Food Microbiology 78 (2019) 194–200

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

High *Clostridium difficile* contamination rates of domestic and imported potatoes compared to some other vegetables in Slovenia

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ARTICLE INFO

Keywords:
Clostridium difficile
Clostridioides difficile
Zoonosis
Food
Long distance transmissions

ABSTRACT

Clostridium difficile, recently reclassified to *Clostridioides difficile*, is among most important causes of intestinal infections in humans. Zoonotic potential and foodborne transmissions are considered to be partially involved in *C. difficile* spread. Here we report prevalence of *C. difficile* in 142 retail and 12 homegrown vegetables in Slovenia between years 2014 and 2017. The overall prevalence of *C. difficile* on vegetables was 18,2% (28/154). A total of 115 isolates were obtained which belonged to 25 PCR ribotypes. Ten of those were toxigenic and PCR ribotype 014/020 was the most prevalent. Most of 25 determined PCR ribotypes were previously reported in humans, animals, soil or water in Slovenia. Among tested vegetables, potatoes had the highest positivity rate (28,0% vs. 6,7% and 9,4% for ginger and leaf vegetables). Altogether 66,7% of *C. difficile* positive potato samples were imported from 12 different countries of three different continents. The origin of contamination could be any point between production and retail store, however, our results suggest a possibility that potatoes represent a transnational and transcontinental way of *C. difficile* transmissions.

1. Introduction

Clostridium difficile, recently reclassified to *Clostridioides difficile* (Lawson et al., 2016; Oren and Rupnik, 2018) is an anaerobic, sporogenic, Gram-positive bacterium. The bacterium is an important cause of intestinal infections in humans with disrupted gut microbiota. *C. difficile* infection (CDI) begins with ingestion of spores which could originate from various sources. Since the description of high prevalence of *C. difficile* in animals, particularly calves and piglets, zoonotic potential and foodborne transmissions have been addressed by numerous studies (Rupnik, 2007; Indra et al., 2009; Gould and Limbago, 2010; Weese et al., 2010; Hensgens et al., 2012; Rodriguez-Palacios et al., 2013; Rodriguez et al., 2016; Knetsch et al., 2018; Rodriguez Diaz et al., 2018).

The bacterium has been detected in diverse range of foods, including meat, seafood and fresh produce (Rodriguez et al., 2016; Rodriguez Diaz et al., 2018). Most of the studies have focused on meat and meat products and reported from 0 to 62,5% positivity rates (Songer et al., 2009; Rodriguez-Palacios et al., 2009; Weese et al., 2010; Limbago et al., 2012). Other food, like milk and seafood was tested only occasionally (Joebstl et al., 2010; Pasquale et al. 2011, 2012). Also reports on *C. difficile* in raw vegetables or ready–to-eat-salads are not numerous (Al Saif and Brazier, 1996; Bakri et al., 2009; Metcalf et al., 2010; Eckert et al., 2013; Lim et al., 2018). The presence of *C. difficile* can be higher for specific types of food; more than half of Italian bivalve molluscs (Pasquale et al. 2011, 2012bib_Pasquale_et_al_2012) and organic potatoes in Australia (Lim et al., 2018) tested positive for *C. difficile*.

While postulated foodborne transmission has not yet been directly shown, there are several observations to support it. Recovered food isolates include PCR ribotypes (subtypes within *C. difficile* species) that are common to humans and animals. Several PCR ribotypes were described in food in Europe, including major human PCR ribotypes 001,014, 078, 015, 002 and 106 (Rodriguez Diaz et al., 2018). In contrast, lower diversity of PCR ribotypes, with 078 and 027 prevailing, was found in North America (Rodriguez-Palacios et al., 2013). Whole genome sequencing, a more discriminatory subtyping technique than PCR ribotyping, has up to date been applied only on human and pig isolates and has detected pairs of indistinguishable *C. difficile* strains (Knetsch et al., 2014; Knight et al., 2017). Finally, a large community outbreak in Australia was discussed to be potentially food related (Eyre et al., 2015).

