Agriculture and Food

AIMS Agriculture and Food, 5(1): 102–128.
DOI: 10.3934/agrfood.2020.1.102
Received: 25 September 2019
Accepted: 24 February 2020
Published: 27 February 2020

http://www.aimspress.com/journal/agriculture

Review

Methods currently applied to study the prevalence of *Clostridioides difficile* in foods

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Abstract: *Clostridioides difficile* is responsible for most cases of antibiotic- and hospital-associated diarrhoea. Several studies have demonstrated the presence of *C. difficile* in different foods such as meat, raw milk, vegetables and seafood, which supports the hypothesis that foods contaminated with spores may be contributing to the exposure to and transmission of *C. difficile*.

Generally, the prevalence of *C. difficile* in foods is low and there is no standard methodology for its isolation. Available methods have been optimized for stool samples rather than foods.

In the majority of the studies, a similar base culture medium has been used and different selective and enrichment compounds are further added, which is, sometimes, controversial. Despite the extensive use of cycloserine and cefoxitin, as well as moxalactam and norfloxacin, many authors believe that the use of these selective supplements had an adverse effect on the recovery of *C. difficile* and only enabled recovery of resistant isolates from food samples. Another example is the use of sodium taurocholate to potentiate the germination of *C. difficile* spores; there are studies reporting that the addition of this component in the enrichment medium did not exert a beneficial effect on *C. difficile* recovery. Variations in sample amounts, dilution factors, incubation times, among others, may also affect the recovery of *C. difficile* from foods.

Numerous studies have recently emerged, since there is increasing interest in *C. difficile* as a potentially foodborne pathogen. Thus, the purpose of this review is to summarize the methodologies currently used on the isolation/detection of *C. difficile* in foods and its subsequent characterization and typing.

Keywords: culture media; detection; enumeration; molecular techniques; recovery

Abbreviations: BB: Brucella broth; BHI: Brain Heart Infusion broth; CCEY: selective media with egg yolk emulsion, cefoxitin and cycloserine; CDI: *Clostridioides difficile* infection; CDMN: *C. difficile* moxalactam norfloxacin; CDSA: *C. difficile* selective agar; CFU: Colony forming units; GDH: Glutamate dehydrogenase; MALDI-TOF MS: Matrix-assisted laser desorption ionization–time of flight mass spectrometry; MB: Mannitol broth; MLST: Multi-locus sequence typing; MLVA: Multi-locus variable-number tandem-repeat analysis; PCR: polymerase chain reaction; PFGE: Pulsed-field gel electrophoresis; RFLP: Restriction fragment length polymorphism; ST: Sequence type number; TB: Thioglycolate broth; TBHI: Brain heart infusion with 0.1% sodium taurocholate; TCCFB: selective media with cycloserine and cefoxitin; TCM: cooked meat medium with 0.1% sodium taurocholate; TCMN: selective media with cysteine hydrochloride, moxalactam and norfloxacin

1. Introduction

Clostridioides difficile (formerly *Clostridium difficile*) [1] is a spore-forming human pathogen that is the main cause of antibiotic- and hospital-associated diarrhoea [2]. Colonization of humans by *C. difficile* spores may promote a toxin-mediated enteric disease designated *C. difficile* infection (CDI). The production of exotoxins A and B and/or binary toxin is the main factor mediating the pathogenesis of *C. difficile* disease [3,4]. Toxins A and B are encoded by tcdA and tcdB genes, respectively, located within a region of the chromosome identified as pathogenicity locus or PaLoc [4]. Binary toxin is encoded by two genes (*cdtA* and *cdtB*) that are outside the PaLoc region at the site of the binary toxin locus in the genome (CdtLoc) [3].

There has been a dramatic change in the epidemiology of *C. difficile* infection in the past decade, with the emergence and spread of new and hypervirulent strains and an increase in the incidence of community-acquired CDI, particularly in populations previously not considered at high risk [5]. Given the absence of clear explanations for such a rapid increase in rates of CDI in recent years, a common vehicle of *C. difficile* spores dissemination such as via food products may not be excluded. Many studies have demonstrated their presence in four food categories: i) meat, such in ground beef [6], pork meat [7], chicken meat [8,9] and poultry meat [10]; ii) dairy products, e.g. raw milk [11]; iii) vegetables, e.g. raw vegetables [12], leafy greens [13], processed vegetables [14] and lettuce [15–17], and iv) seafood, e.g. molluscs [18], shellfish [11,19] and seafood [20]. Contamination of these foods by *C. difficile* spores may be due to the fact that they are susceptible to faecal contamination. The primary mode of transmission of enteric pathogens occurs via the faecal-oral route [21]. As an enteric pathogen, *C. difficile* spores can be disseminated ubiquitously in the environment. Bivalve molluscs filter water and accumulate enteric pathogens when raised in polluted waters [22] and *C. difficile* has been found in seawater [23]. According to Xu et al. [24], *C. difficile* can survive sewage treatments and can be recovered from biosolids along with an effluent discharge, which could explain its detection in vegetables, since the water, manure, compost and/or biosolids are probably contaminated. Romano et al. [11,25] found that wastewater treatment plant effluents represent a potential for spreading of toxigenic *C. difficile* strains.

Clostridioides difficile species comprises several ribotypes [2] and the most commonly identified as a cause of disease in humans have been also found in foods such as retail meat (ribotypes 027 and 078) [26–28], raw milk (ribotype 078) [11] and ready-to-eat salads (ribotypes 001, 078 and 126) [9,11,12].

Food additives are used in the food industry to assure safe foods with an extended shelf life. In order to determine the in vitro susceptibility of *C. difficile* to three food preservatives used in ready-to-eat products—sodium nitrite (E250), sodium nitrate (E251) and sodium metabisulfite (E223)—Lim et al. [29] demonstrated that their use in concentrations complying with the maximum value established by legislation does not prevent the germination of *C. difficile* spores. Therefore, it is doubtful if the use of these additives is a good barrier to the survival of these microorganisms in food.

Since there is still no standard methodology for isolation and/or enumeration of *C. difficile* in foods, different researchers have used different successfully developed methodologies applied for stool samples [30–32]. However, differences in methodologies compromise the comparison of data reported in different studies.

This review attempts to summarize all existing methodologies used for the isolation/detection of *C. difficile* in foods and its subsequent characterization and typing. The intention of the authors is to draw attention to the need for standard methodologies, without proposing the best methodology, but analysing those already used.

2. Recovery of *C. difficile* from food samples

Since there is no standardized methodology for the isolation/detection of *C. difficile* from foods, researchers have been using methodologies based on those used in clinical settings to isolate *C. difficile* from faecal samples.

