Food and Waterborne Parasitology 32 (2023) e00200

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

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

Protozoan parasites and free-living amoebae contamination in organic leafy green vegetables and strawberries from Spain

L. Moreno-Mesonero [a,b,*], L. Soler [a], I. Amorós [a], Y. Moreno [a], M.A. Ferrús [b], J.L. Alonso [a]

[a] Instituto de Ingeniería del Agua y Medio Ambiente (IIAMA), Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[b] Departamento de Biotecnología, Centro Avanzado de Microbiología de Alimentos, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

ARTICLE INFO

Keywords:
Organic fresh produce
Parasitic protozoa
Free-living amoebae
qPCR
Immunomagnetic separation and immunofluorescence assay (IMS-IFA)

ABSTRACT

In this study, the presence of *Acanthamoeba* spp., *Blastocystis* sp., *Cryptosporidium* spp., *Cyclospora cayetanensis, Entamoeba histolytica, Giardia* sp., *Toxoplasma gondii* and *Vermamoeba vermiformis* was assessed in organic leafy green vegetables (lettuce, spinach, cabbage) and fruits (strawberry), which are usually consumed raw. A total of 110 organic samples were collected in Valencia (Spain). Protozoa were concentrated before detection by immunofluorescence (*Cryptosporidium* spp. and *Giardia* sp.) or real-time qPCR (*Acanthamoeba* spp., *Blastocystis* sp., *C. cayetanensis*, *E. histolytica*, *T. gondii* and *V. vermiformis*). The most abundant protozoa in organic vegetables and berry fruits were *Acanthamoeba* (65.5%), followed by *T. gondii* (37.2%), *V. vermiformis* (17.3%), *C. cayetanensis* (12.7%), *Cryptosporidium* spp. (6.8%), *Blastocystis* sp. (1.8%) and *Giardia* sp. (1.7%). *E. histolytica* was not found in any of the organic samples. Thus, results showed that consumers can be exposed to protozoan parasites by consuming organic vegetables and berry fruits. This is the first report in Spain describing the presence of the protozoan pathogens *Acanthamoeba* spp., *Blastocystis* sp., *C. cayetanensis*, *T. gondii* and *V. vermiformis*, *Cryptosporidium* spp. and *Giardia* sp. in organic fresh produce. The results of this research will help determine the risk of foodborne protozoan parasites on organic leafy greens and strawberries that are available at local markets.

1. Introduction

The contamination of fresh produce with intestinal protozoa is now a global public health threat (Badri et al., 2022; Li et al., 2020). The protozoa *Blastocystis*, *Cryptosporidium*, *Cyclospora*, *Giardia* and *Toxoplasma* oocysts and cysts are very robust, which explains their distribution in the environment and food (Caradonna et al., 2017; Martín-Escolano et al., 2023). Although many research studies have examined the prevalence of these parasites on fresh produce in developing regions worldwide, scarce surveillance studies have been performed in developed countries (Dixon, 2016).

Contamination of vegetables with pathogenic protozoa could be due to fertilisation with fresh animal manure, irrigation with polluted water, by infected food handlers and even in some regions because of the use of night soil (Kim et al., 2014; Nasser, 2022).

* Corresponding author at: Research Institute of Water and Environmental Engineering (IIAMA), Universitat Politècnica de València, 46022, Valencia, Spain.
E-mail address: laumome@upv.es (L. Moreno-Mesonero).

https://doi.org/10.1016/j.fawpar.2023.e00200
Received 8 March 2023; Received in revised form 20 June 2023; Accepted 21 June 2023
Available online 22 June 2023

Since organic fertilisers are used in organic foods, these could be more prone to microbiological contamination. The faecal origin of organic fertilisers raises concern about the possibility of contamination by human pathogenic parasites (IFST, Institute of Food Science and Technology, UK, 1999). However, there is limited scientific evidence to uphold this suggestion (Oliveira et al., 2010). Manure and other animal wastes are widely used as organic fertilisers for fruit and vegetable production, particularly in organic production systems. Because of the method of cultivation and processing of organic production, this is considered to constitute a greater public health risk compared to conventional production (i.e., use of chemical fertilisers). This is because natural fertilisers, such as manure, are employed in organic production and chemical treatments to reduce the microbiological load are avoided. In Spain, fertilisers used in vegetable organic production are specified under UNE 142500:2017 regulation. So far, there has been an increase in organic production in the Spanish territory, specifically in the Valencian Community, where the area of organically managed land in 2021 was 153,503 ha. and the agricultural area dedicated to organic farming of vegetables represented 14.5% (CAECV, 2021).