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https://doi.org/10.1016/j.fm.2018.10.017
Received 30 June 2018; Received in revised form 6 September 2018; Accepted 29 October 2018
Available online 30 October 2018
0740-0020/

For better understanding of possible role of *C. difficile* contaminated food in CDI in humans it is important that studies are performed in different countries and that broad range of food sources are covered. Despite considerable reporting of *C. difficile* in Slovenian food animals and environment (Pirs et al., 2008; Zidaric et al., 2008; Avbersek et al., 2009; Janezic et al., 2012, 2016) no extensive studies on *C. difficile* in food have been published so far. Here we present the results of sporadic, long term analysis of the presence of *C. difficile* in vegetables, mainly potatoes and different types of leaf vegetables.

2. Material and methods

2.1. Sampling

A convenience sample of 154 raw vegetables was obtained. Vegetables included fresh leaf vegetables (n = 64; lamb's lettuce (n = 25), green leaf lettuce (n = 22), dandelion leaves (n = 11) young lettuce (n = 2), rucola (n = 2), radicchio (n = 1) and one sample of mixed leaf vegetables), ginger (n = 15) and potatoes (n = 75).

A proportion of collected samples (n = 12) was homegrown, while retail samples (n = 142) were purchased from local supermarkets (n = 121) and food markets (n = 21).

Retail leaf vegetables were sampled on 11 occasions (11 different daily samplings with 2 to up to 8 samples collected) and 12 locations from March to May 2014 and from March to August 2017 (Supplementary table 1). Ginger was sampled three times between June and August 2017 (4 to up to 6 samples per occasion collected) at 6 different locations (Supplementary table 2). Retail potatoes were purchased on 12 different occasions (1 to up to 12 samples) and 15 locations from February 2016 to September 2017. Five potato samples were homegrown, collected at 3 different locations and on 3 occasions in June 2015 and June 2017 (Supplementary table 3).

Where available, the country of origin was noted. Retail leaf vegetables (54 samples) were supplied by national producers (n = 21) and producers from other European countries (n = 30; mostly from Italy (26 out of 30)). Three retail samples were of unknown origin. Altogether 7 samples were homegrown or picked at local meadows (dandelion leaves are traditionally picked for salads in spring time) at 7 different locations on 3 occasions in March and April 2014. Ginger originated from Asia (n = 11), Central (n = 1) and South (n = 2) America. For one ginger sample, origin was unknown. Altogether 12 retail potato samples (17,1%) were of national origin, 34 (48,6%) originated from nine other European countries (mostly France (13 out 34 samples)), 13 (18,6%) samples were from North America (USA), 9 (12,9%) from Africa (Egypt), one from Asia (Israel) and one sample was labeled as USA/Egypt product.

Prepacked vegetables were kept in their original packaging until laboratory processing in order to prevent cross-contamination. Loose vegetable samples were collected into separate plastic bags and properly closed at the place of sampling.

2.2. Cultivation of C. difficile

2.2.1. C. difficile cultivation from leaf vegetables

Altogether 25 g of each leaf vegetable sample was transferred into blender bag (Interscience) and homogenized (BagMixer, Interscience) in approximately 200 mL of BHIST (BHI (Biolife) supplemented with sodium taurocholate (0,1%), yeast extract (0,5%) and with *C. difficile* selective supplement (Oxoid)). After anaerobic incubation at 37 °C for 5–7 days, ethanol treatment of liquid culture (0,9 mL) for spore selection (in volume ratio 1:1; 30 min, room temperature) was performed, followed by centrifugation (10 000 rpm/min, 10 min) and inoculation of pellet onto *C. difficile* selective agar (chromID *C. difficile* agar (bioMerieux) for colony recovery and identification. Presumptive colonies were subcultured on 5% horse blood agar (COH, bioMerieux).