Isolation of *C. difficile* from foods can be influenced by numerous parameters, described below, used for enrichment and/or recovery/isolation and identification. All published studies on the prevalence of *C. difficile* in foods found so far are listed in Table 1, comprising the food product, its origin, number of samples and amount of sample analyzed, the enrichment culture and/or recovery/isolation culture media used and the percentage of positive samples for *C. difficile* and toxigenic *C. difficile* detected.

2.1. Quantitative and enrichment culture methods

Detection following enrichment step was the technique of choice reported in most of the studies listed in Table 1. Direct culture, without previous enrichment step, was conducted as a single technique by the authors of merely four studies [35,53,58,60]. Both culture methods were performed in seven studies [6,9,12,33,38,40,46].

In the studies of Razmyar et al. [35] and Visser et al. [53], the authors reported a prevalence of 15.3% and 6.3% of *C. difficile* by direct culture in chicken meat, respectively. Also by direct culture in cooked kidney and flesh, Kouassi et al. [58] reported a prevalence of 12.4% and Al Said and Brazier [60] reported a prevalence of 2.3% by sampling directly from surfaces of raw vegetables. Different percentages are reported by the authors that used both culture methods. While no *C. difficile* was isolated from poultry and raw meat, respectively, in the studies of Abdel-Glil et al. [33] and Indra et al. [40] nor by direct culture nor by enrichment culture, Weese et al. [6] detected *C. difficile* from ground beef (12%) and ground pork (12%) by enrichment culture (8.7%), by direct culture (1.7%) and by both enrichment and direct culture (1.7%). Something remarkable about this study was the fact that samples positive by direct culture were negative by enrichment culture. The authors explained these results with the hypothesis of possible low-levels of *C. difficile* in meat

samples and its subsequently non-homogeneous distribution [6]. Mooyottu et al. [38] found a prevalence of 0.7% after analyses of 300 ground meat samples, but the authors did not mention if the only two *C. difficile* isolates were recovered by detection or direct culture techniques. Meat samples positive for *C. difficile* reported in the studies of Von Abercron et al. [46], Bakri et al. [12] and Weese et al. [9] were positive only by the enrichment procedure with a prevalence of 2.0, 7.5 and 12.8%, respectively.

In another study about the prevalence of *C. difficile* in uncooked ground meat published by Curry et al. [30], the authors performed enumeration of *C. difficile* by MPN (most-probable-number) but only for all samples that were positive by detection. All 13 meat products positive for *C. difficile* were negative for recovery by enumeration with low level of spore contamination from <0.18 to 0.45 spores/g.

Probably due to the hypothetical low numbers of *C. difficile* in food, detection with an enrichment step is the most widely used technique in published studies.

2.2. Sampling

As with any microbiological analysis, random sampling, numbers of food samples and their amount used for bacteriological examination should be as representative as possible, regardless of the technique used (enumeration or detection). Although most protocols are based on testing for *C. difficile* in stool samples where the pathogen is present at high levels, small amounts of food sample may not reveal the true level of contamination of *C. difficile* due to its non-homogeneous distribution [6]. Different amounts of food samples have been used to assess the presence of *C. difficile*, ranging from 70 mg to 50 g [19,27,33,36,38,42,43,46,55]. Moreover, in eight studies with meat products (Table 1), the authors added phosphate-buffered saline to the respective amount of sample and only then transferred a certain volume of that dilution to an enrichment medium, instead of transferring the sample directly [6,9,10,33–35,37,39]. All these differences may affect the recovery rates of *C. difficile* from foods.

Table 1. Summary of procedures and main results of published studies on the prevalence of *C. difficile* in foods.

Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for*C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Meat									
Poultry meat	Egypt	150	1 mL (1 g in 9 mL PBS)	(1) TCDMN (9 mL) (2) na	7–10 days	(1) CDMN agar (2) CDMN agar (0.1 mL from PBS suspension)	(1) 0.0 (2) 0.0	(1) na (2) na	[33]
Poultry meat	USA	32	Two pieces in 50 mL PBS	A: Weese et al. [9] B:Rodriguez-Palacios et al. [54] C: 10 mL in enrichment broth of procedure B	A: 48 h B: 14 days C: 14 days	A: CDMN agar B: CDMN agar with 5% (v/v) horse blood C: CCFN agar, Brucella agar and TCCFNHB agar	A: 3.1 B: 6.3 C: 12.5	100.0	[10]
Retail chicken	Canada	203	1 mL (entire piece in 50 mL PBS)	(1) TCDMN (9 mL) (2) na	48 h	(1) CDMN agar (2) CDMN agar (0.1 mL from PBS suspension)	(1) 12.8 (2) 0.0	100.0	[9]
Retail chicken meat parts	Turkey	310	1 mL (10 g in 10 mL PBS)	TCDMN (9 mL)	48–72 h	CDMN agar with 7% (v/v) horse blood	8.1	32.0	[34]
Chicken portions	Iran	65	Sample pieces in mL PBS	na	na	A: Columbia agar with 5% (v/v) sheep blood (0.1 mL from PBS suspension) B: CCEY agar (0.1 mL from PBS suspension)	15.3	70.0	[35]

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Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for *C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Meat									
Raw chicken, beef and pork meat	Korea	415	5 g	TCDMN (20 mL)	15 days	CDMN agar and TSA with 5% (v/v) blood agar	10.8	4.4	[36]
Beef and chicken meat	Turkey	101	1 mL (25 g in 25 mL PBS)	TCDMN (9 mL)	48 h	CDMN agar	1.98	0.0	[37]
Ground beef and pork meat, and chicken wings	USA	300	50 g	(1) TCDMN (50 mL) (2) na	48 h	(1) CDMN agar (2) CDMN agar (1 mL from 50 mL TCDMN suspension)	0.7	0.0	[38]
Retail beef, pork and poultry meat	Costa Rica	200	A: 25 g in 225 mL saline solution 0.85% B: 2–3 g	A: na B: BHICC (10 mL)	A: na B: 22 days	A: Pellet after centrifugation of ethanol treated culture in CCF agar (5 days) and FAB (15 days) B: CCF agar	2.0	100.0	[39]
Retail beef, chicken, pork and turkey meat	USA	303	10 g	TCDMNHB (50 mL)	10 days	CDMN agar	10.2	81.0	[28]
Raw beef, pork and chicken meat	Austria	84	5 g	(1) TB (20 mL) (2) na	(1) 12 days (2) na	(1) CCFN agar (2) CCFN agar (from TB suspension before incubation)	0.0	na	[40]

