International Journal of Food Microbiology 379 (2022) 109847

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Survey of the occurrence of *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts in green leafy vegetables marketed in the city of Valencia (Spain)

María Trelis [a,b,*], Sandra Sáez-Durán [a], Pablo Puchades [a], Nicole Castro [a], Ana Miquel [a], Mónica Gozalbo [c], Màrius Vicent Fuentes [a]

[a] *Parasite & Health Research Group, Department of Pharmacy, Pharmaceutical Technology and Parasitology, Faculty of Pharmacy, University of Valencia, Burjassot, 46100, Valencia, Spain*
[b] *Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, University of Valencia-Health Research Institute La Fe, 46026, Valencia, Spain*
[c] *Area of Nutrition and Bromatology, University of Valencia, 46010, Valencia, Spain*

ARTICLE INFO

Keywords:
Lettuce
Cabbage
Ecological farming
Giardia duodenalis
Cryptosporidium spp.
qPCR

ABSTRACT

The role of vegetables usually consumed without prior culinary treatment is known to contribute to the prevalence of foodborne diseases. Cysts and oocysts can contaminate food, which can then be the source of infection in humans. The aim of the study was to assess the occurrence of *Giardia duodenalis* and *Cryptosporidium* spp. (oo) cysts in green leafy vegetables marketed in the city of Valencia (Spain) combining parasitological methods, two real-time qPCRs and light microscopy. An experimental field study was conducted on 129 vegetable samples, 64 from conventional farms and 65 from ecological (organic) farms. The samples were washed with water, and the resulting solution after removing the vegetables, was subjected to 24-hour sedimentation. The concentrated sediment was used for the search for protozoa. A positive result by both real-time PCRs, or a positive result by one qPCR and confirmation by microscopy was established as a positivity criterion. *Giardia duodenalis* was detected in 23.0 % of the samples, and *Cryptosporidium* spp. in 7.8 %. *G. duodenalis* (41.5 %) and *Cryptosporidium* spp. (20.0 %) were more frequent in ecological crops. The high level of contamination detected in organic vegetables may be due to the type of fertilizers and the quality of the water used for their irrigation and reinforces the need to take extreme hygiene measures in vegetables that are consumed raw.

1. Introduction

Food safety is a fundamental fact for the economic and commercial development and establishment of the international prestige of nations. At present, food borne parasitic diseases represent a public health problem worldwide, but in low-income countries their frequency and impact persist due to inadequate sanitary conditions, accelerated urbanization, poor hygiene habits and lack of clean drinking water. There are other determining factors in Western culture, such as the adaptation of new culinary habits and the introduction of new foods from other cultures (WHO, 2008). In the case of green vegetables, few safeguards exist to allow for the prevention. On the other hand, there is access to informative material, food recommendations and prophylactic measures aimed at the consumer regarding the handling and washing of these products, but not on specific legislation for the control and surveillance of parasites (AESAN (Agencia Española de Seguridad Alimentaria y Nutrición), 2021). It is the responsibility of public health agencies to make measures known to consumers, so that they will be able to obtain safe food and avoid what is not optimal (FAO, 2020; Fuentes and Barceló, 2008; Ambiental, 2018).

In the 20th century, ecological farming emerged, defined by the United States Department of Agriculture as a production system that largely avoids or excludes the use of synthetic fertilizers and pesticides (USDA (United States Department of Agriculture), 2015). In this sense, the use of organic fertilizers, such as plant biomass (compost) or fecal matter (sewage sludge, treated sludge, manure, other waste) previously

* Corresponding author at: Parasite & Health Research Group, Department of Pharmacy, Pharmaceutical Technology and Parasitology, Faculty of Pharmacy, University of Valencia, Burjassot, 46100, Valencia, Spain.
E-mail addresses: maria.trelis@uv.es (M. Trelis), sandra.saez@uv.es (S. Sáez-Durán), papuco@alumni.uv.es (P. Puchades), amima2@alumni.uv.es (A. Miquel), monica.gozalbo@uv.es (M. Gozalbo), mario.v.fuentes@uv.es (M.V. Fuentes).

https://doi.org/10.1016/j.ijfoodmicro.2022.109847
Received 1 March 2022; Received in revised form 13 July 2022; Accepted 18 July 2022
Available online 22 July 2022

subjected to a composting process, were chosen to enrich the soil, as a recovery method of degraded soils (USEPA (United States Environmental Protection Agency), 1999).

Agriculture (including irrigation, livestock and aquaculture) is by far the largest consumer of water, accounting for 69.0 % of annual water withdrawals worldwide. In view of the fact that the speed of consumption of water resources does not decrease, it is even expected to increase, it is therefore recommended that water be reused in farming or other secondary manners (fountains, gardens, parks, etc.) (UNESCO, 2019). Although there is a legislative framework in Spain that regulates the basic conditions that treated wastewater must meet for use in irrigation (Spanish Royal Decree 1620/2007), the regulations related to the presence of parasites in water and food exclusively mention helminths, not protozoa. In this sense, due to the lack of legislation and control regarding protozoa, together with their greater capacity to contaminate the environment, the risk of suffering a parasitic infection by consuming contaminated food is greater (Razzolini et al., 2016).

Currently, there are nine recognized species of *Giardia* in various vertebrates including two distinct species from birds, *G. agilis* and *G. psittaci*; three from rodents, *G. muris* and *G. microti* and *G. cricetidarum*; one from amphibians, *G. agilis*; one from reptiles *G. varani*, one from southern brown bandicoot, *G. paramelis*, and a large taxonomic grouping termed *G. duodenalis* with eight established genotypes known as assemblages A to H in a range of mammalian hosts other than mice (Ryan et al., 2021; Sterling, 2018; Thompson, 2004). *Giardia duodenalis* is an extracellular protozoan flagellate with a direct cycle that colonizes the upper intestinal surface and causes the diarrheal disease giardiasis. In humans, *G. duodenalis* reaches prevalences higher than 30 % in low-income countries, while the values range between 2 and 7 % in industrialized countries, where it is considered a re-emerging disease due to its increasing involvement in diarrheal diseases that occur in different settings, such as nurseries, due to a lack of hygiene and water contamination (Chover et al., 2010; Fletcher et al., 2012; Lobo et al., 2009; Plutzer et al., 2008). In the EU/EEA and in Spain, *G. duodenalis* infection is considered the most prevalent water- and foodborne parasitic disease, with reported cases each year (Azcona-Gutiérrez et al., 2017; Carmena et al., 2012; de Lucio et al., 2015; ECDC, 2019; Reh et al., 2019; Vivancos et al., 2018; Wang et al., 2019). The foodborne transmission of *G. duodenalis* involves the use of contaminated water for irrigation of crops or in food preparation, as well as contamination due to poor sanitary and hygienic habits practiced by food handlers; moreover, zoonotic transmission also occurs with regular frequency, resulting from the high prevalence of infection in livestock, pets and wildlife and with an important impact on the water and foodborne routes to humans (Sterling, 2018). In short, giardiasis can be considered a zoonosis, almost exclusively limited to assemblages A and B of *G. duodenalis* (Sterling, 2018), which would largely explain the recent epidemics in cities in industrialized countries, where humans coexist closely with their pets, as well as the growing appreciation for ecologically grown products.