First 34 collected leaf vegetable samples, encompassing all collected samples from year 2014, were washed prior to enrichment in BHIST. In this way we aimed to mimic the preparation of leaf vegetables in domestic environment and to detect possible *C. difficile* removed with washing and remaining *C. difficile* on washed vegetable ready for consumption. Briefly, altogether 25 g of each sample was washed with 100 mL of sterile dH_2O and then twice with 1 L of tap water. Subsequently, rinse from first wash (sterile dH_2O) as well as washed leaf vegetable sample were analyzed. Sample was analyzed as described in previous paragraph. Isolation protocol for water sample started with heat shock (70 °C, 20 min) and filtration through 0.2 µm cellulose nitrate membrane filter (Whatman) using Millipore filtering system. Filters were incubated anaerobically on chromID *C. difficile* agar (bioMerieux) for 3 days. After incubation, up to 10 presumptive *C. difficile* colonies were directly transferred to a fresh blood agar plate (COH, bioMerieux). Remaining bacterial growth was swabbed from the filter, subjected to alcohol shock and plated onto selective chromogenic plate for colony identification.

2.2.2. C. difficile cultivation from ginger and potatoes

The recovery of *C. difficile* from ginger and potatoes was assessed using sterile sponge (Polywipe, MWE). In general, three potatoes per batch were swabbed by a single sponge. If potatoes were covered with extensive amounts of soil, each of the three potatoes was tested separately. In case of ginger samples, only one root was swabbed.

Each sponge was incubated anaerobically for 5–7 days in 30 mL of selective broth BHIST. Enrichment was followed by alcohol shock and plated onto chromogenic selective plates as described in protocol for leaf vegetables (Paragraph 2.2.1.).

2.2.3. Detection threshold of cultivation methods

In order to determine detection thresholds of *C. difficile* in used cultivation methods, 25 g of *C. difficile* negative green leaf lettuce and one negative potato were spiked with 100 µL of two different concentrations of *C. difficile* spores (10–100 CFU/mL and 100–1000 CFU/mL) of nontoxigenic strain (C23). Spiked lettuce and potatoes were processed and cultured as previously described for sample testing (Paragraphs 2.2.1. and 2.2.2.). Experiments were performed in duplicates.

Spore suspension of C23 strain was prepared from 5 days old culture grown on COH plates (bioMerieux). Entire culture was harvested into 1 mL of sterile dH_2O and subsequently centrifuged at 10 000 rpm for 5 min. The washing step was repeated two times. Concentration of spores was determined by inoculation of appropriate dilutions onto chromID *C. difficile* agar (bioMerieux).

2.3. C. difficile identification and characterization

Presumptive *C. difficile* colonies were primarily identified by morphology on chromID *C. difficile* agar. Identification was confirmed by detection of molecular marker *cdd*3 (Zidaric et al., 2008) or mass spectrometry (MALDI-TOF Biotyper System, Bruker).

Isolates were characterized by toxinotyping and PCR ribotyping. Toxinotyping was done by PCR amplification and restriction analysis of A3 and B1 fragments of *tcd*A and *tcd*B genes as previously described. Binary toxin gene was detected by partial amplification of *cdt*B (Rupnik, 2010; Rupnik and Janezic, 2016).

PCR ribotyping was performed by Bidet and Janezic primers and conditions as described by Janezic and Rupnik (2010). PCR ribotype results were analyzed by BioNumerics software (version 7.6 Applied Maths) and compared to ribotypes in our large PCR ribotype library with approximately 300 different PCR ribotypes.

Detected *C. difficile* PCR ribotypes in vegetables were compared to their proportions in our large *C. difficile* strain collection. The collection includes over 7000 *C. difficile* isolates with close to 300 different PCR ribotypes from humans (around 60% of all *C. difficile* strains), animals (e.g. poultry, cattle, pets; 15%), abiotic environment (e.g. soil, water,

shoes, manure, farm environment; 15%) and food (0,7%) collected between years 2006 and 2018 (Janezic et al., 2012).