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Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for *C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Meat									
Retailed raw beef, pork, calf, lamb and chicken meat	Netherlands	500	5 g	TCDMNHB (20 mL)	10–15 days	CDMN agar	1.6	62.5	[41]
Raw beef, minced pork and chicken samples and traditional sausages	Portugal	143	10 g	CDMN with 7% (v/v) horse blood (20 mL)	7 days	CDMN agar	0.0	na	[42]
Raw ground beef, buffalo, chicken, lamb, pork, turkey and veal meat	USA	102	10 g	CCMB-TAL (100 mL)	5 days	TSA with 5% (v/v) sheep blood	12.7	100.0	[30]
Retail beef, chicken and pork meat, and hamburger products	Brazil	80	2 g	TB (10 mL)	7 days	CCFHB agar	0.0	na	[43]
Retail ground pork, beef, chicken, turkey, beef and pork sausages	USA	40	5 g	TCCF (20 mL)	15 days	CCFN agar	7.5	100.0	[44]
Raw beef, pork, chicken, turkey and lamb and processed meat	USA	342	55 g	TCDMN (50 mL)	A: na B: 48 h	A: CDMN agar B: CDMN agar (1 mL of treated sample with ethanol)	0.0	na	[45]

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Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for *C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Meat									
Retail ground beef, pork, sheep, poultry, calf, reindeer, hamburger and cooked sausages and hamburger	Sweeden	82	25 g	(1) CDMN (50 mL) BHI (50 mL) (2) na	10–12 days	(1) CDMNHB agar and FAA (2) CDMNHB agar and FAA (from CDMN and BHI suspensions before incubation)	(1) 2.0 (2) 0.0	100.0	[46]
Ground pork and pork chop	Canada	393	15 g	TCD (50 mL)	7 days	Columbia Blood agar	1.8	85.7	[47]
Raw and ready-to-eat pork	Taiwan	127	25 g	CCBB-TC (50–60 mL)	7 days	CHROMagar™ *C. difficile*	29.9	23.7	[7]
Ground beef	USA	956	1 g	CDMN (9 mL)	15 days	CDMN[1] agar	0.0	na	[48]
Retail ground beef	Canada	60	4–5 g	TCDMNHB (20 mL)	10–15 days	CDMN agar with 5% (v/v) horse blood	20	91.7	[49]
Hamburgers and hamburgers ingredients	Iran	211	5 g	TCDMN (25 mL)	5–7 days	CDMN agar with 7% (v/v) sheep blood	4.2	100.0	[50]
Minced beef and pork meat	Switzerland	46	10 g	A: BHI (20 mL) B: BHI (20 mL and heat shocked)	7 days	CCFN agar	0.0	na	[51]
Ground beef and pork meat	Austria	100	5 g	CDMN (20 mL)	10 days	Schädler agar and CDSS agar	3.0	33.3	[52]
Retail beef[a] and pork meat[b]	Belgium	133[a] 107[b]	10 g	TCCF[1] (90 mL)	3 days	TCCFN agar	2.3[a] 4.7[b]	87.5	[27]

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Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for *C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Meat									
Retail beef and pork	Canada	230	1 mL (25 g in 25 mL PBS)	(1) TCDMN (9 mL) (2) na	48 h	(1) CDMN agar (2) CDMN agar (0.1 mL from PBS suspension)	(1) 8.7 (2) 1.7	100.0	[6]
Retail ground beef and pork meat	USA	48	20 g	na	na	CDMN agar (0.1 mL of treated sample with ethanol)	6.3	100.0	[53]
Ground beef[a] and pork sausages[b]	France	105[a] 59[b]	5 g	TBHICC[1] (100mL)	72 h	Columbia cysteine agar supplemented with cefoxitin-cycloserine, taurocholate and 5% horse blood	1.9[a] na[b]	100.0[a] na[b]	[54]
Beef and mutton meat	Iran	200	5 g	CDMN (25 mL)	7 days	CDMN agar	4.0	100.0	[31]
Retail beef, pork, turkey meat, uncooked pork sausage and chorizo and RTE sausages	USA	88	1 g	TBHIYEC (10 mL)	72 h	TCCFN agar	42.0	100.0	[8]
Ground beef and turkey meat	USA	1231	10g	A: TCDMN (90 mL) B: TBHI (90 mL) C: TBHI (90 mL and heat shocked)	1,3 and 5 days	A: Anaerobic Blood Agar B: TCCFA C: TCDMN agar	0.0	na	[32]

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Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for *C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Meat									
Retail ground beef and veal chop	Canada	214	2 g	A: TCDMN (20 mL) B: TCCF (20 mL)	7 days	A: CDMN agar with 7% (v/v) horse blood B: 5% (v/v) defibrinated sheep blood agar	6.1	80.0	[55]
Raw beef, buffalo, camel, cow, goat and sheep meat	Iran	660	5 g	TCDMNHB (20 mL)	10–15 days	CDMN agar with 7% (v/v) horse blood	2.0	92.3	[56]
Ground veal	USA	50	1 g	TBHIYEC (9 mL)	96 h	TCCFN agar	6.0	100.0	[57]
Raw cow, sheep and goat meat	Saudi Arabia	600	5 g	TCCFN with 5% (v/v) sheep blood	10–15 days	TCCFN with 7% (v/v) sheep blood	1.5	88.8	[26]
Cooked kidney Cooked flesh	Ivory Coast	395	10 g in 90 mL BPW	na	na	TSC agar	12.4	x	[58]
Dairy products									
Raw milk	Austria	50	5 mL	CDMN (20 mL)	10 days	CDMN agar with 7% sheep blood	0.0	na	[52]
Raw milk	Italy	6 *C. difficile* isolates	20 mL	TBHICC[2] (20 mL)	10 days	CDMN agar with 5% (v/v) horse blood	x	100.0	[11]
Seafood									
Seafood and fish	Canada	86	15 g	TCDMN without potassium dihydrogen phosphate (50 mL)	7 days	Blood agar	4.8	80.0	[20]

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Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for *C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Seafood									
Retail seafood	USA	67	70 mg	TCDMN (100 mL)	7 days	CDMN agar with 5% (v/v) laked horse blood	4.5	100.0	[19]
Shellfish	Italy	6	10 g	TCDMN (40 mL)	10 days	CDMN agar with 5% (v/v) horse blood	66.7	50.0	[23]
Bivalve molluscs	Italy	53	10 g	TBHICC[2] (40 mL)	10 days	CDMN agar with 5% (v/v) horse blood	49.1	57.7	[59]
Shellfish	Italy	1 *C. difficile* isolate	10 g	TBHICC[2] (40 mL)	10 days	CDMN agar with 5% (v/v) horse blood	x	100.0	[11]
Bivalve molluscs	Italy	925	10 g	TBHICC[2] (40 mL)	10 days	CDMN agar with 5% (v/v) horse blood	3.9	52.0	[18]
Vegetables									
Raw vegetables	Wales	300	surface	na	na	TCCFN (contact plates)	2.3	71.4	[60]
Ready-to-eat salads (baby leaf spinach, organic lettuce and organic mixed leaf salad)	Scotland	40	50 g	(1) TCDMNHB (20 mL) (2) na	10–15 days	(1) CDMN agar with 5% (v/v) horse blood (2) CDMN agar with 5% (v/v) horse blood (from TCDMNHB suspension before incubation)	7.5	100.0	[12]
Ready-to-eat raw vegetables	France	44	20 g	TBHICC[2] (75 mL)	72 h	TBHICC agar with 5% (v/v) defibrinated horse blood	2.9	100.0	[15]
Lettuce	USA	297	40 g	TBHICC[2] (40 mL)	10 days	CDSA	47.1	26.1	[16]

