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A review of *Clostridioides [Clostridium] difficile* occurrence through the food chain

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ABSTRACT

The epidemiology and transmission of *Clostridioides difficile*, particularly for community-associated infections, are not completely understood. Although there have been no confirmed cases of any foodborne disease caused by *C. difficile,* its occurrence in livestock and foods suggests that contaminated food products with spores could be a vehicle to spread *C. difficile* infection. This review proposes potential sources of *C. difficile* infection in the community and contamination routes of food products. Based on European research, it also summarizes the occurrence and organism characterization of *C. difficile* in animals at slaughterhouses and in human foods. Most of the analyzed literature reported prevalence in retail foods of less than 8%, including microorganism belonging to the ribotype 078, an important hypervirulent strain involved in disease in humans. This prevalence in Europe is underestimated, being lower that reported in North America (rates up to 42%), probably due of the lack of an ISO procedure for the detection of *C. difficile* in food products that preclude the comparison of prevalence data from different studies. The survival and growth of vegetative *C. difficile* cells and the resistance of its spores in foods are discussed as well as the risk factors of acquisition CDI from food products.

1. Introduction

Clostridium difficile, recently renamed *Clostridioides difficile* (Lawson et al., 2016), is an important spore-forming human pathogen associated with serious enteric diseases worldwide, being the major causative agent of nosocomial diarrhea and pseudomembranous colitis (Lyerly et al., 1982). *C. difficile* produces two potent exotoxins, toxin A and B, secreted into the colonic environment that are the major virulence factors responsible for its pathogenesis (Martin-Verstraete et al., 2016). In large subpopulation of strains is also present an additional toxin (the binary toxin or CDT), that has been associated with more severe disease (Barbut et al., 2005), although not proven to cause disease on its own (Eckert et al., 2015). Emphasis has been placed on the clinical epidemiology and virulence properties of PCR ribotype 027 (Valiente et al., 2014), although other *C. difficile* strains - ribotypes 0126, 018, 056, 078, and 244- also have a propensity to cause outbreaks with increased severity, high relapse rate, and significant mortality (Quesada-Gómez et al., 2015; Rupnik et al., 2009).

Traditionally, *C. difficile* infection (CDI) has been associated with patients who were given broad-spectrum antibiotics in hospitals. However, from 2000 onwards, the epidemiology of *C. difficile* has been changing and reviews describe an increase in community-associated CDI that is not linked to traditional risk factors (recent antibiotic therapy, older age, significant comorbidity, or previous hospitalization) (Bauer et al., 2009; Bignardi, 1998; Wilcox et al., 2008). Comparative genomics supports these observations that a large proportion of CDI originate from non-hospital sources (Hoover and Rodriguez-Palacios, 2013; Janvilisri et al., 2009; Knight et al., 2017; Kuijper et al., 2006; Weese et al., 2009). This epidemiological perspective aims to investigate the environment, animals, and food products as new possible routes of transmission of *C. difficile*. In consideration with these aspects, the growth and resistance of *C. difficile* in food products, patient susceptibility, and molecular relationships among the strains isolated from food and humans, should be taken into account to assess the risk of the presence of *C. difficile* in food products.

2. *Clostridioides difficile* introduction in food chain

2.1. Possible sources and contamination routes of Clostridioides difficile to food products

Literature presents several hypotheses about *C. difficile* transmission but, considering its obligate anaerobic nature, the ingestion of the aerotolerant, metabolically dormant endospores seems to be responsible for the transmission of CDI (Deakin et al., 2012; Otten et al., 2010). Food products could be a vehicle for the spread of *C. difficile*

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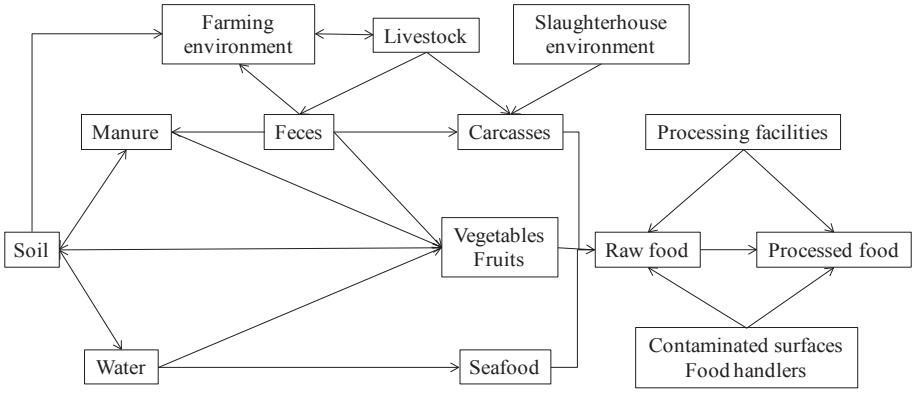

Fig. 1. Flow diagram of the main routes of *C. difficile* spore contamination of foods.

Table 1
Review of presence and organism characterization of *Clostridioides difficile* at slaughterhouses, from European studies.