Hitherto, 44 validated species of *Cryptosporidium* and > 120 genotypes are described, of which 19 species and four genotypes have been reported in humans: *Cryptosporidium hominis*, *C. parvum*, *C. meleagridis*, *C. canis*, *C. felis*, *C. ubiquitum*, *C. cuniculus*, *C. viatorum*, *C. muris*, *C. andersoni*, *C. erinacei*, *C. tyzzeri*, *C. bovis*, *C. suis*, *C. scrofarum*, *C. occultus*, *C. xiaoi*, *C. fayeri*, *C. ditrichi*, *Cryptosporidium* chipmunk genotype I, mink genotype, skunk genotype and horse genotype. *Cryptosporidium hominis* and *C. parvum*, are responsible of near 95 % of human infections, followed by *C. meleagridis*, *C. felis* and *C. canis* (Ryan et al., 2021).

Cryptosporidium spp. are widely distributed, highly transmissible and infectious human parasites, frequently associated with contaminated or untreated, drinking or recreational, waters (Vivancos et al., 2018). *Cryptosporidium* spp. is an intracellular protozoan with a direct life cycle that colonizes the small intestine, preferably the duodenum and causes the diarrheal disease cryptosporidiosis. In immunocompromised individuals it can cause very profuse watery diarrhea, abdominal pain, vomiting and fever. In contrast, in immunocompetent subjects after an acute phase of diarrhea, it tends to self-limit spontaneously (Fayer and Xiao, 2008). Several studies have shown that the infective dose for humans is very low, the ingestion of 1–30 oocysts can cause infection. The main route of transmission is considered water due to oocyst resistance to traditional disinfectants (Carmena et al., 2007; Huang et al., 2004).

Human giardiasis and cryptosporidiosis have traditionally been diagnosed by light microscopy, with and without specific staining. However, skillful microscopists, capable of morphologically identifying parasites by microscopy, are increasingly scarce in non-endemic countries, even in European reference centers. Therefore, less investigator-dependent modern molecular assays for the detection of protozoa have been shown to be more reliable compared to microscopy or antigen-based diagnosis (Weinreich et al., 2021).

In detail, the aim of the present study is to assess the level of contamination with *G. duodenalis* and *Cryptosporidium* spp. in green leafy vegetables marketed in the city of Valencia and to analyze the potential influence of the farming type (ecological or conventional), the growth season and the type of vegetable. Thereafter, the degree of fecal contamination and its role as a vehicle for foodborne parasitic diseases was evaluated.

2. Materials and methods

2.1. Vegetable samples

A total of 129 green leafy vegetable samples were analyzed in this field study, including 44 Romaine *(Lactuca sativa* var. longifolia), 35 Oak leaf (*Lactuca sativa* var. crispa) and 35 Iceberg (*Lactuca sativa*) and 15 Kale cabbages (*Brassica oleracea* var. sabellica). In total, 64 green vegetables from conventional farming (Romaine and Iceberg lettuces) and a second group of 65 organic vegetables from ecological farms (Romaine and Oak leaf lettuce, and Kale cabbage) were analyzed. Likewise, the sampling was carried out throughout the four annual seasons (Table 1).

Vegetables for analysis were purchased in supermarkets where their suppliers show the origin of the product on their labels (various Spanish provinces) to provide additional information to the consumer; and a second group of ecological farming was randomly purchased from local markets in the Valencia metropolitan area, coming from small producers. As a summary of the origins of the analyzed vegetables: 89 samples came from the Valencian Community, 21 from Murcia, 10 from Andalusia, 7 from Navarre and 2 from Catalonia.

2.2. Sample processing prior to analysis

Six processing protocol for the parasitological analysis of foods described to date were reviewed (Álvarez et al., 1981; Bailenger, 1962; Bier, 1991; Cook et al., 2007; Downes and Ito, 2001; Vaerewijck et al., 2011). After the review and as a starting point, the protocol of Álvarez et al. (1981) was applied with slight modifications (Rivero de Rodríguez et al., 1998), considering it the most reproducible, in terms of the

Table 1
Number of vegetables analyzed by type of farming and season.

Type of farming	Season	Type of vegetable			
		Romaine	Oak leaf	Iceberg	Kale
Conventional ($n = 64$)	Spring	–	–	20	–
	Summer	–	–	15	–
	Autumn	12	–	–	–
	Winter	17	–	–	–
Ecological ($n = 65$)	Spring	–	21	–	–
	Summer	–	14	–	–
	Autumn	11	–	–	13
	Winter	4	–	–	2
	TOTAL	44	35	35	15

amount of processed samples, necessary equipment and materials.

For the processing of the samples, 75 g of stem and outermost leaf were taken and added to 500 mL of previously filtered and boiled water. The content was stirred with the help of a magnetic stirrer for 1 h, the remains of the vegetable were removed and the solution left to stand for 24 h. Subsequently, the solution was decanted into a separation funnel and the first fraction was collected in 15 mL tubes to be subjected to centrifugation for 5 min at 800 xg. Once the concentrate or sediment was separated, the supernatant was discarded, and the precipitate was reconstituted in 400 μL of saline solution (0.9 %). For molecular analysis, 200 μL of the precipitate were kept at −80 °C, and a few drops of 10 % formalin were added to the rest as a fixative and it was kept at room temperature until microscopic analysis.