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Food product	Country	Sampling		Enrichment culture		Recover/isolation culture medium	% Positive for *C. difficile*	%Toxigenic *C.difficile*	Reference
		N° ofsamples	Amount	Medium and volume	Incubationtime				
Vegetables									
Retail root vegetables	Australia	300	10 g	TBHICCYEC (9 mL)	10 days	ChromID *C. difficile*	≥10.0–30.0	51.2	[61]
Carrots, eddoes and Ginger	Canada	111	Smaller sized	TCDMN (50 mL)	7 days	Columbia blood agar	4.5	100.0	[14]
Fresh vegetables	USA	125	15 g	TCCFC (50 mL)	7 days	CCF agar	2.4	100.0	[17]
Ready-to-eat salads	Italy	3 *C. difficile* isolates	10 g	TBHICC[2] (40 mL)	10 days	CDMN agar with 5% (v/v) horse blood	x	100.0	[11]
Raw vegetables	Slovenia	154	25 g	TBHICCYE (200 mL)	5–7 days	CCF agar	18.2	8.7	[62]
Ready-to-eat salads	Iran	106	20 g	TCDMN (30 mL)	10–15 days	CDMN agar with 5% (v/v) horse blood	5.66	16.7	[13]
Others									
Ready-to-eat foods	Iran	368	5 g	TCDMNSB (20 mL)	10–15 days	CDMN agar with 7% (v/v) defibrinated sheep blood	1.36	100.0	[63]
Meals	Belgium	188	50 g	TCCF[1] (150 mL)	3 days	CCFN agar	0.53	100.0	[64]

(1) Detection; (2) Enumeration; na: Not applicable; x: Not available; RTE: Ready-to-eat;

PBS: Phosphate-buffered saline (NaCl, KCl, Na_2HPO_4, KH_2PO_4);

BPW: Buffered peptone water;

CD: Proteose peptone (40 g/L), disodium hydrogen phosphate (5 g/L), potassium dihydrogen phosphate (1 g/L), magnesium sulphate (0.1 g/L), sodium chloride (2 g/L) and fructose (6 g/L);

CDMN: CD supplemented with CDMN selective supplement (cysteine hydrochloride (0.5 g/L), moxalactam (32 mg/L) and norfloxacin (12 mg/L);

CDMN[1]: Special peptone (23.0 g/L), starch (1.0 g/L), sodium chloride (5.0 g/L), agar (10.0 g/L), yeast extract (5 g/L), fructose (6 g/L), p-hydroxyphenylacetic acid (1 g/L), L-cysteine HCl (0.5 g/L), hemin (5 mg/L), vitamin K_1 (10 mg/L), sodium taurocholate (1.0 g/L), moxalactam (32 mg/L), norfloxacin (12 mg/L) and defibrinated horse blood (5g/L);

CDMNHB: CDMN supplemented with 5% (v/v) of horse blood;

TCD: CD with 0.1% (w/v) sodium taurocholate;

TCDMN: CDMN with 0.1% (w/v) sodium taurocholate;

TCDMNHB: TCDMN supplemented with 5% (v/v) of horse blood;

TCDMNSB: TCDMN supplemented with 5% (v/v) of sheep blood;

BHI: Brain heart infusion from (Solids) (8.0 g/L), peptic digest of animal tissue (5.0 g/L), pancreatic digest of casein (16.0 g/L), sodium chloride (5.0 g/L), glucose (2.0 g/L), disodium hydrogen phosphate (2.5 g/L);

TBHI: Brain heart infusion with 0.1% (w/v) sodium taurocholate;

TBHICC[1]: BHI with D-cycloserine (250 mg/L) and cefoxitin (10 mg/L) and 0.1% (w/v) sodium taurocholate;

TBHICC[2]: BHI with D-cycloserine (250 mg/L) and cefoxitin (8mg/L) and 0.1% (w/v) sodium taurocholate;

TBHIYEC: BHI supplemented with 0.5% (w/v) yeast extract, 0.05% (w/v) DL-cysteine and 0.1% (w/v) taurocholate; TBHICCYE: TBHICC[2] supplemented with 0.5% (w/v) yeast extract;

TBHICCYEC: TBHICC[2] supplemented with 0.5% (w/v) yeast extract and 0.05% (w/v) DL-cysteine

CCF: CD with *C. difficile* selective supplement (D-cycloserine (250 mg/L) and cefoxitin (8 mg/L);

CCFN: CCF with neutral red (0.03 g/L);

TCCFN: CCFN with 0.1% (w/v) sodium taurocholate;

TCCFNHB: TCCFN with 7% (v/v) horse blood;

CCFHB: CCF supplemented with 7% (v/v) horse blood;

TCCF: CCF with 0.1% (w/v) sodium taurocholate;

TCCF[1]: CD with *C. difficile* selective supplement (D-cycloserine (350 mg/L), cefoxitin (4 mg/L) and 0.1% (w/v) sodium taurocholate;

TCCFC: TCCF supplemented with 0.05% (w/v) L-cysteine;

CCEY agar: Peptone mix (23.0 g/L), sodium chloride (5.0 g/L), soluble starch (1.0 g/L), agar (12.0 g/L), sodium bicarbonate (0.4 g/L), glucose (1.0 g/L), sodium pyruvate (1.0 g/L), cysteine HCl (0.5 g/L), haemin (0.01 g/L), vitamin K (0.001 g/L), L-arginine (1.0 g/L), soluble pyrophosphate (0.25 g/L), sodium succinate (0.5 g/L), cholic acid (1.0 g/L), p-hydroxyphenylacetic acid (1.0 g/L), egg yolk emulsion (4% v/v), cefoxitin (8 µg/ mL) and cycloserine (250 µg/ mL);

CCMB-TAL: Cycloserine (500 mg/L), cefotixin (15.5 mg/L), mannitol broth with 0.1% (w/v) taurocholate and 0.5% (w/v) lysozyme;

Brucella agar: Tryptone (10.0 g/L), peptone (10.0 g/L), yeast extract (2.0 g/L), glucose (1.0 g/L), sodium chloride (5.0 g/L), sodium bisulphite (0.1 g/L) and agar (15.0 g/L);

