-	-	‡	-	-	-	‡
06	-	-	-	‡	-	‡	-	-	-	-	‡	-	‡	‡	-	-	‡
07	-	-	-	‡	‡	-	-	-	-	‡	-	-	-	-	‡	-	‡
08	‡	-	-	H	H	H	H	-	‡	-	-	-	-	‡	-	‡	‡
09	-	‡	‡	-	‡	‡	‡	-	-	-	‡	-	-	-	‡	-	-
10	-	‡	-	‡	-	-	‡	-	-	-	-	-	‡	-	-	‡	‡
12	-	-	-	-	‡	-	-	-	‡	-	-	-	-	-	‡	-	-
13	D	D	-	-	‡	-	-	-	‡	-	-	-	‡	-	‡	‡	‡
14	-	-	-	-	‡	‡	‡	-	-	-	‡	-	‡	-	‡	‡	‡
15	E	E	E	-	‡	D	D	D	D	D	D	D	D	D	‡	‡	D
16	-	-	-	-	-	-	†	†	†	†	†	†	†	†	†	†	†
17	-	-	-	‡	-	-	-	D	-	-	-	-	‡	-	‡	‡	‡
18	‡	‡	‡	‡	D	‡	D	‡	D	‡	‡	D	‡	‡	E	‡	E
19	E	E	E	‡	‡	‡	D	‡	-	-	E	‡	D	‡	-	‡	‡
20	-	‡	-	‡	‡	-	‡	‡	-	‡	‡	‡	‡	‡	-	‡	‡
21	‡	-	-	-	-	-	‡	-	‡	-	-	‡	‡	‡	-	‡	-
22	-	-	-	-	-	-	†	†	†	†	†	†	†	†	†	†	†
23	-	‡	-	-	-	‡	-	-	-	-	-	-	-	‡	-	-	-
24	‡	‡	D	‡	E	-	‡	E	‡	-	‡	-	‡	-	‡	‡	-

Resident number 11 was excluded from the study

D: Positive results detected without enrichment

E: Positive results detected after 3 days of enrichment

-: Negative results for *C. difficile* presence

‡: Sample was not available

H: resident hospitalized

†: The resident passed away during the study period

Table 2. Clinical characteristics of the 23 residents enrolled in the study and molecular type of the isolates

Resident number	Age (years)	Genre	Status	Room Floor	Diarrhea	Hospital stay	Antibiotic treatment	Probiotic treatment	C. difficile culture	PCR-ribotype	N° isolates	CE	tcdA tcdB	cdtA cdtB	tcdC MUT	gyrA MUT
01	77	M	SD	1	+	-	+	-	+	UCL16a	9	+	+	-	-	-
02	78	F	D	2	+	-	-	-	-	-	-	-	-	-	-	-
03	92	F	D	2	-	-	-	-	-	-	-	-	-	-	-	-
04	88	F	D	1	+	-	+	+	-	-	-	-	-	-	-	-
05	93	F	D	1	+	-	+	-	-	-	-	-	-	-	-	-
06	86	F	D	2	-	-	+	-	-	-	-	-	-	-	-	-
07	92	F	SD	3	-	-	+	+	-	-	-	-	-	-	-	-
08	91	F	SD	3	-	+	+	-	-	-	-	-	-	-	-	-
09	88	F	D	2	-	-	+	-	-	-	-	-	-	-	-	-
10	78	F	SD	2	-	-	-	-	-	-	-	-	-	-	-	-
12	87	F	D	2	+	-	-	-	-	-	-	-	-	-	-	-
13	65	M	D	1	-	-	+	-	+	UCL36	4	-	-	-	-	-
14	76	F	SD	1	-	-	-	+	-	-	-	-	-	-	-	-
15	50	F	D	2	+	-	-	+	+	027	22	+	+	+	+	+
16	94	F	D	3	+	-	+	-	-	-	-	-	-	-	-	-
17	63	F	D	3	+	-	+	-	+	UCL36	1	+	+	-	-	-
									+	027	1	+	+	+	+	+
18	86	M	D	2	+	-	-	-	+	027	10	+	+	+	+	+
19	89	F	SD	3	+	-	+	-	+	UCL36	2	+	+	-	-	-
										UCL46	2	+	+	-	-	-
										027	4	+	+	+	+	+
20	81	F	SD	1	-	-	+	-	-	-	-	-	-	-	-	-
21	82	F	D	1	+	-	-	-	-	-	-	-	-	-	-	-
22	83	F	D	1	-	-	-	-	-	-	-	-	-	-	-	-
23	88	F	D	2	-	-	-	-	-	-	-	-	-	-	-	-
24	81	F	D	1	-	-	+	+	+	UCL36	4	-	-	-	-	-

M: masculine
F: feminine
SD: semi-dependant residents
D: dependant residents
CE: cytotoxicity assay using MRC-5 cells
tcdC MUT: Presence of deletions in the regulator gene *tcdC* (118bp-39bp-17bp)
gyrA MUT: Presence of mutation in the *gyrA* gene associated with moxifloxacin resistance

Characterization of *C. difficile* isolates

Four different PCR-ribotypes (UCL16a, UCL36, UCL46 and 027) were identified among the 38 isolates. In one resident (number 19), different PCR-ribotypes were found in different sampling days while in another subject (number 17) two different PCR-ribotypes were detected in the same sampling day (direct culture: PCR-ribotype 027; 3 days of stool enrichment: PCR-ribotype UCL36). Only in one resident (number 015), all but one samples obtained were positive for *C. difficile* and the isolated strains were all identified as PCR-ribotype 027. Three out of these four different PCR-ribotypes had toxic activity. All toxigenic isolates encoded toxin A and B, while PCR-ribotype 027 also contained the binary toxin. In addition, all types 027 contained an 18-base pair deletion, a deletion at 117 of the *tcdC* gen and *gyrA* mutation associated with moxifloxacin resistance (Table 2).

C. difficile MLVA analysis

MLVA was performed in order to provide further insight into the clonal relatedness of the *C. difficile* isolates and cross-infection between patients. A total of 59 isolates have been obtained during the study. Among them, 44 toxigenic and non-toxigenic isolates were further analysed by MLVA. Selection of these strains was based on the inclusion of a representative number of isolates from each classified PCR ribotype. In order to determine if the seven VNTR loci were stable over time or if subjects harboured more than one *C. difficile* type, isolates obtained from the same resident on direct culture and after 3 enrichment days and on different weeks were also studied by MLVA. Thirty-one different MLVA profiles were identified. However, a high degree of genetic relatedness was observed among most of the strains with the same PCR-ribotype (summed tandem repeat difference at all loci ≤ 2). The C6 and A6 were the most diverse VNTR loci. Regarding the strains identified as PCR-ribotype 027, most of them were closely related. Furthermore, several isolates from patients 15, 18 and 19 had an identical MLVA profile (Table 3).

Table 3. MLVA profile of the isolates obtained from each nursing home resident

PCR-Ribotype	MLVA Profile								Resident Number	No. of isolates	Week
	A6	B7	C6	E7	G8	CDR5	CDR60	Total			
027	22	9	38	10	17.5	3.9	7.2	107.6	15	4	1[E] 2[E] 6[E]
									18	1	7[E]
											5[E]
	23	9	38	10	17.5	3.9	7.2	108.6	15	1	3[E]
	22	9	39	10	17.5	3.9	7.2	108.6	15	1	7[D]
	22.2	9	37.8	10	17.5	3.9	7.2	107.6	15	3	9[D] 10[E]
									18	1	14[E]
									19	1	15[E]
											12[D]
	22.2	9	37.8	10	17.5	3.8	7.2	107.5	15	1	8[E]
	22	9	37.8	10	17.5	3.8	7.2	107.3	15	1	9[E]
	23	9	37.8	10	17.5	3.8	7.2	108.3	15	1	12[D]
	22.2	9	26.5	10	18.5	3.9	7.2	97.3	15	1	12[E]
	23	9	40	10	17.5	3.9	7.1	110.5	18	1	2[E]
	22	9	36.8	10	17.5	3.9	7.2	106.4	18	1	7[D]
	22.2	9	37.8	10	17.6	3.9	7.2	107.7	18	1	7[E]
	23	9	36.8	10	17.5	3.8	7.2	107.3	18	1	9[E]
	22	9	37	10	17.5	3.9	7.2	106.6	19	1	3[E]
	22.2	9	36.8	10	17.5	3.8	7.2	106.5	19	2	7[E] 11[E]
UCL16a	30.8	14.1	23.5[a]	5	10.8	6.8	3.2	94.2	1	1	1[E]
	30.8	14	23.5	5	10.8	6.8	3.2	94.1	1	2	2[E] 16[E]
	30.8	14	24.5	5	10.8	6.8	3.2	95.1	1	1	10[E]
	30.7[b]	14[c]	11.3	5	10.8	6.8	3.2	81.8	1	1	11[E]
	29.8	14	23.5	5	10.8	6.8	3.2	93.1	1	2	12[E] 16[D]
	31.8[d]	14	23.5	5	10.8	6.8	3.2	95.1	1	1	14[D]
UCL46	28.8	21.1	22.3	14	8	8.8	2.2	105.2	19	1	1[E]
	28.8	21.1	22.5	14	8	8.8	2.3	105.5	19	1	2[E]
UCL36	19.2	17	42.8	8	9.9	4.9	10.2	112	13	1	1[D]
	18.3	16	42.8	8	9.9	4.9	10.2	110[e]	13	1	1[E]
	18.3	16	36.8	8	9.9	4.9	10.2	104.1	13	1	2[D]
	19.2	16.1[f]	41.8	8	9.9	4.9	10.2	110.1	13	1	2[E]
	30.8	17	34.7	8	10.8	4.9	10.2	117.4	17	1	8[E]
	31.8	17.1	34.7	8	10.8	4.9	10.2	118.5	19	1	7[D]
	31.8	17	34.8	8	10.8	4.9	10.2	118.5	24	1	3[D]
	31.8	17	34.7	8	10.8	4.9	10.2	118.4	24	1	5[E]
	31.8	18.1[g]	35.8	8	10.8	4.9	10.2	120.6	24	1	8[E]

Differences found in the results after one or more repetitions: [a]24.5; [b]30.8; [c]17.3; [d]26.9; [e]10.3; [f]16; [g]18

[E] Strain isolated after 3 days of feces enrichment

[D] Strain isolated after direct culture of the feces

Analysis of the residents' faecal microbiota by barcoded pyrosequencing

Among the 23 residents, available faecal samples from 13 residents (6 *C. difficile* negative and 7 *C. difficile* positive, in total 118 faecal samples) were selected for 16S profiling of their faecal microbiota. A total of 433,815 final reads were attributed to 3,940 species level OTUs among 118 samples (Additional file 1). The analysis showed that the major phyla found in patients were Firmicutes and Bacteroidetes followed by the Verrucomicrobia and the Proteobacteria (Fig. 1). On the family level, the major populations were consistent with previous human studies, *Bacteroidaceae*, *Ruminococcaceae* and *Lachnospiraceae* being dominant. The *Verrucomicrobiaceae*, *Porphyromonadaceae* and *Rikenellaceae* were subdominant (Fig. 1). The 6 major genera were *Bacteroides*, *Akkermansia*, *Parabacteroides*, *Alistipes* and two populations undefined at the genus level belonging respectively to the *Lachnospiraceae* and the *Ruminococcaceae* (Additional file 2).
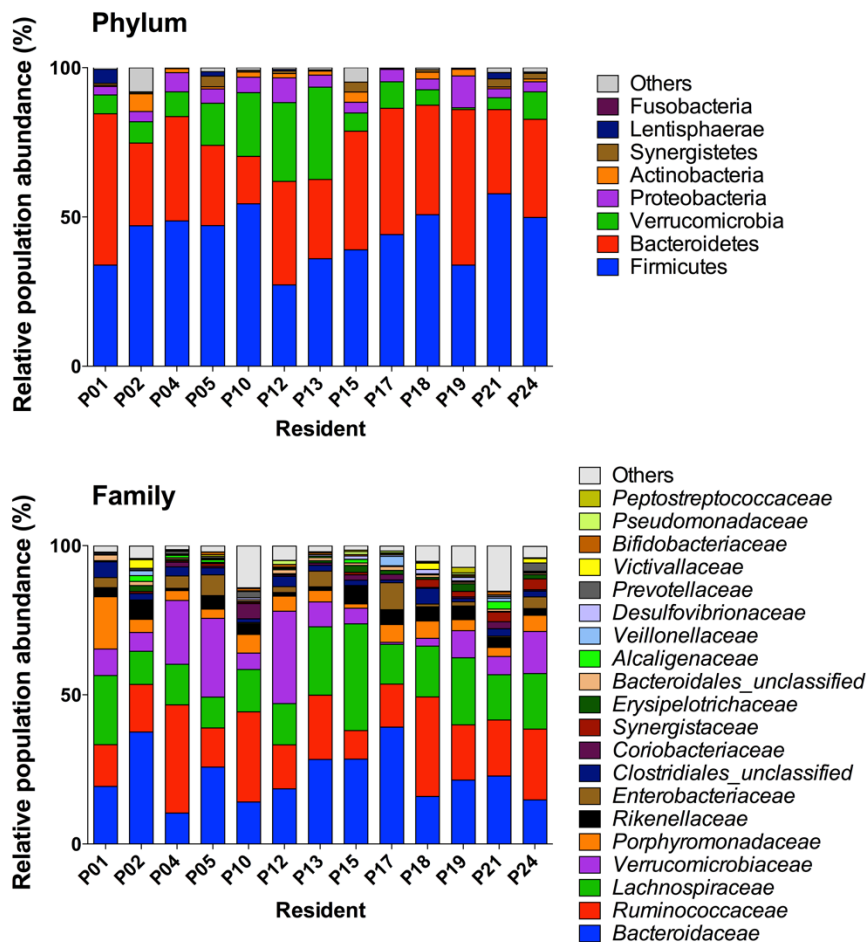
Figure 1. Taxonomical distribution deduced by 16S rDNA profiling. Bart chart detailing the mean cumulated relative abundance of the major phyla and families for each resident

The mean alpha diversity and richness is variable between residents (Additional file 4), though no resident mean values are statistically different from the rest of the cohort (Fig. 2). Moreover, the analysis of the microbiota species structure and composition showed that each patient has his own microbiological imprint during the study as revealed by weighted UNIFRAC analysis of phylotypic distribution of the samples based on a Bray-curtis distance matrix (Fig. 2 and Additional file 3).
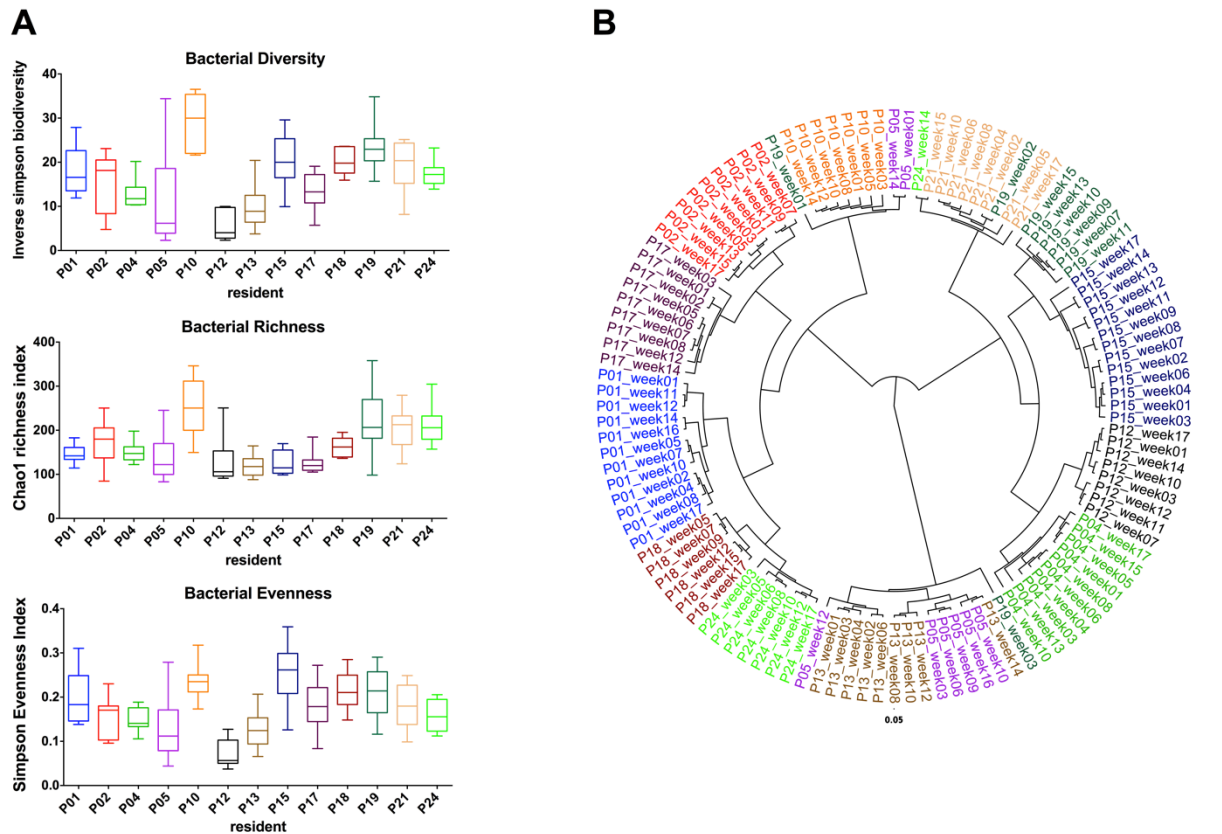
Figure 2. Species bacterial diversity and species phylotypic tree based on Bray-Curtis distance matrix

(A) Bacterial diversity (inverse Simpson biodiversity index), bacterial richness (Chao1 richness index) and bacterial evenness (Deduced from Simpson index). Bacterial diversity indexes are expressed as a box plot of the mean from subsampled datasets, whiskers represent minimum and maximum value. Median is shown as a line inside the box. (B) Phylotype tree of the 118 subsampled datasets built upon a Bray-Curtis distance matrix at the species taxonomical level (average tree is shown, 1000 iterations).

Among the 118 samples, 24 samples were detected positive for *C. difficile* by 16S rRNA gene analysis (Fig. 3). Reads sharing minimum 99% of identity to the *Clostridium difficile* 16S rRNA sequence were easily identified as such. Indeed nearest known species (*Clostridium glycolicum*, *Terrisporobacter mayombei* and *Romboutsia lituseburensis*) share less than 99% of nucleotidic identity on the V1-V3

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hypervariable region with *C. difficile* 16S rRNA sequence. On these specific 118 samples analysed by 16S rRNA gene analysis, 37 were labelled *C. difficile* positive by culture microbiology. Among the positive samples, 18 samples were detected by both methods, 19 samples were positive only by culture and 6 were positive only by 16S rRNA profiling.
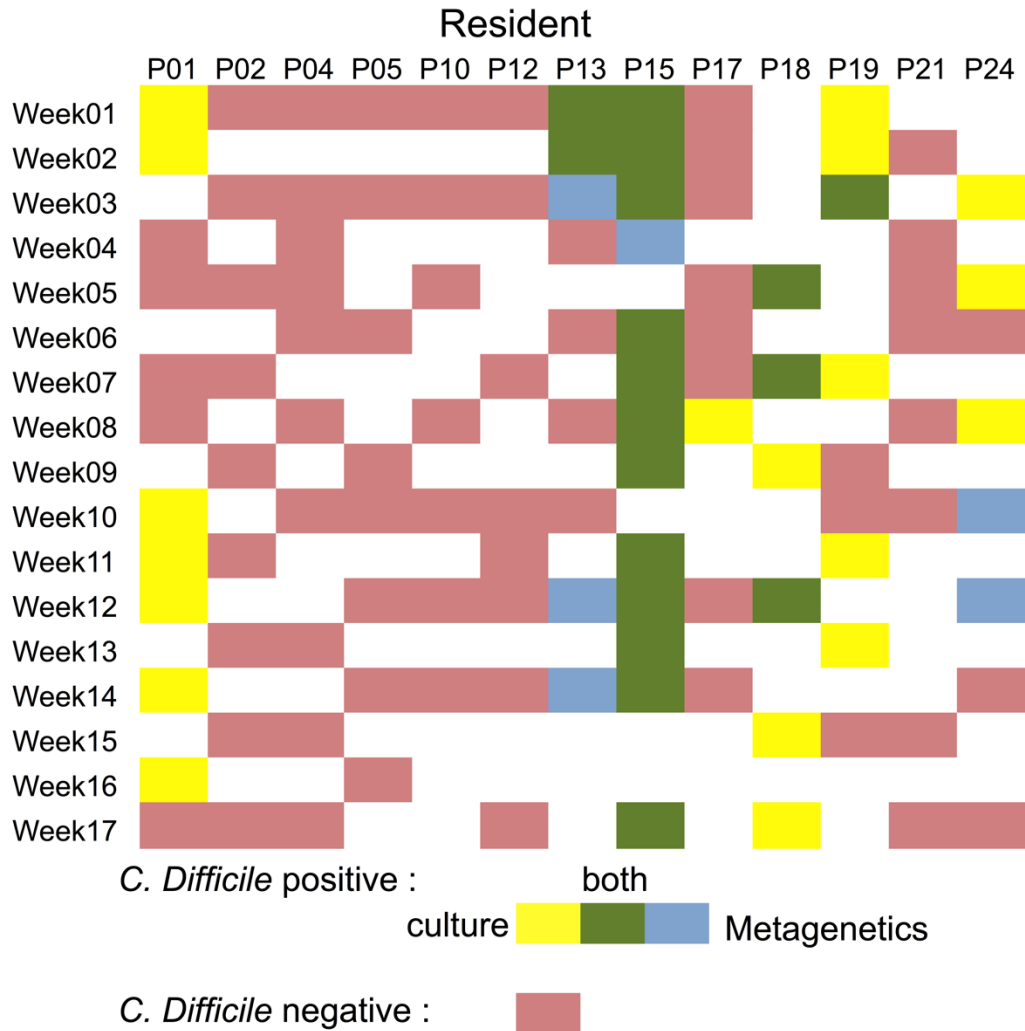
Figure 3. *C. difficile* detection results for the 118 samples analysed by culture and metagenetics
Grid detailing the detection results for the samples analysed by both methods. For classic microbiology, samples are positive if either direct or enrichment culture is positive. For metagenetics, samples are positive if at least one sequence read is identical to *C. difficile* V1-V3 16S rDNA sequence. Red – negative sample; white – non-analysed sample; yellow – *C. difficile* positive culture; blue – *C. difficile* metagenetic detection and green – *C. difficile* positive for both methods.

Link between *C. difficile* colonization and faecal microbiota

In order to explore the link between *C. difficile* colonization and the resident microbiota, residents negative and positive for *C. difficile* detection were grouped. As the inter-individual variability is the

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main driving factor for the sample clustering, the grouping has been made by resident instead of strict positive and negative samples. Figure 4 shows the major mean genus relative abundance for both groups. Statistical analysis revealed that only four genus populations have significant relative abundance between both groups (Fig 4). Indeed *Blautia* (Firmicutes) and *Flavonifractor* (Firmicutes) and the Lachnospiraceae_unclassified (Firmicutes) appeared more abundant in the *C. difficile* positive group, whereas *Akkermansia* (*Verrucomicrobiaceae*) abundance was higher in the *C. difficile* negative group. In order to better understand these differences, both groups were further splitted into diarrheic (> 1 diarrheic feces sample) or non-diarrheic residents. If the decrease in *Verrucomicrobiaceae* is still linked to *C. difficile* positive groups (data not shown), the higher abundance of *Lachnospiraceae* family is specific to *C. difficile* positive diarrheic residents compared to other groups (p<0.05) (Additional file 5).

The analysis of mean alpha diversity of both groups showed that *C. difficile* positive group exhibited a higher evenness (inverse Simpson index) . This difference was not seen in the species richness (Chao index) or microbial biodiversity (Fig. 4).
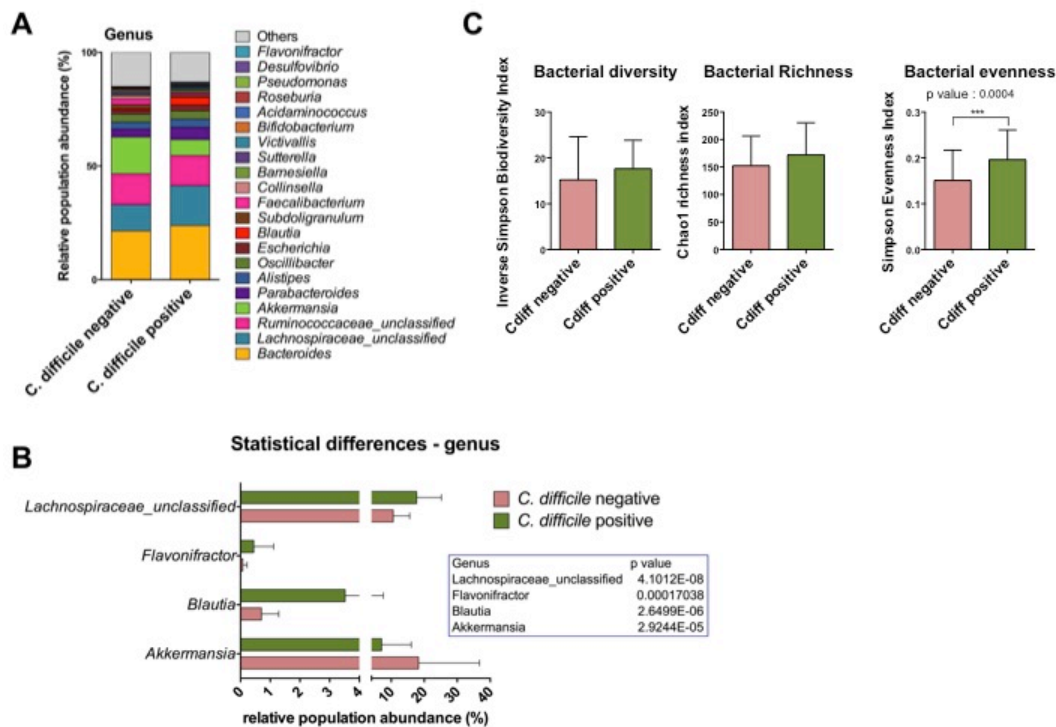
Figure 4. Microbiota comparison between *C. difficile* negative and positive residents

(A) Mean cumulative relative abundance distribution for the major bacterial genera (>1%) for *C. difficile* negative and positive residents. (B) Changes in microbial genus populations between *C. difficile* negative and positive residents. Populations whom relative abundance is statistically different are expressed as mean relative abundance ± standard error of the mean (p < 0.05 according to multiple

unpaired t-test with Benjamini-Hochberg False Discovery Rate). (C) Bacterial diversity (inverse Simpson biodiversity index), bacterial richness (Chao1 richness index) and bacterial evenness (Deduced from Simpson index). Bacterial diversity indexes are expressed as a box plot of the mean from subsampled datasets, whiskers represent minimum and maximum value. Median is shown as a line inside the box. Statistical differences are represented by asterisks (p< 0.05 according to non parametric Mann-Whitney test) after Bonferroni corrections.

Discussion

The gut microbiota ecosystem plays a critical role in resistance to colonisation by pathogenic organisms, infection and recurrence [11]. *C. difficile* colonisation has been described as ten times higher in elderly nursing home residents than in the general population living outside long-term care facilities [13]. The deteriorating health status of nursing home residents, their frequent hospitalisation and the cohabitation in the same contaminated environment promote bacterial colonisation and dissemination [14,15]. The aim of this study was to evaluate the presence of *C. difficile* in a short cohort of elderly nursing home residents and to evaluate the global evolutions of their faecal microbiota.