2.3. Parasitological assessment

DNA was extracted from 200 μL of the concentrated sediment obtained with the QIAamp DNA Stool Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions with slight modifications consisting of the sonication step of 1 h in a bath to favor the disruption of cysts and oocysts. Total DNA was eluted in a volume of 100 μL.

The samples were analyzed by two types of real-time quantitative PCR (qPCR) for the accurate detection of human protozoa in stool samples: a) a multiplex PCR designed for the detection of *Giardia duodenalis*, *Entamoeba histolytica*, *Cryptosporidium* spp. (*C. hominis*, *C. parvum* and *C. meleagridis*, and not *C. muris*), *Blastocystis hominis*, *Dientamoeba fragilis*, and *Cyclospora cayetanensis* (Allplex Gastrointestinal Panel-Parasite Assay, Seegene Inc., Seoul, Korea) (Autier et al., 2020; Kim et al., 2020); b) and individual or singleplex LightMix Modular Assays for *Giardia* spp. and *Cryptosporidium* spp. (manufactured by TIBMOLBIOL, distributed by Roche Diagnostics, Mannheim, Germany) (Friesen et al., 2018). In this manner, the two protozoan species of interest were tested by two different commercially available methods.

Multiplex PCR was performed on the CFX96TM Real Time PCR System (Bio-Rad, Marnes-la-Coquette, France), and managed with CFX Manager IVD 1.6 software, in a 25 μL reaction volume containing 20 μL reaction mix (5 μL Primers (MOM), 5 μL Anyplex PCR master mix (EM2), 8 μL of DNase/RNase-free water and 2 μL of internal control (IC)) and 5 μL of DNA. Negative (ultrapure water) and positive controls provided by the manufacturers for each of the parasites tested were included in each assay. Results were analyzed using the Seegene Viewer V2.1 software optimized for multiplex assays. Samples were considered positive for specific parasites if the cycle threshold (Ct) was below 43 cycles according to the manufacturer's instructions.

The LightMix Modular Assays (LMix) provided the primers and probe for each protozoan. For a final volume of 20 μL, each reaction mix contained 0.5 μL specific LMix assay, 10 μL of 2× master mix (PerfeCTa qPCR ToughMix, Quanta Biosciences, Gaithersburg, MD, USA), 4.5 μL of DNase/RNase-free water, and 5 μL of DNA. Negative (ultrapure water) and positive controls provided by the manufacturers for each of the parasites tested were included in each assay. The PCR was carried out on StepOnePlus Real-Time PCR System (Applied Biosystems, USA) linked to StepOne software version 2.3., following the protocol indicated by the manufacturer. For *G. duodenalis* the target of PCR amplification was a 62 bp fragment of the *18S* rRNA gene, detected with an FAM labeled hydrolysis probe. This gene was chosen by manufacturers as the genome has multiple copies, which favors amplification during the analysis and increases the sensitivity of the diagnostic technique (Azcona-Gutiérrez et al., 2017; Reh et al., 2019; Verweij et al., 2003). According to the information provided by the manufacturer, this assay will detect human *G. duodenalis* and animal *G. microti* species; but may not detect *G. ardeae*, *G. muris* and *G. psittaci*. In case of *Cryptosporidium* spp., the targets of PCR amplification were two fragments of 73 and 118 bp of the oocyst wall protein (*COWP*) gene, detected with a LC610 labeled hydrolysis probe. According to the manufacturer's instructions, this test detects *Cryptosporidium hominis, C. parvum, C. meleagridis, C. tyzzeri, C. wrai, C. erinace, C. cuniculus, C. ferret* and *C. viatorum*, but it does not detect *C. ubiquitum*, which constitutes a limitation in the study of the frequency of this parasite in the sample. Samples were considered positive for specific parasites if the cycle threshold (Ct) was less than or equal to 43, as indicated by the manufacturer, considering Ct values between 20 and 35 to be optimal.

As a confirmatory method, to accompany the results obtained by qPCR techniques, the sediments were analyzed by light microscopy. Analysis started from the concentrate sediment of each sample fixed in formalin. This concentrate was observed under the microscope (10× and 40×) with saline solution and with Lugol solution for the identification of cysts of *G. duodenalis* in quadruplicate. Likewise, four fecal smears per sample were prepared for acid-fast staining for *Crytosporidium* spp. oocysts detection (modified Zielh-Neelsen) and subsequent microscopic assessment (100×) (Tahvildar-Biderouni and Salehi, 2014).

2.4. Statistical methods

In the case of *G. duodenalis*, the standard non-parametric test χ^2 was applied to analyze the influence of the type of farming, type of lettuce and season of the year on the parasites frequencies. Statistical significance was established at $p < 0.05$. With the small number of concordant samples available for *Cryptosporidium* spp. the statistics were not meaningful.

The IBM SPSS Statistics 26 (IBM Corporation, Amonk, New York, NY, USA) for Windows software package was used for statistical analysis.

3. Results

For the two protozoa studied in the present work, obtaining a positive result by both real-time PCRs (single- and multiplex), or a positive result by one qPCR and confirmation by microscopy, was established as a criterion of positivity to classify a sample as "positive" in terms of occurrence and for statistical analysis. On the other hand, there were samples that were classified as "doubtful", when testing positive in one of the qPCRs, but which could not be confirmed by microscopy. Finally, those that obtained a negative result in the three assays were classified as "negative".

3.1. Detection of Giardia duodenalis in green leafy vegetables

Of the 129 green leafy vegetables analyzed, 30 (23.3 %) were considered contaminated with *G. duodenalis* and classified as "positive". In addition, six (4.7 %) samples were considered "doubtful", since their positivity could not be confirmed (Table 2).

When comparing the two qPCR assays, 31 positive results were obtained with the singleplex LMix, while 33 with multiplex Allplex. Therefore, combining these results, 28 samples were considered as "positive". Finally, of the 8 samples with a positive result for only one qPCR, two could be confirmed by microscopy.

Comparing the mean threshold cycle (Ct) obtained in the qPCR assays, it was equally high in both (37), and with similar ranges, slightly wider for the LMix (35–42 LMix vs. 35–39 Allplex).

Analyzing the level of contamination with *G. duodenalis* cysts, just in case of confirmed "positives" (Table 3), the parasite was more frequent

Table 2
Contamination results in the 129 samples analyzed according to the positivity criterion.