CCBB-TC: Brucella broth with cefoxitin (8 µg/mL), cycloserine (250 µg/mL), sodium bicarbonate, D-mannitol, sodium taurocholate, lysozyme, vitamin K_1, hemin, thioglycolic acid and L-cysteine;

Schädler agar: Tryptic soy broth (10.0 g/L), enzymatic digest of casein (2.5 g/L), enzymatic digest of animal tissue (2.5 g/L), yeast extract (5 g/L), dextrose (5g/L), Tris (hydroxymethyl) aminomethane (3 g/L), hemin (0.01 g/L), L-cystine (0.4 g/L) and agar (13.5 g/L);

TB: Thioglycolate broth (pancreatic digest of casein (15.0 g/L), dextrose (5.0 g/L); yeast extract (5.0 g/L), sodium chloride (2.5 g/L), sodium thioglycolate (0.5 g/L), L-cysteine (0.25 g/L) and agar (0.75 g/L);

FAA: Fastidious anaerobe agar (peptone (23 g/L), sodium chloride (5 g/L), soluble starch (1 g/L), sodium bicarbonate (0.4 g/L), glucose (1 g/L), sodium pyruvate (1 g/L), L-cysteine HCl·H_20 (0.5 g/L), sodium pyrophosphate (0.25 g/L), L-arginine (1 g/L), sodium succinate (0.5 g/L), hemin (0.01 g/L), vitamin K (0.001 g/L) and agar (12 g/L));

FAB: FAA without agar;

TSA:Tryptic soy agar (tryptone (17 g/L), soytone (3 g/L), dextrose (2.5 g/L), NaCl (5.0 g/L), K_2HPO_4 (2.5 g/L), agar (15 g/L));

TSC agar: Triptose sulphite cycloserine agar (tryptose (15 g/L), peptone from soymeal (5 g/L), yeast extract (5.0 g/L), sodium disulfite (1 g/L), ammonium iron (III) citrate (1.0 g/L), cycloserine (0.4 g/L) and agar (12 g/L));

CDSA: Peptic digest of animal tissue (32.0 g/L), magnesium sulphate (0.1 g/L), monopotassium phosphate (1.0 g/L), disodium phosphate (5.0 g/L), sodium chloride (2.0 g/L), cefoxitin (0.016 g/L), cycloserine (0.25 g/L), growth factors (3.3 g/L), neutral red (0.03 g/L), mannitol (6.0 g/L) and agar (20.0 g/L);

ChromID *C. difficile*: Meat peptone (porcine) (8.0 g/L), taurocholate (bovine) (1.0 g/L), yeast extract (3.5 g/L), sodium chloride (6.0 g/L), selective mixture (0.27 g), chromogenic mixture (0.3 g/L), agar (13.0 g/L).

CHROMagar™*C. difficile*: (CHROMagar™, Paris, France).

2.3. Selective enrichment

As low numbers of *C. difficile* are expected in food samples, the use of enrichment broths is indicated.

2.3.1. Composition of enrichment broths used

The composition of the enrichment broths used for the detection of *C. difficile* from food samples is often similar to those used for clinical samples, with the same base composition and the same added compounds [6,14,49,56].

Many studies have in common the culture medium mainly composed of proteose peptone, fructose, disodium phosphate, sodium chloride, potassium dihydrogen phosphate and magnesium sulphate [27,28,65,66–69]. Lister et al. [70] used different enrichment culture media for isolation of *C. difficile* from stool samples and detected growth in all the samples enriched in medium containing fructose. According to the authors, fructose is considered the preferred carbon source of *C. difficile* and therefore may be an essential component of the culture media [70]. Other compounds may be added to the above-mentioned base medium. In several studies, selective agents have been used to inhibit the growth of other microorganisms present in samples being examined [9,15,56]. Supplementation of the medium with cycloserine and cefoxitin, named *C. difficile* selective supplement, is used to inhibit the growth of most contaminants, present in large numbers in normal faecal microbiota [71] as well as in different foods such as meats and vegetables [15,44]. An alternative is the supplementation of the culture medium with moxalactam and norfloxacin, named *C. difficile* moxalactam norfloxacin (CDMN) selective supplement [38,48,63], which is composed by 32 mg/L moxalactam, 12 mg/L norfloxacin and also with 0.5 g/L cysteine hydrochloride (Thermo Fisher Scientific, Massachusetts, USA). The function of cysteine hydrochloride is primarily to reduce the redox potential and protect cells against oxidative stress, enabling the growth of *C. difficile* while moxalactam and norfloxacin act as selective agents able to reduce more contaminating microorganisms than cycloserine or cefoxitin [65]. The authors from 28 studies (Table 1) used the above-mentioned base culture medium supplemented with moxalactam and norfloxacin as enrichment broth against nine studies in which the base culture medium supplemented with cycloserine and cefoxitin was used [11,17,26,27,44,55,60,62,64].

In addition to the selective agents mentioned above, other components such as lysozyme and bile salts (cholate and its derivatives) could be added to the base culture medium, to increase germination [71]. According to Warriner et al. [22], one of the reasons *C. difficile* cannot grow in foods and therefore cannot be considered as a typical foodborne pathogen, is their lack of ability to germinate in the absence of bile salts. For this reason, bile salts are essential compounds of the medium to isolate *C. difficile*. However, according to Limbago et al. [32], the addition of sodium taurocholate to the enrichment medium did not have a beneficial effect, but the authors also did not exclude the hypothesis that some strains of *C. difficile* can be stimulated. In the study by Curry et al. [30], the authors used, simultaneously, two agents in the enrichment medium to stimulate germination—lysozyme and taurocholate. As the authors did not perform a comparison using the components individually, it is not possible to conclude whether the combination of the two components has, indeed, increased germination.

Horse blood, normally 5 to 7% (v/v) could also be added to supplement the enrichment culture

media. In only two studies [26,63] the authors used 5% (v/v) sheep blood instead of horse blood.

Besides the aforementioned base medium, other enrichment broths have been used (Table 1), such as Brucella broth (BB) [7], Mannitol broth (MB) [30], Thioglycolate broth (TB) [40, 43], and, to a greater extent, Brain Heart Infusion broth (BHI) [7,8,11,15,16,18,39,46,51,54,57,59,61,62]. With the exception of four studies in which the authors only used TB [40,43] and BHI [46,51], all authors added some of the selective and/or enrichment compounds already discussed above to their enrichment culture media.