Fresh produce is a generalized term for a group of farm-grown crops, including vegetables and fruits (Maffei et al., 2016). Globally, the presence of protozoan parasitic contamination in vegetables and fruits varies between 1.9% and 9.3% (Li et al., 2020). Among the 24 parasites included in the multicriteria-based ranking of foodborne parasites (FAO/WHO, 2014), the most relevant protozoa transmitted by food, in descending order, were *Toxoplasma gondii*, *Cryptosporidium* spp., *Entamoeba histolytica*, *Trypanosoma cruzi*, *Giardia duodenalis*, *Cyclospora cayetanensis* and, *Balantidium coli*. All these protozoa have the potential to be transmitted through fresh produce (FAO/WHO, 2014). Fresh vegetables have also been identified as contributors to *Blastocystis* foodborne transmission (Jinatham et al., 2023). In the last decades, due to the general increasing interest in organic foods, the presence of different relevant protozoa, such as *T. gondii*, *Cryptosporidium* spp., *G. duodenalis*. and *E. histolytica*, has been evaluated in different organic vegetables (Ferreira et al., 2018; Lilly and Webster, 2021; Marchioro et al., 2016; Trelis et al., 2022).

Outbreaks linked to protozoa have been associated with fruits and vegetables eaten raw. The microorganisms most frequently related to outbreaks are *Cryptosporidium*, *Cyclospora* and *Giardia* (SCF/CS/FMH/SURF, 2002). Fresh produce is not routinely tested for the presence of protozoa despite the risk of protozoan foodborne diseases. There is only a standardized microscopy-based method for detecting *Cryptosporidium* and *Giardia* (ISO 18744, 2016) and a real-time PCR method for *C. cayetanensis* validated for regulatory testing (FDA BAM, 2022). There are no other standardized methods for the detection of parasites on berries and other fresh produce.

Free-living amoebae (FLA) include various genera frequently found in soil and water habitats such as vegetable farms (Pazoki et al., 2020; Reyes-Batlle et al., 2021). FLA have not only been reported to be causal agents of several opportunistic diseases but also are of interest as they are predators and hosts for obligate and facultative pathogenic foodborne microorganisms affecting humans and animals' health. Hence, they play a role in pathogen dissemination and, therefore, are of interest to Public Health Authorities (Balczun and Scheid, 2017; Rayamajhee et al., 2021; Sousa-Ramos et al., 2022). For example, *Acanthamoeba* is responsible for granulomatous amoebic encephalitis (GAE) and cutaneous lesions in immunocompromised individuals, amoebic keratitis in healthy individuals, mainly in contact-lens wearers, and also serve as hosts for microbial pathogens (Visvesvara et al., 2007). *V. vermiformis* is a pathogen reservoir and has been also reported as responsible for an ulcer adjacent to the eye and a case of amoebic keratitis (Siddiqui et al., 2021).

Considering the importance of the consumption of leafy green vegetables and berry fruits in the diet and the need to be free of pathogenic protozoa, the aim of this study was to determine the occurrence of relevant protozoan parasites transmitted by food: *Cryptosporidium* spp., *Giardia* sp., *E. histolytica*, *T. gondii*, *C. cayetanensis*, *Blastocystis* sp. and the FLA *Acanthamoeba* spp. and *V. vermiformis* in organic leafy green vegetables and strawberries that are typically consumed raw.

2. Material and methods

2.1. Sample collection

A total of 110 samples, including 28 lettuce, 30 spinach, 22 strawberry and 30 cabbage samples, were collected from 22 locations (eleven supermarkets, four herbalist shops, three eco-friendly shops and four local markets), all located in Valencia (Spain). Samples were collected between November 2020 through May 2022 (Supplementary table S1).