2.4. Statistical analysis

Prevalence of *C. difficile* in food samples was compared with Fisher's exact test. Statistical significance was held at $p < 0,05$.

3. Results

The detection threshold for both, potato and leaf vegetable protocols, was between 1 and 10 CFU per tested sample in all duplicates.

The overall prevalence of *C. difficile* in 154 tested vegetable samples was 18,2% (28 samples). For each *C. difficile* positive sample up to 10 randomly selected *C. difficile* colonies were characterized. Subsequently, a total of 115 isolates were obtained in pure culture and all were characterized by PCR ribotyping. Only one isolate per PCR ribotype per sample was included in further analysis and stored at −80 °C.

3.1. Prevalence of C. difficile on leaf vegetables, ginger and potatoes

C. difficile was the most prevalent on potatoes (28,0% positive samples), followed by leaf vegetables (9,4%) and ginger (6,7%).

Retail samples represented the majority of analyzed samples, while only 12 were homegrown. Out of all 12 collected homegrown vegetable samples, *C. difficile* was detected in one out of 7 (14,3%) leaf vegetables collected in March 2014 and in 3 out of 5 (60,0%) potato samples that were collected on two occasions in June 2015. None of retail leaf vegetable samples tested *C. difficile* positive in 2014, while five (16,7%) were contaminated with *C. difficile* in year 2017 (Table 1, Supplementary table 1). Four out of five *C. difficile* positive retail leaf vegetables originated from Italy (15,4% of all Italian samples) and one from Slovenia (8,3% of all Slovenian samples) (Table 1, Supplementary table 1).

As mentioned, a part of the leaf vegetable samples was prewashed once with sterile water and twice with nonsterile tap water to test *C. difficile* contamination of vegetables after minimal processing that is generally used in domestic environment before consumption. Additionally, a rinse from first wash was used to detect possible *C. difficile* removed with washing. Although one out of 34 washed samples tested positive, *C. difficile* was not detected in any of 34 tested dH_2O water samples from their first wash. Tap water was not tested either before or after use, therefore the conclusion on contamination source is not possible. The sample might have become contaminated during the wash, however, these results could also indicate that washing is not sufficient for the removal of *C. difficile* spores from contaminated vegetables.

Ginger was tested only in 2017 and *C. difficile* was detected in a single sample originating from Peru (1/15 samples (6,7%); Table 1, Supplementary table 2).

C. difficile was detected in 18 (25,7%) out of 70 retail potato samples. Percentage of *C. difficile* positive samples ranged from 0,0% to 50,0% per country of origin (Table 2), however, 50,0% positivity rate was determined only in countries with small number of tested samples (n = 2) and is not representative. Most of tested retail potato samples originated from France (n = 13), USA (n = 13) and Slovenia (n = 12) where isolation rate of 30,8%, 23,1% and 33,3% was determined, respectively (Table 2). *C. difficile* was present in 55,0% of samples tested in year 2016 and in 14,0% of samples tested in year 2017 (Table 1).

Statistical analysis showed no significant difference in *C. difficile* contamination between retail and homegrown vegetables (24 positive out of 142 tested vs. 4 positives out of 12 tested). There was also no significant difference in *C. difficile* prevalence between leaf vegetables and root samples, however, leaf vegetables exhibited significantly ($p = 0,009$) lower proportion of *C. difficile* positive samples than potatoes. Statistical analysis on retail potatoes showed a significant difference ($p > 0,001$) in *C. difficile* prevalence between years 2016 and 2017 (55,0% vs. 34,6%). Retail leaf vegetables showed no significant difference according to the year of sampling. Potatoes covered with soil were more likely to be *C. difficile* positive than clean potatoes (53,8% positive vs. 22,6%; $p = 0, 038$).

3.2. Characterization of C. difficile isolates

In total, 115 *C. difficile* isolates were obtained. Altogether 43 isolates were cultivated from leaf vegetables, 10 from a single ginger and 62 from potatoes.