To understand what type of enrichment medium was the most effective for the recovery of *C. difficile* from chopped beef inoculated with 100 colony forming units (cfu)/g *C. difficile*, Chai et al. [72] used two non-selective media: TBHI (brain heart infusion with 0.1% sodium taurocholate) and TCM (cooked meat medium with 0.1% sodium taurocholate); and two selective media: TCCFB (containing cycloserine and cefoxitin) and TCMN (supplemented with cysteine hydrochloride, moxalactam and norfloxacin). The authors concluded that both selective media, TCCFB and TCMN, had a negative effect on bacterial growth since, in addition to the stationary phase being reached later (after 18h), the number of *C. difficile* decreased approximately 3 log cfu/mL. The non-selective media TBHI and TCM were those that allowed a higher growth rate (log (cfu/mL)/h), but it was in TCM that a higher recovery was observed; that is, *C. difficile* grew more than in all other enrichment media used. Although there is a benefit in the use of selective agents to reduce the growth of background microorganisms, these authors believe that the use of cycloserine and cefoxitin, as well as moxalactam and norfloxacin, had an adverse effect on the recovery of *C. difficile*, even though these microorganisms are resistant to these antibiotics [72].

Based solely on enrichment medium, Hofer et al. [51], Houser et al. [57] and Songer et al. [8] reported a prevalence of *C. difficile* of 0%, 6% and 42%, respectively, using non-selective BHI broth supplemented with taurocholate, yeast extract and cysteine. Von Abercron et al. [46] reported a prevalence of 2% of *C. difficile* in retail ground meat, but the authors did not specify if *C. difficile* was isolated from BHI or CDMN enrichment broths. Limbago et al. [32] reported better recovery of *C. difficile* from inoculated meat samples in TBHI than in CDMN independently of 1, 3 or 5 enrichment days. This means that use of selective agents in enrichment broths may not be essential, and perhaps even having an adverse effect, to recover *C. difficile* from foods, but more studies are needed to confirm this hypothesis.

2.3.2. Volumes of enrichment broths and incubation times

In addition to different compositions of enrichment broths, also different volumes have been used varying from 9 to 200 ml [12,13,15,27,37,38,43,50,54,59,62,64]. The use of different volumes along with different amounts of food sample (section 2.2) result in highly variable dilution factors.

Enrichment time is another factor that should be taken into consideration. In most of the studies shown in Table 1, the enrichment time chosen by the authors was 7 [7,14,17,19,20,42,43,47,51,55] and 10 days [11,16,18,23,28,52,59,61], but several authors also opted for incubation times longer than 10 days [10,36,39,40,44,48,56,63]. Limbago et al. [32] compared the recovery of *C. difficile* (a single spore suspension at a rate of approximately 100 spores/gram) after three different times—1, 3 and 5 days—in three types of broth enrichment—CDMN broth + 0.1% taurocholate without heat shock, Brain Heart Infusion (BHI) broth + 0.1% taurocholate (TBHI) without heat shock and TBHI with heat shock. The authors verified that better recoveries were obtained after 3 and 5 days for all

tested broth enrichments and, moreover, recovery in TBHI without heat shock was higher, since all inoculated meat samples were positive for *C. difficile* [32]. Enrichment time of 5 days was used in only one study from Table 1 [30], and five authors used 3 days of incubation [8,15,27,54,64].

2.4. Heat and ethanol shock

Alcohol shock treatment is recommended to replace the possible inhibitory effect caused by selective antibiotics, since it not only stimulates spores germination but also eliminates possible vegetative cells of contaminating microorganisms [71]. Briefly, after incubation ethanol is added to an aliquot of the enriched broth (1:1) and after 1 h at room temperature, this suspension is centrifuged and the pellet inoculated onto the isolation culture medium.

Heat shock is another spore selection technique that can be used, allowing the inactivation of vegetative cells of contaminants present in the sample. This technique is performed immediately after sampling, i.e. the amount of sample is placed in tubes with an enrichment medium and the tube is heated at 80 ºC prior to enrichment incubation.

Songer et al. [8] did not find any difference between heat-shocked and non-heat-shocked samples since positive cultures were obtained by both methods. In the study by Marler et al. [68], the ethanol treatment allowed the recovery of a higher number of isolates compared to heat shock treatment. Indeed, alcohol shock was the treatment of choice in the majority of the studies (Table 1).

2.5. Composition of recovery solid culture media used

A certain volume of enriched broth or washed pellet (after alcohol treatment) is pour plated on solid culture media to recover *C. difficile* isolates. In the majority of the studies, the culture medium is the same as the enrichment, but with added agar (~15 g/L). Some culture media also contain neutral red as a pH indicator, which changes the color of the medium when pH increases due to the breakdown of peptones by *C. difficile* [8,10,44,57]. The addition of other components such as lysozyme and taurocholate to increase germination is not so common, unlike 5–7% (v/v) horse or sheep blood that was added to increase the recovery of *C. difficile* in many of the studies. Also to increase the recovery of *C. difficile*, egg yolk emulsion may be added to the culture medium, but since the 70's it is known that horse blood allows a greater recovery than egg yolk emulsion [67]. In fact, egg yolk emulsion was used in merely one study out of 54 listed in Table 1 (CCEY agar) [35].

Also other recovery culture media were used such as Blood agar [20,55], Columbia agar supplemented with 5% (v/v) sheep blood [14,35,47] and also with cysteine and taurocholate [54], Brucella agar [10], Tryptic soy agar with 5% (v/v) horse blood [30], Tryptose sulfite cycloserine agar [58], Schädler agar [52], Fastidious anaerobe agar [46], Anaerobic Blood agar [32]; CHROMagarTM *C. difficile* [7], *C. difficile* selective agar (CDSA) [16] and ChromID [61].

But with so many studies using different components, the question arises: Will the presence of these selective agents be essential in a culture medium to recover *C. difficile* from foods? Rodriguez-Palacios et al. [55] conducted a study using a total of 214 meat samples that were cultured using both selective agents—CDMN and C. difficile selective supplement. Despite the higher sensitivity obtained when culture medium supplemented with CDMN was used (39%) compared with culture medium with *C. difficile* selective supplement (23%), no reproducibility of *C. difficile* recovery was observed between duplicates. However, in a more recent study, the same authors

mentioned that they did not use the selective agents moxalactam and norfloxacin since these antibiotics belong to the fluoroquinolones group and are commonly used in the treatment of *C. difficile* infections in humans. Thus, the authors believe that the use of these supplements only enabled recovery of resistant isolates from food samples [17].

In only three out of the 54 studies listed in Table 1 [8,43,57], the authors used nonselective enrichment media only, despite the subsequent use of selective recovery culture media. It is not possible to understand if there are methodologies better than others, due to the high number of variables in the reported studies, including the number of samples, type of enrichment and recovery culture media, incubation time, among others. For instance, in the studies of Songer et al. [8] and Houser et al. [57], the authors used the same enrichment and recovery culture media but different times of enrichment.