Country	Year(s)[a]	% Positive (N positive samples/N tested samples)	% Toxigenic (N toxigenic/N all isolates)	PCR-ribotypes[b]	Reference
Intestinal content: Pig					
The Netherlands	2009	28% (14/50)	100% (14/14)	015	Hopman et al. (2011)
	2009–2010	8.6% (58/677)	NT	013, 014, 078	Keessen et al. (2011a)
	2009–2010	ND (0/100)	–	–	Koene et al. (2012)
Austria	2008	3.3% (2/61)	100% (2/2)	AI-50, 126	Indra et al. (2009)
Belgium	2011–2012	1% (1/100)	100% (1/1)	078	Rodriguez et al. (2013)
	2011	ND (0/194)	–	–	Rodriguez et al. (2012)
Switzerland	2010	ND (0/165)	–	–	Hoffer et al. (2010)
Intestinal content: Beef cattle					
Belgium	2011–2012	9.9% (10/101)	100% (10/10)	078, 029	Rodriguez et al. (2013)
	2011	6.9% (14/202)	69.2% (9/13)	002, 014, 081	Rodriguez et al. (2012)
The Netherlands	2009–2010	6% (6/100)	100% (6/6)	012, 033	Koene et al. (2012)
Austria	2008	4.5% (3/67)	66.7%% (2/3)	AI-225, 014/0	Indra et al. (2009)
Switzerland	2010	0.5% (1/204)	100% (1/1)	078	Hoffer et al. (2010)
Intestinal content: Chicken					
Austria	2008	5% (3/59)	66.7% (2/3)	001, 446	Indra et al. (2009)
The Netherlands	2009–2010	5% (5/100)	80% (4/5)	003, 014, 056	Koene et al. (2012)
Carcasses: Pig					
Belgium	2011–2012	7% (7/100)	–	014, 081, UCL36	Rodriguez et al. (2013)
Carcasses: Beef cattle					
Belgium	2011–2012	7.9% (8/101)	–	UCL5a, UCL16u	Rodriguez et al. (2013)

ND: Not determined, NT: Not tested, -: Data not available or not applicable.
[a] Year(s) when the study was conducted or year when the study was published.
[b] Main PCR-ribotypes found with standard Cardiff nomenclature.

endospores and there are many scenarios that might cause contamination within the food chain as shown in Fig. 1.

The many reports on *C. difficile* demonstrate that this organism is ubiquitous in natural settings, including soils and water, both fresh and sea (Pasquale et al., 2011; Salf and Brazier, 1996; Zidaric et al., 2010), as well as in untreated and treated water from waste water treatment plants (Romano et al., 2012a,b; Xu et al., 2014). The presence of *C. difficile* in wastewater treatment plants, which can be introduced into water courses, might also constitute a potential source of community-associated CDI (Nikaeen et al., 2015; Romano et al., 2012b). Seafood, predominantly edible shellfish, known for their capacity to concentrate various pathogens from water through filter feeding, has been shown to contain *C. difficile* (Romano et al., 2012a,b). An inverse relationship between *C. difficile* and *Salmonella* spp. and *Escherichia coli* has been reported, suggesting a possible environmental contamination of the samples with polluted water rather than a contamination with human waste (Troiano et al., 2015).

Horse and pig manures, traditional organic fertilizers for crops, can also contain spores of *C. difficile* (Medina-Torres et al., 2011; Usui et al., 2017). Thus, the application of composted manure to land poses a possible risk of *C. difficile* transfer to the food chain. Contamination of vegetables and fruits could occur as a result of irrigation or washing with contaminated water (Rupnik and Songer, 2010). Moreover, spore transfer to fresh produce via fertilizer is a realistic probability even if good agricultural practices are followed (Usui et al., 2017). Then, minimally processed or uncooked vegetables or fruits could be potential vehicles in CDI.

Food animals are recognized carriers of *C. difficile* (Dubberke et al., 2011; Freeman et al., 2010; Keel et al., 2007) and several reports demonstrate the shedding of *C. difficile* in animals at slaughter (Rodriguez-Palacios et al., 2011b; Weese et al., 2011). Contamination of carcasses and meats could be a result of gut content spillage during evisceration or perhaps accumulation of spores within the slaughterhouse environment (EFSA, 2013).

If livestock are a potential source of *C. difficile*, food contaminated with the feces of colonized or infected animals could also be one of the transmission routes from animals to humans via the food chain. The majority of studies have focussed on retail meats - especially beef, pork, and poultry - but *C. difficile* has been found in a range of different foods, from vegetables to seafood, taken directly from grocery stores all over the world (Gould and Limbago, 2010; Pirs et al., 2008; Quesada-Gomez et al., 2013; Rahimi et al., 2014; Weese et al., 2010a,b,c).

The presence of spores in end products can be explained by initial contamination of raw materials, cross-contamination in the food industry, and production of spores during food processing (Gauvry et al., 2016; Heyndrickx, 2011). Regarding the domestic environment, the

presence of spores on kitchen surfaces and in refrigerators may indicate transfer to food products (Weese et al., 2010a).

2.2. *Clostridioides difficile* occurrence in slaughterhouses

For a better understanding of the contamination of meats with *C. difficile*, it would be interesting to determine its ocurrence in samples of fully-grown animals at slaughterhouses. Table 1 summarizes the presence of *C. difficile* and the associated PCR ribotypes at European slaughterhouses. In the viscera processing area of the slaughter line, *C. difficile* was detected in the intestinal contents of up to 28% of pigs (Hopman et al., 2011), up to 9.9% of beef cattle (Rodriguez et al., 2013), and 5% of broiler chickens (Indra et al., 2009; Koene et al., 2012). Furthermore, *C. difficile* was detected in 7% of pig carcasses just after fast chilling in the chilling room (Rodriguez et al., 2013). Similarly, *C. difficile* has also been described in 7.9% of veal carcasses (Rodriguez et al., 2013).

The prevalence of *C. difficile* in intestinal contents at slaughterhouses in Europe is higher than that reported from North America (the United States and Canada) or Australia. In these countries, *C. difficile* was detected in the intestinal contents of up to 6.9% of pigs (Norman et al., 2011; Susick et al., 2012) and up to 3.8% of beef cattle (Knight et al., 2016; Rodriguez-Palacios et al, 2011a, 2011b). However, *C. difficile* presence in carcasses in Europe are slighty lower than those reported in North America, where *C. difficile* has been described in 2.5–15% of pork carcasses (Hawken et al., 2013; Norman et al., 2011; Susick et al., 2012) and 4–16.7% of beef carcasses (Houser et al., 2012; Knight et al., 2016). In other studies no carcass or intestinal content tested positive, evidencing a low contamination of the production chain (Kalchayanand et al., 2013; Knight and Riley, 2013; Rodriguez-Palacios et al., 2011c).