In the present study, 30.4% (7/23) residents were positive to *C. difficile*. In previous studies conducted in Germany, UK, Ireland, Australia or Canada, the prevalence of positive residents reported ranges between 0.80% and 10% [13,16-19]. This prevalence is much higher in other reports in USA, varying between 6.4% and 54.8%. The differences in results obtained among studies may be due to geographical or methodological variations [20]. The same scenario was reported for the incidence of CDI in Belgian hospitals when compared with other hospitals in Europe and USA [21,22]. In addition, in the present study residents were not only sampled in one occasion but also tracked weekly during a total of 4 months, which could explain the higher prevalence found in comparison with the other studies conducted in Europe. In our study, only one resident was diagnosed with a CDI. However, other residents presented symptoms (diarrhoea) and either stool test positive for toxigenic *C. difficile*. Therefore, the lack of clinical diagnosis or request do not exclude that other residents suffered CDI during the study period [23]. On the other hand, positive residents to *C. difficile* without any signs of disease were also detected.

Results obtained from PCR-ribotyping and MLVA showed that there was a clonal dissemination within the nursing home residents. Therefore, even if some authors have refuted the theory of person-to person transmission to explain the increase incidence of CDI within hospital awards [24,25], it seems that in this nursing homes the situation is different. Only four different PCR-ribotypes were identified and three of them were toxigenic (UCL16a, UCL46 and 027). Surprisingly, none of them were among the five PCR-ribotypes most commonly identified in Belgian hospitals in 2013 and 2014

[22]. Since 2011, decline in the prevalence of the PCR-ribotype 027 has been reported in different European countries. Furthermore, in Belgium, the proportion of hospitals with the hypervirulent PCR-ribotype 027 decreases from 34% in 2009 to 15% in 2013. The same situation is described for PCR-ribotype UCL46 [21]. Unfortunately, there are no recent studies in the literature conducted in nursing homes and therefore is not possible to compare the PCR-ribotype distribution found in Belgium with those present in other countries. One way of introduction new *C. difficile* strains in nursing homes is by incoming patients, visits, by the transfer of residents to hospitals when they suffer an acute clinical problem [8,14] or by foods or animals visitation [25,26]. Then nursing home population is more closed and restricted and changes in the prevalence of PCR-ribotypes come later than in hospitals. Therefore, it could be hypothesised that the most prevalent PCR-ribotypes today in hospitals (PCR-ribotypes 078 and 014/020) [22] will be in a few years predominant in nursing homes.

The 16S rRNA profiling of faecal microbiota has been applied to a small cohort of this longitudinal study. Although the elderly gut microbiota is thought to be different from that of the healthy adult, the number of publications addressing this topic with "omics" approaches is still low [27,28]. We chose to base our 16S amplicon design on the V1-V3 hypervariable region as it provides precise taxonomical assignment to the genus level and beyond [29,30].

In previous studies on elderly gut microbiota, Bacteroidetes and Firmicutes have been reported to dominate with a marked preponderance of Bacteroidetes over Firmicutes [31-33]. In the present study, the major bacterial phyla identified in residents' microbiota are the Firmicutes followed by the Bacteroidetes. We also found a higher abundance of Verrucomicrobia than previously observed [31-32]. The predominance of Firmicutes and Bacteroidetes has also been highlighted in a large cohort study in Belgium [34], although the overall prevalence of Bacteroidetes in our study is higher than the mean value on a large scale population level (34% in our study vs 25% in the Belgian Flemish Gut Flora Project). This increase in Bacteroidetes relative proportion in elderly gut microbiota compared to a matched cohort of younger adults has already been described [31]. The analyses of the bacterial genera distribution in this study indicate significant differences in the major population compared to a previous study on elderly with western lifestyle and diet [31]. These differences are mainly in the Firmicutes phylum, with a higher abundance of genus *Blautia* and a lower abundance of *Faecalibacterium*. Among notable genera, *Escherichia* abundance is surprisingly high in our results.

It has been recently underlined that longitudinal survey of microbiota in elderly and long-stay residents did not support a model of unstable microbiota and diversity [35]. The longitudinal analysis of the bacterial diversity of community composition showed that bacterial diversity and richness is variable between residents but did not reveal any evolution during the study. Moreover, inter-individual microbiota variability is known to be greater than temporal variability [32] and has been confirmed by community structure analysis.

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There are a growing number of publications on the gut microbiota exploration and CDI. Some of them focus on the idea that commensal bacterial populations can protect from CDI [36]. Although no candidate population has emerged, loss of some bacterial genera like *Bacteroides* has been associated with CDI [37]. Other studies on hospitalized CDI patients described a significant alteration of gut microbiota during CDI along with decreased biodiversity and richness [27,28,36,39]. This alteration includes a rise in *Proteobacteria* and a decrease in *Lachnospiraceae* and other butyrate-producing bacteria. However, it should be noted that these alterations do not appear to be specific to CDI, but are also observed in patients with non *C. difficile* diarrhoea [36]. In a first extensive study on elderly and CDI, Rea et al. [28] showed that there was little difference regarding the microbiota composition between CDI subjects and asymptomatic *C. difficile* carriers. Moreover, only minor bacterial taxon showed a statistically different abundance between *C. difficile* positive subjects and negative individuals.

The 16S rDNA profiling has been performed on a limited cohort of *C. difficile* negative and positive residents. Even if it was longitudinal, we did not focus on the pathology or on the antibiotic use that might have occurred during the survey. We centred this analysis on the hypothesis that in these long term stay residents, *C. difficile* persistent or recurrent colonisation might be associated with more pronounced differences in microbiota between both groups. Significant changes have been observed in *C. difficile* positive individuals in the relative abundance of bacterial populations, but these are limited to the *Lachnospiraceae* and *Verrucomicrobiaceae*. Surprisingly, *Lachnospiraceae* and specifically genus *Blautia* abundance is higher in *C. difficile* positive individuals, which is quite different from previous reports [37,38]. Even if CDI diagnosis was not specifically performed during the study, we further split both residents groups regarding the presence of diarrheic feces and observed that this bacterial family abundance is significantly higher in *C. difficile* positive residents having diarrheic feces compared to diarrheic *C. difficile* negative individuals. *Verrucomicrobiaceae* (genus *Akkermansia*) is known to be linked to gut health and its abundance seems to be reduced in context of gut inflammation [40]. Even if gut inflammatory status of the residents has not been investigated, it is a known risk factor for *C. difficile* colonization and could therefore be responsible for this negative correlation.

Positive *C. difficile* status is not associated with microbiota richness or biodiversity reduction in our study. It appears that impact on gut microbiota structure is associated with actual diarrheic episodes instead of *C. difficile* positive status [28]. Recent studies have demonstrated that stool consistency is a dominant factor associated with microbiota composition and species richness is negatively correlated with stool looseness [34,41].

The major limitation of this is the relatively low number of volunteers. Microbiota analysis has been marked by a strong inter-individual variability, which can certainly influence comparisons between *C.*

difficile negative and positive groups. It has been recently shown that several types of microbiota composition might increase susceptibility to CDI [37]. Further studies on long-term stay residents will be needed to improve our knowledge of the *C. difficile* reservoir and susceptibility in nursing homes.

Conclusion

This study aimed to investigate an elderly closed community, classically considered at risk of being colonised and developing CDI. In addition, we have studied whether the recruitment in a healthcare setting can have an impact on the evolution of the intestinal microbiota communities. *C. difficile* colonisation is higher in nursing homes than in hospitals, with a predominance of the hypervirulent PCR-ribotype 027. Variations in the abundance of taxa have been observed in the faecal microbiota of colonised residents. Positive *C. difficile* status is not associated with microbiota richness or biodiversity reduction in our study. Notably, a decrease of *Akkermansia* in positive subjects to the bacterium has repeatedly found. The link between *Akkermansia,* gut inflammation and *C. difficile* colonisation merits further investigations

Materials and Methods

Resident recruitment and sampling

The study was conducted at the Saint-Joséphine (ACIS) nursing home, in the province of Liège (Theux), Belgium. This local nursing home has a total capacity of 110 beds with a total of 73 employees. Written informed consent was obtained from all of the participants or their next of kin in case of cognitive impairment. Data collected included gender, age, clinical status, medical history, recent history of diarrhoea, recent hospitalization, medication, including nonsteroidal anti-inflammatory drugs (NSAIDs) or antibiotics, probiotics and changes in diet. The study was approved by the Hospital-Faculty Ethics Committee of the University of Liège (707).

During a 4-month period, from March through June 2013, stool samples from a group of 23 elderly care home residents were collected weekly. Most of the subjects were aged 65 years and older. Faecal sampling was performed from Thursday until early Friday. Two samples per person were collected. The first sample was collected in an individual identified sterile 50 ml tube for further culture to detect *C. difficile.* The second one was collected using the Stool DNA Stabilizer (PSP[R] Spin Stool DNA Plus Kit 00310; Invitek, Westburg b.v., Netherlands) to study the microbial biodiversity of the faeces content by amplicon sequencing. Samples obtained were scored as normal, diarrhoea or bloody diarrhoea faeces. They were kept at 4°C for a maximum of 48 hours until their arrival in the laboratory for immediate culture or DNA extraction.

***C. difficile* culture, identification and characterization**

Culturing of faeces (with and without a phase of enrichment), isolation and identification of *C. difficile* colonies were performed as previously described [42]. Toxic activity of the isolated strains was confirmed by a cytotoxicity assay using confluent monolayer MRC-5 cells as described previously [43].

Molecular typing of *C. difficile* isolates

C. difficile isolates were tested using Genotype Cdiff system (Hain Lifescience, Nehren, De) for the presence of the *tpi* gen, toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB*, deletions in the regulator gene *tcdC* and *gyrA* mutation, according to the manufacturer's instructions.

PCR-ribotyping was performed using the primers and conditions described by Bidet et al. [44]. An international number was used for *C. difficile* strains that presented a PCR-ribotype profile matching the Cardiff ribotypes from the strain collection available in our laboratory. Otherwise, strains were identified with an internal nomenclature.

MLVA

The DNA extraction was performed using a chelex 100 solution 5% (Biorad, Nazareth, Be) as described previously [45]. For MLVA, seven variable-number-tandem repeat (VNTR) loci (A6, B7, C6, E7, G8, CDR5, CDR60) were studied as previously described [46]. Isolates with MLVA STRD ≤ 2 were indicative of a high degree of genetic relatedness [47].

16S rDNA pyrosequencing and data analysis

Total bacterial DNA was extracted from the stool samples with the PSP® Spin Stool DNA Plus Kit 00310 (Invitek), following the manufacturer's recommendations. 16S rDNA profiling, targeting V1-V3 hypervariable region and sequenced on Roche GS Junior was performed as described previously [42]. Briefly, libraries from 20 samples were run in the same titanium pyrosequencing reaction using Roche multiplex identifiers, and amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche). A total of six sequencing runs were necessary to obtain the data for the 118 samples.

Sequence reads processing was treated as previously described [29] using respectively MOTHUR software package v1.35, Pyronoise algorithm and UCHIME algorithm for alignment and clustering, denoising and chimera detection [48-50]. 16S rRNA Reference alignment and taxonomical assignation in MOTHUR were based upon the SILVA database (v1.15) of full-length 16S rRNA sequences [51]. Clustering distance of 0.03 was used for OTU generation. Subsample datasets were obtained and used to evaluate ecological indicators, Richness estimation (Chao1 estimator), microbial biodiversity (reciprocal Simpson index), and the population evenness (derived from Simpson index) at the phylotype species level using MOTHUR. Population structure and community membership were

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assessed with MOTHUR using distance matrice based on Bray-Curtis dissimilarity index (a measure of community structure which considers shared OTUs and their relative abundances [52,53]. abundances).

Weighted UNIFRAC test implemented in MOTHUR v1.35 was used to assess differences regarding bacterial community structure between residents. Statistical differences in bacterial biodiversity, richness and evenness between residents and between *C. difficile* positive and *C. difficile* negative groups were respectively assessed using one way-ANOVA and Mann-Whitney test using PRISM 6 (Graphpad Software), and Bonferroni analysis. In order to highlight statistical differences in the bacterial population abundance between groups, multiple unpaired t-test with Benjamini-Hochberg False Discovery Rate were performed using PRISM 6 (Graphpad Software). Differences were considered significant for a p-value of less than 0.05.

All the biosample raw reads have been deposited at the National Center for Biotechnology Information (NCBI) and are available under the Bioproject PRJNA315622.

References

1. George RH, Symonds JM, Dimock F, Brown JD, Arabi Y, Shinagawa N, Keighley MR, Alexander-Williams J, Burdon DW. Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. Br Med J. 1978;1:695.

2. George WL, Sutter VL, Goldstein EJ, Ludwig SL, Finegold SM. Aetiology of antimicrobial-agent-associated colitis. Lancet 1978;1:802–803.

3. Larson HE, Price AB, Honour P, Borriello SP. *Clostridium difficile* and the aetiology of pseudomembranous colitis. Lancet 1978;1:1063–1066.

4. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, *et al*. Update of *C. difficile* infection due to PCR-ribotype 027 in Europe, 2008. Euro Surveill. 2008;13:31.

5. Barbut F, Jones G, Eckert C. Epidemiology and control of *C. difficile* infections in healtcare settings: an update. Curr Opin Infect Dis 2011;24:370-376.

6. Nanwa N, Kendzerska T, Krahn M, Kwong JC, Daneman N, Witteman W, *et al.* The Economic Impact of *Clostridium difficile* Infection: A Systematic Review. Am J Gastroenterol. 2015;110:511–519.

7. Jones AM, Kuijper EJ, Wilcox MH. *Clostridium difficile*: a European perspective. J Infect. 2013; 66:115–128.

8. Rodriguez C, Korsak N, Taminiau B, Avesani V, Van Broeck J, Delmée M, Daube G. *Clostridium difficile* infection in elderly nursing home residents. Anaerobe 2014;30:184-187.

9. Hensgens M, Dekkers OM, Goorhuis A, Lecessie S, Kuijper EJ. Predicting a complicated course of *Clostridium difficile* infection at the bedside. Clin Microbiol Infect. 2014;20:0301-0308.

10. Kelly CP. Can we identified patients at high risk of recurrent Clostridium difficile infection? Clin Microbiol Infect. 2012;18:21-27.

11. Theriot CM, Young VB. Microbial and metabolic interactions between the gastrointestinal track and *Clostridium difficile* infection. Gut Microbes 2014;5:86-95.

12. Rodriguez C, Taminiau B, Van Broeck J, Delmée M, Daube G. *Clostridium difficile* infection and intestinal microbiota interactions. Microb Pathog. 2015;89:201-209.

13. Arvand M, Moser V, Schwehn C, Bettge-Weller G, Hensgens MP, Kuijper EJ. High prevalence of *Clostridium difficile* colonisation among nursing home residents in Hesse, Germany. PlosOne 2012;7:e30183.

14. Mylotte JM, Russell S, Sackett B, Vllone M, Antelek M. Surveillance for *Clostridium difficile* infection in nursing homes. J Am Geriatr Soc. 2013;61:122-125.

15. Hopkins MJ, Sharp R, Macfarlane GT. Variation in human intestinal microbiota with age. Dig Liver Dis Off J Ital Soc Gastroenterol Ital Assoc Study Liver 2002;34 Suppl 2:S12-S18.

16. Marwick CA, Yu N, Lockhart MC, McGuigan CC, Wiuff C, Davey PG, et al. Community associated *Clostridium difficile* infection among older people in Tayside, Scotland, is associated with antibiotic exposure and care home residence: cohort study with nested case-control. J Antimicrob Chemother. 2013;68:2927-2933.

17. Ryan J, Murphy C, Twomey C, Ross RP, Rea MC, McSharry J, et al. Asymptomatic carriage of *Clostridium difficile* in an irish community care institution for the elderly: prevalence and characteristics. Ir J Med Sci. 2010;179:254-250.

18. Stuart RL, Kotsanas D, Webb B, Vandergraaf S, Gillespie EE, Hoog GG, et al. Prevalence of antimicrobial-resistance organisms in residential aged care facilities. Med J Aust. 2011;7:530-533.

19. Simor AE, Yake SL, Tsimidis K. Infection due to *Clostridium difficile* among elderly residents of a long-term-care facility. Clin Infect Dis. 1993;17:672-678.

20. Wesse JS. *Clostridium difficile* in food-innocent bystander or serious threat? Clin Microbiol Infect. 2010;16:3-10.

21. Neely F, Boudewijn C, Lambert ML. Epidemiology of *Clostridium difficile* infection in Belgium. Report 2014. ISP-WIW 2014.

http://www.nsih.be/download/CDIF/CDIF-AR-2014-EN.pdf. Accessed 31 Mars 2016.

22. Neely F, Lambert ML. Epidemiology of *Clostridium difficile* infection in Belgium. Report 2015. ISP-WIW 2015. http://www.nsih.be/download/CDIF/CDIF-AR-2015-EN.pdf. Accessed 31 Mars 2016.

23. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, et al. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol. 2010;31:431-455.

24. Eyre DW, Golubchik T, Gordon NC, Bowden R, Piazza P, Batty EM, et al. A pilot study of rapid benchtop sequencing of *Staphylococcus aureus* and *Clostridium difficile* for outbreak detection and surveillance. BMJ Open 2:pii:e001124.

25. Rodriguez-Palacios A, Borgamann S, Kline TR, LeJeune JT. *Clostridium difficile* in foods and animals: history and measures to reduce exposure. Anim Health Res Rev. 2013;14:11-29.

26. Rodriguez C, Korsak N, Taminiau B, Avesani V, Van Broeck J, Brach P, et al. *Clostridium difficile* from food and surface samples in a Belgian nursing home: an unlike source of contamination. Anaerobe 2015;32:87-89.

27. Zhang L, Dong D, Jiang C, Li Z, Wang X, Peng Y. Insight into alteration of gut microbiota in *Clostridium difficile* infection and asymptomatic *C. difficile* colonization. Anaerobe 2015;34:1-7.

28. Rea MC, O'Sullivan O, Shanahan F, O'Toole PW, Stanton C, Ross RP, et al. *Clostridium difficile* carriage in elderly subjects and associated changes in the intestinal microbiota. J. Clin. Microbiol. 2012;50:867-875.

29. Rodriguez C, Taminiau B, Brévers B, Avesani V, Van Broeck J, Leroux A, et al. Faecal microbiota characterisation of horses using rDNA barcoded pyrosequencing, and carriage rate of *Clostridium difficile* at hospital

admission. BMC Microbiol. 2015;15:181.

30. Conlan S, Kong HH, Segre JA. Species-Level Analysis of DNA Sequence Data from the NIH Human Microbiome Project. Plos One 2012;7:e47075.

31. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc. Natl. Acad. Sci. USA 2011;108: Suppl 1:4586–4591.

32. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature 2012;488:178–184.

33. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, Doré J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. 2009;9:123.

34. Finacane MM, Sharpton TJ, Laurent TJ, Pollard KS. A taxonomic signature of obesity in the microbiome? Getting the guts of the matter. Plos One 2014;9:e84684.

35. Sepp E, Loivukene K, Julge K, Voor T, Mikelsaar M. The association of gut microbiota with body weight and body mass index in preschool children of Estonia. Microb. Ecol. Health Dis. 2013;24.

36. O'Toole PW, Jeffery IB. Gut microbiota and aging. Science 2015;350:1214–1215.

37. Antharam VC, Li EC, Ishmael A, Sharma A, Mai V, Rand KH, et al. (2013) Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhoea. J. Clin. Microbiol. 2013;51:2884–2892.

38. Schubert AM, Rogers MAM, Ring C, Mogle J, Petrosino JP, Young VB, et al. Microbiome Data Distinguish Patients with *Clostridium difficile* Infection and Non-*C. difficile*-Associated Diarrhoea from Healthy Controls. MBio 2014;5:e01021–14–e01021–14.

39. Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, et al. Decreased Diversity of the Fecal Microbiome in Recurrent *Clostridium difficile*–Associated Diarrhoea. J Infect. Dis. 2008;197:435–438.

40. Derrien M, Belzer C, de Vos WM. *Akkermansia muciniphila* and its role in regulating host functions. Microb. Pathog. 2016;9:1–11.

41. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, et al. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut 2015, 65: 57–62.

42. Rodriguez C, Taminiau B, Brévers B, Avesani V, Van Broeck J, Leroux AA, et al. Carriage and acquisition rates of *Clostridium difficile* in hospitalized horses, including molecular characterization, multilocus sequence typing and antimicrobial susceptibility of bacterial isolates. Vet. Microbiol. 2014;172:309-317.

43. Rodriguez C, Taminiau B, Van Broeck J, Avesani V, Delmée M, Daube G. 2014. *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 2014;18: 621-625.

44. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC. Development of a new PCR-ribotyping method based on ribosomal RNA gene sequencing. FEMS Microbiol. Lett. 1999;175: 261-266.

45. O'Neill GL, Ogunsola FT, Brazier JS, Duerden BI. Modification of a PCR ribotyping method for application as a routine typing scheme for *Clostridium difficile*. Anaerobe 1996;2:205-209.

46. Manzoor SE, Tanner HE, Marriott CL, Brazier JS, Hardy KJ, Platt S, Hawkey PM. Extended multilocus variable-number tandem-repeat analysis of *Clostridium difficile* correlates exactly with ribotyping and enables identification of hospital transmission. J. Clin. Microbiol. 2011;49**:**3523-3530.

47. Marsh JV, O'Leary MM, Shutt KA, Pasculle AW, Johnson S, Gerding DN, Muto CA, Harrison LH. Multilocus variable-number tandem-repeat analysis for investigation of *C. difficile* transmission in hospitals. J. Clin. Microbiol. 2006;44:2558-2566.

48. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 2009;75:7537–7541.

49. Quince C, Lanze´n A, Curtis TP, Davenport RJ, Hall N, Head IM, et al. Accurate determination of microbial diversity from 454 pyrosequencing data. Nat. Methods 2009;6: 639–641.

50. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 2011;27:2194–2200.

51. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 2007;35:7188–7196.

52. Bray, J. R. and J. T. Curtis. An ordination of upland forest communities of southern Wisconsin. Ecological Monographs. 1957; 27:325-349.

53. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 1988;26:2465-2466.

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Additional file 1. Quality analysis of the 16S rRNA gene analysis for the 118 human faecal samples analysed.

Item	Total number (or %)	Mean read length, nucleotide
Raw reads	590,067	512
Postdenoising/Postchimeric	433,815	454
Loss in Treatment (%)	26	
OTU 0.03[a]	10,458	
Phylotype species	3,940	
Phylotype genus	208	

[a] OTU clustering distance

Genus
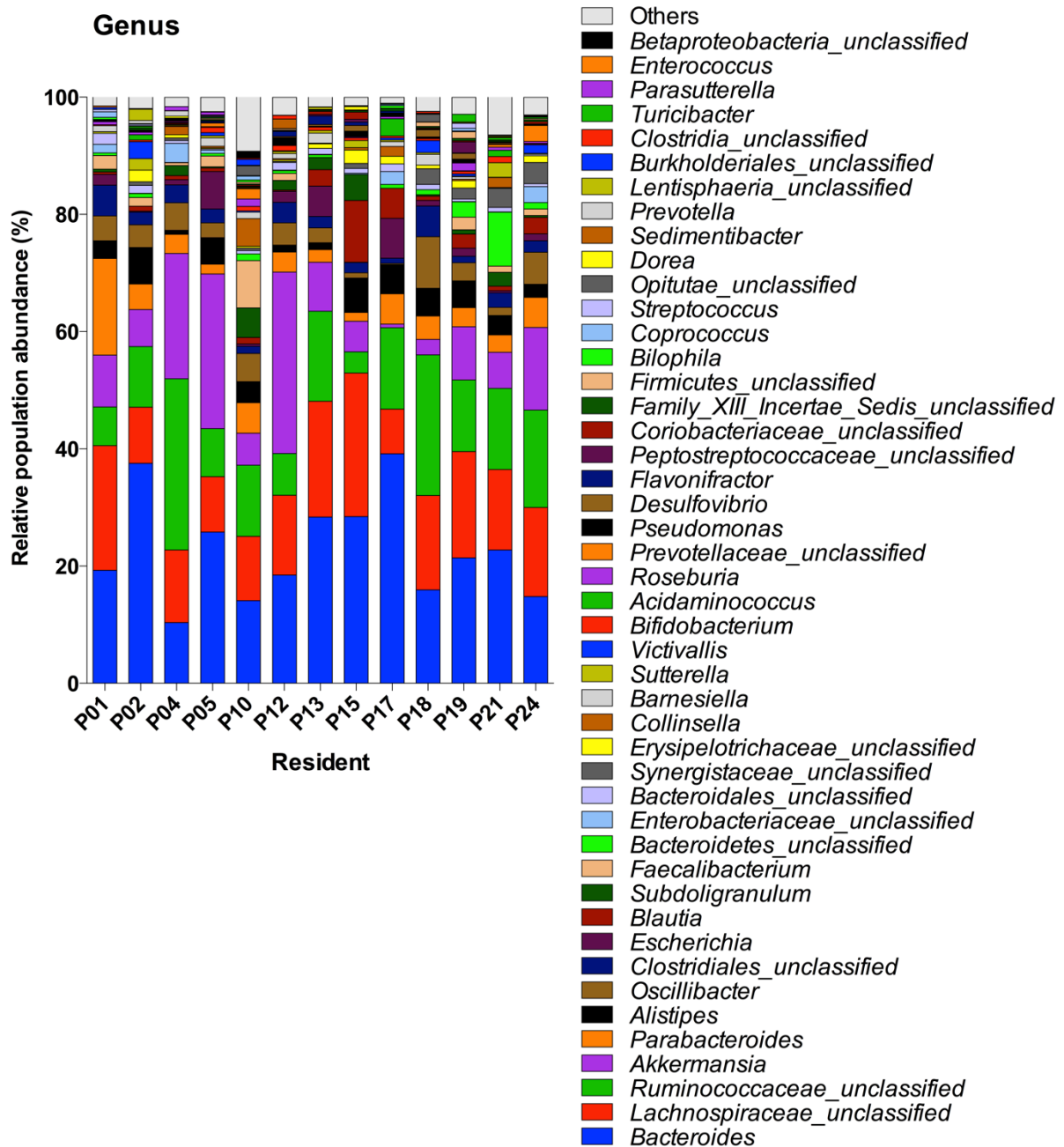
Additional file 2. Taxonomical distribution deduced by 16S rDNA profiling. Bart chart detailing the mean cumulated relative abundance of the major genera for each resident

Bart chart detailing the mean cumulated relative abundance of the major genera for each resident.
Additional file 3

W Score

	P01	P02	P04	P05	P10	P12	P13	P15	P17	P18	P19	P21
P01												
P02	1											
P04	1	1										
P05	0.989227	0.989227	0.994597									
P10	1	1	1	0.975752								
P12	1	1	1	0.994597	1							
P13	1	1	1	0.811885	1	1						
P15	1	1	1	0.994597	1	1	1					
P17	1	1	1	0.989227	1	1	1	1				
P18	1	1	1	0.989227	1	1	1	1	1			
P19	0.978402	0.978402	0.91147	0.987315	0.976086	0.91147	0.997421	0.898462	0.978402	0.978402		
P21	1	1	1	0.994597	1	1	1	1	1	1	0.893297	
P24	0.953487	0.969149	0.996232	0.989227	0.980789	0.996232	1	0.996232	0.969149	0.905936	0.937105	0.93807

W significance

	P01	P02	P04	P05	P10	P12	P13	P15	P17	P18	P19	P21
P01												
P02	<0.001											
P04	<0.001	<0.001										
P05	<0.001	<0.001	<0.001									
P10	<0.001	<0.001	<0.001	<0.001								
P12	<0.001	<0.001	0.011	<0.001	<0.001							
P13	<0.001	<0.001	0.076	0.075	<0.001	0.040						
P15	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001					
P17	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
P18	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001			
P19	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
P21	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
P24	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Additional file 3. Unifrac weighted score and significance between patients

UNIFRAC weighted score (W score) and significance for patients clustering based on Bray-Curtis dissimilarity distance matrix.