3. Identification and/or typing of *C. difficile* isolates and detection of their toxins

Classical microbiological methods are essential to recovering *C. difficile* colonies for further identification and typing methods. The presumptive identification of *C. difficile* is based on phenotypic characteristics, but additional biochemical and genotypic tests are essential for accurate identification.

3.1. Clostridioides difficile methods of identification

3.1.1. Biochemical methods for *C. difficile* identification

After growth on recovery culture media, suspected colonies are confirmed biochemically by the L-proline aminopeptidase test. This is a sensitive, specific, fast and inexpensive method based on colorimetric detection of the enzyme L-proline aminopeptidase (Pro-disk, Hardy Diagnostics).

API Rapid ID 32A (bioMérieux, Inc., Marcy l'Etoile, France) was also used in a few studies to identify *C. difficile* isolates [18,19,39] as well as Api 20A [37,58].

A Latex Agglutination test was also used by nine authors [23,27,40,41,46,53,59,60,64] for the detection of *C. difficile*. This assay is based on the detection of the common glutamate dehydrogenase (GDH) antigen which is produced and preserved by *C. difficile* isolates, in both toxin producers and non-producers [73].

3.1.2. Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) for *C. difficile* identification

This technology provides a rapid and precise tool for identification of pathogens [74] and has been reported to be useful for the identification of distinctive *C. difficile* genotypes [75]. Despite the advantages of this emerging technique, such as testing performed from single colonies on primary culture plates and their accurate identification in minutes without the need of previous knowledge of microorganism type, the cost of the instrument might be one of the major limitations. In one recent study, the authors used MALDI-TOF MS to identify presumptive colonies of *C. difficile* [62], but no information about the results obtained with this technique was described.

Once identified as *C. difficile*, additional tests are performed to confirm the production of toxins

and also, methods of typing for epidemiological purposes, which are presented below.

3.2. Non-genotypic methods to detect toxin production by C. difficile

As mentioned before in this review, there are no standardized procedures for the detection of *C. difficile* in food samples. However, there are other tests that, although more complex, provide more information and are based on the detection of toxin proteins.

3.2.1. Toxigenic culture and cell culture cytotoxicity neutralization assay

The toxigenic culture methodology is based on the isolation of *C. difficile* on culture media with subsequent confirmation of whether the *C. difficile* isolate is a toxin-producing strain. This is often achieved using a culture supernatant of the *C. difficile* isolate, which will be tested with toxin detection kits [37,46] or from which its genomic DNA will be extracted and a subsequent polymerase chain reaction (PCR) amplification applied [17,23,56].

The cell culture cytotoxicity neutralization assay method, as its name implies, uses cell lines (Hep2 cells, human diploid fibroblasts, human foreskin fibroblasts, McCoy cells, MRC-5 lung fibroblasts and Vero cells) and is widely applied for clinical samples, using faeces filtrates. The method consists of the inoculation of a culture filtrate on the cell line and, after the proper incubation time (24–48h), the induced cytopathic effect by toxins is observed. If this effect is verified, it is necessary to perform a neutralization test with *Clostridium sordellii* or *C. difficile* antiserum (reviewed by [76]). In this way, it is assured that the induced cytopathic effect is due to *C. difficile* toxins. This assay detects toxins A and B, although toxin A is only detectable to some extent [77]. Perhaps because it is a more complex and time-consuming method, the authors of only four studies from those listed in Table 1 used it to detect the presence of toxin B [27,39,58,64]. In the study of Von Abercron et al. [46], the authors used Vero tissue culture cells to detect toxin B in two *C. difficile* isolated from ground beef and verified its presence by neutralization of the cytopathic effect on Vero cells by *C. difficile*-specific antitoxin (Techlab, Blacksburg, VA). Quesada-Gómez et al. [39] used four *C. difficile* culture supernatants on HeLa cells and all strains were able to induce cytopathic effects, thus being toxin producers. Cytotoxicity test in the studies of Rodriguez et al. [27,64] were performed in MRC-5 cells and the specificity of the cytotoxic activity by neutralization was confirmed for eight *C difficile* isolates using a specific *C. difficile* antitoxin-B kit (T500, TechLab, Virginia, USA).

3.2.2. Enzyme immunoassays

The use of enzyme immunoassays, which are based on the use of antibodies, monoclonal or polyclonal, directed against the toxins (reviewed by [76]) is rapid, simple and low-cost method. However, as the sensitivity of these assays varies largely from ~40 to 100%, its use alone is not recommended [78]. Mooyottu et al. [38] used a commercially available enzyme immunoassay *C. difficile* Tox A/B II kit (TechLab, Blacksburg, VA, USA) to detect the presence of toxins A and B in two *C. difficile* isolates from pork samples and confirmed the negative results using a multiplex-PCR for genes of toxins TcdA, TcdB, CdtA, CdtB and TcdC deletion [79]. Rahimi et al. [63] used an ELISA detection kit (RIDASCREEN, R-Biopharm AG, Darmstadt, Germany) and confirmed that the four out of five toxigenic *C. difficile* strains isolated from ready-to-eat foods were also positive for

tcdA and *tcdB* toxin genes by PCR assay [80]. The presence of *C. difficile* toxins A or B were reported by Pasquale et al. [23] using Xpect *C. difficile* Toxin A/B test (Thermo Fisher Scientific, Remel Products, Lenexa, KS, USA) and confirmed by PCR assays [80]. The same Xpect *C. difficile* Toxin A/B test was used by Ersöz and Coşansu [37], but the authors did not confirm the negative results obtained for non-toxigenic *C. difficile* isolates.

Two other enzyme immunoassays were used alone to detect toxin production by *C. difficile*. Lee et al. [36] used VIDAS-CDAB Kit (bioMérieux, Marcy L'Etoile, France) to detect toxins A and B and found two of 45 *C. difficile* isolates producers of both toxins. Von Abercron et al. [46] also reported *C. difficile* producers of both toxins using *C. difficile* toxin A test (TD 0970A, Oxoid, Hampshire, United Kingdom).

3.3. Genotypic methods to detect toxin production by C. difficile and strain typing

Detection of *C. difficile* toxin genes or characterization of *C. difficile* isolates by genotypic profiles can be done using nucleic acid amplification techniques such as conventional PCR, real-time PCR, PCR-ribotyping, among others.

3.3.1. Conventional PCR assay and multiplex-PCR

As already mentioned above, genes *tcdA* (toxin A) and *tcdB* (toxin B) are commonly found in the PaLoc region, but also three other genes - *tcdR*, *tcdC* and *tcd*E – are included in this locus [81,82]. Proteins involved in the transcriptional regulation of the toxin genes are encoded by *tcdR* and *tcdC* genes [83,84] and *tcdE* product is essential for the efficient secretion of TcdA and TcdB toxins [85]. Besides genes *tcdA* and *tcdB* [86,87], genes encoding the two-component toxin, *cdtA* (enzymatic domain) and *cdtB* (binding domain), and *tcdBv* (toxin B variant) may be detected by molecular assays [88].