It should be noted that young swine, calves, and poultry on farms are colonized intestinally by *C. difficile* more frequently than fully-grown animals at slaughterhouses (Rodriguez et al., 2016). Previous studies reported spore or toxin detection ranging between 1.4 and 96% in piglets with normal feces and this prevalence decreases with age, varying from 0 to 23% at finishing on the farm or at slaughter (Rodriguez et al., 2016). Similarly, prevalence of up to 56% has been reported in healthy calves aged less than three months old (Rodriguez et al., 2016). For poultry, more than 60% carry *C. difficile* early in production, but by the time of slaughter incidence rates decline to between 6 and 12% (Zidaric et al., 2008). With or without signs of enteric disease, a decrease in the prevalence rate of *C. difficile* is observed in adult animals (Rodriguez et al., 2016). The reason for this age effect is still unknown. A probable explanation is that the bacterium is better able to colonize and proliferate in the intestinal tract of younger animals, where the gut microbiota is less developed (Rodriguez-Palacios et al., 2006).

Fig. 2 shows the factors that may influence the ocurrence level and concentration of *C. difficile* in food of animal origin at the farm and during processing at slaughterhouse. Scarce data are available about the carcass contamination with *C. difficile* spores, their germination and inactivation during the recycling process, and the potential for accumulation during the operations. *C. difficile* was detected in 3.5% of swabs taken from the environment around hamburger-processing plants in Iran (Esfandiari et al., 2014b). The authors suggested that this environmental contamination might be due to any residual organic material involved in biofilm formation facilitating the attachment of *C. difficile* spores to meat processed. In contrast, in a study conducted in sausage-manufacturing plants in Texas, sponge swabs collected from the equipment and facilities yielded no *C. difficile* isolates, while meat samples tested positive for the bacterium, indicating meat contamination with *C. difficile* from the intestinal contents (Harvey et al., 2011b). These findings suggest that carcasses and meats could be contaminated by direct contact with fecal material, somewhere during processing, rather by another external source such as via the environment or via the hands of infected personnel (Rodriguez et al., 2016). Some concern has been raised in a Scientific Opinion done by the European Commission to European Food Safety Authority (EFSA) regarding the use of recycled hot water at slaughterhouses and the risk of contamination from heat-resistant spores among others *C. difficile* (EFSA, 2010). For carcass decontamination purposes, only use of potable water is currently allowed in the European Union. However, recycling of water (i.e. reusing after reheating) used for carcass decontamination has been practiced in some countries (e.g. Canada, Denmark) (Porsbo and Agersø, 2016).

2.3. *Clostridioides difficile* occurrence in food products

The detection of genetically similar *C. difficile* strains in livestock, food, and humans has led to an increased awareness of the potential for *C. difficile* as an unspecific foodborne agent (Goorhuis et al., 2008a; Hoover and Rodriguez-Palacios, 2013; Janezic et al., 2014; Knight et al., 2015; Rupnik, 2007; Rupnik et al., 2009; Songer and Anderson, 2006; Steinmuller et al., 2006). The majority of studies have focused on retail meats, but *C. difficile* spores responsible for disease in humans also have been found in other foods including vegetables and seafood.

Table 2 reviews the presence and organism characterization of *C. difficile* in meats reported in European studies. These manuscripts describe values ranging from 1.9 to 6.3% in retail meat samples. As shown in Table 2, the retail ground beef samples were positive from more countries than retail pork or chicken meat, that only were positive in a country. Generally, the reported prevalence of *C. difficile* in meats from Europe is lower than for those from the United States and Canada. In North America, prevalence rates have been reported in retail meat of up to 42% in various categories of meats (Songer et al., 2009). The reasons for this disparity between the results from North America and those from Europe are not completely clear. Rodriguez-Palacios et al. (2009) found higher presence in winter and proposed that seasonal differences may account for the different prevalence data. The sampling strategies, the size of operation, the types of food examined along with methods of culture of the different countries may also hinder the comparison of the prevalence data from the studies.

C. difficile is also isolated from fresh produce and seafood that are minimally processed. Table 3 reviews the presence and organism characterization of *C. difficile* in vegetables, seafood and prepared meal reported in European studies. These works describe values ranging from 2.2 to 7.5% in vegetables and from 3.9 to 49% in seafood. The higher presence was observed in bivalve mollusks, mainly sampled in highly polluted areas of Italy (Pasquale et al., 2011, 2012) The scarce data about the presence of *C. difficile* in seafood and raw vegetables in North America shows that it is similar than for those from Europe. Studies show isolaton rates of 4.5% in seafood in Texas (Norman et al., 2014), and 47.4% in oyster in Louisiana (Montazeri et al., 2015). Metcalf et al. (2010) found *C. difficile* in 4.5% of raw vegetables in Canada.

C. difficile was isolated from only one prepared-meal sample prepared in an Belgian nursing home (Rodriguez et al., 2015). This meal - composed of pork sausage, mustard sauce, and carrot salad - had been prepared in the canteen kitchen and the contamination could have originated from any of the ingredients or as a result of manipulation. In contrast, a study in Texas showed that 25% of the meat meals served to patients in hospital settings were contaminated with *C. difficile* (Koo et al., 2012).

C. difficile PCR ribotypes 017, 027, and 078, which are found in community-associated CDI, are also isolated in food products and livestock (Bauer et al., 2011; Goorhuis et al., 2008a, 2008b; Janezic et al., 2012; Keel et al., 2007; Rodriguez-Palacios et al., 2006; Rodriguez et al., 2014; Weese et al., 2010b). Ribotypes 001 and 014 were the most isolated from several types of food from different European countries, including poultry, vegetables, and shellfish. Ribotype 078, an important hypervirulent strain involved in disease in humans (Indra et al., 2015; Janezic and Rupnik, 2015), has been found in pork and beef from Belgium, in mussels from Italy, and in a prepared meal