Detected parasite	"Positive" samples		"Doubtful" samples		Negative samples	
	n	%	n	%	n	%
Giardia duodenalis	30	23.3	6	4.7	93	72.1
Cryptosporidium spp.	10	7.8	34	26.4	85	65.9

Table 3
Frequencies of *Giardia duodenalis* depending on the variables analyzed.

	Variable	"Positive" samples (n)	%
Type of farming	Ecological	27	41.5*
	Conventional	3	4.7
Type of vegetable	Oak leaf lettuce	26	74.3*
	Romaine lettuce	1	2.3
	Iceberg lettuce	3	8.6
	Kale cabbage	–	–
Harvest season	Spring	22	53.7*
	Summer	7	24.1
	Autumn	1	2.8
	Winter	–	–

n, n° of samples; %, frequency.
* Significant association ($p < 0.05$).

(41.5 %; 27/65) in ecologically grown vegetables ($\chi^2 = 20.964$; $p < 0.0001$; df = 1), in the Oak leaf lettuce (74.3 %; 26/35) ($\chi^2 = 68.886$; p < 0.0001; df = 3), in those samples picked in spring (53.7 %; 22/41) ($\chi^2 = 36.039$; p < 0.0001; df = 1).

3.2. Detection of Cryptosporidium spp. in green leafy vegetables

In case of Cryptosporidium spp., only 10 (7.8 %) samples could be classified as "positive" (Table 2). However, for a large group of samples (34; 26.4 %) the final decision was "doubtful", since they were only positive for one of the two qPCRs. Comparing results of both PCR assays in real time, a low concordance in the number of positives was observed, with 30 positives with LMix and 19 with Allplex, but coinciding only in 5 of them (11.4 %; 5/44).

Analysis of the sediments using microscopy and modified Ziehl-Neelsen was able to confirm 5 "doubtful" cases that had obtained a single positive result by LMix (qPCR for the detection of COWP gene). These cases, along with the positives for both qPCRs, resulted in confirmed contamination for 10 samples. If we had considered "positive" those samples with a positive qPCR result, we would have a total frequency of contamination of 34.1 % (44/129). The mean Ct for the singleplex (LMix) was 33, while for Allplex it was 41. Likewise, individual PCR provided a wider Ct range (21–42) than multiplex (37–42).

Due to the small number of concordant results for Cryptosporidium spp. no statistical analysis was performed. However, the results regarding the variables studied are shown in the Table 4. For confirmed "positive" samples, the frequency between vegetables from organic and conventional farming was almost equal (7.7 % vs. 7.8 %). Regarding the type of vegetable, the "positive" cases predominated in the Kale cabbage (20.0 %; 3/15) from organic farming. Finally, autumn accumulated the highest percentage of positivity (11.1 %; 4/36). However, if "doubtful" samples could be confirmed, the results would be different, with a predominance of the parasite in organic cultivation (41.5 %; 27/65), Oak leaf lettuce (40.0 %; 14/35), Spring (39.0 %; 16/41) as the variables with the highest frequency of the parasite and in consistency with the risk factors of vegetables contaminated with G. duodenalis (Table 4).

Table 4
Frequencies of *Cryptosporidium* spp. depending on the variables analyzed.

	Variable	"Positive" samples (n)	%	"Doubtful" samples (n)	%
Type of farming	Ecological	5	7.7	22	33.8
	Conventional	5	7.8	12	18.8
Type of vegetable	Oak leaf lettuce	–	–	14	40.0
	Romaine lettuce	2	4.5	9	20.5
	Iceberg lettuce	5	14.3	7	20.0
	Kale cabbage	3	20.0	4	26.7
Harvest season	Spring	3	7.3	13	31.7
	Summer	2	6.9	8	27.6
	Autumn	4	11.1	11	30.6
	Winter	1	4.3	2	8.7

n, n° of samples; %, frequency.

3.3. Fecal contamination of green vegetables for human consumption

Finally, a comment considering the presence of both parasites in the analyzed vegetables, in relation to human or animal fecal contamination. Considering all samples with a "positive" result, a total contamination level of 31.0 % (40/129) was obtained. A significant association between the detection of the parasite (cysts, oocysts, or parasite DNA) and the organic farming type (32 ecological vs. 8 conventional) ($\chi^2 = 20.337$; $p < 0.0001$; df = 1) (odds ratio (OR) = 6.8); the Oak leaf lettuce (26 Oak vs. 8 Iceberg, 3 Romaine and 3 Kale) ($\chi^2 = 43.485$; p < 0.0001; df = 3) (with Iceberg OR = 9.8, Romaine OR = 39.4 and Kale OR = 11.6); and the samples harvested in spring (25 spring vs. 9 summer, 5 autumn and 1 winter) ($\chi^2 = 24.107$; p < 0.0001; df = 3) (with summer OR = 3.5, autumn OR = 9.7 and winter OR = 34.4), was demonstrated.

Among all the samples analyzed, only in two cases (both Iceberg lettuce, conventional farming in Murcia and picked in spring) the presence of both parasites was confirmed.

However, it should be noted that among the "doubtful" cases of contamination by *Cryptosporidium* spp., 12 of them were "positive" for *G. duodenalis*, and therefore fecal contamination was demonstrated, so a double infection was more likely. Among these suspected cases of double infection, 11 samples were ecological Oak leaf, picked in spring (7) and summer (4); and one Iceberg lettuce from conventional farming, picked in spring.

The opposite case of a positive sample of *Cryptosporidium* spp. but "doubtful" of *G. duodenalis* occurred only once in a lettuce with the same risk factors as in the last case (Iceberg, conventional and spring).

4. Discussion

The health of humans depends largely on the nutritional quality of the food they consume daily, and the hygienic and sanitary conditions of its production chain, from the field to the consumer. It is a priority that government agencies responsible for the food and health sectors, train all farmers and food handlers and implement actions aimed at making water for irrigation or human consumption drinkable (AQUASTAT, 2021).