In leaf vegetables, a total of 8 different PCR ribotypes were distinguished with up to three different PCR ribotypes per sample (Table 1). One of these PCR ribotypes, SLO 279, originated from Italian lamb's lettuce was new to our PCR ribotype library. PCR ribotypes 014/

Table 1
Prevalence of *C. difficile* in raw vegetables in Slovenia between years 2014 and 2017 and PCR ribotype distribution.

Year of sampling		Number of *C. difficile* positive/number of tested samples (%)					PCR ribotype	Toxinotype	Sample designation[a]	Year of sample collection	Country of origin
		2014	2015	2016	2017	All					
Leaf vegetables	Retail	0/27 (0%)	nt	nt	5/30 (16,7%)	5/57 (8,8%)	011/049	0	B, C	2017	Italy
							014/020	0	C, D	2017	Italy
							SLO 279	0	C	2017	Italy
							SLO 205	Tox-	E	2017	Slovenia
							SLO 214	Tox-	E	2017	Slovenia
							SLO 221	Tox-	F	2017	Italy
	Homegrown	1/7 (14,3%)	nt	nt	0	1/7 (14,3%)	SLO 204	Tox-	A	2014	Slovenia
							SLO 217	Tox-	A	2014	
Ginger	Retail	nt	nt	nt	1/15 (6,7%)	1/15 (6,7%)	SLO 229	Tox-	G	2017	Peru
							SLO 158	Tox-	G	2017	
Potatoes[b]	Retail	nt	nt	11/20 (55,0%)	7/50 (14,0%)	18/70 (25,7%)	17 PCR ribotypes	5 toxinotypes and Tox-	na	See Table 2	See Table 2
	Homegrown	nt	3/3 (100,0%)	nt	0/2 (0%)	3/5 (60,0%)					
Total number		1/34 (2,9%)	3/3 (100,0%)	11/20 (55,0%)	13/97 (13,4%)	28/154 (18,2%)	25 PCR ribotypes	5 toxinotypes and Tox-	na	na	na

na-not applicable; nt-not tested.
Tox- -nontoxigenic strain.
[a] – each letter indicates individual sample; note that more than one PCR ribotype was occasionally found in a given sample.
[b] -detailed description of potato testing is shown in Table 2.

Table 2
Clostridium difficile on potatoes from Slovenian retail stores according to specified country of origin.

Source country	Number of tested samples	Number of *C. difficile* positive samples (%)	PCR ribotype	Toxinotype	Sample designation[a]	Year of strain isolation
Austria	6	2 (33,3%)	053	Tox-	A	2016
			001/072	0	B	2017
			070	0	B	2017
Croatia	3	1 (33,3%)	032	Tox-	na	2017
Cyprus	2	1 (50,0%)	014/020	0	na	2016
			053	Tox-	na	2016
			126	V (BTb+)	na	2016
			150	0	na	2016
Egypt	9	1 (11,1%)	014/020	0	na	2016
			150	0	na	2016
			SLO 187	0	na	2016
France	13	4 (30,8%)	053	Tox-	C, D	2016
			014/020	0	C	2016
			126	V(BTb+)	D	2016
			394	0	E	2017
			SLO 214	Tox-	F	2017
Germany	2	1 (50,0%)	014/020	0	na	2016
			126	V (BTb+)	na	2016
Great Britain	2	1 (50,0%)	014/020	0	na	2016
			027	III (BTb+)	na	2016
			053	Tox-	na	2016
Greece	1	0 (0,0%)	na	na	na	na
Italy	3	0 (0,0%)	na	na	na	na
Israel	1	0 (0,0%)	na	na	na	na
Slovenia	17	7 (41,2%)	012	0	G[b]	2015[b]
			023	IV (BTb+)	H[b]	2015[b]
			014/020	0	I, [b]J	2015, [b]2016
			014/020	I	J	2016
			SLO 129	0	J	2016
			126	V (BTb+)	K	2016
			150	0	K	2017
			009	Tox-	L	2017
			SLO 057	Tox-	M	2017
Spain	2	0 (0,0%)	na	na	na	na
USA	13	3 (23,1%)	014/020	0	N	2016
			053	Tox-	O	2016
			126	V (BTb+)	O	2016
			244	III (BTb+)	P	2017
USA/Egypt	1	0 (0,0%)	na	na	na	na
Total number	75	21 (28,0%)	17	5 and Tox-	na	na