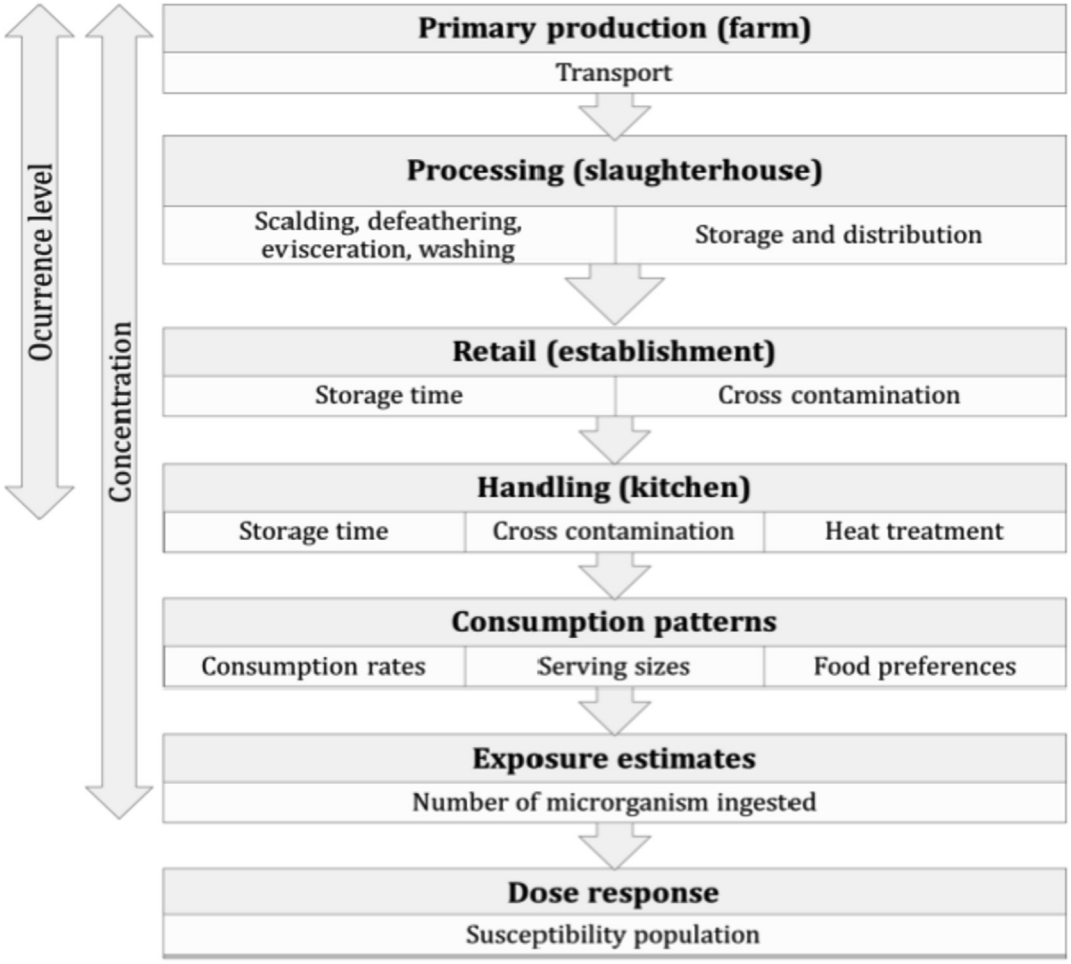

Fig. 2. Elements of a 'farm-to-fork' exposure assessment.

from a Belgian nursing home. In North America, the hypervirulent ribotypes 027 and 078 are the predominant strains isolated from foods (Songer et al., 2009).

3. Survival and growth of vegetative *Clostridioides difficile* cells in food products

Due its anaerobic nature, vegetative cells of *C. difficile* are greatly sensitive to oxygen (Jump et al., 2007). Outside the anaerobic conditions of the large bowel, *C. difficile* has to be in the spore form to survive in food environment and other aerobic surfaces (Gauvry et al., 2016; Kramer et al., 2006; Shaughnessy et al., 2016). The data available concerning the isolation of *C. difficile* in vacuum packaged foods (Bouttier et al., 2010; Broda et al., 1996) and from 4% of the samples of modified-atmosphere-packaged minced beef tested by Atasoy and Gücükoğlu (2017) indicate that removing oxygen from food vacuum-packaging could allow *C. difficile* to survive in food products. Changes in food production which favour anaerobic growth could though influence the occurrence of *C. difficile* and other anaerobic bacteria in the food. This should be taken into consideration, especially in regards to ready-to-eat products produced for consumers with risk factors for CDI (Porsbo and Agersø, 2016).

C. difficile is able to form a biofilm on abiotic surface, embedded in a matrix comprised of DNA, polysaccharides and proteins, including toxins (Dapa et al., 2013; Dawson et al., 2012; Pantaléon et al., 2015; Semenyuk et al., 2014). Biofilms have been shown to enhance the resistance of *C. difficile* cells to oxygen stress as well as to standard sanitation regimes in health care settings, abattoir environments, and industry (Barra-Carrasco and Paredes-Sabja, 2014; Båverud et al., 2003; Dixit et al., 2005; Kramer et al., 2006; Maillard, 2011; Paredes-Sabja et al., 2014). Despite this ability, *C. difficile* spores may become entrapped within biofilms formed by other bacteria, rather than to form biofilms (Esfandiari et al., 2014b).

C. difficile is not still included as a foodborne pathogen. Thus, there are fewer data about the vegetative cell growth and its viability in foods during storage compared with other pathogenic *Clostridium* species. The majority of works about *C. difficile* have studied the stability of spores of a small selection of *C. difficile* strains and it had been carried out in clinical samples or in food models (Deng et al., 2015, 2017; Flock et al., 2016; Redondo-Solano et al., 2016; Rodriguez-Palacios et al., 2010; Rodriguez-Palacios and Lejeune, 2011). Warriner et al. (2016) showed that vegetative cells of ribotype 027 and 078 are sensitive to acid (minimum pH of 6.5), salt (maximum 4%) and are able to grow at 22 °C and 45 °C with an optimum of 37 °C, within the pH range of 7–9. Nevertheless, there remains large knowledge gaps with respect to growth ranges of *C. difficile*, especially in real foods matrices.