Bacterial diversity

Bacterial Richness
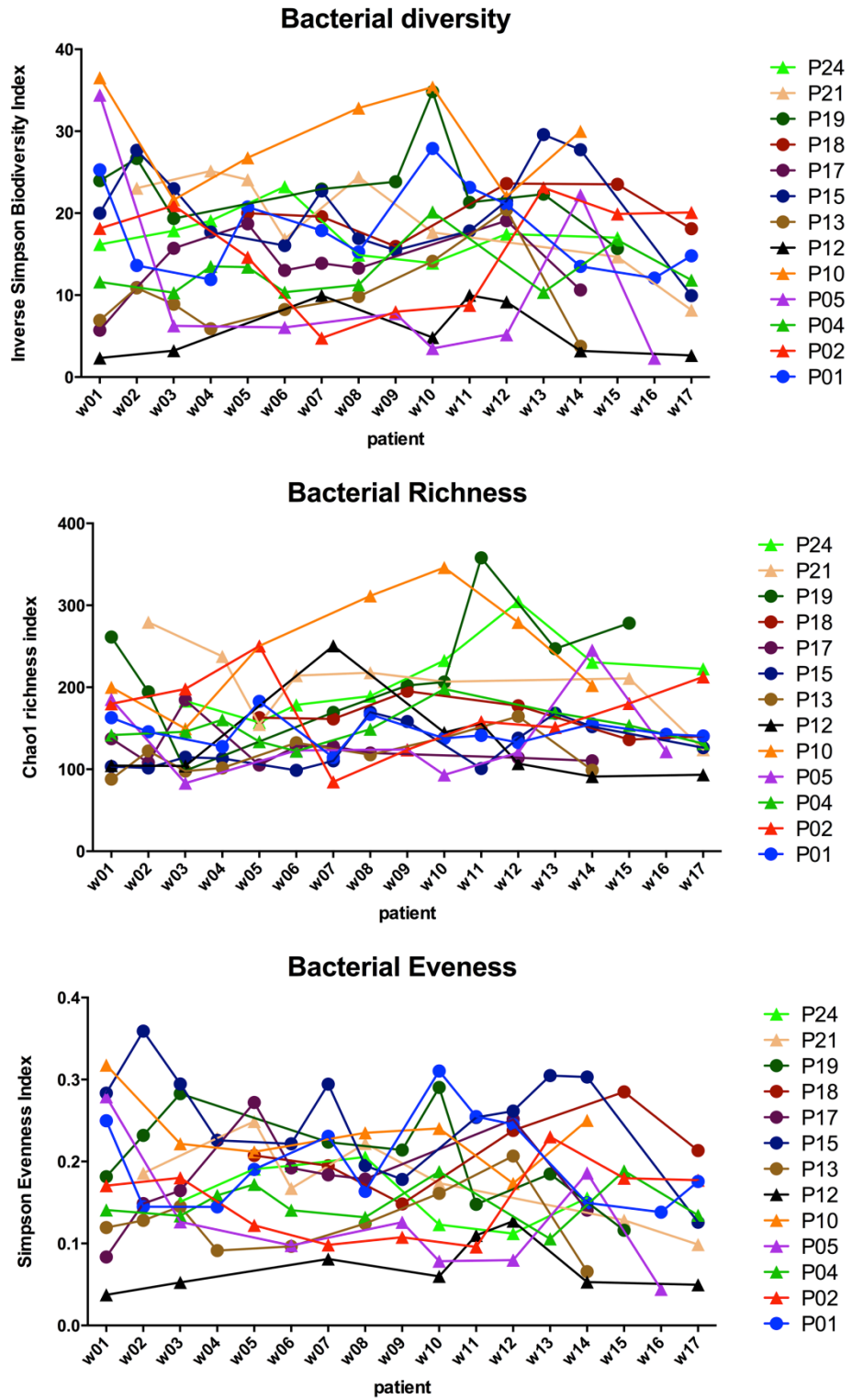
Bacterial Eveness

Additional file 4. Longitudinal distribution of the ecological indicators

Bacterial diversity (inverse Simpson biodiversity index), bacterial richness (Chao1 richness index) and bacterial evenness (Deduced from Simpson index) expressed for each analysed samples.

Lachnospiraceae relative abundance
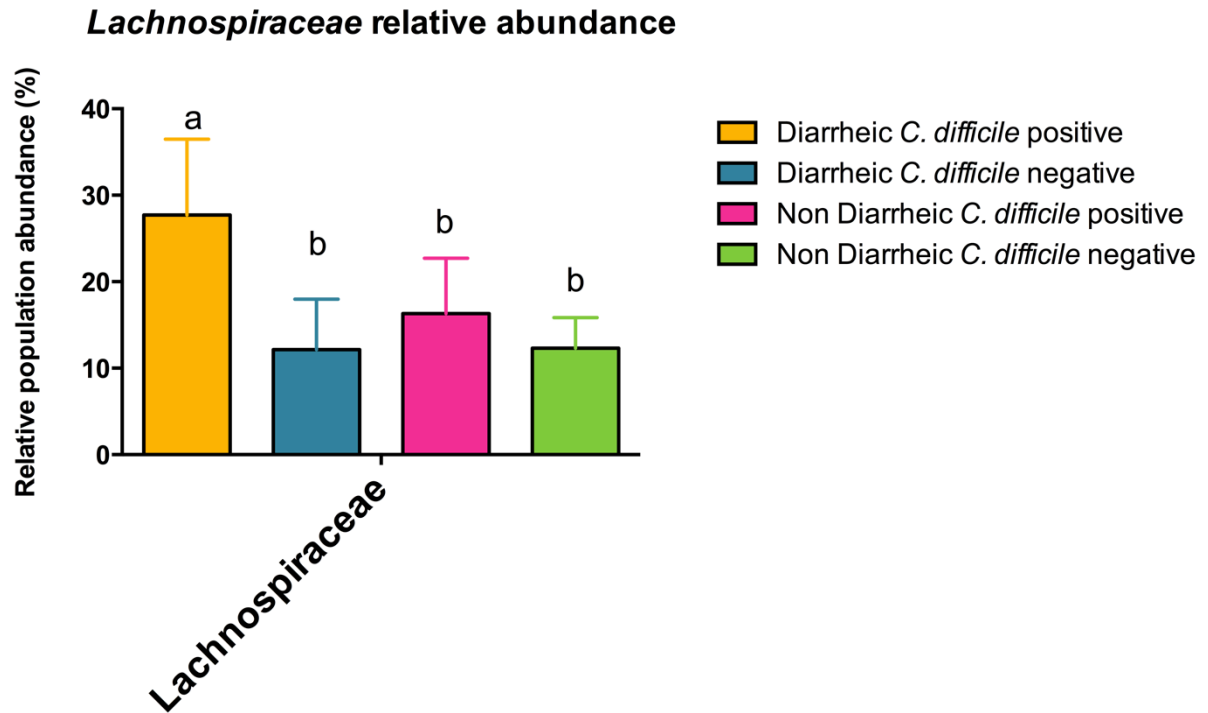
Additional file 5. Relative abundance of Lachnospiraceae between groups of diarrheic/non diarrheic and *C. difficile* status.

Lachnospiraceae relative abundance is expressed as mean relative abundance ± standard error of the mean. Different superscript letters correspond to statistical difference according to one way ANOVA with Tukey-Kramer post-hoc test ($p < 0.05$).

3.5 *C. difficile* in hospitalised patients with diarrhea

When this study first began in 2010, there was a global increase in incidence, severity and mortality associated with CDI. This increase was attributed with the spread of the epidemic strain *C. difficile* PCR-ribotype 027. However, from 2014 in North America and in some European countries, it was reported that PCR-ribotypes 078 and 014 were becoming more prominent and associated with a higher rate of complications compared to other ribotypes. Furthermore, in Belgium the last epidemiological report has revealed a decreasing prevalence of PCR-ribotype 027, with a large variety of circulating ribotypes. Other European countries include slightly different definitions of CDI cases and different typing protocol within their surveillance and therefore, comparisons between countries are not always consistent. Some preliminary international studies have revealed that Belgium has incidence rates of CDI lower than reported in the United States but in the mid-range of other European countries.

To complete this study the final goal was to survey the *C. difficile* circulation in an European hospital (located in Spain) and to compare the ribotype distribution with that observed in a second hospital located in Belgium, during the same study period. Data obtained shows that even if the total number of samples analysed per month in the Belgian hospital was triple the number of samples analysed in the Spanish hospital, the prevalence is lower in the Belgian hospital (9.3% versus 12.3%). These results may reflects the efforts of the Belgian hospital to improve the management of CDI, and a probable misdiagnosis of CDI in Spain due to the lack of clinical suspicion and request. The most common PCR-ribotypes reported in Europe were found in the two hospitals, including 078 and 014. The great variety of PCR-ribotypes detected underlined the absence of regional or hospital spread of one specific clone. Furthermore, the strain diversity may indicate that there are multiple pathways of transmission. In addition, it was found that the same PCR-ribotypes commonly detected in animals and foods were the predominant types circulating in the Belgian and in the Spanish hospital (PCR-ribotypes 078, 014, 020, 002). Once again, these results evidence the potential role of animals and foods as reservoirs of CDI in the community.

Clostridium difficile presence in Spanish and Belgian hospitals

in preparation for submission

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Abstract

Clostridium difficile is recognised worldwide as the main cause of infectious bacterial antibiotic-associated diarrhoea in hospitals and other healthcare settings. The aim of this study was to first survey *C. difficile* prevalence during the summer of 2014 at the Central University Hospital of Asturias (Spain). By typing the isolates obtained, it was then possible to compare the ribotype distribution at the Spanish hospital with results from the St Luc University Hospital in Belgium over the same period. The prevalence of positive cases reported in Spain and Belgium was 12.3% and 9.3% respectively. The main PCR-ribotypes previously described in Europe were found in both hospitals, including 078, 014, 012, 020 and 002. In the Spanish hospital, most of the *C. difficile*-positive samples were referred from oncology, acute care and general medicine services. In the Belgian hospital the majority of positive samples were referred from the paediatric service. However, a high percentage of isolates from this service were non-toxigenic. This study finds that the presence and detection of *C. difficile* in paediatric and oncology services requires further investigation.

Keywords

Clostridium difficile; infection; hospitals; PCR-ribotype distribution

Introduction

Clostridium difficile is currently one of the most largely studied pathogenic bacteria in the world and is considered the major cause of nosocomial antibiotic-associated diarrhoea and colitis in industrialised countries[1]. Clinical manifestations of *C. difficile* infection (CDI) range from mild or moderate diarrhoea to fulminant and sometimes fatal pseudomembranous colitis[2]. Normally, the diarrhoea has been described to appear 48-72 h post infection and is characterised as non-haemorrhagic and watery, accompanied with abdominal pain, fever and leucocytosis[3]. However, the worst outcome is sepsis and death, which is observed in 17% of CDI cases[4]. The highest incidence and mortality rate is usually reported among patients of advanced age who have had a stay in a healthcare setting[5].

A recent review of CDI cost-of-illness attributes a mean cost ranging from $8,911 to $30,049 per hospitalized patient in the USA[6] and around €3,000 million total per annum in Europe[7]. In addition, in many hospitals the diagnosis strategy remains suboptimal and a proportion of infections may remain undiagnosed[8]. In the past decade, an increase in the incidence and severity of the infection has been reported in various healthcare settings among many countries[9]. This situation was attributed to the emergence of a new epidemic and hypervirulent *C. difficile* strain, identified as PCR-ribotype 027 (North American pulsed field type 1)[10]. Since 2003, in the United States and Canada, studies have shown an increase in the number and severity of CDI cases, including an increase in the case fatality,

mortality and colectomy rates. The change in the epidemiology of *C. difficile* was also attributed to the spread of the NAP1/027 strain[11]. The situation presented by studies in North America is mirrored in Europe. In 2008, the PCR-ribotype 027 was detected in 16 European countries and caused outbreaks in Belgium, Germany, Finland, France, Ireland, The Netherlands, Switzerland and the United Kingdom[11,12]. However, in a further epidemiology study conducted in Europe, the most prevalent PCR-ribotypes were identified as 014/020 (15%), 001 (10%) and 078 (8%), while PCR-ribotype 027 was less prevalent (5%)[11]. Surveillance data for Belgium from 2008 to 2010 showed a stable incidence of CDI in Belgian hospitals, and even a decrease in 2010. In addition, PCR-ribotype 027 was the most prevalent type during the years 2007-2009[13]. A further study reporting CDI ribotype distribution in Belgian hospitals between 2008 and 2010 described a decrease in cases caused by PCR-ribotype 027 (from 55% in 2008 to 28% in 2010). In contrast, the proportion of other PCR-ribotypes involved in CDI increased, such as ribotype 014 (from 20% in 2008 to 33% in 2010) and ribotype 078 (from 11% in 2009 to 23% in 2010)[14]. Meanwhile, a prospective study conducted in 2009 in the region of Barcelona (Spain) identified the main PCR-ribotypes associated with CDI as 241 (26%), 126 (18%), 078 (7%) and 020 (5%), while PCR-ribotype 027 was not detected[15]. In a later study conducted in the region of Madrid (Spain) from January to June 2013, most of the isolates associated with a CDI case possessed binary toxin and were classified as PCR-ribotype 078/126 (90.7%)[16]. Consistent with these reports, Weber et al.[17] studied *C. difficile* clinical isolates recovered at the reference hospital of the Balearic Islands (Spain) between August 2007 and April 2011. The authors detected a total 43 different PCR-ribotypes with a higher prevalence of types 014 (34%), 078 (13%) and 001 (5%). As in other Spanish studies, none of the isolates were identified as PCR-ribotype 027.

The aim of this study was to survey the *C. difficile* circulation during the summer of 2014 at the Central University Hospital of Asturias (Spain), a provincial hospital located in the North of Spain. By typing of all the isolates obtained, we were able to compare the ribotype distribution with that observed in the St Luc University Hospital (Belgium) during the same period.

Methods

Hospital selection, data and sampling

The Central University Hospital of Asturias (HUCA) located in Oviedo (Asturias, Spain), is the referral hospital of the Health Service of the Principality of Asturias. Overall, the hospital has 17 buildings with a total of 1,324 beds, 29 operating rooms, 203 consultation rooms (for outpatients) and 123 emergency rooms.

During the 4-month period from July to October 2014, all samples from outpatients and hospitalised patients suspected of being infected with *C. difficile* were tested. Stool consistency of samples was evaluated using the Bristol Stool Chart (BSC). Samples were documented for data relating to clinical

history, diagnosis and treatment received, including the prescription of antimicrobial agents. Numerical identification was used for all samples to guarantee patient anonymity.

C. difficile rapid detection

Initial screening for *C. difficile* presence was performed using a rapid membrane enzyme immunoassay for the simultaneous detection of *C. difficile* glutamate dehydrogenase antigen and toxins A and B (Cdiff QuickChek Complete® TechLab, Blaclsburg, USA). In the case of doubtful results or glutamate dehydrogenase antigen testing positive and toxins A and B testing negative, GenomEra CDX System *C. difficile* (Abacus Diagnostica, Turku, Finland) was performed for rapid identification of toxin B. These tests were applied only in semisolid, mushy stools and watery/entirely liquid faeces (Bristol stool chart levels 4 to 7) while samples outside this range were discarded. This analysis constituted the routine protocol followed in the hospital laboratory for the diagnosis of CDI. If various stool samples were received from the same patient, a second analysis was only performed if the first *C. difficile* screening was made at least one month prior.

Culture, identification and characterisation

All specimens received in the laboratory for *C. difficile* testing were cultured regardless of their classification in the Bristol stool chart. Culture was carried out as described previously[18]. Briefly, approximately 0.1 g of faeces was spread directly on cycloserine cefoxitin fructose agar taurocholate medium (CCFAT), freshly prepared in the laboratory. Plates were incubated anaerobically for 72 h at 37 °C. The anaerobic atmosphere in the jar was created using AnaeroGen™ sachet (Oxoid, Dardilly, FR) and checked using an anaerobic indicator BR0055B (Oxoid). An enrichment step was also performed. One gram of faeces was inoculated into 9 ml of CCFT (cycloserine cefoxitin fructose taurocholate) broth and incubated anaerobically for 72 h at 37 °C. A 10 µl aliquot of the enriched broth was spread on CCFT plates and incubated anaerobically at 37 °C for three days. One presumptive colony per plate was subcultured onto blood agar 5 % Sheep Blood (Biorad, Temse, BE) and checked using a *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid). Detection of a species-specific internal fragment of the *tpi* gene, toxin A and B genes, and CDT (*cdtA*) was performed according to the multiplex PCR protocol[18]. Further toxin profile characterisation, deletions in the regulator gene *tcdC,* and *gyrA* mutation (a gene associated with moxifloxacin resistance) were determined using the Genotype Cdiff system (Hain Lifescience, Nehren, DE) according to the manufacturer's instructions. The supernatant from each pure culture was tested for cytotoxicity assay (TcdB) using confluent monolayer MRC-5 cells, as previously described[18].

All strains were ribotyped as described by Bidet et al[19]. Amplicon sizes were analysed by capillary electrophoresis and profiles obtained were compared with those of reference strains from the European

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collection (Cardiff International number, Brazier classification) and with our own database (nomenclature beginning with UCL).

Antibiotic resistance

Susceptibility of the isolates to metronidazole, moxifloxacin and tetracycline was determined by Etest strips (Lucron ELITech Group, Zottegem, BE) on Brucella Blood Agar with hemin and vitamin K1 (Becton-Dickinson Benelux NV, Erembodegem, BE) according to the manufacturer's instructions. Plates were anaerobically incubated at 37° C for 48 h. The resistance (r) breakpoints for metronidazole (Met r ≥ 32 µg/ml), moxifloxacin (Mox r ≥ 8 µg/ml) and tetracycline (Tet r ≥ 8 µg/ml) were those recommended by the Clinical and Laboratory Standard Institute (CLSI)[20]. *Bacteroides fragilis* ATCL was included as a quality control.

Surveillance data in Belgium

During the same study period (from July to October 2014) analysis of *C. difficile* ribotype distribution was made at the St Luc University Hospital (Brussels, Belgium) in order to compare PCR-ribotypes with those obtained in Spain. The Belgian hospital is an academic acute care hospital with a total of 1,000 beds. All stools received in the laboratory were tested for the presence of *C. difficile*. Unlike the Spanish hospital, multiple stool samples from the same patient were all tested, without regard for the date of the first analysis. Initial screening was made using Cdiff QuickChek Complete® (TechLab). Culture of positive samples was performed on CHROMagar *C. difficile* Colorex™ (CHROMagar, BioTrading, Keerbergen, BE) in order to isolate the strain (without enrichment, planting faeces directly on agar). Plates were incubated anaerobically for 24 h at 35 °C. All cultures were read with a binocular stereomicroscope, with the light beam through the Petridish under a certain angle. Strains were ribotyped as described above. The toxin gene profile of the strains and PCR-ribotype distribution in the Belgian hospital were then compared with those found in the Spanish ward.

Results

C. difficile detection and strain characterisation in HUCA, Spain

During the four-month study period, a total of 249 samples were screened for *C. difficile* presence using both the rapid enzyme test and culture analysis. Twelve additional samples were only examined by culture because they were classified outside of the range established (between 4 and 7) on the Bristol stool chart. The overall prevalence of *C. difficile* in the faecal microbiota of patients studied was 12.3% (32/261). Of these, 69% were from adults aged more than 65 years old. Only following clinical suspicion, and a positive result for toxins A and/or B by rapid-test detection (Cdiff QuickChek

Complete® or GenomEra CDX System *C. difficile*), was the patient was considered to suffer from infection. With this approach, 22 patients were diagnosed with CDI.

Altogether, 7 of the 32 *C. difficile*-positive samples detected (22%) were referred from the oncology unit. However, the medical services which sent the most samples for the screening of *C. difficile* during the study period were the acute care unit (28/261; 10.7%) and general medicine service (37/261; 14.2%) (Table 1). From these two services, *C. difficile* was isolated from six and five patients respectively. Regarding the type of faeces, six patients (2.3%) suspected of CDI presented bloody stools but all tested negative for the bacterium. Most of the positive patients had mushy, watery or liquid stools (n=24). However, two patients with formed stools were also colonised with toxigenic *C. difficile* strains (Table 1).

Using rapid detection, 22 isolates tested positive for *tcdB* gene while 6 isolates were found to be non-toxigenic. Characterisation of colonies obtained after culture of samples showed 27 toxigenic isolates (presence of toxins A and B). Of these 27 toxigenic isolates, 6 had binary toxin genes. None of the isolates presented a single base deletion at position 117 in the regulator *tcdC* gene. Two isolates showed an 18 bp deletion and eight presented a 39 bp deletion in the regulator *tcdC* gene. Twenty different PCR-ribotypes were detected. Only nine isolates had a ribotype profile associated with a Cardiff collection reference number (002 (n=3), 078 (n=2), 012, 070, 023 and 020). The remaining isolates were associated with an internal nomenclature (UCL), with a total of 14 different PCR-ribotypes identified. The only non-toxigenic PCR-ribotype was associated with the ribotype UCL9. In addition, this ribotype was the only that presented three types of deletions in the regulator *tcdC* gene (117 bp, 39 bp and 18 bp deletions) (Table 2). The same results (the presence of *C. difficile* in the sample with the same PCR-ribotypes) were obtained with and without enrichment of faeces. None of the patients were identified as carriers of more than one PCR-ribotype.

Table 1. Clinical data comparison between *C. difficile*-colonised and non-colonised patients

	C. difficile-negative patients (%)	*C. difficile*-positive patients (%)
Total (%)	229 of 261 (87.7)	32 of 261 (12.3)
Mean age in years	60.5	63,6
Sorted by age		
>65 years	137 (60)	22 (68.8)
20-<65 years	76 (33.2)	7 (22)
>10-20 years	7 (3.1)	3 (9.4)
>3, ≤10 years	4 (1.7)	0 (0)
≤3 years	5 (2.2)	0 (0)
Sorted by gender		
Male	125 (54.6)	22 (68.8)
Female	104 (45.4)	10 (31.3)
Sorted by service		
Oncology	4 (1.7)	7 (22)
Acute Care Unit	22 (9.6)	6 (18.8)
General Medicine	32 (14)	5 (15.6)
Nephrology	14 (6.1)	2 (6.3)
Digestive	20 (8.7)	2 (6.3)
Haematology	5 (2.2)	2 (6.3)
General Emergencies	11 (4.8)	2 (6.3)
Paediatric Emergencies	6 (2.6)	1 (3.1)
Surgery	1 (0.4)	1 (3.1)
Urology	2 (0.9)	1 (3.1)
Other	112 (48.9)	3 (9.4)
Sorted by type of sample		
Bloody stools	6 (2.3)	0 (0)
Mushy, watery or entire liquid stools (Bristol Stool Chart 6-7)	163 (62.5)	24 (75)
Smooth and soft stools (Bristol Stool Chart 4-5)	82 (31.4)	6 (18.8)
Formed stools (Bristol Stool Chart 1-3)	10 (3.8)	2 (6.3)

None of the isolates showed resistance to metronidazole. For tetracycline, eight isolates were fully resistant: PCR-ribotype 078 (two isolates), PCR-ribotype UCL16b (one isolate), PCR-ribotype UCL5a (four isolates) and PCR-ribotype 36a (one isolate). Resistance for moxifloxacin was detected in all isolates of PCR-ribotypes 078, UCL5a and UCL9. All of these isolates (PCR-ribotype 078, UCL9 and UCL5) presented a mutation in *gyrA* gen (Table 2).

Table 2. Detailed information on *C. difficile* isolates at the HUCA hospital (Spain), including molecular characterisation and antibiotic resistance of the isolates

Isolate Number	Rapid detection GDH	Rapid detection Toxin B[c]	Rapid detection Toxin B[d]	Culture detection	PCR-ribotype	CE	tcdA tcdB	cdtA cdtB	tcdC[a] MUT117	tcdC[a] 39bp	tcdC[a] 18bp	gyrA[b] MUT	Metronidazole	Moxifloxacin	Tetracycline
10404	+	-	+	+	UCL23b	+	+	-	-	-	-	-	-	-	-
10405	+	+	+	+	002	+	+	-	-	-	-	-	-	-	-
10406	-	-	NT	+	078	+	+	+	-	+	-	Mut1A	-	+	+
10407	+	+	-	+	UCL16b	+	+	-	-	-	-	-	-	-	+
10408	+	+	+	+	UCL16*	+	+	-	-	-	-	-	-	-	-
10409	+	-	-	+	UCL9	-	-	-	+	+	+	Mut1A	-	+*	-
10410	+	-	+	+	012	+	+	-	-	-	-	-	-	-	-
10411	+	+	+	+	078	+	+	-	-	+	-	Mut1A	-	+	+
10412	+	-	-	+	UCL16*	+	+	-	-	-	-	-	-	-	-
10413	+	+	+	+	UCL160	+	+	-	-	-	-	-	-	-	-
10414	+	+	+	+	UCL55a	+	+	-	-	-	-	-	-	-	-
10415	+	+	+	+	070	+	+	-	-	-	-	-	-	-	-
10419	+	+	+	+	014	+	+	-	-	-	-	-	-	-	-
10420	+	+	+	+	002	+	+	-	-	-	-	-	-	-	-
10421	+	-	+	+	UCL489	+	+	-	-	-	-	-	-	-	-
10422	+	+	+	+	UCL5a	+	+	+	-	+	-	Mut1A	-	+	+
10423	+	+	+	+	002	+	+	-	-	+	-	-	-	-	-
10454	+	+	+	+	023	+	+	+	-	+	+	-	-	-	-
10425	+	-	-	+	UCL5a	+	+	+	-	+	-	Mut1A	-	+	+
10426	+	-	+	+	UCL36a	+	+	-	-	-	-	-	-	-	-
10427	-	-	NT	+	UCL499	+	+	-	-	-	-	-	-	-	-
10428	+	+	+	+	UCL16i	+	+	-	-	-	-	-	-	-	-
10429	+	+	+	+	UCL5a	+	+	+	-	+	-	Mut1A	-	+	+
10430	+	+	+	+	UCL108	+	+	-	-	-	-	-	-	-	-
10431	-	-	NT	+	UCL5a	+	+	+	-	+	-	Mut1A	-	+	+
10432	+	-	+	+	020	+	+	-	-	-	-	-	-	-	-
10433	+	+	+	+	UCL483	-	-	-	-	-	-	-	-	-	-
10155	-	-	NT	+	UCL283	+	+	-	-	-	-	-	-	-	-
10559	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
10497	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10584	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10287	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Presence of deletions in the regulator gene *tcdC* (118bp-39bp-17bp)
[b] Presence of mutation in the *gyrA* gene associated with moxifloxacin resistance
[c] Cdiff QuickCheck Complete TechLab
[d] GenomeEra CDX System C. difficile
MUT: mutation; CE: cytotoxicity assay using confluent monolayer MRC-5 cells; NT: not tested

C. difficile detection and strain characterisation in St Luc University Hospital, Belgium

Between July 2014 and October 2014 a total of 880 stool specimens were analysed from patients of the St Luc University Hospital suspected of having CDI. The national prevalence for *C. difficile* reported from the Belgian Reference Centre was 9.3%. A total of 127 *C. difficile*-positive samples were obtained from 87 patients. Seventeen of these positive patients (19.5%) were referred from the paediatric service (including eight from the paediatric haematology unit and four from the intensive neonatology unit). The other medical services with significant numbers of *C. difficile*-positive patients were general internal medicine (n=9; 10.3%), consultation (n=8; 9.2%), pneumatology-gastroenterology (n=8; 9.2%), nephrology-neurology (n=7; 8%), surgery (n=5; 5.7%) and subacute geriatrics (n=5; 5.7%). The oncology service referred three positive patients (3.4%). Twenty-one patients were *C. difficile*-positive in more than one sampling. The mean age of positive patients was 45 years old. However, 19 positive patients were children less than 10 years old, with a mean age of 1 year and 6 months in this group, and 9 of these patients were less than 1 year old. If the paediatric group is analysed separately, the mean age of positive adult patients was 60 years old (Table 3).