The foodborne protozoan parasites of particular concern with respect to public health include *Cyclospora cayetanensis*, *Cryptosporidium* spp., *G. duodenalis* and *Toxoplasma gondii* (Dixon et al., 2011). Among them, in the case of *Cryptosporidium* spp. and *G. duodenalis* transmission through contaminated water should be highlighted (Dixon, 2016; Efstratiou et al., 2017; Lane and Lloyd, 2002). However, food transmission has also been cited and they are considered foodborne parasites associated with numerous disease outbreaks (ECDC, 2019; Ryan et al., 2019; Sterling, 2018; Xiao and Cama, 2018). Previous studies in green vegetables (Alemu et al., 2020; Amorós et al., 2010; Barnabé et al., 2010; Dixon, 2016; Dixon et al., 2013; Doménech et al., 2018; Montanher et al., 2007), lettuces and cabbage for human consumption confirmed the presence of these parasites. Specifically, a study carried out by Amorós et al. (2010) in salad products and irrigation waters from Valencia (Spain), a high level of parasitic contamination of *Cryptosporidium* (63.1 %; 12/19) and *Giardia* (52.6 %; 10/19) was reported, with a predominance of contamination detected in lettuce with respect to cabbage. ISO 18744 method is the only standard method for the detection of *Cryptosporidium* and *Giardia* in green leafy vegetables and berries based on surface washing, immunomagnetic separation and concentration, and detection and quantification by immunofluorescence microscopy, but this method is not suitable for routine analysis or monitoring in a hazard analysis plan and critical control points (Chalmers et al., 2020) neither for determine species or genotypes.

After the detection of intestinal protozoa in a third of the samples

analyzed, fecal contamination is obvious, with Oak leaf lettuces, ecological farming and harvest in spring being the most frequently contaminated, so that their raw consumption poses a greater risk. The contamination of vegetables runs from primary production to consumption, associated with the use of human or animal manure as fertilizers, the use of contaminated water from irrigation, the direct contamination by food handlers, consumers, rodents and peridomestic and wild mammals, and indirect contamination by insects (flies, cockroaches, beetles) (Alemu et al., 2020).

According to the scientific literature, *G. duodenalis* infection does not show a clear seasonal pattern (Carmena et al., 2007; ECDC, 2019). However, the annual epidemiological report of the ECDC (2019) shows an increase in cases in the period from August to October in the EU/EEA, between 2013 and 2017, suggesting greater transmission of the disease during the hottest months. Despite these claims, the samples with the highest positivity were detected in spring, followed by summer. This fact could be related to the scarce rainfall and the resulting need of additional irrigation during these seasons. During these seasons, greater activity of animals takes place and the fecal contamination of water could be favored, also increasing the direct contact between the animals and the crops. Specifically, in a study carried out by the Universitat Politècnica de València in which *G. duodenalis* cysts were quantified in water, in untreated influents and treated effluents, from treatment plants located in eastern Spain observed that the highest influent counts are obtained in summer and in effluents in winter (Amorós et al., 2010). Regarding the type of lettuce, Oak leaf is the one with the highest rate of contamination. As a lettuce that grows in width, it is likely to be entirely covered when watered, and also its deformable leaves with recesses allow water to enter the internal layers.

In our study, it is shown that the food route can play an important role in the transmission of *G. duodenalis*, since the cysts were found in the 23.3 % of samples. Although the general data are lower than those reported in previous studies in the city of Valencia (52.6 %) (Amorós et al., 2010), we believe that it is due to the fact that a more heterogeneous and numerous group of samples has been analyzed. In support of this transmission route, the risk of parasites posed by the consumption of ecological vegetables has been confirmed by our research team in two studies carried out in two Valencian adult populations, one suffering from chronic gastrointestinal symptoms (Trelis et al., 2019), and the other being morbidly obese (Caudet-Esteban et al., 2021). Of the risk factors analyzed, the one that stood out for its significance was precisely the regular consumption of ecological grown products, from small producers and even their own orchards.

Cryptosporidium spp. oocysts have been isolated from several foodstuffs (Ursini et al., 2020), and have been associated with green vegetables in several countries worldwide, such as Norway, Spain and Ghana (Amorós et al., 2010; Duedu et al., 2014; Robertson and Gjerde, 2001). Direct contamination of food by fecal materials from animals or food handlers has been implicated in several foodborne outbreaks of cryptosporidiosis in industrialized nations. It is estimated that about 8 % of *Cryptosporidium* spp. infections in the United States are foodborne (Scallan et al., 2011). The results regarding the detection of *Cryptosporidium* spp. oocysts in our samples are somewhat controversial, if we use a very restrictive positivity criterion that requires a double positive result, only a small proportion of vegetables appeared contaminated, but leaving a significant number of samples as "doubtful" and possibly also contaminated, because they tested positive once. In "positive" cases exclusively, predominance in Kale cabbage, organically grown and picked in autumn was detected. It is noteworthy that among the *Cryptosporidium* spp. cases considered "doubtful", there were 12 vegetables suspected of fecal contamination due to the detection of *G. duodenalis* on them. If these cases could be confirmed, the level of contamination of both protozoa would be closer.

In our case, light microscopy analysis detected a limited number of positives, showing poor sensitivity, even after prior concentration by sedimentation and using acid-fast staining for *Cryptosporidium* spp. However, microscopy was useful in "doubtful" cases by molecular techniques for confirmation, since (oo)cyst detection is at genus level and its sensitivity is not subjected to specific variations.

At present, fast and precise molecular detection techniques are prioritized that also allow establishing an epidemiological trace of the analyzed parasites, genotyping or subtyping species and recognizing possible routes of infection to which the consumer is exposed (Resendiz-Nava et al., 2020). In addition, the reduction of cost and time to obtain results are crucial in industrial monitoring plans and food outbreak investigations (Chalmers et al., 2020). Regarding the level of agreement between the two real-time PCR techniques, it should be said that just as for *G. duodenalis*, both the single- and the multiplex PCR have practically had the same efficacy for the detection of positives, since both should have the same molecular target. However, in the case of *Cryptosporidium* spp., individual LMix and the gene encoding the oocyst wall protein has shown a higher sensitivity than Allplex multiplex panel; perhaps because a different molecular target is analyzed, or because LMix PCR is designed to amplify two fragments of the same gene gaining sensitivity with it.

Despite detecting the protozoa in concentrated sediment, the low parasite load disabled the characterization of the species involved in food contamination and the subsequent epidemiological analysis that would clarify the origin of the contaminating parasites and transmission routes.