It is generally accepted that there is a need that *C. difficile* endospores germination should be initiated prior to grow out as vegetative cells and produce toxins. Like most spores, the germination can be enhanced by heat treatments at between 60 and 100 °C (Ghosh et al., 2009; Rodriguez-Palacios and Lejeune, 2011), as used in preparing end products. In contrast with *Bacillus* and other *Clostridium* spores which sense nucleosides, sugars, amino acids, and ions (Ghosh et al., 2009; Paredes-Sabja et al., 2011), *C. difficile* spores germinate in response to the combination of specific bile salts (taurocholate, glycocholate, cholate and deoxycholate) and L-glycine acting as a co-germinant (Paredes-

Table 2
Review of presence and organism characterization of *Clostridioides difficile* in meats, from European studies.

Country	Sample material	% Positive (N positive samples/N tested samples)	% Toxigenic (N toxigenic/N all isolates)	PCR-ribotypes[a]	References
Retail beef					
Sweden	Ground beef, hamburger	5% (2/40)	100% (2/2)	NT	Von Abercron et al. (2009)
Belgium	Ground beef, hamburger	2.3% (3/133)	100% (3/3)	014, 078	Rodriguez et al. (2014)
France	Ground beef	1.9% (2/105)	100% (2/2)	012	Bouttier et al. (2010)
The Netherlands	Beef	ND (0/145)	–	–	de Boer et al. (2011)
Austria	Beef, ground beef	ND (0/51)	–	–	Indra et al. (2009)
Switzerland	Ground beef	ND (0/46)	–	–	Hoffer et al. (2010)
Austria	Ground beef	ND (0/30)	–	–	Jöbstl et al. (2010)
Retail pork					
Belgium	Ground pork, pork sausage	4.7% (5/107)	80% (4/5)	014, 078, UCL57, UCL378	Rodriguez et al. (2014)
The Netherlands	Pork	ND (0/63)	–	–	de Boer et al. (2011)
France	Pork sausage	ND (0/59)	–	–	Bouttier et al. (2010)
Switzerland	Ground pork	ND (0/46)	–	–	Hoffer et al. (2010)
Austria	Ground pork	ND (0/27)	–	–	Jöbstl et al. (2010)
Austria	Pork, ground pork	ND (0/27)	–	–	Indra et al. (2009)
Sweden	Ground pork	ND (0/11)	–	–	Von Abercron et al. (2009)
Retail beef/pork					
Austria	Ground beef/pork	3% (3/100)	33.3% (1/3)	057	Jöbstl et al. (2010)
Sweden	Ground beef/pork, hamburger, cooked sausages	ND (0/19)	–	–	Von Abercron et al. (2009)
Poultry products					
The Netherlands	Chicken meat	2.7% (7/257)	57.1% (4/7)	001, 003, 087, 071	de Boer et al. (2011)
Austria	Chicken meat	ND (0/6)	–	–	Indra et al. (2009)
Sweden	Poultry, cooked sausages	ND (0/4)	–	–	Von Abercron et al. (2009)
Retail lamb					
The Netherlands	Lamb	6.3% (1/16)	100% (1/1)	045	de Boer et al. (2011)
Sweden	Sheep	ND (0/7)	–	–	Von Abercron et al. (2009)

ND: Not determined, NT: Not tested, -: Data not available or not applicable.
[a] Main PCR-ribotypes found with standard Cardiff nomenclature.

Sabja et al., 2014; Warriner et al., 2016). Generally, the food matrices do not meet these bile salts requeriments, thus it is doubtful that *C. difficile* spores would be able to germinate in food products. To our knowledge, there have been no reports published in the literature on *C. difficile* spore germination in food systems (Warriner et al., 2016).

Neither are there enough data about the lability of *C. difficile* toxins in food systems. A few works report that toxins could be stable from −20 to 37 °C for up to 4 weeks stored in a *in vitro* models (Sullivan et al., 1982; Weese et al., 2000). Sullivan et al. (1982) showed that there was a 99% loss of toxin activity within 6 min at 56 °C for both toxins in a *in vitro* model. Thus, it should taken into account the pre-formed toxin presence in raw or poorly cooked food. Anyway, both

Table 3
Review of presence and organism characterization of *Clostridioides difficile* in other foods, from European studies.

Country	Sample material	% Positive (N positive samples/N tested samples)	% Toxigenic (N toxigenic/N all isolates)	PCR-ribotypes[a]	References
Vegetables					
Scotland	Ready-to-eat salads	7.5% (3/40)	100% (3/3)	001, 017	Bakri et al. (2009)
France	Ready-to-eat salads	3.3% (2/60)	100% (2/2)	001, 014/020/077	Eckert et al. (2013)
France	Ready-to-eat vegetables	2.2% (1/44)	100% (1/1)	015	Eckert et al. (2013)
UK	Raw vegetables	2.3% (7/300)	71.4% (5/7)	NT	Salf and Brazier (1996)
Seafood					
Italy	Bivalve molluscs	49% (24/49)	58% (14/24)	001, 002, 003, 010, 012, 014/020, 018, 045, 070, 078, 106	Pasquale et al. (2012)
Italy	Bivalve molluscs	43% (9/21)	56% (5/9)	003, 005, 009, 010, 056, 066	Pasquale et al. (2011)
Italy	Bivalve molluscs	3.9% (36/925)	52% (19/36)	001, 009, 010, 017, 018, 031, 051, 073, 078, 085, 100, 106, 120, 126, 204	Troiano et al. (2015)
Other foods					
Wales	Fish gut content	ND (0/107)	–	–	Salf and Brazier (1996)
Austria	Raw milk	ND (0/50)	–	–	Jöbstl et al. (2010)
Prepared meal					
Belgium	Meal (canteen kitchen)	0.5% (1/188)	100% (1/1)	078	Rodriguez et al. (2015)

ND: Not determined, NT: Not tested, -: Data not available or not applicable.
[a] Main PCR-ribotypes found with standard Cardiff nomenclature.