The polymerase chain reaction has been used by almost all researchers to detect, essentially, toxin A and B genes and *cdtB* binary toxin gene [41,50,63]. These genes can be detected individually or simultaneously by multiplex-PCR. The protocol developed by Lemee et al. [89] is used in most studies. These authors designed a multiplex-PCR toxigenic culture approach which allows simultaneous identification and toxigenic type characterization of *C. difficile* isolates by amplification of a species-specific internal fragment of the triose phosphate isomerase (*tpi*) gene and internal fragments of toxin A (*tcdA*) and toxin B (*tcdB*) genes [89].

3.3.2. Real-time PCR

Real-time PCR combines conventional PCR assay with a probe-based mechanism of fluorescence. Specificity and sensitivity of this methodology are very high allowing earlier detection of targets and in a mere single step, with no need for further analysis of the PCR product [90]. Only in three studies (Table 1) the authors used a multiplex real-time PCR to detect simultaneously *tpi*, *tcd*A and *tcdB* genes [41] and *tcdA*, *tcdB*, *cdtA* and *cdtB* genes [28]. Still with several limitations, real-time PCR technology has more advantages compared to the conventional PCR and represents a powerful tool in microbial diagnostics.

3.3.3. Restriction fragment length polymorphism (RFLP)-PCR

Toxinotyping of *C. difficile* is an RFLP-PCR-based method using a combination of restriction profiles obtained with amplification of the *tcdA* and *tcdB* genes for determination of toxinotype [91]. In 15 out 54 studies found, the authors performed toxinotyping of *C. difficile* strains isolated from different food products analyzed [6,8–10,14,19,20,23,44,48,49,52,54,55,59]. This technique allows the differentiation of *C. difficile* strains according to the changes in the PaLoc region, which code for toxins A and B. Although the several techniques available to detect *C. difficile* strains with variant toxin genes without the need of amplification of the toxin gene fragments, none other than toxinotyping (except whole-genome sequencing) allow the detection of all variant strains [91].

3.3.4. PCR-Ribotyping

This typing method is based on the amplification of DNA fragments using primers in both 16S rRNA and 23S rRNA genes. The profiles of PCR DNA fragments of different sizes obtained correspond to the different alleles of the rRNA operon on the *C. difficile* chromosome [92]. Because it is fast, easy and reproducible, it is currently one of the most widely used methods. Almost all researchers from the studies listed in Table 1 used this typing method to identify the PCR ribotypes of *C. difficile* isolates and to visually compare with PCR ribotypes previously identified [6,9,23,38,41,49,55,59].

Several *C. difficile* ribotypes that have been associated with a potential cause of disease in humans have also been found in foods, such as ribotype 001 [15,23], ribotype 014 [59,64] and ribotype 078 [9,18,27,59].

3.3.5. Pulsed-field gel electrophoresis (PFGE)

PFGE is a very discriminatory and reproducible technique widely used to characterize *C. difficile*. Several authors performed PFGE for typing *C. difficile* food isolates [8,10,14,19,20,44,49,53]. This method requires the preparation of undigested DNA, DNA digestion using a restriction endonuclease, fragment separation in a gel matrix by an electric field that periodically changes direction, and visualization and interpretation of band patterns [93]. Despite offering good results (typeability, discriminatory ability, etc), PFGE is still a labor-intensive technique when compared with other techniques such as RFLP-PCR or PCR-ribotyping [94].

3.3.6. Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) is a typing method that allows discrimination of *C. difficile* isolates through nucleotide sequences of housekeeping gene fragments, wherein a sequence type number (ST) is given to each combination [95]. Despite being a very common technique for *C. difficile* isolated from humans, only two authors used MLST to study *C. difficile* isolated from food [7,27]. By MLST analysis, Rodriguez et al. [27] identified 4 different STs among 8 *C. difficile* meat isolates and only one isolate was not clustered in the same lineage as human isolates, also included in the study. Wu et al. [7] found 4 different STs among 40 *C. difficile* isolates, but the only human isolate belonged to a different lineage to the 39 meat isolates studied.

3.3.7. Multi-locus variable-number tandem-repeat analysis (MLVA)

According to Killgore et al. [94], multi-locus variable-number tandem repeat analysis is the most discriminatory method, followed by PFGE, PCR-ribotyping and MLST, being able to discriminate strains with identical PCR ribotypes [96].

Curry et al. [30] suggested environmental contamination in a retail meat processing facility based on MLVA genotypes of *C. difficile* isolated from different ground pork products collected over 5 months from this facility. Also closely related MLVA genotypes of *C. difficile* ribotype 126 from a slaughterhouse, pig stool, colons, carcasses and scalding water suggested cross-contamination in the study of Wu et al. [7].

It is important to underline that besides each genotyping method mentioned above is being used to detect toxin production by *C. difficile* and strain typing, the detection of housekeeping genes also allows evaluation of the occurrence of *C. difficile* in food samples. Furthermore, the genetic characterization of the isolates has an important role in epidemiological studies aimed to emphasize the correlation among food and clinical strains.

4. Conclusions

The purpose of this study was to present an overview of the methodologies that have been used to recover *C. difficile* from food samples; despite several studies having been reported, there is no widely accepted methodology for the detection/enumeration of this bacterium in foods. Current methodologies are only focused on classical microbiological methods of isolation/detection, followed by molecular tests to confirm the toxigenic potential of the suspected colonies. Nonetheless, several culture media are used with the same base, but with the addition of different selective and enrichment components, the role of which is, sometimes, controversial.

In contrast to studies with clinical strains, which were derived from original stool samples, in studies with foods, only culture methods followed by molecular analysis of the suspected colonies are used. If molecular methods were applied as the first approach, would the prevalence of *C. difficile* in foods be higher? In theory, the values would be higher since the studies mentioned above showed that techniques like real-time PCR are very sensitive. However, more tests are needed, to adapt and validate the use of these molecular methods in food samples.

Since *C. difficile* is a human pathogen and as several studies have reported its presence in foods, more studies are necessary in order to define an appropriate methodology which could, ideally, become standardized.

Acknowledgments

The authors are grateful to Dr. Paul Gibbs for the English edition. We would also like to thank the scientific collaboration under the *Fundação para a Ciência e Tecnologia*(FCT) project UID/Multi/50016/2019.Financial support for author J. Barbosa was provided by a post-doctoral fellowship SFRH/BPD/113303/2015 (FCT).

Conflict of interest

All authors declare no conflicts of interest in this paper.

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