Tox- -nontoxigenic strain.
BTb + - binary toxin gene (*cdtB*) positive.
na-not applicable.
[a] -sample designation-specified only for source countries with multiple *C. difficile* positive samples.
[b] -specified *C. difficile* positive samples were homegrown while all other were purchased at supermarket or food market.

020 and 011/049 were each detected twice (in retail lamb's lettuces from Italy). Samples with detected ribotype 011/049 were collected at the same sampling location (store) and year, however, not in the same month. Samples with detected PCR ribotype 014/020 were collected at two different sampling locations on 2 different occasions (same year, different month). Only three out of 8 PCR ribotypes detected in leaf vegetables were toxigenic and were determined as toxinotype 0 (binary toxin negative (BTb−)) (Table 1).

In ginger, two nontoxigenic PCR ribotypes, SLO 158 and SLO 229 were determined.

Altogether 62 isolates were recovered from potatoes and distributed into17 different PCR ribotypes and five different toxinotypes (0, I, III, IV, V). Five detected PCR ribotypes were nontoxigenic. The most common PCR ribotype was 014/020 (in 8 out of 21 *C. difficile* positive samples), followed by nontoxigenic PCR ribotype 053 (6/21), PCR ribotype 126 (5/21) and PCR ribotype 150 (3/21). The remaining 13 PCR ribotypes were detected only once. PCR ribotypes 014/020, 053 and 126 were often present in samples that were collected in February and March 2016 (Supplementary table 3). They had shared sampling time or location of sample collection (6/8 samples with strains of PCR ribotype 014/020; 5/6 samples with strains of PCR ribotype 053 samples and 3/5 samples with strains of PCR ribotype 126).

Despite extensive variability of PCR ribotypes and country of origin, no new PCR ribotypes were identified. The characterization of up to 10 colonies per sample showed that almost half (47,6%) of *C. difficile* positive potato samples contained multiple PCR ribotypes (up to 4 different PCR ribotypes) (Table 2, Supplementary table 4). The analysis of individual potatoes (Supplementary Table 4) revealed that *C. difficille* contamination is variable within a specific batch. While some potato tubers in specific batch tested *C. difficile* negative, up to 3 different PCR ribotypes could be identified on other tubers from the same batch.

4. Discussion

Meat and meat products have often been tested for *C. difficile* contamination, while studies on vegetables are rare. In this study, we describe overall prevalence of 18,2% for *C. difficile* in raw vegetables that were purchased or homegrown in Slovenia. The positivity rate is somewhat higher than described in other countries with prevalence ranging from 2,3% to 7,5% (Al Saif and Brazier, 1996; Bakri et al., 2009; Metcalf et al., 2010; Eckert et al., 2013), mostly because of high proportion of *C. difficile* positive potatoes (28,0% vs. 9,4% in leaf vegetables). Root vegetables in general seem to be more often contaminated with *C. difficile* than other types of vegetables, which is in

Table 3
C. difficile PCR ribotypes isolated in this study from vegetables and comparison to their proportions in our large strain collection.