Table 4
Methods of culture used for the isolation of *Clostridioides difficile* in European studies.

Reference	Amount of sample	Volume of enrichment broth	Germinant used in enrichment broth	Time of incubation in the enrichment broth	Treatment for spore recovery	Solid medium
Salf and Brazier (1996)	NA	NU	NU	NU	NU	Brazier's Medium
Bakri et al. (2009)	50 g	NA	NA	NA	NU	CDMN
Indra et al. (2009)	5 g	20 mL	NU	12 days	NU	CCFA
Von Abercron et al. (2009)	25 g	50 mL	NU	10–12 days	Alcohol shock	FAA *C. difficile* agar
Bouttier et al. (2010)	5 g	100 mL	Taurocholate	3 days	Alcohol shock	Columbia agar (supplemented with cefoxitin, cycloserine, taurocholate, and horse blood)
Hoffer et al. (2010)	20 g	20 mL	NU	7 days	Heat shock (80 °C, 10 min)	*C. difficile* agar
Jöbstl et al. (2010)	5 g	20 mL	NU	10 days	NU	Schädler agar CCFA
de Boer et al. (2011)	5 g	20 mL	Taurocholate	10–15 days	Alcohol shock	CDMN
Pasquale et al. (2011)	10 g	10, 40 and 80 mL	Taurocholate	10 days	Alcohol shock	*C. difficile* agar (supplemented with moxolactam, norfloxacin, and horse blood)
Pasquale et al. (2012)	10 g	40 mL	Taurocholate	10 days	Alcohol shock	CCEY
Eckert et al. (2013)	20 g	75 mL	Taurocholate	3 days	Alcohol shock	TCCA
Rodriguez et al. (2014)	10 g	90 mL	Taurocholate	3 days	Alcohol shock	NA
Rodriguez et al. (2015)	50 g	150 mL	Taurocholate	10 days	NU	CCFT
Troiano et al. (2015)	10 g	40 mL	Taurocholate	10 days	Alcohol shock	CCEY

NA: Not available; NU: Not used.
Brazier's Medium (Cefoxitin, cycloserine and sodium salt of cholic acid); CDMN (*Clostridium difficile* Moxolactam, Norfloxacin); CCFA (Cycloserine, Cefoxitin, Fructose Agar); FAA (Fastidious Anaerobe Agar, non-selective medium); *C. difficile* agar (Cefoxitin, Cycloserine); CEY (Cefoxitin, Cycloserine, Egg Yolk); TCCA (Taurocholate, Cycloserine, Cefoxitin Agar); CCFT (Cycloserine, Cefoxitin, Fructose, Taurocholate).

toxins were inactivated at pH 2.0 (Sullivan et al., 1982), consequently might be expect that preformed toxins would also be destroyed at acid gastric juice of the host stomach after its ingestion (Fordtran, 2006), and would be almost impossible to get the intestine in an active form.

Even in the unlikely case of the spore germination and growth of *C. difficile* in foods, measures applied to control either *C. perfringens* or *C. botulinum* would also be effective against the growth of vegetative *C. difficile* cells (Lund and Peck, 2015). *C. difficile* growth showed susceptibility to the preservative nisin (Le Lay et al., 2016) and other natural products as vegetal extracts or essential oils (Aljarallah, 2017, 2016; Roshan et al., 2017). Nevertheless, *C. difficile* can survive in the presence of other preservatives commonly applied to control clostridia - namely nitrite, nitrate, and sodium metabisulfite - at concentrations higher than their maximum levels allowed in commercial products (Lim et al., 2016).

4. Resistance of *Clostridioides difficile* spores in food products

There are no established guidelines for recommended maximum levels of spores in food products or in animals at slaughterhouses. In the case of *C. perfringens*, a major cause of foodborne illness, the growth of the bacterium to give $> 10^5$/g in the food consumed is considered to result in food poisoning (Heredia and Labbé, 2013). In the case of *C. difficile*, direct plating method indicate that the number of spores is low, being 7 cfu/cm^2 for veal calves carcasses (range 3–33 cfu/cm^2), 30 spores/g (range 20–60 spores/g) for pork, and 100 spores/g (range 20–240 spores/g) for beef (Knight et al., 2016; Weese et al., 2009) (Weese et al., 2009). Despite these low levels of spores that were detected, in comparison with other pathogens, the key feature of *C. difficile* in foods is that its spores are highly resistant to extreme physical conditions.

Viability of *C. difficile* spores subjected to chilling, freezing and cooking was investigated by Flock et al. (2016). Results revealed that chilling for a week or freezing for 12 weeks did not affect the survival of *C. difficile* spores in ground beef. Moreover, Flock (2017) indicated that *C. difficile* spores can survive the acidity and cooking in fermented pork summer sausage over a period of 60 days at 4 °C.

The recommended cooking temperature to kill bacteria (71 °C, 160 °F) are ineffective to inactivating its spores (Flock et al., 2016; Rodriguez-Palacios et al., 2010). An inhibitory effect on *C. difficile* spores has been observed after a heat shock at > 96 °C (> 204.8 °F) for 15 min (Rodriguez-Palacios and Lejeune, 2011). However, Redondo-Solano et al. (2016) found that underestimation of the thermal resistance of *C. difficile* spores can occur when water or other liquid media are used to determine the thermal destruction parameters of spores. They suggested that the use of a specific food matrix for the determination of thermal resistance of *C. difficile* spores is necessary to accurately calculate or predict the lethality of a thermal process during food processing.

5. Methods for detection of *Clostridioides difficile* in food products

There is no gold standard or ISO procedure for the detection of *C. difficile* in food products, leading to the use of different methods and culture techniques for its detection (Rupnik and Songer, 2010). These methodological variations preclude the comparison of prevalence data from different studies.