Eighty-three isolates (65%) were positive for toxigenic culture and toxins A and B. Forty-four isolates were identified as non-toxigenic. Overall, 37 different PCR-ribotypes were detected. Eight of these had ribotype profiles associated with the Cardiff collection under reference numbers 015 (n=1), 078 (n=14), 106 (n=8), 014 (n=5), 020 (n=9), 056 (n=13), 012 (n=6) and 002 (n=4). The remaining isolates were associated with an internal nomenclature (UCL), with a total of 29 different PCR-ribotypes identified, including all the non-toxigenic isolates (Table 3).

Table 3. Detailed information on *C. difficile* positive patients at the St Luc University hospital (Belgium), including molecular characterisation of the isolates

Patient Number	Age	Genre	Medical service	*C. difficile* Isolation date	PCR-ribotype	CE	*tcdA tcdB*
01	4 years	Male	Paediatric haematology	01/07/2014	UCL36	-	-
				04/07/2014	UCL36	-	-
				08/07/2014	UCL36	-	-
				17/07/2014	UCL36	-	-
06	28 years	Male	Consultation	09/07/2014	UCL36	-	-
11	62 years	Male	Medical surgical intensive care	25/08/2014	UCL36	-	-
15	1 year	Male	Paediatric haematology	10/10/2014	UCL36	-	-
56	51 years	Female	Gastroenterology	25/08/2014	UCL36	-	-
59	81 years	Female	General internal medicine	02/10/2014	UCL36	-	-
				09/10/2014	UCL36	-	-
61	88 years	Female	Not specified	15/10/2014	UCL36	-	-
63	48 years	Female	Consultation	17/10/2014	UCL36	-	-
38	6 years	Male	Paediatric haematology	12/09/2014	UCL36a	+	+
82	35 years	Female	Urgency	01/10/2014	UCL36a	+	+
02	4 years	Male	Paediatric haematology	01/07/2014	UCL9	-	-
03	62 years	Male	Cardiovascular surgery	07/07/2014	UCL9	-	-
04	64 years	Male	Nephrology neurology	07/07/2014	UCL9	-	-
07	20 days	Male	Intensive neonatology	28/07/2014	UCL9	-	-
08	5 months	Male	Outpatient emergency	04/08/2014	UCL9	-	-
12	2 years	Male	Paediatric haematology	26/08/2014	UCL9	-	-
				01/09/2014	UCL9	-	-
				22/09/2014	UCL9	-	-
13	10 days	Male	Intensive neonatology	16/09/2014	UCL9	-	-
14	2 months	Male	Intensive neonatology	22/09/2014	UCL9	-	-
19	2 years	Male	Not specified	23/10/2014	UCL9	-	-
16	60 years	Male	Outpatient dialysis	13/10/2014	UCL9	-	-
49	9 months	Female	Consultation	01/07/2014	UCL9	-	-
50	14 years	Female	Consultation	18/07/2014	UCL9	-	-
53	15 days	Female	Intensive neonatology	28/07/2014	UCL9	-	-
54	84 years	Female	Subacute geriatrics	04/08/2014	UCL9	-	-
58	8 years	Female	Paediatric haematology	22/09/2014	UCL9	-	-
60	8 months	Female	Paediatric	13/10/2014	UCL9	-	-
05	66 years	Male	Pneumatology gastroenterology	07/07/2014	UCL110	-	-
21	35 years	Male	Pneumatology gastroenterology	01/07/2014	UCL100b	+	+
				02/07/2014	UCL100b	+	+
62	25 years	Female	Abdominal surgery	17/10/2014	UCL122	-	-
09	79 years	Male	Subacute geriatrics	05/08/2014	UCL257	-	-
			Intensive Care Unit Neuro-traumatology	16/10/2014	UCL257	-	-
55	66 years	Female	Haematology	13/08/2014	UCL384	-	-
10	47 years	Male	Orthopaedics	19/08/2014	UCL46d	-	-
				25/08/2014	UCL46d	-	-
34	94 years	Male	Urgency	01/09/2014	UCL48	+	+
20	76 years	Male	General internal medicine	29/10/2014	UCL122	-	-
22	40 years	Male	General internal medicine	14/07/2014	UCL 23f	+	+
23	46 years	Male	Urgency	14/07/2014	UCL86	+	+
39	33 years	Male	Consultation	16/09/2014	UCL14	+	+
32	82 years	Male	Not specified	19/08/2014	UCL5a	+	+
86	68 years	Female	General internal medicine	29/10/2014	UCL26	+	+
72	78 years	Female	General internal medicine	18/08/2014	UCL16r	+	+
44	63 years	Male	Not specified	03/10/2014	UCL16u	+	+
30	81 years	Male	General internal medicine	18/08/2014	UCL16b	+	+
77	79 years	Female	Subacute geriatrics	08/09/2014	UCL16L	+	+
31	5 years	Male	Paediatric intensive care	18/08/2014	UCL16L	+	+
70	58 years	Female	Cardiovascular and thoracic surgery	13/08/2014	UCL16L	+	+
				29/08/2014	UCL16L	+	+
				01/09/2014	UCL16L	+	+
				01/09/2014	UCL16L	+	+
				07/10/2014	015	+	+
29	1 year	Male	Not specified	11/08/2014	UCL16o**	+	+
				12/08/2014	UCL16o**	+	+
				13/08/2014	UCL16o**	+	+
				02/09/2014	UCL16o**	+	+
				03/09/2014	UCL16o**	+	+
				24/09/2014	UCL16o**	+	+

				27/10/2014	UCL16o**	+	+
57	17 years	Female	Paediatric haematology	30/08/2014	UCL266	-	-
68	36 years	Female	Maternal Intensive Care	23/07/2014	UCL381	+	+
52	77 years	Female	Nephrology neurology	22/07/2014	UCL468	-	-
			Nephrology neurology	04/08/2014	UCL468	-	-
			Subacute geriatrics	30/08/2014	106	+	+
51	34 years	Female	Stomatology neurology	22/07/2014	UCL471*	-	-
28	11 years	Male	Paediatric haematology	11/08/2014	UCL475*	+	+
45	50 years	Male	Nephrology neurology	06/10/2014	UCL477*	+	+
17	78 years		Subacute geriatrics	17/10/2014	UCL478*	-	-
18	6 months	Male	Ambulatory emergency	17/10/2014	UCL479*	-	-
27	55 years	Male	Pneumatology Gastroenterology	31/07/2014	UCL472*	+	+
				07/08/2014	078	+	+
64	78 years	Female	Surgery, orthopaedics and traumatology	02/07/2014	078	+	+
			Subacute geriatrics	23/07/2014	078	+	+
71	64 years	Female	Haematology	18/08/2014	078	+	+
				28/08/2014	078	+	+
40	67 years	Male	Pneumatology Gastroenterology	18/09/2014	078	+	+
				18/09/2014	078	+	+
47	30 years	Male	Pneumatology Gastroenterology	17/10/2014	078	+	+
				17/10/2014	078	+	+
				20/10/2014	078	+	+
75	53 years	Female	Oncology	01/09/2014	078	+	+
			Medical surgical intensive care	29/09/2014	078	+	+
76	60 years	Female	Nephrology neurology	01/09/2014	078	+	+
78	57 years	Female	Nephrology neurology	14/10/2014	078	+	+
				23/10/2014	078	+	+
80	92 years	Female	General internal medicine	29/09/2014	078	+	+
24	67 years	Male	Nephrology neurology	17/07/2014	014	+	+
25	90 years	Male	Subacute geriatrics	30/07/2014	014	+	+
				12/08/2014	014	+	+
33	44 years	Male	Medical surgical intensive care	27/08/2014	014	+	+
81	54 years	Female	Gastroenterology	29/09/2014	014	+	+
41	29 years	Male	Oncology	24/09/2014	020	+	+
42	11 months	Male	Paediatric transplantation	25/09/2014	020	+	+
48	1 year	Male	Consultation	20/10/2014	020	+	+
65	2 years	Female	Paediatric transplantation	04/07/2014	020	+	+
73	1 year	Female	Consultation	26/08/2014	020	+	+
79	68 years	Female	General internal medicine	09/09/2014	020	+	+
				10/09/2014	020	+	+
				12/09/2004	020	+	+
87	7 months	Female	Paediatric transplantation	30/10/2014	020	+	+
35	82 years	Male	Pneumatology Gastroenterology	08/09/2014	056	+	+
83	84 years	Female	Not specified	06/10/2014	056	+	+
				14/10/2014	056	+	+
36	62 years	Male	Oncology	09/09/2014	106	+	+
				11/09/2014	106	+	+
				12/09/2014	106	+	+
37	71 years	Male	Urgency	10/09/2014	106	+	+
46	92 years	Male	Cardiology	07/10/2014	106	+	+
26	60 years	Male	Consultation	30/07/2014	106	+	+
84	64 years	Female	Nephrology neurology	20/10/2014	106	+	+
43	76 years	Male	Neurology	01/10/2014	012	+	+
				10/10/2014	012	+	+
				27/10/2014	012	+	+
67	85 years	Female	Pneumatology Gastroenterology	16/07/2014	012	+	+
85	9 months	Female	Consultation	24/10/2014	012	+	+
66	90 years	Female	Pneumatology Gastroenterology	09/07/2014	002	+	+
			Cardiology	05/08/2014	002	+	+
69	21 years	Female	General internal medicine	11/08/2014	002	+	+
74	56 years	Female	Gastroenterology	01/09/2014	002	+	+

* New PCR-ribotype
CE: cytotoxicity assay using confluent monolayer

Discussion

C. difficile continues to be the most common cause of healthcare-associated infection in the developed world. A previous European *C. difficile* infection hospital-based survey has shown that the incidence of CDI and the distribution of causative PCR-ribotypes differed greatly between hospitals[21]. In Spain, the number of toxin-positive cases reported varied between 5.5%- 5.6% (2008)[22], 9% (2008)[21] and 6.0%-6.5% (2013)[22]. In this study the prevalence was higher than has been previously found in Spain. The number of *C. difficile*-positive specimens was 32 (12.3%), but in 1 of these a non-toxigenic strain was identified. In addition, two other positive cases detected only by rapid test were toxin-negative. Therefore, the final percentage of toxin-positive cases in the Spanish hospital was established as 11.1% (29/261). While in the other surveys[21,23] *C. difficile* was more commonly detected in females, in this study 68.8% of positive samples were from male patients.

All diarrhoeal non-duplicate specimens submitted to the diagnostic laboratory were tested, even if they were discarded from the routine *C. difficile* detection protocol due to their consistency (samples labelled outside levels 4 to 7 in the BSC). Two positive samples were detected in the analysis of these additional samples (n=12); however, the overall prevalence was almost the same (12.3% (32/261); 12% (30/249)). In a recent study conducted in Australia, while the number of *C. difficile*-positive specimens increased with the analysis of all diarrhoeal specimens (including non-requested samples), the overall prevalence with the analysis of all samples was lower than that identified by routine testing[23]. In a further study conducted in Spain, the authors found that CDI remained a highly neglected disease because of the absence of clinical suspicion or the lack of sensitive diagnostic testing in some institutions. They also observed that underdiagnosis most frequently affected younger patients and patients with community-acquired CDI[22]. In the present study, no positive patients were detected in the paediatric group (less than 10 years old). However, during the study period only 9 samples were received from this service. This data may reflect that in this Spanish hospital a specific request for the diagnosis of CDI from the clinician is less common in the paediatric service than in others. The mean age of all patients studied (62.5 years old) corroborates this observation. Recent reports warn that the incidence of CDI has increasingly risen among paediatric patients[24]. Collins et al.[23] reported in one survey conducted in Western Australia that undiagnosed CDI cases only occurred among paediatric patients, and 32.3% of all CDI cases were aged <20 years. A further study also conducted in Spain showed that the isolation of *C. difficile* was common in children hospitalised for diarrhoea, especially in patients younger than 2 years old with chronic disease. Furthermore, in the same study the authors reported that the clinical picture observed in children with CDI was characterised by mild symptoms and low clinical severity[25], which may contribute to underdiagnosis in this population. In a previous survey that assessed risk factors and outcomes in children with *C. difficile*-associated diarrhoea, only 12.5% of positive samples were identified as bloody stools while 79% of positive samples were watery stools[26]. In a further study conducted in Calcutta to investigate

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the major clinical features of *C. difficile*-induced diarrhoea, only 17.6% of *C. difficile* cases reported bloody stools compared to 84.2% reporting watery diarrhoea[27]. These reports suggest that bloody stools are not the most common samples associated with CDI. Concurrent with these findings, in this study it was observed that all bloody stools tested negative for the bacterium.

In the study of Alcala et al.[22], the second cause proposed for undiagnosed or misdiagnosed CDI was the lack of sensitive diagnostic tests in some institutions. In the present study, four specimens were identified as negative for *C. difficile* and its toxins using rapid tests, but were found to be positive for toxigenic *C. difficile* strains following the culture of samples. In three additional samples toxins were not detected by rapid test but culture, isolation and characterisation of the isolates revealed the presence of at least one of the two toxins A and B. Enzyme immunoassay detection of GDH as initial screening for *C. difficile* presence has been suggested as a potential strategy. However results appear to differ based on the GDH kit used and therefore this approach remains an interim recommendation[2]. While the GenomEra *C. difficile* assay has been described to be an excellent option for toxigenic *C. difficile* detection in faecal specimens[28], in the present study toxigenic *C. difficile* strains were isolated by culture from three samples that were found negative for the toxin using this method. However, the results obtained confirm that GenomEra *C. difficile* assay is more sensitive than EIA testing for *C. difficile* toxin B, as described previously[2]. In contrast, four samples were only positive for *C. difficile* by rapid tests. Two of these positive samples were toxin negative by EIA and Genome *C. difficile* assay. Ethanol shock was not used in the course of this study, nor was alcohol selection of microorganisms conducted, and cultured colonies were observed in high numbers. Therefore, the high contamination of samples by other bacteria species may explain the failure to isolate these four strains by classical culture. For the two non-toxigenic samples identified by rapid screening, false-positive GDH test results are also plausible[29]. The use of the enrichment step in this study was shown to be not useful in the clinical samples tested, as all of the samples that tested positive after 3 enrichment days were already positive by direct culture and the same PCR-ribotypes were isolated.

The surveillance data in Belgium reported a lower prevalence than in Spain (9.3%). It should be noted that the Spanish and Belgian results must be compared with caution. In the case of the Belgian laboratory, all diarrhoeal faecal specimens were analysed, including duplicate samples from the same patient. In the Spanish hospital only non-duplicate specimens were analysed. Nevertheless, despite this important difference in the routine protocol among laboratories, the prevalence of *C. difficile* is likely to be genuinely lower in Belgium than in Spain. While incidence varies considerably between hospitals and regions, an increase in the proportion of community-associated cases and a decrease in the proportion of hospital-acquired cases of CDI between the years 2008 to 2014[30] have been reported in Belgium. This data may reflect the efforts of Belgian hospitals to improve the management and prevention of CDI.

Another important difference found is the mean age of positive patients. In Spain, the mean age found in *C. difficile*-positive cases was 63.6 years old, which correlates with other surveys conducted in the country[22]. In contrast, in Belgium the mean age of adult positive patients was 45 years old. However, if the paediatric group is evaluated separately, the mean age in Belgium is 60 years old. A significant number of positive samples in the Belgian survey were from children less than ten years old (n=19). However, only eight of the positive patients harboured toxigenic *C. difficile* strains. It has been described that during early infancy the gut microbiota complexity is poor and asymptomatic colonisation by *C. difficile* is common[31,32]. However, all the paediatric patient samples analysed in this study were diarrhoeal. These findings corroborate with previous suggestions[23] that the surveillance and the significance of *C. difficile* in paediatric groups requires further investigation.

In both Spain and Belgium, *C. difficile*-positive patients referred from oncology services all carried toxigenic strains. In a previous study, a great diversity of *C. difficile* strains associated with CDI was detected among paediatric oncology patients[33]. A further study found a probable association between certain types of tumours, the use of antibiotics and CDI incidence. The authors also emphasised the urgent need for early recognition and diagnosis of CDI in adult cancer patients[34]. PCR-ribotypes 078, 014, 012, 020, 002, UCL36a, UCL5a, UCL16b and UCL9 were isolated in both hospitals. In previous surveys in hospitals in Spain, PCR-ribotypes 078/126, 014 and 001 were the most prevalent[22]. As in previous years, PCR-ribotype 014 remains the most common in Belgium, increasing in proportion to other ribotypes and in the number of hospital sites affected since 2014. The other PCR-ribotypes more commonly detected in Belgian hospitals in 2014 were BR020 and BR078[30]. In the Spanish hospital studied, there were no commonly-encountered PCR-ribotypes, suggesting there is neither regional infection nor contamination in the hospital. On the contrary, a great variety of toxigenic PCR-ribotypes was identified. Consistent with the European survey which reported that PCR-ribotype 027 was less prevalent than others[21], this ribotype was not detected either in Spain or Belgium during the present study period.

In conclusion, the data obtained shows that even with three times the number of samples analysed per month, the prevalence of *C. difficile* is lower in the Belgian hospital than the Spanish one. This data may reflect the efforts of the Belgian hospital to improve the management and prevalence of CDI, and, as previously reported, misdiagnosis or underdiagnosis of CDI in Spain due to a lack of clinical suspicion. The most common PCR-ribotypes reported in Europe were found in both hospitals. The great variety of PCR-ribotypes detected suggests there is neither regional infection nor contamination within the hospital. This study finds that the presence of *C. difficile* in paediatric and oncology services requires further investigation.

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Declaration of interest

None

References

1   Brazier JS. Clostridium difficile: from obscurity to superbug. *British Journal of Biomedical Science* 2008;**65**(1):39–44.

2   Stuart H. Cohen M., Dale N. Gerding M., Stuart Johnson M., et al. Clinical Practice Guidelines for Clostridium difficile Infection in Adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA) 2010.

3   Squire MM., Riley T V. *One Health: The Human-Animal-Environment Interfaces in Emerging Infectious Diseases*. vol. 365. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013.

4   Crogan NL., Evans BC. Clostridium difficile: an emerging epidemic in nursing homes. *Geriatric Nursing (New York, NY)* **28**(3):161–4.

5   Arvand M., Moser V., Schwehn C., Bettge-Weller G., Hensgens MP., Kuijper EJ. High prevalence of Clostridium difficile colonization among nursing home residents in Hesse, Germany. *PloS One* 2012;**7**(1):e30183. Doi: 10.1371/journal.pone.0030183.

6   Nanwa N., Kendzerska T., Krahn M., et al. The Economic Impact of Clostridium difficile Infection: A Systematic Review. *The American Journal of Gastroenterology* 2015;**110**(4):511–9. Doi: 10.1038/ajg.2015.48.

7   Jones a M., Kuijper EJ., Wilcox MH. Clostridium difficile: a European perspective. *The Journal of Infection* 2013;**66**:115–28. Doi:

10.1016/j.jinf.2012.10.019.

8   Tattevin P., Buffet-Bataillon S., Donnio P-Y., Revest M., Michelet C. Clostridium difficile infections: do we know the real dimensions of the problem? *International Journal of Antimicrobial Agents* 2013;**42 Suppl**:S36–40. Doi: 10.1016/j.ijantimicag.2013.04.009.

9   Evans CT., Safdar N. Current Trends in the Epidemiology and Outcomes of Clostridium difficile Infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America* 2015;**60 Suppl 2**(suppl_2):S66–71. Doi: 10.1093/cid/civ140.

10 McDonald LC., Killgore GE., Thompson A., et al. An epidemic, toxin gene-variant strain of Clostridium difficile. *The New England Journal of Medicine* 2005;**353**(23):2433–41. Doi: 10.1056/NEJMoa051590.

11 Freeman J., Bauer MP., Baines SD., et al. The changing epidemiology of Clostridium difficile infections. *Clinical Microbiology Reviews* 2010;**23**(3):529–49. Doi: 10.1128/CMR.00082-09.

12 Kuijper EJ., Barbut F., Brazier JS., et al. Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. *Euro Surveillance : Bulletin Européen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin* 2008;**13**(31).

13 Van Esch G., Van Broeck J., Delmée M., Catry B. Surveillance of Clostridium difficile infections in a long-term care psychogeriatric facility: outbreak analysis

and policy improvement. *Archives of Public Health = Archives Belges de Santé Publique* 2015;**73**(1):18. Doi: 10.1186/s13690-015-0067-y.

14 Viseur N., Lambert M., Delmee M., Van Broeck J., Catry B. Nosocomial and non-nosocomial Clostridium difficile infections in hospitalised patients in Belgium: compulsory surveillance data from 2008 to 2010. *Euro Surveillance : Bulletin Européen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin* 2011;**16**(43).

15 Rodríguez-Pardo D., Almirante B., Bartolomé RM., et al. Epidemiology of Clostridium difficile infection and risk factors for unfavorable clinical outcomes: results of a hospital-based study in Barcelona, Spain. *Journal of Clinical Microbiology* 2013;**51**(5):1465–73. Doi: 10.1128/JCM.03352-12.

16 REIGADAS E., ALCALÁ L., MARÍN M., MARTÍN A., IGLESIAS C., BOUZA E. Role of binary toxin in the outcome of Clostridium difficile infection in a non-027 ribotype setting. *Epidemiology and Infection* 2015:1–6. Doi: 10.1017/S095026881500148X.

17 Weber I., Riera E., Déniz C., Pérez JL., Oliver A., Mena A. Molecular epidemiology and resistance profiles of Clostridium difficile in a tertiary care hospital in Spain. *International Journal of Medical Microbiology* 2013;**303**(3):128–33. Doi: 10.1016/j.ijmm.2013.02.016.

18 Rodriguez C., Taminiau B., Van Broeck J., Avesani V., Delmée M., Daube G. Clostridium difficile in

young farm animals and slaughter animals in Belgium. *Anaerobe* 2012;**18**(6):621–5. Doi: 10.1016/j.anaerobe.2012.09.008.

19 Bidet P., Barbut F., Lalande V., Burghoffer B., Petit JC. Development of a new PCR-ribotyping method for Clostridium difficile based on ribosomal RNA gene sequencing. *FEMS Microbiology Letters* 1999;**175**(2):261–6.

20 Standards - CLSI :CLSI. Available at http://clsi.org/standards/. Accessed March 4, 2015.

21 Bauer MP., Notermans DW., van Benthem BHB., et al. Clostridium difficile infection in Europe: a hospital-based survey. *Lancet (London, England)* 2011;**377**(9759):63–73. Doi: 10.1016/S0140-6736(10)61266-4.

22 Alcalá L., Reigadas E., Marín M., Martín A., Catalán P., Bouza E. Impact of clinical awareness and diagnostic tests on the underdiagnosis of Clostridium difficile infection. *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology* 2015;**34**(8):1515–25. Doi: 10.1007/s10096-015-2380-3.

23 Collins DA., Riley T V. Routine detection of Clostridium difficile in Western Australia. *Anaerobe* 2015. Doi: 10.1016/j.anaerobe.2015.11.007.

24 Sammons JS., Toltzis P. Pitfalls in Diagnosis of Pediatric Clostridium difficile Infection. *Infectious Disease Clinics of North America* 2015;**29**(3):465–76. Doi: 10.1016/j.idc.2015.05.010.

25 Santiago B., Guerra L., García-Morín M., et al. [Clostridium difficile isolation in children hospitalized with diarrhea]. *Anales de Pediatría (Barcelona, Spain : 2003)* 2015;**82**(6):417–25. Doi: 10.1016/j.anpedi.2014.07.015.

26 Morinville V., McDonald J. Clostridium difficile-associated diarrhea in 200 Canadian children. *Canadian Journal of Gastroenterology = Journal Canadien de Gastroenterologie* 2005;**19**(8):497–501.

27 Bhattacharya MK., Niyogi SK., Rasaily R., et al. Clinical manifestation of Clostridium difficile enteritis in Calcutta. *The Journal of the Association of Physicians of India* 1991;**39**(9):683–4.

28 Hirvonen JJ., Mentula S., Kaukoranta S-S. Evaluation of a new automated homogeneous PCR assay, GenomEra C. difficile, for rapid detection of Toxigenic Clostridium difficile in fecal specimens. *Journal of Clinical Microbiology* 2013;**51**(9):2908–12. Doi: 10.1128/JCM.01083-13.

29 Goldenberg SD., Cliff PR., French GL. Glutamate dehydrogenase for laboratory diagnosis of Clostridium difficile infection. *Journal of Clinical Microbiology* 2010;**48**(8):3050–1; author reply 3051. Doi: 10.1128/JCM.01074-10.

30 Neely F., Marie Lambert L. Epidemiology of Clostridium difficile infection in Belgium Report 2015. Epidemiology of Clostridium Difficile Infection in Belgium Report 2015. Available at http://www.nsih.be/download/CDIF/CDIF-AR-2015-EN.pdf. Accessed November 27, 2015.

31 Rousseau C., Levenez F., Fouqueray C., Doré J., Collignon A., Lepage P. Clostridium difficile colonization in early infancy is accompanied by changes in intestinal microbiota composition. *Journal of Clinical Microbiology* 2011;**49**(3):858–65. Doi: 10.1128/JCM.01507-10.

32 Rodriguez C., Taminiau B., Van Broeck J., Delmée M., Daube G. Clostridium difficile infection and intestinal microbiota interactions. *Microbial Pathogenesis* 2015;**89**:201–9. Doi: 10.1016/j.micpath.2015.10.018.

33 Dantes R., Epson EE., Dominguez SR., et al. Investigation of a cluster of Clostridium difficile infections in a pediatric oncology setting. *American Journal of Infection Control* 2015. Doi: 10.1016/j.ajic.2015.09.004.

34 Rodríguez Garzotto A., Mérida García A., Muñoz Unceta N., et al. Risk factors associated with Clostridium difficile infection in adult oncology patients. *Supportive Care in Cancer : Official Journal of the Multinational Association of Supportive Care in Cancer* 2015;**23**(6):1569–77. Doi: 10.1007/s00520-014-2506-7.