PCR ribotype	Number of *C. difficile* positive vegetable samples with specified PCR ribotype	Number of *C. difficile* strains in collection								Other (number od isolates (year of isolation)- source)
		2007–2013				2014–2017				
		human	animal	soil	water	human	animal	soil	water	
014/020	10	301	63	5	160	239	3	7	2	26 (2014, 2016)-shoes, slippers 4 (2016) -dog paws 3 (nd)- manure 3 (2008, 2009)-poultry litter
053[a]	6	1	–	–	13	1	–	–	–	–
126	5	8	–	–	2	11	–	–	–	–
150	3	27	15	–	6	40	6	–	–	23 (2009)- poultry litter 3 (2015, 2016)- pig farm environment
011/049	2	42	5	–	7	39	–	–	–	1 (2016)-shoes
SLO 214	2	–	–	1	–	–	–	1	–	–
001/072	1	77	6	1	3	101	–	–	–	2 (2016, 2017)-shoes
009	1	3	–	–	9	6	1	–	–	3 (2015, 2016, 2017)-shoes
012	1	43	–	1	1	12	1	1	–	6 (2014)-shoes
023	1	62	4	3	2	50	–	–	–	12 (2009)- poultry litter 7 (2014), 1(2016)-shoes 1 (2016)-dog paws 1 (nd)- manure
027	1	240	–	–	16	256	–	–	–	9 (2014,2016)- shoes
032	1	6	–	–	6	2	–	–	–	1-animal[b]
070	1	30	4	–	7	28	–	–	–	1 (2009)-poultry litter
244	1	2	1	–	1	–	–	–	–	–
394	1	6	19	–	3	9	–	–	–	1 (2015)- dust
SLO 057	1	3	1	1	12	3	1	–	–	–
SLO 129	1	2	–	–	–	–	–	–	–	–
SLO 158	1	–	–	–	2	1	–	–	–	–
SLO 187	1	–	–	–	1	1	–	–	–	–
SLO 204	1	–	–	1	1	–	–	4	–	–
SLO 205	1	–	–	1	–	–	–	2	–	–
SLO 217	1	–	–	–	–	–	–	1	–	–
SLO 221	1	–	–	–	–	–	–	–	1	–
SLO 229	1	–	–	–	–	–	–	2	–	–
SLO 279	1	–	–	–	–	–	–	–	–	–

nd-no data.

[a] - only nontoxigenic PCR ribotype 053 strains were detected in this study and are therefore compared only against RT 053 strains with identical nontoxigenic profile; 4 and 5 toxinogenic strains of PCR ribotype 053 wered detected in humans in both respective time intervals.

[b] -isolation year not known. Number of strains isolated only in Slovenia from humans, animals, soil or water is shown for two time intervals; prior to vegetable sampling and overlapping to vegetable sampling.

concordance with soil as a contamination source. For example, in Canada, only root vegetables tested positive for *C. difficile* among distinct types of vegetables, but potatoes were not sampled (Metcalf et al., 2010). Four out of 7 samples of root vegetables were positive in a study published by Al Saif and Brazier (1996). In a recent Australian study (Lim et al., 2018), *C. difficile* contamination of organic and non-organic potatoes, organic carrots, organic onions and organic beetroot was studied. Potatoes comprised almost half (45,0%) of all collected samples. A positivity rate of 30,0% was determined in all tested samples and positivity rate of 53,3% in potatoes only.

Low *C. difficile* spore counts in vegetables were previously suggested (Bakri et al., 2009). Numerous methodologies and media have been used in detection of *C. difficile* in foods, frequently using enrichment protocols that enable detection of even less than 10 spores per 20 g of salad (Eckert et al., 2013) and less than 10 spores per 1 g of meat (Weese et al., 2009). Methods used in our study also supported detection of less than 10 CFU per tested sample. As there is no consensus and no standardized method in cultivation of *C. difficile* from food samples, evaluation of the detection threshold for chosen method is necessary in order to provide a reliability of the data on *C. difficile* contamination of different products. However, the relevance of exposure to such low doses for CDI in humans remains unknown.