Contamination with *C. difficile* spores may be relatively common in food products although spore numbers tend to be low. A few works have employed quantitative culture methods to provide information about the number of *C. difficile* in food samples (Knight et al., 2016; Weese et al., 2009). However, these enumeration methods may not detect very low spore numbers of *C. difficile* in samples. The enrichment method that was used by Weese et al. (2009) has a detection threshold of ≤10 spores/g, while the detection threshold of the quantitative procedure is somewhat higher (20 spores/g. In some samples, the test for *C. difficile* only gave a positive result on enrichment culture and the use of other detection methods with lower sensitivity may have underreported the prevalence (Weese et al., 2010a,b,c). Thus, the use of enrichment broth prior to plating on solid medium is recommended to maximize the *C. difficile* recovery (Arroyo et al., 2005a,b; Blanco et al., 2013).

Table 4 summarizes the different critical points of the methods used to culture *C. difficile* isolated from retail food in European studies. As any foodborne microbe, variations in the amount of sample analyzed could also affect the recovery rates. A small amount of sample may not reflect the real contamination due to a non-homogeneous distribution of the *C. difficile* spores in the sample. In some North American studies, instead of using a portion of the sample as the inoculum, a rinse of the object of interest with phosphate-buffered saline is taken and then, mixed by hand in a sterile plastic bag (Harvey et al., 2011a; Weese et al., 2010a; b; c; Weese et al., 2009). Different volumes of enrichment broth and distinct times of incubation have been used. The possibility of competition for the broth's nutrients with other microorganisms present in the sample, influenced by the volume of broth and the days of incubation, also could affect the growth and survival of *C. difficile*.

Another critical point is if the enrichment broth is supplemented or not. Lysozyme and specific bile salts such a cholate and its derivatives (taurocholate, glycocholate, cholate, and deoxycholate) enhance the recovery of *C. difficile* spores (Sorg and Sonenshein, 2008; Wilson et al., 1982). It should be noted that the spore response to taurocholate is diminished subsequent to thermal treatment. The use of broth supplemented with lysozyme may improve the recovery of heat-injured spores and, thus, is more suitable for studying the thermal resistance of *C. difficile* in food products, especially those that rely on thermal processing alone (Kamiya et al., 1989; Redondo-Solano et al., 2016).

In order to facilitate the isolation of *C. difficile,* different selective methods are used. Termic treatment or ethanol shock, are based on the high survival capacity of the spores at high temperatures and ethanol, against the inactivation of the vegetative forms of contaminants present in the sample (Koransky et al., 1978; Marler et al., 1992; Rodriguez-Palacios and Lejeune, 2011). Selective antibiotics employed in solid medium, such cefoxitin and cycloserine (CCFA; Indra et al., 2009) or moxalactam and norfloxacin (CDMN; Weese et al., 2009), could also be affecting *C. difficile* isolation.

A consensus protocol using 10 g of sample in 90 mL of BHI enrichment broth supplemented with taurocholate, for 3–5 days, followed by ethanol shock and plating to selective and non-selective media was proposed for the isolation of *C. difficile* (Limbago et al., 2012).

Traditional culture provides an isolate for further typing methods, wich are essential for epidemiological studies (Avbersek, 2017; Knetsch et al., 2013). Historically, restriction enzyme analysis and pulsed-field gel electrophoresis have been the methods of choice in North America, whereas PCR ribotyping has primarily been used in Europe. As a result, epidemic strains are often indicated with multiple typing designations and comparison of results between laboratories is unreliable (Tenover et al., 2011). Capillary gel-based electrophoresis PCR ribotyping has been standardized and validated in a collaborative effort of the European Centre for Disease Control and Prevention, the US Centers for Disease Control and Prevention and the Public Health Agency of Canada, and is likely to become common place throughout the world (Fawley et al., 2015). However, PCR ribotyping may not provide sufficient discrimination to differentiate closely related populations (Huber et al., 2013). Whole genome sequencing and phylogenetic analysis are necessary to track the evolutionary relationships between various *C. difficile* strains of livestock, food products and humans (Knetsch et al., 2013; Knight et al., 2017; Marsh, 2013).

6. Risk of adquisition of *Clostridioides difficile* infection from food products

The presence of toxigenic *C. difficile* strains on ready-to-eat foods as

well as the demonstrated potential for spores to survive freezing and cooking processes suggests that ingestion of these spores from contaminated food products is a realistic scenario. Fig. 2 shows the factors that may influence the ocurrence level and concentration of *C. difficile* during retailing and handling as well as the role of consumption patterns, exposure (infectious dose of spores), and population response (susceptibility population).

At present there are no documented cases of CDI related with a consumption pattern. Søes et al. (2014) reported that, in patients aged 52 years, consumption of beef was associated with CDI when multivariable analysis conditional logistic regression was performed [odds ratio 5.5, 95% confidence interval 3.1–23]. However, further studies are needed to advise vulnerable consumers not to eat beef to avoid CDI. Following a request from the EFSA, the scientific reports conducted by the Panel on Biological Hazards and the Panel on Contaminants in the Food Chain regarding the hazards of adquire CDI from different types of meat confirm the insufficient knowledge in the area and conclude that the risk is low based on the limited available evidence (Porsbo and Agersø, 2016).

Infectious dose of spores that results in colonization and possibly disease has not been established for *C. difficile*. Spores, rather than vegetative cells, need to be ingested to cause CDI (Warriner et al., 2016). Thus, the germination and growth of *C. difficile* in food products prior to their ingestion is not a requisite for the pathogen to cause foodborne illness. Even in the unlikely case of the food matrices meet the optimal growth conditions (lack of oxigen, ph, temperature) for as long as necessary, the vegetative cells and toxins have marginal survival in gastric content at low pH. However, it should be taken into account that the risk presumably varies according to susceptibility population.