CHAPTER 4: General discussion

Background, hypothesis and aims

Since its discovery in 1935, the bacterium *C. difficile* has been largely investigated, as well as all the aspects regarding its infection in humans and animals. Nowadays, *C. difficile* is recognised as the most important cause of antibiotic associated hospital-acquired diarrhea worldwide. The infection has been classically associated with advanced age, antibiotic treatment such as fluoroquinolones, hospitalisation or stays in other healthcare settings, like nursing homes (Kelly and LaMont, 1998). In the last decade the circulation of *C. difficile* in the community has been repeatedly reported (Tracey *et al.,* 2015) but little is known about the sources of contamination. Humans have been proposed as the main reservoir, with the hypothesis that *C. difficile* is most often acquired from asymptomatic carriers; the infection is developed in the community and subsequently spread to healthcare facilities (Bauer and Kuijper, 2015). A second hypothesis was also suggested: direct or indirect contacts with animals and ingestion of contaminated foods have both been proposed as possible sources of community-acquired CDI (Gould and Limbago, 2010). Therefore, *C. difficile* transmission is a complex problem that must be addressed taking into account the circulation of the bacterium in both humans and animals, in order to have a global vision of the situation.

The main objectives of this dissertation included:

- Understanding the dissemination of *C. difficile* in companion animals (horses) and the changes in the gut microbiota in presence of *C. difficile* colonisation and/or diarrhoea *(Goal 1)*
- Understanding the dissemination of *C. difficile* in food animals (pigs and cattle) on farms and at slaughter, and the risk of carcass and meat contamination *(Goal 2)*
- Investigation of genetic relationships between *C. difficile* isolates from humans, animals, carcasses and meats *(Goal 3)*
- Assessing the risk of ingestion of *C. difficile* spores in ready-to-eat meals *(Goal 4)*
- Assessing the environmental contamination of *C. difficile* in a healthcare setting *(Goal 5)*
- Understanding the dissemination of *C. difficile* in a closed population (nursing home residents) and the changes in the gut microbiota in presence of *C. difficile* colonisation or/and diarrhoea *(Goal 6)*
- Assessing the *C. difficile* circulation in two different European healthcare settings *(Goal 7)*

Goal 1: Horses as an example of *C. difficile* presence in companion animals

Currently, *C. difficile* is considered one of the most important causes of diarrhea and enterocolitis in horses and foals (Arroyo *et al*., 2006; Weese *et al*., 2006; Uzal *et al*., 2012), but these animals can also carry the bacterium without showing signs of disease (Diab *et al*., 2013). As the major risk factors for the development of CDI in horses are antimicrobial treatment and hospitalisation, two preliminary surveys were conducted at an equine medical teaching clinic.

C. difficile prevalence in hospitalised horses and main PCR-ribotypes

The prevalence of *C. difficile* observed in hospitalised horses ranged between 3.7% and 13.7%. Although these prevalences are both comparable with those reported in other studies (Baverud *et al*. 2003; Medina-Torres *et al.,* 2011), the discrepancy observed between the two studies performed could be explained by the fact that in the latter, animals were sampled at the time of admission but not tracked during their hospitalisation as in the first study (prevalence 13.7%). In addition, both studies differed by their sample size and their study period. A clear seasonality of CDI in humans was observed, with a high number of affected patients in winter months (January-March) and spring, and lower frequencies in summer and autumn (Reil *et al*., 2012; Furuya-Kanamori *et al*., 2015b). In retail meats, Rodriguez-Palacios *et al*., (2009) also reported a highest prevalence of *C. difficile* in winter (January and February). Regarding our two studies, more isolates were obtained in winter than during the remaining months. However, the small number of positive animals made it impossible to conduct an epidemiological analysis. In the future, it will be interesting to conduct a surveillance programme for at least one year to completely assess the carriage of *C. difficile* and the epidemiology of CDI in horses, and to determine if there is a consistent seasonal variation.

Regarding the different PCR-ribotypes identified, the only one detected in both studies was PCR-ribotype 014. Since 2011, this PCR-ribotype become the most frequent one in hospitalised human patients (range from 12.1% in 2011 to 8.8% in 2013) (Taori *et al*., 2014; Neely and Lambert, 2015). In addition, other PCR-ribotypes found in horses (notably PCR-ribotype UCL16L, UCL5a and UCL23f) were also among the most frequently identified PCR-ribotypes in patients in Belgium between 2011 and 2013, with a percentage of 3.3-2.2%, 0.87-2.5% and 2.0-2.3% respectively (Van Broeck *et al*., 2015). These results suggest the possibility, albeit not proven, of zoonotic transmission through direct contact or close proximity with infected horses (Thean *et al.,* 2011), as previously hypothesised for piglets (Squire and Riley, 2013). Close contact of horses with infected humans could also turn into a source of infection for animals. Therefore, it is also possible that some strains of *C. difficile* moved from humans to animals in the last decade. This anthroponotic transmission has not been previously described in horses but, in healthy dogs visiting hospitalised patients, high rates of *C. difficile* carriage were detected*,* suggesting that infected humans or their contaminated environment can be a source of the bacterium for assistance dogs (Lefebvre *et al*., 2009). To solve this question and to understand the dissemination of *C. difficile*, further studies conducted in closely related populations of humans and horses, as horse stud farms, are needed.

C. difficile disease in horses

Two horses positive for *C. difficile* presented clinical signs of diarrhoea. Therefore, it seems that in most of the cases *C. difficile* colonisation in these animals does not cause disease. Similarly, any association between antibiotic usage and *C. difficile* colonisation or diarrhea in horses is less

documented than in humans. In one experimental study, it was demonstrated that erythromycin can induce sever colitis associated with the proliferation of *C. difficile* in mature horses (Baverud, 2002). Regarding the clinical presentation of CDI in horses, acute and watery diarrhoea, anorexia and severe dehydration are the most common clinical sings. Hyperemic mucous membranes, pyrexia, tachycardia, tachypnea, tympanitic abdominal distension and mild to moderate or to severe colic are often associated with diarrhoea (Zhang *et al.,* 2014). Respiratory disorder, lung abscess, leucopenia, mucosal necrosis in the caecum and colon were also reported in thoroughbred racehorses with postoperative CDI (Niwa *et al.,* 2013). Several hypothesis have been formulated to understand the etiopathogenesis of *C. difficile* bacteremia, including direct transfer of polymicrobial gut flora to the bloodstream through a site of injury in the intestinal mucosa or translocation of bacteria from the intestine (after disruption of the normal intestinal mucosal barrier) to the lymph nodes, peritoneum, and blood (Kazanji *et al.,* 2015). An animal model of acute and chronic *C. difficile* illness reported that *C. difficile* toxins rather than bacteria are responsible for the systemic complications of *C. difficile* disease (Steele *et al*., 2010).

C. difficile and diarrhoea in horses: gut microbiota characterisation by metagenetics

The gastrointestinal ecosystem is a fundamental component of health and gut bacterial populations play a critical role in the resistance to infection by pathogenic organisms like *C. difficile* (Theriot and Young, 2014). Equine gut microbiota is poorly characterised, as are interspecies interactions and their contribution to animal health (Dougal *et al*., 2013). In this study, high-throughput 16S rDNA amplicon sequencing analysis was used to provide further information on the nature of bacterial communities present in horses developing diarrhea, through comparison with faeces from horses without diarrhea. As reported in a previous study (Daly *et al*., 2001), a great part of the sequences found (60%) were not identical (more than 1% mismatch) to sequence entries present in SILVA database (v1.15). In addition, even among the sequences identical to known entries, the species name was seldom taxonomically defined. These findings show the complexity of horses' gut microbiota, the lack of knowledge and the need for further research on fundamental microbiology either at taxonomic as well as at the functional level. A remarkable finding is the presence of *Akkermansia* in 90% of horses studied. This bacterium is an appealing candidate to become a human probiotic, on the grounds of established mechanisms of preventive treatment of obesity and diabetes (Shin *et al*., 2014). Regarding the other microbiota's communities, a recent study described an increasing diversity in the bacterial composition towards the distal gut in horses, in comparison with the gastric environment (Costa *et al*., 2015). In the present study, the composition of the faecal microbiota of all horses (with and without diarrhea) was dominated by the four same phyla (*Firmicutes*, *Bacteroidetes*, *Verrucomicrobia* and Bacteria_unclassified). However, differences were observed in their cumulative mean relative abundance. The predominance of *Firmicutes* in horse faecal samples found in this study is in agreement with previous reports (Dougal *et al*., 2013; Costa *et al.,* 2015). The second most prevalent

phylum found was *Bacteroidetes*, which is consistent with some studies (Costa *et al.,* 2012; Dougal *et al.,* 2013) but in disagreement with three recent studies that found *Verrucomicrobia* as the second most abundant phylum (Shepherd *et al.,* 2012; Steelman *et al.,* 2012; Costa *et al.,* 2015). In this study *Verrucomicrobia* was the third most predominant phylum. The reasons for the discrepancy observed between studies are unclear. Costa *et al.,* (2015) suggested that these differences are explained by the use of different methods, as each study used different regions of the 16S rRNA gene and different sequencing platforms, but they can also be due to differences in animal diet or age. Unfortunately, the study has failed to answer whether any alterations of the gut microbiota composition can favour *C. difficile* colonisation, because of the lack of clinical manifestations of CDI during the study period. In addition, the limited size of the analysed cohort reduces the strength and the scope of the results obtained. Large cohort studies using multi-omic analysis to improve the knowledge on diarrhea impact and other gastrointestinal disorders on horse microbiota must be secured.

Goal 2: Understanding *C. difficile* dissemination in food animals and risk of food contamination

One major concern of the last decade was the possibility of human exposure to *C. difficile* spores via the contact with colonised food animals or the ingestion of contaminated meat or meat products from such animals.

Shedding of C. difficile in farms

This study found that at farms, apparently healthy piglets and calves harboured *C. difficile* with a prevalence of 78.3% and 22.2% respectively. Most of the isolates were toxigenic and identified as PCR-ribotypes 078, 002 and 015. These findings are comparable to the reported prevalence in the literature (Alvarez-Perez *et al*., 2009; Costa *et al*., 2011). In piglets, the presence of *C. difficile* toxins in the colon has been associated with profuse diarrhoea, colitis and systematic sepsis (Yaeger *et al.,* 2007). In calves, the bacterium and its toxins have also been associated with diarrhoea, although the reported prevalence of CDI is lower than in piglets. Why some colonised piglets and calves with toxigenic strains of *C. difficile* do not develop any signs of disease remains unclear. In humans, both toxigenic and non-toxigenic *C. difficile* strains are common in infants during the first two years of life, but in most of the cases this colonisation seems to be transient and rarely associated with CDI (Bolton *et al.,* 1984). Potential explanations, although not proven, are the absence of toxin receptors, poorly developed cellular signalling pathways in the immature gut mucosa, or the presence of protective factors in the gut (Jangi and Lamont, 2010; Adlerberth *et al.,* 2014). By analogy, the reason that some toxin-positive piglets and calves remain uninfected may be related with humoral immunity. Colostrum intake and colostrum antibody concentration could be crucial in the development (or not) of the disease (Squire and Riley, 2013). Regarding hyperimmune bovine colostrum, it has been shown to be effective for the treatment of CDI in human patients (Steele *et al.,* 2013) and in piglets (Sponseller *et*

al., 2015). Therefore, a natural protective effect of this first milk in calves is plausible (Rodriguez-Palacios *et al*., 2007). On the other hand, non-toxigenic strains of *C. difficile* and parental and mucosal immunization with toxoids have been suggested as prophylaxis and/or therapy for *C. difficile* disease in piglets. In these cases, protection against CDI is apparently associated with high serum concentration of toxin-neutralizing antibodies (Songer *et al.,* 2000). Bacteriophage and phage-like particles have great potential therapy for CDI. No *C. difficile* phage receptors have been identified to date, but the S-layer and cell wall polysaccharides are likely candidates (Kirk *et al.,* 2016). In humans, at an early stage of life, the presence of *C. difficile* has been associated with some bacterial species in the gut microbiota (*Ruminococcus gnavus* and *Klebsiella pneumoniae*) while lactobacilli and *Bifidobacterium longum* has been associated with non-colonised infants (Naaber *et al.,* 1997; Rousseau *et al.,* 2011; Naaber *et al.,* 2004). Studies of the gut microbiota in colonised, infected and non-colonised piglets and calves have not been previously conducted. However, it is probably that the presence of some bacterial communities has a crucial role against CDI. This hypothesis merits further investigations with the aid of up-to-date microbiota exploration strategies.

Prevalence of C. difficile in pigs and cattle at harvest

The carriage of *C. difficile* has been shown to decrease with the age. We reported a prevalence of 0%-1% in pigs and 6.6%-9.9% in cattle just prior slaughter (6 months and older). While the reason for this age affect is still unknown, a probable explanation is that the bacterium is better able to colonise and proliferate in the intestinal tract of younger animals, where the gut microbiota is less developed or less stable (Rodriguez-Palacios *et al*., 2006). To verify this effect, it could be possible to perform faecal transplantation with immature microbiota in adult cattle, for example, and to study the susceptibility to CDI. Some studies have also described how diet appears to modulate the presence and growth of *C. difficile* in the gut of humans and animals (Rodriguez-Palacios, 2011; Lizuka *et al.*, 2004; Moore *et al*., 2015; Hamper *et al*., 2016). A further hypothesis could be that environmental changes affect susceptibility to *C. difficile* colonisation. On the other hand, faecal samples were collected directly from the colon, in the viscera processing area. A recent study describing the dynamics of *C. difficile* in the murine gastrointestinal tract showed that spore germination occurs in the small intestine, while vegetative *C. difficile* establishment occurred in the distal gastrointestinal tract. In contrast, disease was only localized in the large intestine (Koenigsknecht *et al*., 2015). A further study in horses investigated the presence of *C. difficile* in different intestinal compartments of healthy adult horses. The study showed a good correlation between rectal samples and intestinal compartments. In addition, right dorsal colon and small colon yielded the highest number of isolates (Schoster *et al*., 2012). Although it will be interesting to investigate the presence of *C. difficile* in different intestinal samples of food animals (duodenum, jejunum, ileum, caecum, colon and rectum samples), available data suggest that colon samples are representative for the presence of *C. difficile* in the intestinal tract, but probably not representative for a specific strain.

As previously reported, it is still uncertain if the presence of *C. difficile* in faecal samples resulted from short-term successful bacterial colonisation and proliferation or from intestinal passage of ingested dormant spores originating from feed or environmental sources (Rodriguez-Palacios, 2011). In humans, it has been proposed that asymptomatic *C. difficile* colonisation has a protective effect against disease progression through an immune-mediated response. Asymptomatic human patients colonised with *C. difficile* have shown highest IgG levels than diarrheic patients. Furthermore, many children and adults have IgG and IgA antibodies to *C. difficile* toxins, even when they tested negative for the bacterium (Furuya-Kanamori *et al*., 2015a). This argument might be applied to livestock animals as the stimulation of antibodies during early stages of life may result in a protection against CDI in adulthood and explains the absence of clinical signs in all of the positive pigs and cattle detected in our studies.

Some PCR-ribotypes associated with CDI in humans (014, 078, 023, 002 and 020) were detected in pig and cattle samples. PCR-ribotype 078 has been described as predominant in swine populations and cattle (Rodriguez-Palacios, 2011). It has been hypothesised that *C. difficile* PCR-ribotype 078 is a strain coming from animals that have expanded in livestock and then to humans (from the community to the hospitals) (Dahms *et al*., 2014).

Carcass contamination at slaughter line

The presence of toxigenic *C. difficile* strains in intestinal contents of pigs and cattle at slaughter indicated that there was a risk of carcass and meat contamination at slaughterhouse. In this study, *C. difficile* was detected on 7% of pig and 7.9% of cattle carcasses. Three different PCR-ribotypes were isolated from pork carcass samples (014, 081 and UCL36) while beef carcass samples were contaminated with a wide variety of PCR-ribotypes, including 023 and 015 among others. Surprisingly, none of the carcass isolates was identified as PCR-ribotype 078. This high prevalence of *C. difficile* found in pig carcasses suggests that cross contamination during processing might occur. Furthermore, the PCR-ribotypes found in the intestinal contents were not identical to those isolated from carcasses of the same animal, corroborating that there is environmental contamination at slaughter or, indicating that more than one PCR-ribotype is present in the intestinal contents of animals. However, all intestinal samples were cultured twice (after 3 enrichment days and after 30 enrichment days) and, in most of the cases, the same PCR-ribotypes were obtained, making the second scenario less likely. It is obvious that carcass contamination is associated with the prevalence and load of faecal shedding by live animals at the time of harvest (Rodriguez-Palacios, 2011). As it was reported an enhanced shedding in younger animals, the age at slaughter can also play a critical role in carcass contamination. In this context, a recent study conducted in Australia showed a *C. difficile* prevalence of 60% in feces and 25.3% on carcasses of neonatal calves (Knight *et al.,* 2016), which is much higher than that obtained in this study or in other reports in the literature, may due to this age

effect. Microbiological sampling of carcasses by excision or swabbing has been evaluated in a previous study. Authors found that swabs were more suitable than excision for use in carcass sampling. However, for the recovery of some bacteria, the person who collected the samples also had a significant effect on the results obtained (Martinez *et al.,* 2010). There are no studies to compare different sampling methods for *C. difficile* detection on carcasses. A previous study detected toxin genes of *C. difficile* in one carcass swab by multiple real-time PCR only, but viable cultures were not recovered (Houser *et al.,* 2012). Therefore, different sampling techniques for detection of *C. difficile* contamination in carcasses should be evaluated. On the other hand, several studies have addressed the importance of good slaughter hygiene. Furthermore, in the USA, some slaughterhouses have conducted decontamination interventions along the slaughter line. A recent Norwegian study did not find a clear correlation between hide dirtiness in cattle and carcass contamination for *Escherichia coli*, *Enterobacteriaceae* and total aerobic bacteria. However, the authors stated that these results are valid when carcasses from dirty animals are handily carefully during slaughter and dressing (Hauge *et al*., 2015). The extent of such an association for *C. difficile* remains unknown. An additional study reported that biofilm formation in the surfaces of the slaughterhouse could facilitate the attachment of *C. difficile* spores, and therefore be a source of contamination (Esfandiari *et al*., 2014).

Detection of contaminated meats in retail markets

The evidence that carcass contamination occurs inside the slaughterhouse reinforces the hypothesis about a potential risk of foodborne infections linked to the ingestion of foods contaminated with *C. difficile* spores. Raw ground beef and pork were found to be contaminated with *C. difficile* with a frequency ranging from 2.3% to 4.7% respectively, and the main PCR-ribotypes identified were 078 and 014 in both types of samples. The reason for the presence of these two PCR-ribotypes in most of the samples is not clear, considering the great variety of strains found in intestinal contents and carcasses. One possible explanation is the differences in the sporulation frequencies and susceptibility to external agents among the different PCR-ribotypes. However, there is little data in the literature to support this hypothesis. A previous study showed that *C. difficile* strains isolated from calves at later points of production (identified as PCR-ribotypes 078 and 126) presented higher early sporulation efficiencies *in vitro* than other strains isolated at the beginning of production, suggesting their persistence in the environment or in the host (Zidaric *et al.,* 2012). A further study assessing the sporulation and susceptibility of three PCR-ribotypes (027, 012 and 017) to four classes of disinfectants reported that PCR-ribotype 017 showed the highest sporulation frequency under the test conditions (Dawson *et al.,* 2011). This feature may contribute to the survival of only some PCR-ribotypes until the end of the meat supply chain (distribution in retail markets). Another hypothesis to explain why most of the PCR-ribotypes found in meats were identified as 014 and 078 is that animals are not the sole origin of *C. difficile* meat contamination. In 2014, year in which the study in meat was conducted, the most frequently isolated PCR-ribotypes in Belgian hospitals were PCR-ribotypes 014,

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020, 078 and 002 (sorted by % of identified strains) (Neely *et al.,* 2015). Recent data suggest that PCR-ribotypes found in hospitals are circulating in the community and they are introduced in healthcare settings by incoming patients (Rodriguez-Palacios *et al.*, 2013). Therefore, the source may involve contamination during processing or in retail markets.

Goal 3: Genetic relationships between *C. difficile* isolates from humans, animals and meats

In order to investigate genetic relationships between *C. difficile* isolates from humans, animals and foods, MLVA and MLST analysis were performed. The sampling areas for each type of sample are indicated in Figure 1. A first study compared meat and humans isolates by MLST and it was shown that they cluster in the same lineage. Unfortunately, meat isolates were not compared by MLST with animal isolates obtained at slaughter or in farms.

When it was compared *C. difficile* isolates from animals with those isolated from hospitalised patients by MLST, high correlation between isolates with the same PCR-ribotype was revealed, regardless their origin (carcass, intestinal contents or human faeces). However, after a second characterisation of all strains by MLVA, it was shown that MLST has less discriminatory power (Marsh *et al.,* 2010). MLVA clearly distinguishes between human and animal isolates and therefore interspecies transmission could not be demonstrated.

In relation to antimicrobial sensitivities, results show similar susceptibilities for the majority of antibiotics tested for isolates from human and animal or meat sources. In addition, no association could be established between antimicrobial resistance and toxigenic strains. However, the number of isolates tested was small and does not allow to argument further conclusions. The *C. difficile* genome contains approximately 11% of mobile genetic elements, many of them involved in antibiotic resistance. Both genetic elements and mutations in the molecular targets of antibiotics can be mantained and may persist in absence of antibiotic selective pressure, as described for fluoroquinolones resistance. A rapid identification of new phenotypic and genotypic traits is needed to prevent the spread of *C. difficile* antibiotic resistant strains (Mullany *et al*., 2015; Spigaglia, 2016)

To elucidate the potential of *C. difficile* zoonotic transmission, the next step will be the study of very closely related populations (for example farmers and their animals or pets and their owners). However, considering that the prevalence of *C. difficile* in domestic and adult food animals is below 10% (Rodriguez-Palacios *et al*., 2013) and that intestinal *C. difficile* colonisation rates of human healthy adults are estimated between 2.4% and 13% (Perras *et al.,* 2011), the sample size required (samples from humans and their animals) makes it difficult to perform such studies. In addition, MLVA typing method has shown some limitations with some loci that seem to be unstable and are absent in some isolates. Recent studies showed that whole genome sequencing provides a greater discriminatory

power in elucidating dissimilarity between *C. difficile* strains, with an affordable cost (Dominguez *et al*., 2015; Gerding, 2016).

Figure 1. Sampling areas in Belgium.

📍 Faecal samples from humans (from a hospital in Brussels and from a nursing home in Theux-Liège)

📍 Intestinal samples and carcass samples from pig and cattle at slaughter (Bressoux-Liège)

📍 Meat samples collected from different shops in Liège

📍 Faecal samples from hospitalised horses (Sart Tilman- Liège)

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Goal 4: Risk of ingestion of *C. difficile* with meals in a community at risk (elderly)

Even with the evidence that *C. difficile* is present in meats, to date there are no conclusive data supporting the development of CDI due to the consumption of contaminated foods. A recent study reporting the thermal resistance of *C. difficile* spores to minimum recommended cooking temperatures (70°C-75°C) gives more strength to the hypothesis of foodborne *C. difficile* infections (Rodriguez-Palacios and Lejeune, 2011). In this context, subjects over 65 years are more prone to suffer CDI. As defined by Chen et al. (2014), frailty is a genetic syndrome characterised by age-associated declines in physiologic reserve and function across multiorgan systems, leading to increased vulnerability for adverse outcome. In addition, old people are more commonly hospitalised and under antibiotic treatment, and they suffer changes in the gut microbiota, which make this population less resistant to *C. difficile* colonisation and infection (Shin *et al.,* 2016). Possible foodborne transmission was reported in 1982 in a case of pseudomembranous colitis due to *C. difficile* in a 78-year-old woman without a previous history of antibiotic therapy. The woman had consumed canned salmon before the onset of disease. However, the salmon was not cultured to demonstrate the presence of *C. difficile* spores (Gurian *et al.,* 1982). A recent study described a marked thermal inhibitory effect at 96°C of *C. difficile* spores (reduction of $6Log_{10}$ within 1 to 2 min) (Rodriguez-Palacios and Lejeune, 2011). The same study reported that thermal treatment at 85°C inhibited cell division but not *C. difficile* germination and that this inhibitory effect can be reversed during incubation in broth. Therefore, canned salmon could be the source of CDI if the heat treatment was insufficient or if the contamination occurred once the canned salmon was opened. A further study identified the susceptibility of *C. difficile* to food preservatives sodium nitrate, sodium nitrite and sodium metabisulphite (Lim *et al.,* 2016). The results demonstrated that *C. difficile* can survive in the presence of these food preservatives at concentrations higher that the current maximum permitted levels allowed in ready to eat meat. Therefore, there is a potential for foodborne transmission on *C. difficile* through commercially produced ready-to-eat meals such as cured, fermented or smoked meats.

After the investigation of *C. difficile* in freshly prepared food in the kitchen area of a nursing home, only one strain (PCR-ribotype 078) was isolated from a pork sausage served with salad. It is interesting to note that none of the circulating clinical *C. difficile* isolates among the nursing home residents were identified as PCR-ribotype 078. Therefore, even if it is not clear what was the source of contamination of the positive meal (contamination of meat at slaughterhouse, contamination during processing or contamination by the food handlers) it can be suggested that foods can be a vector of *C. difficile* strains circulating in the community to be introduced to nursing homes and other healthcare settings, although the frequency and likelihood of this phenomenon is low.

Goal 5: Environmental contamination of *C. difficile* in a healthcare setting

The increasing number of CDI cases in hospitalised patients has lead to suspect that environmental contamination is the main cause of contamination inside healthcare settings. However, in our study nursing home environment was not detected as a source of contamination, as none of the surfaces tested positive for the bacterium. It is likely that the implemented cleaning program to control the spread of spores in this healthcare setting is effective. But it is also important to note that culture methodology for environmental samples might be suboptimal at the time, since other similar studies in hospital environment yielded no *C. difficile* spores or a minor contamination (Rodriguez-Palacios *et al.,* 2013; Sjörberg *et al.,* 2014). In this context, more frequent sampling of different healthcare settings using different swabbing methods could provide more information about the best sampling method, but also about the environmental contamination in these close communities and its implication in the propagation of the infection.

Goal 6: *C. difficile* in elderly: presence and gut microbiota interactions

Results of our studies suggested that food or environments are not the main sources of contamination in the nursing home investigated. Therefore, the next concern was to evaluate and follow the presence of *C. difficile* among the elderly residents and to characterise the isolates in order to demonstrate if the positive status was due to the same strains (person-to-person transmission). Furthermore, barcoded pyrosequencing was used to characterize the faecal microbiota of the elderly residents, to evaluate the global evolution of the total microbiota, and to identify possible relations between certain bacterial populations and *C. difficile* colonization.