5. Conclusions

The high level of contamination detected in organic lettuces may be due to the type of fertilizers and water used for their cultivation. Our identification of *G. duodenalis* and *Cryptosporidium* spp., protozoa with possible zoonotic potential, in green vegetables points to foodborne transmission as a source of human infection, and reinforces the need to take extreme hygiene measures in the consumption of raw vegetables and highlight a substantial public health concern.

Multiplex panels for the simultaneous detection of *G. duodenalis* and *Cryptosporidium* spp. will hopefully provide an improvement in diagnosis in clinical parasitology laboratories, and at the same time, find application in environmental and food analyses offering useful information for public health specialists. In addition, the use of molecular tools for the determination of the species/assemblages/genotypes of *Giardia* spp. and *Cryptosporidium* spp. isolated from food will be very useful for tracking sources of food contamination.

The present study has demonstrated the usefulness of human commercial diagnostic kits to the study of food samples. In the future, once the procedure for sampling, conservation, preparation and extraction of the sediments has been optimized, together with the selection of the most accurate qPCR, a high performance in detection can be aspired to monitor foodborne protozoa.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

None.

Acknowledgments

Not applicable.

References

AESAN (Agencia Española de Seguridad Alimentaria y Nutrición), 2021. Spanish Agency for Food Safety and Nutrition. Retrieved from. https://www.aesan.gob.es. (Accessed 29 November 2021).

Alemu, G., Nega, M., Alemu, M., 2020. Parasitic contamination of fruits and vegetables collected from local markets of Bahir Dar City, Northwest Ethiopia. Res. Rev. Trop. Med. 11, 17–25. https://doi.org/10.2147/RRTM.S244737.

Álvarez, M., Colina, M., Rodríguez, H., 1981. Recuperación de formas evolutivas de enteroparásitos en legumbres del mercado Las Pulgas, Maracaibo, Venezuela. Universidad del Zulia, Facultad de Medicina, Escuela de Bioanálisis, Maracaibo, Venezuela. Unpublished dissertation degree's thesis.

Ambiental, Higiene, 2018. Higiene alimentaria: Parásitos transmitidos por los alimentos, un riesgo poco controlado todavía. Retrieved from. https://higieneambiental.com/higiene-alimentaria/parasitos-transmitidos-por-los-alimentos-un-riesgo-poco-controlado-todavia. (Accessed 29 November 2021).

Amorós, I., Alonso, J.L., Cuesta, G., 2010. Cryptosporidium oocysts and Giardia cysts on salad products irrigated with contaminated water. J. Food Prot. 73 (6), 1138–1140. https://doi.org/10.4315/0362-028x-73.6.1138.

AQUASTAT, 2021. FAO's Global Information System on Water and Agriculture. Retrieved from. http://www.fao.org/aquastat/en/. (Accessed 29 November 2021).

Autier, B., Gangneux, J.P., Robert-Gangneux, F., 2020. Evaluation of the AllplexTM gastrointestinal panel–parasite assay for protozoa detection in stool samples: a retrospective and prospective study. Microorganisms 8, 569. https://doi.org/10.3390/microorganisms8040569.

Azcona-Gutiérrez, J.M., de Lucio, A., Hernández-de-Mingo, M., García-García, C., Soria-Blanco, L.M., Morales, L., Aguilera, M., Fuentes, I., Carmena, D., 2017. Molecular diversity and frequency of the diarrheagenic enteric protozoan Giardia and Cryptosporidium in a hospital setting in northern Spain. PloS One 12 (6), e0178575. https://doi.org/10.1371/journal.pone.0178575.

Bailenger, J., 1962. Valuer compare des ethodes denrichissement en coprologie parasitarie. Étude des facteurs de concentration dans les méthodes a l'éther. Pharm. Biol. 3, 249–259.

Barnabé, A.S., Ferraz, R.R., Pincinato Ede, C., Gomes, R.C., Galleguillos, T.G., Cerqueira, M.Z., Soares, E.G., Lage, P.S., Araújo, C.X., Szamszoryk, M., Massara, C.L., 2010. Análisis comparativo de los métodos Para la detección de parásitos en las hortalizas Para el consumo humano. Rev. Cubana Med. Trop. 62 (1), 24–34.

Bier, J.W., 1991. Isolation of parasites on fruits and vegetables. Southeast Asian J. Trop. Med. Public Health Suppl, 144–145.

Carmena, D., Aguinagalde, X., Zigorraga, C., Fernández-Crespo, J.C., Ocio, J.A., 2007. Presence of giardia cysts and cryptosporidium oocysts in drinking water supplies in northern Spain. J. Applied. Microbiol. 102 (3), 619–629. https://doi.org/10.1111/j.1365-2672.2006.03193.x.

Carmena, D., Cardona, G.A., Sánchez-Serrano, L.P., 2012. Current situation of giardia infection in Spain: implications for public health. World J. Clin. Infect. Dis. 2 (1), 1–12. https://doi.org/10.5495/wjcid.v2.i1.1.

Caudet-Esteban, J., Trelis, M., Cifre, S., Soriano, J.M., Merino-Torres, J.F., 2021. Presence and significance of intestinal unicellular parasites in a morbidly obese population. Int. J. Obes. 46, 220–227. https://doi.org/10.1038/s41366-021-00980-6.

Chalmers, R.M., Robertson, L.J., Dorny, P., Jordan, S., Karssin, A., Katzer, F., La Carbona, S., Lalle, M., Lassen, B., Mladineo, I., Rozycki, M., Bilska-Zajac, E., Schares, G., Mayer-Scholl, A., Trevisan, C., Tysnes, K., Vasilev, S., Klotz, C., 2020. Parasite detection in food: current status and future needs for validation. Trends Food Sci Technol. 99, 337–350. https://doi.org/10.1016/j.tifs.2020.03.011.

Chover, L., Borrás, M., Gozalbo, M., Muñoz-Antoli, C.T., Manrique, I., Puchades, O., Salazar, A., Esteban, J.G., 2010. Intestinal parasites in school children in the city of Valencia: prevalence survey. Boletín Epidemiológico Semanal 18 (7), 69–72.