The risk of adquisition of CDI from food products varies greatly between healthy individuals and vulnerable population with risk factors for developing CDI (Cózar-Llistó et al., 2016), being the factor determining the imbalance of the microbiota. Buffie et al. (2015) and Theriot and Koenigsknecht, 2014 studied the importance of the microbiota in maintaining bile acid homeostasis and resistance to outgrowth of pathogenic bacteria. In normal health, in upper ileum, bile acids are deconjugated by microbial-derived bile salt hydrolases into primary bile acids such as cholate, taurocholate, and glycocholate (Ridlon et al., 2006), which can stimulate the germination of *C. difficile* spores after its ingestion (Bhattacharjee et al., 2016; Francis et al., 2013). On the other hand, in lower ileum, secondary bile acids (deoxycholic acid) inhibit *C. difficile* growth, protecting against CDI. With a disrupted microflora, the primary bile acids increase due to they are not metabolized to secondary bile acids, thereby activating the germination of *C. difficile* that then undergoes overgrowth (Allegretti et al., 2016). Moreover, the perturbations of the commensal flora and therefore decreasing their colonization resistance, could leading to the outgrowth of vegetative *C. difficile* cells in the colon and cecum, where enterotoxins are produced.

Antimicrobial therapy is the most widely reported risk factor for CDI in humans (Bloomfield and Riley, 2016; Delaney et al., 2007). It is scientifically accepted that an exposure to antibiotics disrupting the colonic microbiota in the intestine and leading to an overgrowth by *C. difficile*. However, international studies have shown CDI cases had no previous antibiotic exposure (Collins et al., 2014; Dial et al., 2005). Other factors may play a role in community-acquired CDI, such the intake of proton pump inhibitors (PPIs) (Gupta and Khanna, 2014; Kuntz et al., 2011). The induced decrease of stomach acid by PPIs can facilitate the survival of spores, vegetative cells, and toxins in gastric contents at pH > 5 allowing them to directly colonise the intestinal tracts of susceptible hosts (Jump et al., 2007). Indeed, long-term PPIs use has an effect on the ratio of *Firmicutes* to *Bacteroidetes*, that may predispose to the development of CDI (Clooney et al., 2016). There is also evidence that PPIs disrupt the host immune system that would ordinarily reduce the risk of CDI (Larcombe et al., 2016). However, other studies report contradictory results about PPIs as a risk factor for CDI (Bavishi and DuPont, 2011; Hensgens et al., 2011; Naggie et al., 2011).

In adittion to the decrease of stomach acid and microbiota disruption, the ability of *C. difficile* to cause colitis depends on further steps, including adherence to intestinal epithelium, colonization, toxin endocytosis, toxin-mediated intestinal damage, and activation of the host inflammatory immune response (Albert et al., 2016). Toxins are the major virulence factor responsable for CDI. Toxin A, also called enterotoxin, causes fluid accumulation in several animal models, and toxin B has been described as a cytotoxin, which induce diarrhea, inflammation, and damage of colonic mucosa (Voth and Ballard, 2005). Toxins A and B are chromosomally encoded in a region termed the pathogenicity locus (PaLoc), which also contains regulatory genes, and is absent in non-toxigenic strains (Elliott et al., 2014; Martin-Verstraete et al., 2016). Non-toxigenic strains have been isolated from human, animal, and environmental samples, including food products. Some studies demonstrated a protective effect of non-toxigenic *C. difficile* colonization against toxigenic infection in the hamster model. However, with human patients there are several as yet unanswered questions (Natarajan et al., 2013). It should be noted that other putative virulence factors have been described, including the production of other toxins (Janoir, 2016). Moreover, approximately 11% of the *C. difficile* genome is made up of mobile genetic elements. This includes transfer of antibiotic resistance and other factors that allow the organism to survive challenging environments, modulation of toxin gene expression, transfer of the toxin genes themselves, and the conversion of non-toxigenic strains into toxin producers (Brouwer et al., 2013; Mullany et al., 2015; Peng et al., 2017). Roy Chowdhury et al. (2016) suggest that toxin-negative strains of *C. difficile* that are efficient colonisers of the host gastrointestinal tract may readily acquire the PaLoc via lateral gene transfer and evolve to become future hypervirulent strains. Studies in animal models are needed to examine the pathogenic potential of toxin-negative strains of *C. difficile* and to determine the frequency by which toxin-negative strains may acquire the PaLoc and express clinically relevant levels of toxins.

Given our current knowledge, it is difficult to develop an exposure assessment to analyze the risk of exposure of food contamination by *C. difficile* and to develop risk management strategies that reduce the risk of transmission via such a route. What is certain is that changes in food-preparation protocols inside and outside health care settings deserve attention, especially to protect vulnerable people during periods of high risk (i. e., neutropenia) (Lund, 2014). Food should be ensured to be heated to more than 85 °C as a simple and important intervention to reduce the risk of inadvertent ingestion of *C. difficile* spores (Rodriguez-Palacios and Lejeune, 2011). As *C. difficile* could still survive cooking temperatures and multiply in heated foods, it is also recommended that foods should be properly chilled and stored as indicated for other other clostridia foodborne pathogens (Porsbo and Agersø, 2016).

Contamination of food products with *C. difficile* spores does not necessarily mean foodborne disease although this is one of several potential routes for CDI. In fact, not all strains found in foods are present in CDIs, and vice versa (Walk et al., 2012). Some authors have reported no molecular relationship between clinical human and meat isolates and, therefore, that sources other than meat may be responsible for CDI (Esfandiari et al., 2014a).

7. Conclusions

Although the data regarding *C. difficile* in food are compelling, care must be taken when interpreting the currently available studies. Only a limited number of studies have been published, and these have typically involved a small number of geographical regions. Most of the worldwide studies report less than 20% of food contaminated with *C. difficile* while in some European studies most of the works analyzed reported a prevalence of less than 8%, that is lower than that reported in North America (rates up to 42%). The establishment of an ISO procedure for the isolation of *C. difficile* from food products is indeed necessary to

evaluate inter-region variation in prevalence.

There is no conclusive evidence that the presence of *C. difficile* in food products a risk to the consumer and hence *C. difficile* is currently regarded as an unspecified foodborne agent. Further research is needed to clarify the knowledge gaps with respect to germination and growth ranges of *C. difficile* in real foods matrices. Even exposed to *C. difficile*, the risk of community-associated CDI depends on individual circumstances and on the type of the *C. difficile* strain.

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