C. difficile dispersion among nursing home residents

Nursing home residents commonly have risk factors for CDI, including hospitalisation, an age over 65 years and antibiotic exposure. *C. difficile* was isolated from 7/23 (30.4%) nursing home residents. In other countries, the prevalence of *C. difficile* colonisation varied from 0.8% to 54.8% between nursing homes (Rodriguez *et al.,* 2014). In this study no association between probiotic administration or antibiotic therapy and *C. difficile* colonisation could be established. The application of probiotics to CDI prevention and treatment is controversial (Allen *et al*., 2013; Ehrhardt *et al.,* 2016). Current treatment guidelines for CDI express that there is insufficient evidence to conclusively recommended probiotics as preventive treatment. Studies have demonstrated the importance of bile acid metabolism by gut microbiota in providing moderate *C. difficile* colonisation resistance (Koenigsknecht *et al.,* 2015). Specific strains with defined beneficial properties are being targeted including *Lactobacillus plantarum* and *Sccharomyces boulardii* (Spinler *et al.,* 2016) Further efforts in determining specific probiotic formulations and doses are needed to solidify clear recommendations for probiotic applications. On the other hand, PCR-ribotype 027 was the most prevalent type. Interestingly, this

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PCR-ribotype was among the five more commonly identified in Belgian hospitals in the past years. However, since 2011 a decline in its prevalence has been described (Cairns *et al.,* 2012; Neely and Lambert, 2015). Results suggest that residents of nursing homes are at high risk of colonisation by hypervirulent strains. MLVA revealed that there was a clonal dissemination of the strains within the nursing home residents, probably due to person-to person transmission. As previously cited, nursing home environment samples tested negative for the bacterium. Therefore, the isolation of elderly in nursing homes has an important impact on the epidemiology of *C. difficile* infection. Furthermore, this state of closure makes nursing home residents a valuable population to conduct further studies in the dynamics of the infection, in the spread of different *C. difficile* strains and also to investigate changes in the gut microbiota when there is a bacterial colonisation and/or infection.

Gut microbiota changes in elderly C. difficile colonised subjects

Regarding gut microbiota modifications with *C. difficile* colonisation, the preliminary metagenomic analysis revealed that microbial evenness is slightly but significantly higher in *C. difficile* positive residents. In contrast, bacterial diversity and richness are not significantly higher in *C. difficile* positive residents after statistical Bonferroni correction (McDonald, 2014). Richness is defined as the estimated number of bacterial species in the biotope. Evenness refers to how close in numbers each species is in the biotope. Diversity encompass both richness and evenness, and it is a measure of species biodiversity taking into account the number of species as well as their relative abundance (Beals *et al.,* 2000). The findings of this study are may be due to the fact that the survey was conducted in elderly people in a closed environment for long-term stay, but its repeatability in other populations must be investigated. In addition, results obtained suggest that the gut microbiota structure is associated with diarrheic episodes instead of *C. difficile* positive status, as previously hypothesised (Antharam *et al.,* 2013). A further study also reported a little difference regarding the microbiota composition of *C. difficile* colonised subjects suffering CDI and asymptomatic carriers (Rea *et al*., 2012). A decrease of *Verrucomicrobiaceae* (genus *Akkermansia*) in subjects positive for the bacterium has found. The genus *Akkermansia* is linked to gut health and its abundance seems to be reduced when there is gut inflammation (Derrien *et al.,* 2016). The association between *Akkermansia* and *C. difficile* colonisation is a novel important finding that merits further investigation.

Goal 7: Epidemiology of *C. difficile* in two different European healthcare settings

It has been shown that the nursing home studied is a closed community with clonal dissemination of a single or few strains, and it does not reflect the transmission current in the community. However, in hospitals the situation is not the same. Recent studies refuted the theory of person-to person transmission in hospitals and suggested that *C. difficile* strains appear to be introduced in hospital by incoming patients or even by visitation animals or foods (Rodriguez-Palacios *et al*., 2013). Therefore, hospitals are a better scenario than nursing homes to study what are the main strains circulating in the

community. The collaboration with the National Reference Centre for *C. difficile* in humans has allowed us to know the prevalence and the main PCR-ribotypes implicated in CDI in Belgium and to compare them with animal and food isolates. The next concern was to determine if the situation in Belgium regarding CDI was similar in other countries. Therefore, a final study was conducted in a hospital located in Spain, in order to survey the *C. difficile* circulation and to compare the ribotype distribution with those observed in a second hospital located in Belgium during the same study period. As previous reported in an European *C. difficile* infection hospital-based survey, the incidence of CDI and the distribution of causative PCR-ribotypes differed greatly between hospitals (Bauer *et al*., 2011). The prevalence of positive cases reported in Spain and Belgium was 12.3% and 9.3% respectively. However, during the same study period the total number of samples analysed in Belgium was triple the number of samples analysed in the Spanish hospital. According to another study (Alcalá *et al.,* 2015), it seems that CDI in Spain may be misdiagnosis or undiagnosed due to the lack of clinical suspicion and requests. A great variety of PCR-ribotypes were detected in both hospitals, including PCR-ribotypes 078, 014 and 020. In previous surveys in Belgium and Spain PCR-ribotypes 078 and 014 remained the most prevalent since 2014 (Alcalá *et al.,* 2015; Neely and Lambert, 2015). PCR-ribotype 027 was not detected either in Spain or Belgium during the present study period. These findings corroborate our previous hypothesis that changes in the prevalence of PCR-ribotypes in nursing homes come later than in hospitals or in the community. A high number of positive patients were referred from paediatric and oncology units, which indicated, as previously suggested (Collins and Riley, 2015), that the presence of *C. difficile* in these two medical services is a new important finding that requires further surveillance and investigation.

CHAPTER 5: Conclusions and perspectives

C. difficile is a worldwide public health concern. The bacterium is considered the leading cause of antibiotic associated disease, responsible for mild-to-severe diarrhea and colitis in healthcare settings, but the infection is also increasingly expanding in the community. In the last decade, the emerging of a new hypervirulent strain, PCR-ribotype 027, and the high number of severe CDI cases with fatal outcome caused a general alarm, which led to reinforce the infection control measures and the creation of new surveillance initiatives in hospitals, in order to reduce the incidence of the infection. Under this situation, during 2000s, research was focused on transmission, rapid diagnostic and treatment of CDI in hospitals, but little was reported on other possible sources of exposure. Indeed, at that time, the increasing number of infections inside healthcare settings maintained the attention (almost exclusively) on human close-contact transmission, but several cases began to appear also in the community, in populations previously considered a low risk, such as healthy children and peripartum women, patients without an antibiotic treatment or without a history of hospital exposure. The sources of CDI and risk factors in the community are not well understood, which makes difficult the control of transmission. Only with the identification of these potential vectors it would be possible the development of new strategies to prevent contamination.

The main hypothesis of this study was that food animals could act as vectors of toxigenic *C. difficile* strains and therefore contribute to expand the infection in the community. In Belgium, there were no data about the intestinal carriage of *C. difficile*, the risk for carcass contamination at slaughter or the contamination of foods with its spores. To address these important gaps of knowledge, this study investigated the circulation of *C. difficile* in humans, animals and foods, in order to have a global vision of the situation and to better understand the potential of the bacterium as a zoonotic or foodborne infectious agent.

Results obtained revealed that animals are carriers of *C. difficile* in farms and at slaughter, and carcass contamination occurs inside the slaughterhouse. Spores of *C. difficile* were also detected in retail meats, indicating a potential risk of human foodborne transmission.

Why is the presence of C. difficile in animals and food relevant to human CDI?

Regarding the results obtained, it can be deduced that the most important role of animals and foods in human CDI is not their potential to produce disease but their contribution to the changing epidemiology of the infection. The decrease of PCR-ribotype 027 in hospitals has lead to the increase in the numbers of other PCR-ribotypes causing CDI, like PCR-ribotypes 014, 020 and 078. As observed in our studies, these strains are now commonly found in both animals and foods. While zoonotic infection has been not demonstrated, we can say that animals contributed to the spread of these emerging PCR-ribotypes in the community, either by food contamination as previously described, or by direct or indirect contact with humans and their environments. In this context, in the

future it will be very interesting to study close related human and animal populations (farmers in close contact with their animals or pets of human patients with community-acquired CDI).

This dissertation has also shown that virulent strains of *C. difficile* are present in meats, either by a contamination from the intestinal contents of carrier animals at slaughter or by contamination from the environment and/or from colonised food handlers during processing. But even if spores are present, their prevalence in meats is not very high and much less important in prepared meals, maybe due to the low thermal resistance of these spores compared with the spores of other clostridial species. In this context, in the future it will probably be more interesting to study the presence of *C. difficile* in raw foods consumed directly (as raw meats or fish consumed without thermal treatment), in bio products (as fruits or vegetables, normally grown with the help of organic fertilizers), or in traditional food products of developing countries (where the hygienic procedures are not always the best). Maybe in these cases the prevalence and the counts of spores are sufficiently important to present a potential risk of foodborne infection attributed to these products.

On the other hand, in our studies, the bacterium was detected after an enrichment step, which indicates that the initial spore load is small. Therefore, even if at present the human infectious dose for *C. difficile* is not known, it can be supposed that in the food products studied, spores ingested by healthy people with normal intestinal microbiota do not cause greater impact. *C. difficile* foodborne infection would result from a very unlikely scenario in Belgium, which will be the repeated consumption of contaminated foods with virulent strains by vulnerable subjects with gastrointestinal perturbations. We cannot rule out this possibility, but this situation does not seem to be as common today. We think that to determine a foodborne transmission of *C. difficile*, the only feasible way would be the study of clinical CDI community cases at hospitals. Only with a good anamnesis of the patient linked with the analysis of suspected ingested foods (if it is the case) it will be possible to trace the origin of the contamination (the food product as hypothesised here). This survey will be possible with a close collaboration between laboratories, clinicians, and patients. The apparition of several problems must be taken into account: the patients can not remember or don't associate what they have eaten with the infection, the foods that they have consumed are not longer available for analysis, or there is no clinical suspicion of foods as sources of CDI. Therefore the study period and the number of cases studied should be considered to overcome these problems.

After analysis of all the data obtained, what type of measures could be established to prevent this contamination from farm-to-table?

Our findings evidenced that animals are able to shed spores of toxigenic *C. difficile* without showing any signs of disease. Therefore, it is very difficult to predict or notice which animals are carriers of the bacterium. A rapid screening test before slaughter is neither optimal nor affordable. The use of good hygiene practices through the slaughter operations to produce visibly clean carcasses will be the most

reasonable measure to reduce the contamination, as indicated for other foodborne pathogens. Regarding meats, in our study *C. difficile* was found in samples with good organoleptic quality, which makes impossible to notice the presence of the bacterium by visual examination. As *C. difficile* spores cannot survive to high temperature, a good thermal treatment (85°C during 10 min; based on the literature data) is the best strategy to reduce the risk of foodborne transmission. Furthermore, it is an easy household practice that must be emphasized, as it is also useful to eliminate other pathogens present in foods.

Have the current typing methods sufficient discriminatory power to compare the strains?

We have evidenced that the most commonly used typing techniques (PCR-ribotyping, MLST and MLVA) are not discriminating enough to determine the clonality of strains. Therefore it is necessary to change the use of these methods when we compare *C. difficile* strains from different origins. Next generation typing techniques, notably whole genome sequencing analysis has been shown to provide the best resolution to study DNA variations such as single nucleotide variations, copy number variations, insertions, deletions, inversions or translocations. This powerful and promising tool must be applied in the future to study the relatedness of strains, in order to finally demonstrate the zoonotic or anthroponotic transmission of *C. difficile*.

Link between the epidemiology of C. difficile in humans and animals

The presence of a great variety of PCR-ribotypes in CDI human cases without an apparent hospital ward contamination confirms that community plays an essential role in the epidemiology of CDI. It may be interesting to conduct further studies in healthy subjects (children and adults) to know what strains are circulating in the community, the prevalence of *C. difficile* colonisation in asymptomatic subjects and the relatedness of these human strains with animal strains. This information would help to better understand the transmission of *C. difficile*, the reservoir of the bacterium, as well as anticipate potential emerging strains that can cause serious outbreaks in hospitals. On the other hand, we have observed that changes in the prevalent PCR-ribotypes in closed nursing home populations come later than in hospitals. Our studies concluded that this population is more static because strains high prevalent in hospitals five years ago are now the more prevalent in these healthcare settings. Therefore, nursing home residents could be a perfect controlled population to study changes in the faecal microbiota, changes in the CDI epidemiology and to study the different factors promoting colonisation and infection.

Population dynamics and C. difficile colonisation: further perspectives

The last important concern of this dissertation was to apply the new "omic" techniques, in our case metagenetics, to determine changes in gut microbiota composition with CDI or colonisation. Unfortunately, in both humans and animals, the study did not provide conclusive results because the

number of colonised or infected subjects was very low. But two observations of these studies must be considered to conduct further investigations. The first one is the presence of *Akkermansia* in all horses and humans studied and its decrease in *C. difficile* positive humans in relation with no colonised residents. The genus *Akkermansia* has been linked to gut health and its abundance seems to be reduced when there is gut inflammation. The association between *Akkermansia* and *C. difficile* colonisation and its presence in the gut microbiota of horses is a novel important finding that merits further investigations. The second important observation is that positive *C. difficile* status was not associated with microbiota richness or biodiversity reduction in our study. These findings should not be dismissed as they provide a first insight about the impact of *C. difficile* colonisation in the gut microbiota.

On the other hand several animal models, including mice, rats, rabbits, hares, guinea pigs, prairie dogs and quails, have been used to explain the mechanism whereby *C. difficile* colonises the intestinal tracts of humans in both with and without diarrhea. However, results vary substantially with the choice of model and in most of the cases they are not comparable or reproducible. Now, the in vitro models offer the advantages of greater control and number of replicates, easier manipulations, data comparison and the respect of life and animal welfare. We are convinced that the metagenomic approach coupled with animal and in vitro models will be key to the future management of the disease, not only for the treatment of infection (as faecal transplantation), but also to understand the factors that help or hinder the colonisation of *C. difficile* in the gut microbiota and the development of the infection. Furthermore, it will be possible to elucidate the factors contributing to the asymptomatic status in animals and human neonates or adults.

CHAPTER 6: Summary- Résumé

SUMMARY

Clostridium difficile is a spore-forming anaerobic bacterium recognised as a major human pathogen responsible for pseudomembranous colitis and nosocomial-antibiotic associated diarrhea. Traditionally, hospitals were considered the main reservoirs for infection. However, in the last years the incidence, deaths, complications and costs of *C. difficile* infection (CDI) have been rising, not only in healthcare facilities, but also within the community. In the community, it has been detected in a growing number of CDI cases in previously healthy individuals without antimicrobial exposure, hospital stay or any other classical risk factors. Furthermore, the disease has been repeatedly described in younger patients, including children. Some hypotheses have been proposed to explain this peak of community cases, the most obvious being that nowadays more attention is given to CDI surveillance. In the last years, diarrhea due to *C. difficile* disease might have gone undiagnosed, and in many cases went unreported, particularly in the community.

Since 2004, severe outbreaks of CDI have been documented increasingly in the United States, Canada and in Europe. These outbreaks have been associated with the emergence of a novel strain, known as PCR-ribotype 027, characterised by higher than usual levels of toxins A and B production, and the presence of a third toxin named CDT or binary toxin. This strain type is also characterised by its resistance to both erythromycin and fluoroquinolones (i.e. moxifloxacin, gatifloxacin and levofloxacin). However, according to the latest hospital surveillance studies in Europe, since 2010 there is a decrease in the incidence rates of PCR-ribotype 027 while other PCR-ribotypes, including PCR-ribotypes 014, 020, 001, 002, 078 and 015 are increasing.

Person to person contact is one source proposed for the spread in the community, occurring after visiting hospitalised patients or residents in long-term care facilities. Employees of these health-care settings can also carry spores and contaminate their entourage. The second hypothesis is contamination from the environment, following visits to a potentially contaminated place, such as hospitals or nursing homes. The two most important potential sources of CDI in the community, which have been demonstrated by investigations in the last decade are animals and foods.

While *C. difficile* is also known as enteric pathogen in some food producing and companion animal species, there are several reports describing the presence of the bacterium in the intestinal contents of apparently healthy animals. Moreover, data published recently suggests animals as an important source of human CDI, which can spread disease through environmental contamination, direct or indirect contact, or food contamination, including carcass and meat contamination at slaughter or in the case of crops, through the use of organic animal manure. By definition, zoonoses are infectious

diseases that can be transmitted directly or indirectly between animals and humans, through direct contact or close proximity with infected animals, or through the environment. Foodborne zoonotic pathogens are transmitted via the consumption of contaminated food or drink water. The first description of *C. difficile* in domestic animals and their environments dates from 1974 and possible foodborne transmission was reported for the first time in 1982. However, nowadays the importance of *C. difficile* as zoonotic disease remains largely unknown.

The "One Health" concept is a worldwide strategy, which recognises that the health of humans and animals is connected and also depends on the environment. The present dissertation is a 5 year national study that has investigated the presence of *C. difficile* in animals and food, from "farm-to-table". The study was also extended to humans resident in a nursing home and in two hospitals in Belgium and in Spain. The characterisation of the isolates obtained has ultimately allowed comparison of the PCR-ribotype distribution in the different European hospitals, as well as with the PCR-ribotype distribution found in animals and foods. This work explores how *C. difficile* spreads among human patients, animals, foods and the environment to better understand the potential of the bacterium as a zoonotic or foodborne infectious agent.

To start the survey, *C. difficile* was first investigated in hospitalized horses at an equine medical teaching clinic. In both foals and horses, the bacterium has been identified as an important agent of diarrhea and enterocolitis. As in the case of human infections, hospitalization, antibiotic therapy or changes in diet have been described to contribute to the development of CDI. In a preliminary study, the objective was to assess the carriage of *C. difficile* in hospitalised horses and the possible influence of some risk factors in colonization. Ten out of a total of 73 horses (13.7%) tested positive for *C. difficile* but only two presented clinical signs of diarrhea associated with CDI. Seven different PCR-ribotypes were identified (014, UC16L, UCL16a, UCL228, UCL9 UCL261, UCL5a), and 5 of them were toxigenic. Multi-locus sequencing typing (MLST) analysis revealed a clear concordance between some PCR-ribotypes and the sequence types attributed. Nonetheless, no relationships could be established between the acquisition of *C. difficile* in the hospital and a particular circulating strain, or between the presence of diarrhea and one particular type. This study showed that horses were frequently colonized by *C. difficile* regardless of the reason for hospitalization, however the development of diarrhea was more unusual. Therefore, an appropriate infection prevention strategy, for example to avoid gut microbiota disruptions may be able to reduce the incidence of the associated disease.

Regarding these data obtained, the next main question was about the relationships between *C. difficile* and the rest of the intestinal flora of horses, which had experienced an episode of diarrhea. To address this concern, a second study was performed in the same equine clinic to attempt to determine whether the presence of diarrhea and/or the isolation of *C. difficile* were related to changes in the composition

of the faecal microbiota. High-throughput sequencing analysis revealed that bacterial diversity of the gut microbiota in diarrhoeic horses was lower than in non-diarrhoeic horses in terms of species richness and in population evenness. Some taxa like *Fusobacteria, Actinobacillus* and *Porphyromonas* were detected more abundantly in horses with diarrhea, while *Akkermansia* was found in all of the horses studied. For a great variety of bacterial species the currently available systems were not able to confidently assign taxonomy, which showed how complex and still unknown the equine microbiome is. The overall prevalence found for *C. difficile* colonization was lower than in the previous study (3.7%). However, animals were only sampled at the time of admission but not tracked during their hospitalisation as in the previous study. Five different PCR-ribotypes were identified: 014, UCL237, UCL49, UCL23f and UCL36 (the only non-toxigenic PCR-ribotype). None of these positive animals suffered an episode of diarrhea, which suggests that *C. difficile* infection was transient in horses studied without overgrowth to trigger infection. Therefore, no association between CDI in horses and a specific modification of the microbiota could be demonstrated.

The second objective was to study the carriage of the bacterium in food-producing animals in farms and at slaughter, and to determine if carcasses were contaminated at the slaughter line. In our study, *C. difficile* was found more prevalent in piglets (78.3%) and calves (22.2%) on farms than in adult animals (pigs 0%-1%; cattle 6.6%-9.9%) just prior slaughter. Carcasses of pigs and cattle were shown to be contaminated with a prevalence of 7% and 7.9% respectively. However, characterisation of the isolates showed a variety of *C. difficile* PCR-ribotypes on carcasses that were not found in the intestinal samples from the same animals, suggesting environmental contamination at slaughter. Among the PCR-ribotypes isolated from intestinal contents and carcasses, the greatest variety of PCR-ribotypes was found in slaughter cattle, possibly explained by the fact that in cattle there was larger herd diversity than in pigs. This study further documented that animals were carriers of *C. difficile* at slaughter, and that carcass contamination occurred inside the slaughterhouse. Moreover, the most common PCR-ribotypes involved in human CDI in Belgium were identified in animals and their carcasses (including the PCR-ribotypes 014, 078, 023, 081, UCL16L, UCL16U, UCL11 et UCL5a).

In order to investigate genetic relationships between *C. difficile* isolates from human hospitalized patients, pig and cattle intestinal and carcasses samples in the same geographic region and during the same time period, MLST and multilocus variable-number tandem-repeat analysis (MLVA) were performed. The study revealed that *C. difficile* strains PCR-ribotype 078, 014, 081, UCL16U, UCL11 and UCL5a circulate among the same animal species and among human patients. MLST showed that animal and human strains clustered in the same lineage. MLVA, more discriminating, revealed close relationships between isolates from different animal species (pig and cattle), but less genetic similarity among human and animal isolates. These data suggest a wide dissemination of clones at hospitals, breeding-farms and at the slaughterhouse.

To clarify the concern about foodborne transmission through consumption of contaminated animal products, a further study was conducted to evaluate the presence of *C. difficile* in retail meat sold in market places in Belgium. *C. difficile* was isolated from 2.3% retail beef samples and 4.7% retail pork samples. However, the contamination of meats may involve faecal or environmental contamination of carcasses at slaughterhouse but also during human processing post-slaughter. A total of 4 different PCR-ribotypes were identified with a large percentage of types 078 and 014. Furthermore, by MLST, PCR-ribotype, toxin activity, toxin genes and antimicrobial resistance strains isolated were indistinguishable from the human strains isolated during the same period in Belgian hospitals.

Knowing that this bacterium is present in foods, what are the risks for the consumers? Spores of *C. difficile* are heat resistant and can survive gentle cooking of foods (70-75 °C). Even if the spore numbers in foods are usually low, a small dose ingested with an altered gut microbiota may be able to trigger infection, and patients over 65 years can therefore be particularly susceptible to outbreaks. The following study aimed to assess the presence of *C. difficile* on freshly prepared food in the kitchen area of a nursing home and also to examine the presence of spores in the environment of this retirement home. *C. difficile* was isolated from only one meal sample composed of pork sausage, mustard sauce and salad, suggesting that the contamination in prepared meals is lower than in raw foods. Nevertheless, the isolate was identified as PCR-ribotype 078, which has also been considered as an emergent hypervirulent strain in human beings. Regarding the environment, none of the surfaces sampled were positive for *C. difficile,* but it is probable that results vary significantly between retirement and healthcare establishments according to the implemented cleaning program to control the spread of the bacterium. At the time of the study on food and surface samples, the prevalence of *C. difficile* among the residents of the same nursing home was evaluated. Twenty-three residents were monitored weekly during 7 different weeks. Prevalence of *C. difficile* was 30.4%, with 7 residents positive for *C. difficile* at least once. The bacterium was detected in 43.3% episodes of diarrhea, but there was only one patient diagnosed with CDI during the study period. Four different PCR-ribotypes were identified but the most common type isolated was the hypervirulent ribotype 027. MLVA showed clonal relatedness of the *C. difficile* isolates and cross-infection between patients. Barcoded pyrosequencing was used to characterize the faecal microbiota of the elderly residents, to evaluate the global evolutions of the total microbiota and to identify possible relationships between some bacterial populations and *C. difficile* colonization, diarrhea and antibiotic treatment. The study of the microbial phylotype composition of the samples showed that almost of the samples were clustered in a sub-tree corresponding to a single resident, which means that each resident studied had their own bacterial imprint and that it was stable during the entire study. Furthermore, residents positive for *C. difficile* by classical microbiology showed by metagenetic analysis an important proportion of *C. difficile* sequences, which shows a good correlation between *C. difficile* detection by classical microbiology and barcoded pyrosequencing analysis. However, it does not seem that carriage and shedding of *C.*

difficile was associated with a specific microbiota. The study further evidences the importance of high-throughput amplicon sequencing analysis to investigate the intestinal microbiota interactions in patients suffering CDI.

Once the presence and characterisation of *C. difficile* circulating strains was examined in companion and food animals, carcasses, foods and humans in Belgium, a final study was conducted in a hospital located in Spain. The aim of this study was to survey the *C. difficile* circulation and to compare the ribotype distribution with those observed in a second hospital located in Belgium, during the same study period. Data obtained shows that even if the total number of samples analysed per month in the Belgian hospital was triple the number of samples analysed in the Spanish hospital, the prevalence was lower in the Belgian hospital (9.3% versus 12.3%). These results may reflect the efforts of the Belgian hospital to improve the management of CDI, and a probably misdiagnosis of CDI in Spain due to the lack of clinical suspicion and request. The most common PCR-ribotypes reported in Europe were found in the two hospitals, including 078, 014, 020, 012 and 002. The great variety of PCR-ribotypes detected did not suggest regional spread or hospital ward contamination by a particular strain. However, the same PCR-ribotypes detected in animals and foods were isolated from human hospitalised patients, including PCR-ribotypes 014 and 078.

In conclusion, this dissertation documented that companion and food animals harbour toxigenic *C. difficile*, but the development of diarrhea is more rarely reported than in the case of humans. Food animals are carriers of *C. difficile* at harvest and carcass contamination occurs at the slaughter line. *C. difficile* spores are also present in pork and beef retail meat. A massive use of antibiotics, like fluoroquinolones or cephalosporins in animal production could be responsible for an expansion of the bacterium with an increased presence of its spores at farms, and in consequence at slaughterhouses and food processing plants. Prepared meals and nursing home environment have not been identified as a potential source of contamination. However, the isolate obtained from prepared meals was identified as one hypervirulent type. This dissertation also documented the prevalence and the ribotype distribution in healthcare settings in two European countries, Belgium and Spain. The main PCR-ribotypes found in humans were also isolated from animals and foods. While genomic techniques used in this work did not always reveal clonality in the isolates found in humans and animals, they are closely related. The analysis of *C. difficile* strains isolated from different sources by whole genome sequencing analysis will definitively confirm the absence of host tropism of certain strains.

These findings documented the actual zoonotic and foodborne transmission risk of CDI and the relevance to develop further strategies to reduce the dissemination of the bacterium at farms and to reduce the risk of food contamination.

Finally, the investigation of the gut's microbial communities by metagenetic analysis in *C. difficile* infected humans and animals made it possible to discern whether any alteration of the gut microbiota

composition could favour *C. difficile* colonisation, as well as if there were some microorganisms able to reduce susceptibility to the infection. Unfortunately, the present project has failed to answer this question because of the lack of clinical manifestations of CDI in humans and horses during the study period. This new approach will be critical in the future for further understanding the pathogenesis of *C. difficile* disease and to develop new successful prevention measures to reduce the presence and the number of CDI in both humans and animals.

RESUME

Clostridium difficile est une bactérie anaérobie sporulée reconnue comme un agent pathogène humain majeur, responsable de colites pseudomembraneuses et diarrhées nosocomiales associées à la prise d'antibiotiques. Traditionnellement, les hôpitaux étaient considérés comme les principaux réservoirs de l'infection. Toutefois, ce schéma a évolué à la fin du siècle dernier avec une hausse de l'incidence, des décès, des complications et des coûts des infections à *C. difficile* (ICD), ainsi que l'apparition d'infections communautaires. Ces dernières ont pour particularités l'absence d'exposition précédente à des antimicrobiens, à un séjour à l'hôpital ou à d'autres facteurs de risque classiques. En outre, l'infection a été décrite à plusieurs reprises chez des jeunes patients, y compris des enfants. Certaines hypothèses ont été proposées pour expliquer cette augmentation de cas communautaires. Il faut cependant mettre cette augmentation en perspective avec une conscientisation plus importante à cette problématique et donc un nombre de dépistage plus élevé.