The variety of detected PCR ribotypes was considerable. We also observed some examples where identical PCR ribotype was isolated from samples with shared sampling time or location (6/8 for 014/020 positive samples, 5/6 for 053 positive samples and 3/5 for 126 positive samples). This could indicate potential cross-contamination in the retail store, during the transport to the laboratory or subsequent laboratory cross-contamination. Alternatively, strains of the same PCR ribotype are not necessarily identical and whole genome sequencing would be needed to elucidate this further.

All but two of 25 PCR ribotypes found on vegetables in this study have already been reported in different reservoirs in Slovenia before (Table 3). As already observed in one of our previous reports (river water isolates; Zidaric et al., 2010), an unexpected toxin profile was determined for the second most common PCR ribotype in this study-PCR ribotype 053. PCR ribotype 053 primarily correlates to toxinotype 0, however, nontoxigenic status was determined for all potato isolates with ribotype profile identical to the profile of 053 reference strain. Besides environmental strains, nontoxigenic 053 strains can sporadically be found also in humans (Table 3).

The most prevalent PCR ribotypes in this study (014/020, 126, 150, 053) belong to major human PCR ribotypes responsible for CDI in multiple countries (Freeman et al., 2015; Davies et al., 2016). PCR ribotype 014/020, which was detected in almost half of positive vegetable samples represents the predominant PCR ribotype in humans in Slovenia and is also frequently encountered in animals and other environments such as soil, water, shoes or manure (Table 3). Interestingly, some clinically important PCR ribotypes, such as 027, 001/072, 023, 070, 012, were rare on vegetables (Table 3). Only the minority of

obtained PCR ribotypes have not yet been isolated from humans, however, they have been associated either with animals, soil or water in Slovenia in our previous studies. From one potato sample of USA origin, PCR ribotype 244 was obtained. This PCR ribotype suddenly emerged in Australia in 2011 and caused severe community acquired infection but is currently still rare in Slovenia (Table 3).

Although contamination of vegetables is possible by various sources, including fertilizer, soil, water, food production process and marketing, considerable overlap of PCR ribotypes between vegetables and soil (9 of 17) suggests that growth environment could be the important source of contamination. However, further studies on this hypothesis are required.

More than half of *C. difficile* positive samples were imported (mostly from European countries) and in these, 17 different PCR ribotypes were detected. Although the high number of positive samples may mirror large number of imported vegetable samples that are available in Slovenia and although contamination point is not clear, our results suggest that vegetables could be an important vector for transmission of *C. difficile* over large geographical areas resulting in spread of some globally prevalent PCR ribotypes and introducing new ribotypes into import countries. The burden of imported vegetables contaminated with *C. difficile* was also noticed by some other researchers (Bakri et al., 2009; Metcalf et al., 2010).

One of the limitations of this study is that sampling method used was not systematic, with different food items sampled at different times (months/years) and from different types of locations (retail, homegrown). Additionally, some clusters of PCR overlap were detected for potato samples that could point to cross contamination. This potential contamination was detected only in February and March 2016 and could in part explain higher *C. difficile* positivity rates. On the other hand, the results on positivity and negativity of individual samples and tubers within one sample (Supplementary table 4) indicate that laboratory contamination was not present most of the time. Despite this limitation, this report adds missing information on *C. difficile* contamination of domestic and imported vegetables in Slovenia.

To conclude, although *C. difficile* infection has not yet been directly linked to contaminated food, our results endorse the potential of fresh produce as a possible source of *C. difficile* introduction into domestic environment and an important vehicle of *C. difficile* transnational and long-distance spread. Potatoes seem to form a specific vegetable subgroup when compared to other types of vegetables. They are more likely to be *C. difficile* positive and contaminated with several PCR ribotypes concurrently. As they are distributed across large geographical areas, they could therefore markedly impact *C. difficile* epidemiology by introducing new PCR ribotypes into import countries or even by disseminating epidemic PCR ribotypes such as 027 or 244.

Accknowledgement

Part of this study was supported by Slovenian Research Agency grant J3-4298.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.017.

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