Cook, N., Nichols, R.A.B., Wilkinson, N., Paton, C.A., Barker, K., Smith, H.V., 2007. Development of a method for detection of giardia cysts on lettuce and for simultaneous analysis of salad products for the presence of Giardia cysts and Cryptosporidium oocysts. Appl. Environ. Microbiol. 73 (22), 7388–7391. https://doi.org/10.1128/AEM.00552-07.

de Lucio, A., Martínez-Ruiz, R., Merino, F.J., Bailo, B., Aguilera, M., Fuentes, I., Carmena, D., 2015. Molecular genotyping of giardia isolates from symptomatic individuals attending two major public hospitals in MadridSpain. PloS One 10 (12), e0143981. https://doi.org/10.1371/journal.pone.014398.

Dixon, B., Parrington, L., Cook, A., Pollari, F., Farber, J., 2013. Detection of cyclospora, cryptosporidium, and giardia in ready-to-eat packaged leafy greens in OntarioCanada. J. Food Prot. 76 (2), 307–313. https://doi.org/10.4315/0362-028X.JFP-12-282.

Dixon, B.R., 2016. Parasitic illnesses associated with the consumption of fresh produce - an emerging issue in developed countries. Curr. Opin. Food Sci. 8, 104–109. https://doi.org/10.1016/j.cofs.2016.04.009.

Dixon, B.R., Fayer, R., Santín, M., Hill, D.E., Dubey, J.P., 2011. Protozoan parasites: Cryptosporidium, Giardia, Cyclospora, and Toxoplasma. In: Hoorfar, J. (Ed.), Rapid Detection, Characterization and Enumeration of Food-borne Pathogens. ASM Press, Washington, DC, pp. 349–370. https://doi.org/10.1128/9781555817121.ch24.

Doménech, E., Amorós, I., Moreno, Y., Alonso, J.L., 2018. Cryptosporidium and giardia safety margin increase in leafy greens vegetables irrigated with treated wastewater. Int. J. Hyg. Environ. Health 221, 112–119. https://doi.org/10.1016/j.ijheh.2017.10.009.

Downes, F.P., Ito, K., 2001. Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington DC.

Duedu, K.O., Yarnie, E.A., Tetteh-Quarcoo, P.B., Attah, S.K., Donkor, E.S., Ayeh-Kumi, P. F., 2014. A comparative survey of the prevalence of human parasites found in fresh vegetables sold in supermarkets and open-aired markets in AccraGhana. BMC Res. Notes 7 (1), 1–6. https://doi.org/10.1186/1756-0500-7-836.

ECDC, 2019. Giardiasis (lambliasis) - Annual Epidemiological Report for 2017. Retrieved from. https://www.ecdc.europa.eu/en/publications-data/giardiasis-lambliasis-annual-epidemiological-report-2017. (Accessed 17 November 2021).

Efstratiou, A., Ongerth, J.E., Karanis, P., 2017. Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2011–2016. Water Res. 114, 14–22. https://doi.org/10.1016/j.watres.2017.01.036.

FAO, 2020. The State of Food Security and Nutrition in the World 2020. Retrieved from. http://www.fao.org/publications/sofi/2020/es/. (Accessed 17 November 2021).

Fayer, R., Xiao, L. (Eds.), 2008. Cryptosporidium and Cryptosporidiosis, second ed. CRC press, Boca Raton.

Fletcher, S.M., Stark, D., Harkness, J., Ellis, J., 2012. Enteric protozoa in the developed world: a public health perspective. Clin. Microbiol. Rev. 25 (3), 420–449. https://doi.org/10.1128/CMR.05038-11.

Friesen, J., Fuhrmann, J., Kietzmann, H., Tannich, E., Müller, M., Ignatius, R., 2018. Evaluation of the Roche LightMix gastro parasites multiplex PCR assay detecting giardia, Entamoeba histolytica, cryptosporidia, Dientamoeba fragilis, and blastocystis hominis. Clin. Microbiol. Infect. 24 (12), 1333–1337. https://doi.org/10.1016/j.cmi.2018.03.025.

Fuentes, M.V., Barceló, M.E., 2008. Parásitos en alimentos: un problema de salud pública. In: Medicina Operacional. Dirección Nacional de Sanidad de las Fuerzas Armadas, Escuela de Sanidad de las Fuerzas Armadas, Ministerio de Defensa de la República Oriental del Uruguay, Montevideo, pp. 51–60.

Huang, D.B., Chappell, C., Okhuysen, P.C., 2004. Cryptosporidiosis in children. Semin. Pediatr. Infect. Dis. 15 (4), 253–259. https://doi.org/10.1053/j.spid.2004.07.006.

Kim, B., Kim, H.S., Kim, J.J., Park, Y.J., Kim, D., Yong, D., 2020. Detection of intestinal protozoa in korean patients using BD MAX enteric parasite panel and seegene allplex gastrointestinal parasite assay. Lab. Med. Online 10, 221–226. https://doi.org/10.3343/lmo.2020.10.3.221.

Lane, S., Lloyd, D., 2002. Current trends in research into the waterborne parasite Giardia. Crit. Rev. Microbiol. 28 (2), 123–147. https://doi.org/10.1080/1040-840291046713.

Lobo, M.L., Xiao, L., Antunes, F., Matos, O., 2009. Occurrence of cryptosporidium and giardia genotypes and subtypes in raw and treated water in Portugal. Lett. Appl. Microbiol. 48 (6), 732–737. https://doi.org/10.1111/j.1472-765X.2009.02605.x.

Montanher, C.C., de Camargo Coradin, D., Fontoura-da-Silva, S.E., 2007. Avaliação parasitológica em alfaces (Lactuca sativa) comercializadas em restaurantes self-service por quilo, da cidade de Curitiba, ParanáBrasil. Estud. Biol. 29 (66), 63–71. https://doi.org/10.7213/reb.v29i66.22749.

Plutzer, J., Karanis, P., Domokos, K., Törökné, A., Márialigeti, K., 2008. Detection and characterization of giardia and cryptosporidium in hungarian raw, surface and sewage water samples by IFT, PCR and sequence analysis of the SSUrRNA and GDH genes. Int. J. Hyg. Environ. Health 211 (5–6), 524–533. https://doi.org/10.1016/j.ijheh.2008.04.004.

Razzolini, M.T., De Souza-Lauretto, M., Hachich, E.M., Sato, M.I.Z., Nardocci, A.C., 2016. Giardia and cryptosporidium infection risk by simultaneous exposure to drinking water. Microb. Risk Anal. 4, 1–6. https://doi.org/10.1016/j.mran.2016.01.001.