Depuis 2004, des épidémies d'ICD sévères ont été rapportées aux Etats-Unis, au Canada et en Europe. Toutes ces épidémies ont été liées à l'émergence d'une nouvelle souche PCR-ribotype 027, avec une grande virulence due à l'hyperproduction de toxines A et B, et à la production d'une troisième toxine connue comme CDT ou toxine binaire. En outre, ce type de souche est caractérisé par sa résistance à l'érythromycine et aux fluoroquinolones (p.ex. moxifloxacine, gatifloxacine and lévofloxacine). Cependant, selon les dernières études de surveillance des hôpitaux en Europe, depuis 2010, il y a une diminution dans l'incidence du PCR-ribotype 027 alors que d'autres PCR-ribotypes, notamment les PCR-ribotypes 014, 020, 001, 002, 078 et 015 sont en augmentation.

Le contact personne à personne est une des sources proposées pour la propagation dans la communauté, survenant après la visite à des patients hospitalisés ou résidents dans des établissements de soins de longue durée. Les employés de ces établissements peuvent également transporter des spores et contaminer leur entourage. La seconde hypothèse est la contamination par l'environnement, suite à la visite des lieux potentiellement contaminés comme les hôpitaux ou les maisons de repos. Mais les deux sources potentielles d'ICD communautaires les plus étudiées durant la dernière décennie sont les animaux et les aliments.

C. difficile est également connu comme un agent pathogène entérique chez certains animaux de ferme et de compagnie. Mais de nombreuses publications décrivent la présence de cette bactérie dans le microbiote intestinal des animaux apparemment sains. En outre, les études publiées récemment suggèrent les animaux comme une source importante d'ICD humaines, pouvant propager la maladie par la contamination de l'environnement, directe ou indirecte, ou par la contamination des aliments, que ce soit via la contamination de la carcasse et de la viande lors de l'abattage, jusqu'à la contamination de cultures via l'utilisation d'engrais organiques d'origine animale. Par définition, les

zoonoses sont des pathologies infectieuses pouvant être transmises directement ou indirectement entre les animaux et les humains, par contact direct ou proximité avec des animaux infectés, ou via l'environnement. Les agents pathogènes zoonotiques d'origine alimentaire se transmettent par la consommation d'aliments ou d'eau contaminés. La première description de *C. difficile* chez les animaux domestiques et de leurs environnements date de 1974 et la possible transmission d'origine alimentaire a été signalée pour la première fois en 1982. Pourtant aujourd'hui encore, l'importance de *C. difficile* en tant qu'agent zoonotique reste largement inconnue.

Le concept "One Health" est une nouvelle stratégie mondiale, qui reconnaît que la santé des humains et celle des animaux sont intimement liées et avec celle de l'environnement. La présente thèse est une étude nationale de 5 ans portant sur la présence de *C. difficile* chez les animaux et dans les aliments, "de la ferme à la table". L'étude a été étendue également aux humains confinés dans une maison de repos belge et dans deux hôpitaux en Belgique et en Espagne. La caractérisation des isolats obtenus a permis *in fine* de comparer la distribution des PCR-ribotypes des souches issues des deux hôpitaux européens, ainsi qu'avec des PCR-ribotypes retrouvés chez les animaux et dans les aliments. Ce travail explore la circulation de *C. difficile* chez les humains, les animaux, les aliments et l'environnement afin de mieux comprendre le potentiel de transmission de cette bactérie en tant qu'agent infectieux zoonotique ou d'origine alimentaire.

En premier lieu, *C. difficile* a été recherché chez des chevaux hospitalisés dans une clinique vétérinaire universitaire. Chez les poulains et les chevaux, la bactérie a été identifiée comme un agent important de diarrhée et entérocolite. Comme dans le cas d'infections humaines, l'hospitalisation, l'antibiothérapie ou les changements de régime alimentaire sont des facteurs favorisant le développement des ICD. Dans une étude préliminaire, l'objectif était d'évaluer la présence de *C. difficile* chez les chevaux hospitalisés et la possible influence de certains facteurs de risque dans la colonisation. Sur un total de 73 chevaux, 10 animaux (13,7%) ont été testés positifs pour *C. difficile* mais seulement 2 de ces animaux présentaient des signes cliniques de diarrhée compatibles avec une ICD. Sept PCR-ribotypes différents ont été identifiés (014, UCL16L, UCL16a, UCL228, UCL9, UCL261, UCL5a), et 5 d'entre eux étaient toxigènes. Le typage par MLST (multi-locus sequence typing) a révélé une concordance claire entre certains PCR-ribotypes et des clones retrouvés identifiés par MLST. Néanmoins, aucune relation n'a pu être établie, démontrant l'acquisition de *C. difficile* à l'hôpital, l'association avec la présence de la diarrhée ou la présence d'une souche circulante particulière. Cette étude a montré que les chevaux sont fréquemment colonisés par *C. difficile,* quelle que soit la raison de l'hospitalisation, mais que le développement de la diarrhée était rare. Par conséquent, une stratégie appropriée de prévention de l'infection, en particulier en évitant les perturbations du microbiote intestinal, peut être en mesure de réduire l'incidence de la maladie associée.

Sur base de ces données, la question suivante a porté sur les relations entre *C. difficile* et le reste de la flore intestinale des chevaux ayant développé un épisode de diarrhée. Pour répondre à cette question, une seconde étude a été réalisée dans la même clinique équine afin de tenter de déterminer si la présence de la diarrhée et/ou l'isolement de *C. difficile* pouvaient être associés à des changements dans la composition du microbiote fécal. L'analyse par séquençage à haut débit a révélé que la diversité bactérienne du microbiote intestinal chez les chevaux diarrhéiques était plus faible que chez les chevaux non-diarrhéiques en termes de richesse des espèces présentes et de distribution des différentes populations. Alors que le genre *Akkermansia* a été trouvé étonnamment en forte proportion chez tous les chevaux étudiés, certains taxons comme les *Fusobacteria*, *les Actinobacillus* et les *Porphyromonas* ont été détectés en abondance surtout chez les chevaux souffrant de diarrhée. Une forte proportion des populations bactériennes observées n'a pu être identifiée à l'espèce, ce qui souligne la méconnaissance actuelle du microbiote équin. La prévalence de *C. difficile* était plus faible que dans l'étude précédente (3,7%). Toutefois, les animaux ont été échantillonnés seulement au moment de l'admission et non suivis au cours de leur hospitalisation comme précédemment. Cinq PCR-ribotypes différents ont été identifiés: 014, UCL237, UCL49, UCL23f et UCL36 (le seul PCR-ribotype non-toxigène). Aucun des animaux positifs n'avait subi un épisode de diarrhée. L'hypothèse serait que la présence de *C. difficile* était transitoire chez les chevaux étudiés, sans surcroissance aboutissant à une infection. De ce fait, aucune association entre l'ICD chez le cheval et une modification spécifique du microbiote n'a pu être mise en évidence.

Dans un second temps, le portage de *C. difficile* chez les animaux de rente a été étudié dans des exploitations agricoles et à l'abattoir. Dans notre étude, *C. difficile* a été le plus fréquemment isolé chez les porcelets (78,3%) et ensuite chez les veaux (22,2%) par rapport aux animaux en âge d'abattage (porcs 0%-1%; bovins 6,6%-9,9%). Les carcasses de porcs et de bovins ont montré une prévalence de contamination de 7% et 7,9% respectivement. Cependant, la caractérisation des isolats a montré qu'il n'y avait pas de relation entre les PCR-ribotypes identifiés en surface des carcasses et dans le contenu intestinal de l'animal d'origine, ce qui suggère une contamination de la carcasse via l'environnement de l'abattoir. Quant aux PCR-ribotypes isolés de contenus intestinaux et de carcasses, la plus grande diversité a été retrouvée chez les bovins. Cette étude a documenté que les animaux étaient souvent porteurs de *C. difficile* à l'abattoir, et qu'il y a une contamination fréquente de la carcasse sur la ligne d'abattage. Enfin, les PCR-ribotypes les plus communs impliqués dans les infections par *C. difficile* chez les humains en Belgique ont été identifiés chez les animaux et sur leurs carcasses (y compris les PCR-ribotypes 014, 078, 023, 081, UCL16L, UCL16U, UCL11 et UCL5a).

Les techniques MLST et MLVA (multilocus variable-number tandem-repeat analysis) ont ensuite été utilisées afin d'étudier les relations génétiques entre les isolats de *C. difficile* circulant chez des patients humains hospitalisés en Belgique et ceux isolés chez les porcins, bovins et sur les carcasses dans la même région géographique, au cours de la même période. L'étude a révélé que des souches de

C. difficile PCR-ribotype 078, 014, 081, UCL16U, UCL11 et UCL5a circulent à la fois chez les animaux et chez les patients humains. L'analyse MLST a montré que les souches d'origine animale et humaine appartenaient au même groupe. L'analyse MLVA, plus discriminante, a révélé des liens étroits entre les isolats d'espèces animales différentes (de porcs et de bovins), mais une plus grande dissimilarité génétique entre les isolats humains et ceux issus des animaux. Ces données suggèrent une large diffusion de clones dans les hôpitaux, les fermes et les abattoirs.

Afin de déterminer le potentiel de transmission de *C. difficile* via la consommation de produits d'origine animale contaminés, une autre étude a été menée pour évaluer la présence de *C. difficile* dans la viande vendue au détail sur le marché belge. *C. difficile* a été isolé dans 2,3% des échantillons de viande de bovin et dans 4,7% des échantillons de viande de porc analysés. Cependant, la contamination des viandes peut avoir pour origine, soit la contamination fécale des carcasses ou de l'environnement à l'abattoir, soit la contamination par la manipulation humaine post-abattoir. Un total de 4 PCR-ribotypes différents a été identifié avec une majorité des types 078 et 014. En outre, à la fois par MLST, PCR-ribotypage et détection de toxines, de gènes de toxines et de résistance aux antibiotiques, les souches isolées de la viande étaient indiscernables des souches humaines isolées pendant la même période dans les hôpitaux belges.

Sachant que cette bactérie est bien présente dans les denrées alimentaires, quels sont les risques encourus par les consommateurs ? Les spores de *C. difficile* sont résistantes à la chaleur et peuvent survivre à une cuisson douce (70-75 °C). Même si le nombre de spores dans les aliments est généralement faible, une petite dose ingérée peut être en mesure de déclencher l'infection chez les sujets dont le microbiote intestinal est altéré. Il est bien connu que les personnes de plus de 65 ans sont particulièrement sensibles à l'ICD. L'étude suivante visait donc à évaluer la présence de *C. difficile* dans les plats fraîchement préparés dans la cuisine d'une maison de repos et à examiner également la présence de spores dans l'environnement de cet établissement. *C. difficile* a été isolé à partir d'un seul échantillon d'un repas composé de saucisse de porc, de moutarde et de salade, ce qui suggère que la contamination dans les plats cuisinés est plus faible que dans les viandes fraîches. Néanmoins, l'isolat identifié appartenait au PCR-ribotype 078, considéré comme rassemblant des souches hypervirulentes en émergence chez l'homme. En ce qui concerne l'environnement, aucune des surfaces échantillonnées ne s'est révélée positive pour *C. difficile*, mais il est probable que les résultats varient considérablement entre les différentes maisons de repos en fonction des systèmes de nettoyage et de désinfection utilisés pour contrôler la propagation de la bactérie. Lors d'une étude menée en parallèle, la prévalence de *C. difficile* a été évaluée parmi les résidents de la même maison de repos. Vingt-trois volontaires ont été contrôlés chaque semaine pendant 7 semaines. Le taux de contamination par *C. difficile* sur la période a été de 30.4%, avec 7 résidents positifs au moins une fois. La bactérie a été détectée dans 43,3% des épisodes de diarrhée mais il n'y a eu qu'un patient avec un d'épisode d'ICD pendant la période de l'étude. Quatre PCR-ribotypes différents ont été identifiés, le type le plus

fréquent était le ribotype hypervirulent 027. L'analyse MLVA a montré la parenté clonale des isolats et la probable infection croisée entre résidents. Une analyse par pyroséquençage a été réalisée pour caractériser la flore fécale des personnes âgées étudiées, afin d'évaluer les évolutions de l'écosystème bactérien et d'identifier les relations possibles entre certaines populations de bactéries et la colonisation par *C. difficile,* ou la présence de diarrhée. L'étude de la distribution des phylotypes dans les échantillons a montré que les microbiotes se regroupaient en sous-ensembles propres à chaque résident. Cela signifie que chaque résident avait sa propre empreinte bactérienne et qu'elle a été stable au cours de toute la période de l'étude. En outre, il y a une bonne corrélation entre la détection de *C. difficile* par la microbiologie classique et par l'analyse métagénétique. Par contre, il ne semble pas que le portage et l'excrétion de *C. difficile* soient associés à un microbiote spécifique différent des résidents non porteurs. Ces résultats montrent l'importance que prendra à l'avenir l'analyse métagénétique pour étudier et mieux comprendre les interactions dans le microbiote intestinal chez les patients souffrant d'une ICD.

Cette investigation autour de la présence de *C. difficile* chez les animaux, sur leurs carcasses, dans les aliments et chez les humains en Belgique a été complétée par une étude dans un hôpital en Espagne. Le but de cette étude était d'étudier la circulation de *C. difficile* entre les patients et de comparer la distribution des PCR-ribotypes avec celle observée dans un deuxième hôpital situé en Belgique, au cours de la même période. Les données recueillies ont montré que, même si le nombre total d'échantillons analysés par mois à l'hôpital belge est le triple du nombre d'échantillons analysés à l'hôpital espagnol, la prévalence était plus faible en Belgique (9,3% contre 12,3%). Ces résultats reflètent les efforts de l'hôpital belge pour améliorer la gestion des ICD, et une probable sous-estimation des diagnostics d'ICD en Espagne, en raison de l'absence de suspicion clinique et de demande d'analyses. Les PCR-ribotypes les plus fréquemment rapportés en Europe ont été retrouvés dans les deux hôpitaux, y compris les types 078, 014, 020, 012 et 002. La grande variété de ribotypes détectés suggère qu'il n'y a pas de propagation régionale ou une contamination de l'environnement dans les hôpitaux étudiés par une souche particulière. Il est intéressant de noter que les mêmes PCR-ribotypes détectées chez les animaux et les aliments ont été isolés à partir des patients humains hospitalisés, notamment les PCR-ribotypes 014 et 078.

En conclusion, cette thèse révèle que les animaux de compagnie et de production hébergent des *C. difficile* toxigènes et non-toxigènes, mais le développement de la pathologie est plus rare que chez l'homme. Les animaux de production sont porteurs de souches toxigènes à l'abattage et une contamination de leurs carcasses a été démontrée. Des spores de *C. difficile* sont également présentes dans la viande de porc et de bœuf vendue au détail. Une utilisation massive d'antibiotiques, comme les fluoroquinolones ou les céphalosporines dans la production animale pourrait être responsable d'une expansion de la bactérie avec une présence accrue de ses spores dans les fermes, et par conséquent les abattoirs et les usines de transformation alimentaire. Les plats préparés et l'environnement d'une

maison repos n'ont pas été identifiés comme une source importante de contamination. Cependant, l'isolat obtenu à partir de plats préparés était hypervirulent. Cette thèse a également documenté la prévalence et la distribution des PCR-ribotypes dans deux hôpitaux de deux pays européens, la Belgique et l'Espagne. Les principaux PCR-ribotypes retrouvés chez les patients hospitalisés ont également été isolés à partir d'animaux et d'aliments. Bien que les techniques de typage moléculaire utilisées dans ce travail n'aient pas toujours révélé de clonalité des isolats trouvés chez les humains et les animaux, ils sont étroitement liés. Le séquençage et l'analyse du génome complet de souches isolées des différentes sources permettra probablement de confirmer définitivement l'absence de tropisme particulier de certaines souches pour certains hôtes.

Les résultats obtenus indiquent que *C. difficile* est probablement transmis des animaux aux humains provoquant des infections communautaires. Il est donc pertinent d'élaborer des stratégies visant à réduire la diffusion de la bactérie dans les exploitations animales afin de réduire le risque de contamination des aliments et le risque d'une transmission zoonotique.

Enfin, l'étude des communautés microbiennes de l'intestin par l'analyse métagénétique chez les animaux et les hommes infectés par *C. difficile* permet de discerner si une modification de la composition du microbiote intestinal peut favoriser la colonisation par la bactérie ou, à l'inverse, si certains micro-organismes peuvent rendre les sujets moins sensibles à l'infection. Malheureusement, les présentes études n'ont pas permis de répondre à cette question du fait de l'absence de manifestations cliniques caractéristiques des ICD dans les populations humaines et équines étudiées. Cette nouvelle approche sera certainement critique dans l'avenir pour comprendre davantage la pathogenèse des infections à *C. difficile* et pour développer de nouvelles mesures de prévention efficaces pour réduire la présence et le nombre d'ICD chez les humains et les animaux.

CHAPTER 7: References

A

ADLERBERTH I., HUANG H., LINDBERG E., ABERG N., HESSELMAR B., SAALMAN R., NORD C.E., WOLD A.E., WEINTRAUB A. Toxin-producing *Clostridium difficile* strains as long-term gut colonizisers in healthy infants. *J. Clin. Microbiol.*, 2014, **52**, 173-179.

ALCALA L., REIGADAS E., MARIN M., MARTIN A., CATALAN P., BOUZA E. Impact of clinical awareness and diagnostic tests on the underdiagnosis of *Clostridium difficile* infection. *Eur. J. clin. Microbiol. Infect. Dis.*, 2015, **34**, 1515-1525.

ALLEN S.J., WAREHAM K., WANG D., BRADLEY C., SEWELL B., HUTCHINGS H., HARRIS W., DHAR A., BROWN H., FODEN A., GRAVENOR M.B., MACK D., PHILLIPS C.J. A high-dose preparation of lactobacilli and bifidobacteria in the prevention of antibiotic-associated and *Clostridium difficile* diarrhoea in older people admitted to hospital: a multicentre, randomised, double blind, placebo-controlled, parallel arm trial (PLACIDE). *Health Technol. Assess.*, 2013, **17**, 1-140

ALVAREZ-PEREZ S., BLANCO J., BOUZA E., ALBA P., GIBERT X., MALDONADO J., GARCIA M.E. Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets. *Vet. Microbiol.*, 2009, **137**, 302-305.

ANTHARAM V.C., LI E.C., ISHMAEL A., SHARMA A., MAI V., RAND K.H., WANG GP. Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhoea. *J. Clin. Microbiol.*, 2013, **51**, 2884-2892.

ARROYO L.G., HENRY R., STAMPFLI H.R., WEESE J.S. Potential role of *Clostridium difficile* as a cause of duodenitis-proximal jejunitis in horses. *J. Med. Microbiol.*, 2006, 55, 605-608.

B

BAUER M.P., NOTERMANS D.W., VAN BENTHEM B.H.B., BRAZIER J.S., WILCOX M.H., RUPNIK M., MONNET D.L., VAN DISSEL J.T., KUIJPER E.J., ECDIS Study Group. *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet*, 2011, **377**, 63-73.

BAUER M.P., KUIJPER Ed. J. Potential sources of *Clostridium difficile* in human infection. *Infect. Dis. Clin. North Am.*, 2015, **29**, 29-35.

BAVERUD V. *Clostridium difficile* infection in animals with special reference to the horse. A review. *Vet. Q.*, 2002, **24**, 203-219

BAVERUD V., GUSTAFSSON A., FRANKLIN A., ASPAN A., GUNNARSSON A. *Clostridium difficile*: prevalence in horses and environment and antimicrobial susceptibility. *Equine Vet. J.*, 2003, **35**, 465-471.

BEALS M., GROSS L., HARRELL S. Diversity Indices: Shannon's *H* and *E*. 2000, [online], http://www.tiem.utk.edu/-gross/bioed/bealsmodules/shannonDI.html, viewed 24 June 2016.

BOLTON R.P., TAIT S.K., DEAR P.R., LOSOWSKY M.S. Asymptomatic neonatal colonisation by *Clostridium difficile*. *Arch. Dis. Child.*, 1984, **59**, 466-472.

C

CAIRNS M., STABLER R.A., SHETTY N., WREN B.W. The continually evolving *Clostridium difficile* species. *Future Microbiol.*, 2012, **7**, 945-957.

CHEN X., MAO G., LENG S. Frailty syndrome: an overview. *Clin. Interv. Aging*, 2014, **9**, 433-441.

COLLINS D.A., RILEY T.V. Routine detection of *Clostridium difficile* in Western Australia. *Anaerobe,* 2015, **37**, 34-37.

COSTA M.C., STAMPFLI H.R., ARROYO L.G., PEARL D.L., WEESE J.S. *Clostridium difficile* on a veal farm: prevalence, molecular characterization and tetracycline resistance. *Vet. Microbiol.*, 2011, **152**, 379-384.

COSTA M.C., ARROYO L.G., ALLEN-VERCOE E., STAMPFLI H.R., KIM P.T., STURGEON J.S., WEESE J.S. Comparison of the faecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of 16S rRNA gene. *Plos One,* 2012, **7**, e41484.

COSTA M.C., SILVA G., RAMOS R.V., STAMPFLI H.R., ARROYO L.G., KIM P., WEESE J.S. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. *Vet. J.,* 2015, **205**, 74-80.

D

DAHMS C., HUBNER N.O., WILKE F., KRAMER A. Mini-review: epidemiology and zoonotic potential of multi resistant bacteria and *Clostridium difficile* in livestock and food. *GMS Hyg. Infect. Control*, 2014, **9**, Doc21.

DALY K., STEWART C.S., FLINT H.J., SHIRAZI-BEECHEY S.P. Bacterial diversity within the equine large intestine as revealed by molecular

analysis of cloned 16S rRNA genes. *FEMS Microbiol. Ecol.*, 2001, **138**, 141-151.

DAWSON L.F., VALIENTE E., DONAHUE E.H., BIRCHENOUGH G., WREN B.W. Hypervirulent *Clostridium difficile* PCR-ribotypes exhibit resistance to widely used disinfectants. *Plos One,* 2011, **6**, e25754.

DERRIEN M., BELZER C., DE VOS W.M. *Akkermansia muciniphila* and its role in regulating host functions. *Microb. Pathog.,* 2016, **9**, 1-11.

DIAB S.S., SONGER G., UZAL F.A. *Clostridium difficile* infection in horses: a review. *Vet. Microbiol.*, 2013, **167**, 42-49.

DOMINGUEZ S.R., ANDERSON L.J., KOTTER C.V., LITTLEHORN C.A., ARMS L.E., DOWELL E., TODD J.E., FRANK D.N. Comparison of whole-genome sequencing and molecular-epidemiological techniques for *C. difficile* strain typing. *J. Pediatric Infect. Dis. Soc.,* 2015, **23**.

DOUGAL K., DE LA FUENTE G., HARRIS P.A., GIRDWOOD S.E., PINLOCHE E., NEWBOLD C.J. Identification of a core bacterial community within the large intestine of the horse. *Plos One*, 2013, **8**, e77660.

E

EHRHARDT S., GUO N., HINZ R., SCHOPPENS S., MAY J., REISER M., SCHROEDER MP., SCHMIEDEL S., KEUCHEL M., REISINGER E.C., LANGEHEINECKE A., DE WEERTH A., SCHUCHMANN M., SCHABERG T., LIGGES S., EVESLAGE M., HAGEN R.M., BURCHARD G.D., LOHSE A.W. *Saccharomyces boulardii* to prevent antibiotic-associated diarrhea: a

randomized, double-masked, placebo-controlled trial. *Open Forum Infect. Dis.,* 2016, **3**.

ESFANDIARI Z.S., WEESE S., EZZATPANAH H., JALALI M., CHAMANI M. Occurrence of *Clostridium difficile* in seasoned hamburgers and seven processing plants in Iran. *BMC Microbiol.*, 2014, **14**, 283.

F

FURUYA-KANAMORI L., MARQUESS J., YAKOB L., RILEY T.V., PATERSON D.L., FOSTER N.F., HUBER C.A., CLEMENTS A.C.A. Asymptomatic *Clostridium difficile* colonization: epidemiology and clinical implications. *BMC Infect. Dis.*, 2015a, **14**, 15:516.

FURUYA-KANAMORI L., MCKENZIE S.J., YAKOB, L., CLARK J., PATERSON D.L., RILEY T.V., CLEMENTS A.C. *Clostridium difficile* infection seasonality: patterns across hemispheres and continents- a systematic review. *Plos One*, 2015b, **16,** 10(3): e0120730.

G

GERDING D.N. Editorial commentary: whole genome sequencing of *Clostridium difficile*: exquisitely sensitive but not yet optimally applied. *Clin. Infect. Dis*. 2016, **62**, 753-754.

GOULD L.H., LIMBAGO B. *Clostridium difficile* in food and domestic animals: a new foodborne pathogen? *Clin. Infect. Dis.,* 2010, **51**, 577-582.

GURIAN L., WARD T.T., KATON R.M. Possible foodborne transmission in a case of pseudomembranous colitis

due to *Clostridium difficile:* influence of gastrointestinal secretions on *Clostridium difficile* infection. *Gastroenterology*, 1982, **83**, 465-469.

H

HAMPER B.A., BARTGES J.W., KIRK C.A. Evaluation of two raw diets vs a commercial cooked diet on feline growth. *J. Feline Med. Surg.,* 2016, **29**.

HAUGE S.J., NESBAKKEN T., MOEN B., ROTTERUD O.J., DOMMERSNES S., NESTERY O., OSTENSVIK O., ALVSEIKE O. The significance of clean and dirty animals for bacterial dynamics along the beef chain. *Int. J. Food Microbiol.*, 2015, **214**, 70-76.

HOUSER B.A., SOEHNLEN M.K., WOLFGANG D.R., LYSCZEK H.R., BURNS C.M., JAYAICO B.M. Prevalence of Clostridium difficile toxin genes in the feces of veal calves and incidence of ground veal contamination. *Foodborne Pathog. Dis.,* 2012, **9,** 32-36**.**

J

JANGI S., LAMONT J.K. Asymptomatic colonization by *Clostridium difficile* in infants: implications for disease in latter life. *J. Pediatr. Gastroenterol. Nutr.*, 2010, **51**, 2-7.

K

KAZANJI N., GJEORGJIEVSKI M., YADAV S., MERTENS A.N., LAUTER C. Monocrobial vs Polymicrobial *Clostridium difficile* bacteremia: a case report and a review of the literature. *Am. J. Med.*, 2015, **128**, 19-26.

KELLY C.P., LAMONT J.T. *Clostridium difficile* infection. *Annu. Rev. Med.*, 1998, **49**, 375-390.

KIRK J.A., BANERJI O., FAGAN R.P. Characteristics of the *Clostridium difficile* cell envelope and its importance in therapeutics. *Microb. Biotechnol.*, 2016, **17**.

KNIGHT D.R., PUTSATHIT P., ELLIOT B., RILEY T.V. Contamination of Australian newborn calf carcasses at slaughter with *Clostridium difficile*. *Clin. Microbiol. Infect.*, 2016, **22**, 266.e1-266.e7.

KOENIGSKNECHT M.J., THERIOT C.M., BERGIN I.L., SCHUMACHER C.A., SCHLOSS P.D., YOUNG V.B. Dynamics and establishment of *Clostridium difficile* infection in the murine gastrointestinal tract. *Infect. Immun.*, 2015, **83**, 934-941.

L

LEFEBVRE S.L., REID-SMITH R.J., WALTNER-TOEWS D., WEESE J.S. Incidence of acquisition of methicillin-resistant *Staphylococcus aureus*, *Clostridium difficile*, and other health-care-associated pathogens by dogs that participate in animal-assisted interventions. *J. Am. Vet. Med. Assoc.*, 2009, **234**, 1404–1417.

LIM S.C., FOSTER N.F., RILEY T.V. Susceptibility of *Clostridium difficile* to the food preservatives sodium nitrite, sodium nitrate and sodium metabisulphite. *Anaerobe,* 2016, **37**, 67-71.

LIZUKA M., ITOU H., KONNO S., CHIHARA J., TOBITA M., OYAMADA H., TOYOSHIMA I., SASAKI K., SATO A., HORIE Y., WATANABE S. Elemental diet modulates the growth of *Clostridium difficile* in the gut flora. *Aliment. Pharmacol. Ther.*, 2004, **20**, suppl.

1:151-157.

M

MCDONALD J.H. Handbook of biological statistics (3rd ed.). Sparky House Publishing, Baltimore, Maryland, [online], http://www.biostathandbook.com/multiplecomparisons.html, viewed 24 June 2016.

MARSH J.V., O'LEARY M.M., SHUTT K.A., SAMBOL S.P., JOHNSON S., GERDING D.N., HARRISON L.H. Multilocus variable-number tandem-repeat analysis and multilocus sequence typing reveal genetic relationships among *Clostridium difficile* isolates genotyped by restriction endonuclease analysis. *J. Clin. Microbiol.,* 2010, **48,** 412-418.

MARTINEZ B., CELDA M., ANASTASIO B., GARCIA I., LOPEZ-MENDOZA M.C. Microbiological sampling of carcasses by excision or swabbing with three types of sponge or gauze. *J. Food Prot.*, 2010, **73**, 81-87.

MEDINA-TORRES C.E., WEESE J.S., STAEMPFLI H.R. Prevalence of *Clostridium difficile* in horses. *Vet. Microbiol.*, 2011, **152**, 212-215.

MOORE J.H., PINHEIRO C.C., ZAENKER E.I., BOLICK D.T., KOLLING G.L., VAN OPSTAL E., NORANHA F.J., DE MEDEIROS P.H., RODRIGUEZ R.S., LIMA A.A., GUERRANT R.L., WARREN C.A. Defined nutrient diets alter susceptibility to *Clostridium difficile* associated disease in a murine model. *Plos One*, 2015, **16**, 10:e0131829.

MULLANY P., ALLAN E., ROBERTS A.P. Mobile genetic elements in *Clostridium difficile* and

their role in genome function. *Res. Microbiol.,* 2015, **166**, 361-367.

N

NAABER P., KLAUS K., SEPP E., BJORKSTEN B., MIKELSAAR M. Colonisation of infants and hospitalised patients with *Clostridium difficile* and lactobacilli. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.,* 1997, **25**, S189-S190.

NAABER P., SMIDT I., STSEPETOVA T., BRILENE H., ANNUK H., MIKELSAAR M. Inhibition of *Clostridium difficile* strains by intestinal *Lactobacillus* species. *J. Med. Microbiol.,* 2004, **53**, 551-554.

NEELY F., LAMBERT M.L. Epidemiology of *Clostridium difficile* in Belgium. Report 2015, [online], http://www.nsih.be/download/CDIF/CDIF-AR-2015-EN.pdf, viewed 07 March 2016.

NIWA H., KATO H., HOBO S., KINOSHITA Y., UENO T., KATAYAMA Y., HARIU K., OKU K, SENOH M., KURODA T., NAKAI K. Postoperative *Clostridium difficile* infection with PCR-ribotype 078 identified at necropsy in five thoroughbred racehorses. *Vet. Rec.*, 2013, **173**, 607.

P

PERRAS C., TSAKONAS E., NDEGWA S., CONLY J., VALIQUETTE L., FARRAH K. Vancomycin or metronidazole for the treatment of Clostridium difficile Infection: Clinical and Economic Analyses [Internet]. Ottawa: Canadia Agency for Drugs and Technologies in Health; 2011 (Technology report; no. 136), [online], http://www.ncbi.nlm.nih.gov/books/NBK174428/ viewed 21 April 2016.

R

REA M.C., O'SULLIVAN O., SHANAHAN F., O'TOOLE P.W., STANTON C., ROSS R.P., HILL C. *Clostridium difficile* carriage in elderly subjects and associated changes in the intestinal microbiota. *J. Clin. Microbiol.,* 2012, **50**, 867-875.

REIL M., HENSGENS M.P., KUIPER E.J., JAKOBIAK T., GRUBER H., KIST M., BORGMANN S. Seasonality of *Clostridium difficile* infections in Southern Germany. *Epidemiol. Infect.*, 2012, **140**, 1787-1793.

RODRIGUEZ C., KORSAK N., TAMINIAU B., AVESANI V., VAN BROECK J., DELMEE M. *Clostridium* difficile in elderly nursing home residents. *Anaerobe,* 2014, **30**, 184-187.

RODRIGUEZ-PALACIOS A., STAEMPLI H.R., DUFFIELD T., PEREGRINE L.A., TROTZ-WILLIAMS L.A., ARROYO L.G., BRAZIER J.S., WEESE J.S. *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg. Infect. Dis.*, 2006, **12**, 1730-1736.

RODRIGUEZ-PALACIOS A., STAEMPLI H.R., STALKER M., DUFFIELD T., WEESE J.S. Natural and experimental infection of neonatal calves with *Clostridium difficile*. *Vet. Microbiol.*, 2007, **124**, 166-172.

RODRIGUEZ-PALACIOS A., REID-SMITH R.J., STAEMPLI H.R., DAIGNAULT D., JANECKO N., AVERY B.P., MARTIN H., TOMSPON A.D., MCDONALD L.C., LIMBAGO B., WEESE J.S. Possible seasonality of *Clostridium difficile* in retail meat in Canada. *Emerg. Infect. Dis.*, 2009, **15**, 802-805.

RODRIGUEZ-PALACIOS A. Ecology and epidemiology of human pathogen *Clostridium difficile* in foods, food animals and wildlife, 2011, [online], https://etd.ohiolink.edu/ap:10:0::NO:10:P10_ETD_SUBID:74884, viewed 15 March 2016.

RODRIGUEZ-PALACIOS A., LEJEUNE J.T. Moist-heat resistance, spore aging, and superdormancy in *Clostridium difficile*. *Appl. Environ. Microbiol.* 2011, **77**, 3085-3091.

RODRIGUEZ-PALACIOS A., BORGMANN S., KLINE T.R., LEJEUNE J.T. *Clostridium difficile* in foods and animals: history and measures to reduce exposure. *Anim. Health Res. Rev.,* 2013, **14**, 11-29.

ROUSSEAU C., LEVENEZ F., FOUQUERAY C., DORE J., COLLIGNON A., LEPAGE P. *Clostridium difficile* colonization in early infancy is accompanied by changes in intestinal microbiota composition. *J. Clin. Microbiol.*, 2011, **49**, 858-865.

S

SHEPHERD M.L., SWECKER W.S.J., JENSEN R.V., PONDER M.A. Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. *FEMS Microbiol. Lett.,* 2012, **326**, 62-68.

SHIN N.R., LEE J.C., LEE H.Y., KIM M.S., WHON T.W., LEE M.S., BAE J.W. An increase in the *Akkermansia spp.* population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut*, 2014, **63**, 727-735.

SHIN J.H., HIGH K.P., WARREN C.A. Older is not wiser, immunologically speaking: effect of aging on host response to *Clostridium difficile* infection. *J. Gerontol. A. Biol. Sci. Med. Sci.*, 2016, **25**.

SCHOSTER A., ARROYO L.G., STAEMPFLI H.R., SHEWEN P.E., WEESE J.S. Presence and molecular characterization of *Clostridium difficile* and *Clostridium perfringens* in intestinal compartments of healthy horses. *BMC Vet; Res.*, 2012, **8**, 94.

SJORBERG M., ERIKSSON M., ANDERSSON J., NORENT T. Transmission of *Clostridium difficile* spores in isolation room environments and through hospital beds. *APMIS,* 2014, **30**.

SONGER J.G., POST K.W., LARSON D.J., JOST H., GLOCK R.D. Infection of neonatal swine with *Clostridium difficile*. *Swine Health Prod.*, 2000, **8**, 4.

SPONSELLER J.K., STEELE J.A., SCHMIDT D.J., KIM H.B., BEAMER G., SUN X., TZIPORI S. Hyperimmune bovine colostrum as a novel therapy to combat *Clostridium difficile* infection. *J. Infect. Dis.,* 2015, **211**, 1334-1341.

SPIGAGLIA P. Recent advances in the understanding of antibiotic resistance in *Clostridium difficile* infection. *Ther. Adv. Infect. Dis.,* 2016, **3**, 23-42.

SPINLER J.K., ROSS C.L., SAVIDGE T.C. Probiotics as adjunctive therapy for preventing *Clostridium difficile* infection- what are we waiting for? *Anaerobe*, 2016, in press.

SQUIRE M.M., RILEY T.V. *Clostridium difficile* infection in humans and piglets: a 'One Health' opportunity. *Curr. Top. Microbiol. Immunol.*, 2013, **365**, 299-314.

STEELE J., FENG H., PARRY N., TZIPORI S. Piglet models of acute or chronic *Clostridium difficile* illness. J.

Infect. Dis., 2010, **201**, 428-434.

STEELE J., SPONSELLER J., SCHMIDT D., COHEN O., TZIPORI S. Hyperimmune bovine colostrum for treatment of GI infections: a review and update on *Clostridium difficile Hum. Vaccin. Immunother.*, 2013, **9**, 1565–1568.

STEELMAN S.M., CHOWDHARY B.P., DOWD S., SUCHODOLSKI J., JANECKA J.E. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. *BMC Vet. Res.,* 2012, **8**, 1.

T

TAORI S.K., WROE A., HARDIE A., GIBB A.P., POXTON I.R. A prospective study of community-associates *Clostridium difficile* infections: the role of antibiotics and co-infections. *J. Infect.,* 2014, **69**, 134-144.

THEAN S., ELLIOTT B., RILEY T. *Clostridium difficile* in horses in Australia-a preliminary study. *J. Med. Microbiol.*, 2011, **60**, 1188-1192.

THERIOT C.M., YOUNG B.V. Microbial and metabolic interactions between the gastrointestinal track and *Clostridium difficile* infection. *Gut Microbes*, 2014, **5**, 86-95.

TRACEY L., KIRKE A., AMSTRONG P., RILEY T.V. From the hospital to the home—the rise and rise of *Clostridium difficile* infection. *Aust. Fam. Physisian*, 2015, **44**, 712-717.

U

UZAL F.A., DIAB S.S., BLANCHARD P., MOORE J., ANTHENILL L., SHAHRIAR F.,

GARCIA J.P., SONGER J.G. *Clostridium perfringens* type C and *Clostridium difficile* co-infection in foals. *Vet. Microbiol.*, 2012, **156**, 395-402.

V

VAN BROECK J., AVESANI V., NGYUVULA-MANTU E., DELMEE M. Distribution of ribotypes of *C. difficile* in Belgian acute hospitals: surveillance data 2009-2013, 2015, [online], http://icds.si/icds-2015/posters/P34_van%20broeck.pdf, viewed 07 March 2016.

W

WEESE J.S., TOXOPEUX L., ARROYO L. *Clostridium difficile* associated diarrhoea in horses within the community: predictors clinical presentation and outcome. *Equine Vet. J.*, 2006, **38**, 185-188.

Y

YAEGER M.J., KINYON J.M., SONGER J.G. A prospective, case control study evaluating the association between *Clostridium difficile* toxins in the colon of neonatal swine and gross microscopic lesions. *J. Vet. Diagn. Invest.*, 2007, **19**, 52-59.

Z

ZHANG K., MARTINOD S., SUN X. *Clostridium difficile* infection in horses. *Austin J. In Vitro Fertili.,* 2014, **1**, 5.

ZIDARIC V., PARDON B., DOS VULTOS T., DEPREZ P., BROUWER M.S.H., ADAM P., ROBERTS A.P., HENRIQUES A.O., RUPNIK M.

Clostridium difficile PCR-ribotypes 078, 126 and 033 in a single calf farm. *Appl. Environ. Microbiol.,* 2012, **78**, 8515-8522.

ANNEXES

Université de Liège

Publications and communications of
Cristina Rodriguez Diaz [u213982]

1. Dissertations and Theses

1.b. Doctoral thesis

3. Articles in peer reviewed academic journals

3.a. With an international target audience

As first or last author

📄 Rodriguez Diaz, C., Warszawski, N., Korsak Koulagenko, N., Taminiau, B., Van Broeck, J., Delmée, M., & Daube, G. (2016, June). Laboratory identification of anaerobic bacteria isolated on Clostridium difficile selective medium. *Acta Microbiologica et Immunologica Hungarica*.
http://hdl.handle.net/2268/195303
Peer reviewed ✔
ORBi viewed: **50** (11 ULg) ; downloaded: **40** (7 ULg) — SCOPUS®: **-**

📄 Rodriguez Diaz, C., Taminiau, B., Brévers, B., Avesani, V., Van Broeck, J., Leroux, A., Gallot, M., Bruwier, A., Amory, H., Delmée, M., & Daube, G. (2015). Faecal microbiota characterisation of horses using 16 rdna barcoded pyrosequencing, and carriage rate of clostridium difficile at hospital admission. *BMC Microbiology, 15*, 181.
http://hdl.handle.net/2268/186011
Peer reviewed (verified by ORBi) ✔
ORBi viewed: **77** (22 ULg) ; downloaded: **37** (8 ULg) — SCOPUS®: **-**
IF 2015: **?**; last: **2.729**; IF5: **3.251** — EigenF 2015: **?**; last: **0.024** — Article Infl. 2015: **?**; last: **1.0**

📄 Rodriguez Diaz, C., Korsak Koulagenko, N., Taminiau, B., Avesani, V., van Broeck, J., Brach, P., Delmée, M., & Daube, G. (2015). Clostridium difficile from food and surface samples in a Belgian nursing home: An unlikely source of contamination. *Anaerobe, 32*, 87-89.
http://hdl.handle.net/2268/178824
Peer reviewed (verified by ORBi) ✔
ORBi viewed: **36** (5 ULg) ; downloaded: **53** (7 ULg) — SCOPUS®: **0**
IF 2015: **?**; last: **2.479**; IF5: **2.628** — EigenF 2015: **?**; last: **0.005** — Article Infl. 2015: **?**; last: **0.7**

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Publications and communications of Cristina Rodriguez Diaz [u213982]

Rodriguez Diaz, C., Avesani, V., Taminiau, B., Van Broeck, J., Brévers, B., Delmée, M., & Daube, G. (2015). Investigation of clostridium difficile interspecies relatedness using multilocus sequence typing, multilocus variable-number tandem-repeat analysis and antimicrobial susceptibility testing. *Veterinary Microbiology*.

http://hdl.handle.net/2268/186819

Peer reviewed (verified by ORBi) ✔
ORBi viewed: **42** (9 ULg) ; downloaded: **34** (7 ULg) — SCOPUS®: **0**
IF 2015: **?**; last: **2.511**; IF5: **2.870** — EigenF 2015: **?**; last: **0.028** — Article Infl. 2015: **?**; last: **0.8**

Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., Michel, D., & Daube, G. (2015). Clostridium difficile infection and intestinal microbiota interactions. *Microbial Pathogenesis, 89*, 201-209.

http://hdl.handle.net/2268/194859

Peer reviewed ✔
ORBi viewed: **33** (11 ULg) ; downloaded: **1** (1 ULg) — SCOPUS®: **-**
IF: ? — EigenF: ? — Article Infl.: ?

Rodriguez Diaz, C., Taminiau, B., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2014). Multilocus sequence typing analysis and antibiotic resistance of Clostridium difficile strains isolated from retail meat and humans in Belgium. *Food Microbiology, 42*, 166-171.

http://hdl.handle.net/2268/165988

Peer reviewed (verified by ORBi) ✔
ORBi viewed: **61** (23 ULg) ; downloaded: **10** (10 ULg) — SCOPUS®: **4**
IF 2014: **3.331**; last: **3.331**; IF5: **3.675** — EigenF 2014: **0.013**; last: **0.013** — Article Infl. 2014: **1.0**; last: **1.0**

Rodriguez Diaz, C., Korsak Koulagenko, N., Taminiau, B., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2014). Clostridium difficile infection in elderly nursing home residents. *Anaerobe, 30*, 184-187.

http://hdl.handle.net/2268/174178

Peer reviewed (verified by ORBi) ✔
ORBi viewed: **46** (11 ULg) ; downloaded: **105** (5 ULg) — SCOPUS®: **0**
IF 2014: **2.479**; last: **2.479**; IF5: **2.628** — EigenF 2014: **0.005**; last: **0.005** — Article Infl. 2014: **0.7**; last: **0.7**

Rodriguez Diaz, C., Taminiau, B., Brévers, B., Avesani, V., Van Broeck, J., Leroux, A., Amory, H., Delmée, M., & Daube, G. (2014). Carriage and acquisition rates of Clostridium difficile in hospitalized horses, including molecular characterization, multilocus sequence typing and antimicrobial susceptibility of bacterial isolates. *Veterinary Microbiology*.

http://hdl.handle.net/2268/168650

Peer reviewed (verified by ORBi) ✔
ORBi viewed: **28** (6 ULg) ; downloaded: **1** (1 ULg) — SCOPUS®: **1**
IF 2014: **2.511**; last: **2.511**; IF5: **2.870** — EigenF 2014: **0.028**; last: **0.028** — Article Infl. 2014: **0.8**; last: **0.8**

Rodriguez Diaz, C., Avesani, V., Van Broeck, J., Taminiau, B., Delmee, M., & Daube, G. (2013). Presence of Clostridium difficile in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium. *International Journal of Food Microbiology, 166*(2), 256-262.

http://hdl.handle.net/2268/155252

Peer reviewed (verified by ORBi) ✔
ORBi viewed: **107** (12 ULg) ; downloaded: **1** — SCOPUS®: **13**
IF 2013: **3.155**; last: **3.082**; IF5: **3.746** — EigenF 2013: **0.029**; last: **0.026** — Article Infl. 2013: **1.0**; last: **1.0**

Rodriguez Diaz, C., Taminiau, B., Vandebroek, J., Avesani, V., Delmée, M., & Daube, G. (2012). Clostridium difficile in young farm animals and slaughter animals in Belgium. *Anaerobe, 18*(6), 621-625.

http://hdl.handle.net/2268/131955

Peer reviewed (verified by ORBi) ✔
ORBi viewed: **103** (12 ULg) ; downloaded: **4** (1 ULg) — SCOPUS®: **15**
IF 2012: **2.022**; last: **2.479**; IF5: **2.628** — EigenF 2012: **0.004**; last: **0.005** — Article Infl. 2012: **0.7**; last: **0.7**

As co-author

Janezic, S., Zidaric, V., Pardon, B., Indra, A., Kokotovic, B., Blanco, J. L., Seyboldt, C., Rodriguez Diaz, C., Poxton, I., Perreten, V., Drigo, I., Jiraskova, A., Ocepek, M., Weese, J., Songer, J., Wilcox, M., & Rupnik, M. (2014). International Clostridium difficile animal strain collection and large diversity of animal associated strains. *BMC Microbiology, 14*, 173.

http://hdl.handle.net/2268/194874

Peer reviewed (verified by ORBi) ✔
ORBi viewed: **11** (2 ULg) ; downloaded: **0** — SCOPUS®: **25**
IF 2014: **3.251**; last: **2.729**; IF5: **3.251** — EigenF 2014: **0.024**; last: **0.024** — Article Infl. 2014: **1.0**; last: **1.0**

8. Scientific conferences at universities and research centers

Rodriguez Diaz, C. (2015, October 16). *Carriage of Clostridium difficile in hospital patients in Spain, including molecular characterization and antimicrobial susceptibility of the isoates*. Paper presented at 5th Edition Farah Day, Liège, Belgium.
http://hdl.handle.net/2268/194866
ORBi viewed: **15** (3 ULg) ; downloaded: **1** (1 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Korsak Koulagenko, N., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2014, October 17). *Diversity of Clostridium difficile isolates from humans and animals*. Paper presented at Farah Day, Liège, Belgium.
http://hdl.handle.net/2268/194869
ORBi viewed: **12** (2 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., van Broeck, J., Delmée, M., & Daube, G. (2011, December 09). *Clostridium difficile in farm and slaughterhouse animals: prevalence and typing*. Paper presented at 1ST Scientific Meeting of the Faculty of Veterinary Medicine, Liège, Belgium.
http://hdl.handle.net/2268/194873
ORBi viewed: **16** (1 ULg) — SCOPUS®: **-**

9. Scientific congresses and symposia

9.b. On a personal proposal

Oral presentations only or conference poster

With an international target audience

Rodriguez Diaz, C., Oukbir, M., Taminiau, B., & Daube, G. (2016, May 11). *Association of Targeted metagenomic analysis and classical microbiology for Clostridium difficile detection and microbial ecosystem mapping of surfaces hands and foodstuffs in a meat processing plant*. Poster session presented at IAFP's 12th European Symposium of Food Safety, Athens, Greece.
http://hdl.handle.net/2268/198134
ORBi viewed: **17** (2 ULg) ; downloaded: **4**

Rodriguez Diaz, C. (2015, July 28). *Clostridium difficile, a new zoonotic agent. Characterization and relatedness of C. difficile strains isolated from animals, food and humans in Belgium*. Poster session presented at Interna'onal Association of Food Protection, Portland, Oregon.
http://hdl.handle.net/2268/194863
ORBi viewed: **18** (3 ULg) ; downloaded: **3** (2 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., Avesani, V., Fernandez, J., Boga, J. A., Vazquez, F., Delmée, M., & Daube, G. (2015, May 21). *CARRIAGE OF CLOSTRIDIUM DIFFICILE IN HOSPITAL PATIENTS IN SPAIN, INCLUDING MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY OF THE ISOLATES*. Poster session presented at ICDS 5th International Clostridium difficile Symposium, Bled, Slovenia.
http://hdl.handle.net/2268/184962
ORBi viewed: **38** (3 ULg) ; downloaded: **17** (4 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2015, April 21). *Heat survival of Clostridium difficile spores in ground meat during cooking process*. Poster session presented at IAFP's 11th European Symposium on Food Safety, Cardiff, Wales, England.
http://hdl.handle.net/2268/184175
ORBi viewed: **44** ; downloaded: **7** — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2015, April 01). *Association of classical microbiology and 16S rDNA metagenetic analysis to evaluate the presence of Clostridium difficile ina a belgian nursing home*. Poster session presented at 5th International Human Microbiome Congres, Luxembourg, Luxembourg.
http://hdl.handle.net/2268/181320
ORBi viewed: **82** (7 ULg) ; downloaded: **33** (9 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Korsak Koulagenko, N., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2014, May 07). *Comparison and molecular characterization of animal and human Clostridium difficile strains*. Poster session presented at IAFP 2014 European Symposium on Food Safety, Budapest, Hungary.
http://hdl.handle.net/2268/167545
ORBi viewed: **52** (8 ULg) ; downloaded: **36** (1 ULg) — SCOPUS®: **-**

244

Rodriguez Diaz, C., Taminiau, B., Korsak Koulagenko, N., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2013, October 22). *ASSOCIATION OF CLASSICAL MICROBIOLOGY AND TARGETED METAGENOMIC ANALYSIS TO EVALUATE THE PRESENCE OF CLOSTRIDIUM DIFFICILE IN A BELGIAN NURSING HOME*. Paper presented at ClostPath 2013 Meeting, Palm Cove, Tropical North Queensland, Australia.
http://hdl.handle.net/2268/162686
ORBi viewed: **64** (19 ULg) ; downloaded: **77** (4 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., & Daube, G. (2012, September 22). *Clostridium difficile in intestinal contents and on carcasses of pigs and cattle at slaughterhouse in Belgium*. Poster session presented at 4th International Clostridium difficile Symposium, Bled, Slovenia.
http://hdl.handle.net/2268/194871
ORBi viewed: **13** (1 ULg) ; downloaded: **3** — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., Delmee, M., & Daube, G. (2012, April 18). *Clostridium difficile in farm and slaughter animals in Belgium: detection and characterization*. Poster session presented at Biomedica, Liège, Belgium.
http://hdl.handle.net/2268/194870
ORBi viewed: **12** (1 ULg) ; downloaded: **0** — SCOPUS®: **-**

With a national target audience

Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., & Daube, G. (2015, October 16). *Characterization and relatedness of C. difficile strains isolated from animals, food and humans in Belgium*. Poster session presented at 5th Edition Farah Day, Liège, Belgium.
http://hdl.handle.net/2268/194864
ORBi viewed: **11** (2 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Avesani, V., Van Broeck, J., Delmée, M., & Daube, G. (2014, September 18). *Relatedness of human, animal and food Clostridium difficile strains*. Poster session presented at 19th Conference of Food Microbiology, Brussels, Belgium.
http://hdl.handle.net/2268/194867
ORBi viewed: **19** (4 ULg) ; downloaded: **1** (1 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Avesani, V., Korsak Koulagenko, N., Van Broeck, J., Delmée, M., & Daube, G. (2013, November 21). *Clostridium difficile: an emerging zoonotic pathogen ?* Paper presented at Diagnostic et surveillance des maladies infectieuses, Brussels, Belgium.
http://hdl.handle.net/2268/163963
ORBi viewed: **120** (7 ULg) ; downloaded: **57** (2 ULg) — SCOPUS®: **-**

Rodriguez Diaz, C. (2013, October 11). *MOLECULAR CHARACTERIZATION OF CLOSTRIDIUM DIFFICILE STRAINS FROM ELDERLY CARE HOME RESIDENTS*. Poster session presented at 3rd FMV Scientific Meeting 2013 – 11 October 2013 – Liège, Belgium, Liege, Belgium.
http://hdl.handle.net/2268/162685
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Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., & Daube, G. (2013, September 13). *Clostridium difficile in pigs and cattle at slaughterhouse, carcass contamination and prevalence in retail meat in Belgium*. Paper presented at Eighteenth Conference on Food Microbiology, Brussels, Belgium.
http://hdl.handle.net/2268/162720
ORBi viewed: **36** (2 ULg) ; downloaded: **231** (2 ULg) — SCOPUS®: **-**

Taminiau, B., Delhalle, L., Rodriguez Diaz, C., Nezer, C., Crèvecoeur, S., Piccolo, D., & Daube, G. (2013, July 01). *Impact of the use of lac.c/diace.c acids on the microbial diversity of white pudding samples during ageing under various condi.ons, with the use of next-gen sequencing and targeted metagenomic approach*. Poster session presented at Microbial Spoilers in Food, Quimper, France.
http://hdl.handle.net/2268/194868
ORBi viewed: **10** (2 ULg) ; downloaded: **2** — SCOPUS®: **-**

Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmée, M., & Daube, G. (2012, September 20). *Clostridium difficile is present in retail meat in Belgium*. Poster session presented at 17th Conference on Food Microbiology, Brussels, Belgium.
http://hdl.handle.net/2268/194872
ORBi viewed: **13** (1 ULg) ; downloaded: **2** — SCOPUS®: **-**

10. Book reviews

Rodriguez Diaz, C., Taminiau, B., Van Broeck, J., & Delmée, M. (2016). Clostridium difficile in foods and animals: A comprehensive review. *Advances in Experimental Medicine and Biology, 27*.
http://hdl.handle.net/2268/199069
Peer reviewed ✔
ORBi viewed: **0** ; downloaded: **0**
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