Reh, L., Muadica, A.S., Köster, P.C., Balasegaram, S., Verlander, N.Q., Chércoles, E.R., Carmena, D., 2019. Substantial prevalence of enteroparasites Cryptosporidium, Giardia and Blastocystis sp. in asymptomatic schoolchildren in Madrid, Spain, November 2017 to June 2018. Euro Surveill. 24 (43), 1900241 https://doi.org/10.2807/1560-7917.ES.2019.24.43.1900241.

Resendiz-Nava, C.N., Orozco-Mosqueda, G.E., Mercado-Silva, E.M., Flores-Robles, S., Silva-Rojas, H.V., Nava, G.M., 2020. A molecular tool for rapid detection and traceability of Cyclospora cayetanensis in freshberries and berry farm soils. Foods 9 (3), 261. https://doi.org/10.3390/foods9030261.

Rivero de Rodríguez, Z., Fonseca, R., Moreno, Y., Oroño, I., Urdaneta, M., 1998. Detección de parásitos en lechugas distribuidas en mercados populares del municipio Maracaibo. Kasmera 26, 1–16.

Robertson, L.J., Gjerde, B., 2001. Occurrence of parasites on fruits and vegetables in Norway. J. Food Prot. 64 (11), 1793–1798. https://doi.org/10.4315/0362-028x-64.11.1793.

Ryan, U., Hijjawi, N., Feng, Y., Xiao, L., 2019. Giardia: an under-reported foodborne parasite. Int. J. Parasitol. 49 (1), 1–11. https://doi.org/10.1016/j.ijpara.2018.07.003.

Ryan, U.M., Feng, Y., Fayer, R., Xiao, L., 2021. Taxonomy and molecular epidemiology of Cryptosporidium and Giardia – a 50-year perspective (1971–2021). Int. J. Parasitol. 51 (13–14), 1099–1119. https://doi.org/10.1016/j.ijpara.2021.08.007.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States – major pathogens. Emerg. Infect. Dis. 17 (1), 7–15. https://doi.org/10.3201/eid1701.p11101.

Sterling, C.R., 2018. Foodborne giardia duodenalis and trypanosoma cruzi. In: Ortega, Y. R., Sterling, C.R. (Eds.), Foodborne Parasites, second ed. Springer, Cham, pp. 17–40.

Tahvildar-Biderouni, F., Salehi, N., 2014. Detection of cryptosporidium infection by modified ziehl-neelsen and PCR methods in children with diarrheal samples in pediatric hospitals in Tehran. Gastroenterol. Hepatol. Bed Bench 7 (2), 125–130.

Thompson, R.C., 2004. The zoonotic significance and molecular epidemiology of giardia and giardiasis. Vet. Parasitol. 26 (1–2), 15–35. https://doi.org/10.1016/j.vetpar.2004.09.008.

Trelis, M., Taroncher-Ferrer, S., Gozalbo, M., Ortiz, V., Soriano, J.M., Osuna, A., Merino-Torres, J.F., 2019. Giardia intestinalis and fructose malabsorption: a frequent association. Nutrients 11 (12), 2973. https://doi.org/10.3390/nu11122973.

UNESCO, 2019. United Nations World Water Development Report 2019 – Leaving No One Behind. Retrieved from. https://en.unesco.org/themes/water-security/wwap/wwdr/2019. (Accessed 13 March 2003).

Ursini, T., Moro, L., Requena-Méndez, A., Bertoli, G., Buonfrate, D., 2020. A review of outbreaks of cryptosporidiosis due to unpasteurized milk. Infection 48 (5), 659–663. https://doi.org/10.1007/s15010-020-01426-3.

USDA (United States Department of Agriculture), 2015. Introduction to Organic Practices. Retrieved from. https://www.usda.gov/topics/organic. (Accessed 10 September 2021).

USEPA (United States Environmental Protection Agency), 1999. Biosolids Generation Use and Disposal in the United States. Retrieved from. https://www.epa.gov/sites/default/files/2018-12/documents/biosolids-generation-use-disposal-us.pdf. (Accessed 10 September 2021).

Vaerewijck, M.J., Sabbe, K., Baré, J., Houf, K., 2011. Occurrence and diversity of free-living protozoa on butterhead lettuce. Int. J. Food Microbiol. 147 (2), 105–111. https://doi.org/10.1016/j.ijfoodmicro.2011.03.015.

Verweij, J.J., Schinkel, J., Laeijendecker, D., van Rooyen, M.A., van Lieshout, L., Polderman, A.M., 2003. Real-time PCR for the detection of Giardia lamblia. Mol. Cell. Probes 17 (5), 223–225. https://doi.org/10.1016/s0890-8508(03)00057-4.

Vivancos, V., González-Alvarez, I., Bermejo, M., González-Alvarez, M., 2018. Giardiasis: characteristics, pathogenesis and new insights about treatment. Curr. Top. Med. Chem. 18 (15), 1287–1303. https://doi.org/10.2174/1568026618666181002095314.

Wang, Y., Gonzalez-Moreno, O., Roellig, D.M., Oliver, L., Huguet, J., Guo, Y., Feng, Y., Xiao, L., 2019. Epidemiological distribution of genotypes of Giardia in humans in Spain. Parasit. Vectors 12 (1), 432. https://doi.org/10.1186/s13071-019-3692-4.

Weinreich, F., Hahn, A., Eberhardt, K.A., Feldt, T., Sarfo, F.S., Di Cristanziano, V., Frickmann, H., Loderstädt, U., 2021. Comparison of three real-time PCR assays targeting the SSU rRNA gene, the COWP gene and the DnaJ-like protein gene for the diagnosis of cryptosporidium in stool samples. Pathogens 10 (9), 1131. https://doi.org/10.3390/pathogens10091131.

WHO, 2008. WHO initiative to estimate the global burden of foodborne diseases: increasing impact through collaboration. Retrieved from. https://www.who.int/publications/i/item/WHO-HSE-FOS-09.4. (Accessed 10 September 2021).

Xiao, L., Cama, V.A., 2018. Cryptosporidium and cryptosporidiosis. In: Ortega, Y.R., Sterling, C.R. (Eds.), Foodborne Parasites, second ed. Springer, Cham, pp. 73–117.