2.2. Sample processing

2.2.1. Leafy green samples

Leafy green samples were obtained from different locations as specified in Supplementary Table S1. The outer leaves were discarded (Cook et al., 2006) and the remainder was processed as described by Caradonna et al. (2017). Shortly, 100 g of each product type were introduced in a stomacher bag (Bag Page, Intescience, Sant Nom, France) containing 200 ml of detergent solution (PBS 1×, 0.1% Tween 80, 0.1% sodium dodecyl sulfate (SDS) and 0.05% antifoam B emulsion). Bags were mixed well and homogenized for 1 min in a homogenizer. Then, homogenates were collected into four 50 ml tubes and centrifuged (2500 x*g* for 15 min). Supernatants were discarded, and pellets were combined into another 50 ml centrifuge tube (3000 x*g* for 15 min). The resultant pellet was resuspended into 2 ml of buffered detergent solution and divided into two different tubes: one for microscopy (immunomagnetic separation and immunofluorescence assay, IMS-IFA) and the other for molecular investigations (quantitative PCR, qPCR). Each tube was centrifuged at 10,000 x*g* for 10 min, and the supernatants were discarded. Finally, the pellets were resuspended in 500 µl of PBS 1× for microscopy and 978 µl of PBS 1× for molecular investigation. The aliquot for microscopy investigation was tested within 3 days after processing, whereas the aliquot for molecular investigation was stored at −80 °C until DNA extraction was performed.

2.2.2. Strawberry samples

One hundred grams of strawberries were weighed in a stomacher bag (BagPage, Interscience, Sant Nom, France). A total of 200 ml of 1 M glycine buffer at pH 3.5 were added to the bags, according to ISO 18744:2016. Samples were gently agitated for 1 min (e.g., by rolling or slow-speed shaking) to minimize damage to the fruits. Homogenate was transferred to four 50 ml centrifuge tubes for concentration by repeated cycles of centrifugation as described above for leafy greens. Samples' aliquots for microscopy and molecular investigations were also processed as described above.

2.3. Detection of Giardia cysts and Cryptosporidium oocysts by IMS-IFA

Cryptosporidium oocysts and *Giardia* cysts were tested in 59 samples (16 lettuce, 14 spinach, 10 strawberry and 19 cabbage samples, which were randomly selected) according to ISO 18744:2016. After sample concentration, the aliquot obtained for microscopy was transferred to a glass Leighton tube (Invitrogen Dynal AS) and resuspended in 10 ml of distilled water before immunomagnetic separation (IMS). IMS was conducted using the commercially available Dynabeads GC-Combo kit (Applied Biosystems by Thermo Fisher Scientific) according to the manufacturer's instructions. The final concentrate from the IMS was dried overnight at room temperature and labelled with fluorescent monoclonal antibody for *Giardia* and *Cryptosporidium* immunofluorescence assay (IFA) according to the manufacturer's protocol (EasyStain, BioPoint, Australia). After staining, slides were placed in the dark, mounted with mounting medium, and examined at 60× magnification using epifluorescence microscopy (Olympus BX 50, Tokyo, Japan). A FITC (blue) filter block (excitation, 480 nm; emission, 520 nm) was used to detect fluorescein isothiocyanate (FITC)–conjugated MAb-labelled cysts and oocysts. An UV filter block (excitation, 350 nm; emission 450 nm) was used to detect sporozoite and trophozoite nuclei stained with 4′,6′-diamidino-2-phenylindole dihydrochloride dihydrate (DAPI). Results were considered positive when FITC+ and DAPI+.

2.4. DNA extraction

DNA was extracted using the commercial kit FastDNA™ SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA), following the manufacturer's instructions. FastPrep-24® instrument (MP Biomedicals, Irvine, CA, USA) along with Lysing Matrix E tube were used

Table 1
Oligonucleotide sequences of primers and probes, and thermal profile used in this study.

Target gene	Primer name: Sequence (5′→3′)	Amplicon size (bp)	Thermal profile[a]	Reference
Acanthamoeba spp. *18SrRNA* gene	Acant900-F: CCCAGATCGTTTACCGTGAA Acant1100-R: TAAATATTAATGCCCCCAACTATCC Acant1000-P: 6-FAM-CTGCCACCGAATACATTAGCATGG-BHQ1	180	95 °C for 10 min 1 cycle, 95 °C for 10 s, 63 °C for 8 s, 72 °C for 7 s 40 cycles	Qvarnstrom et al. (2006)
Blastocystis sp. *18S rDNA* gene	Blasto FWD-F5: GGTCCGGTGAACACTTTGGATTT Blasto R-F2: CCTACGGAAACCTTGTTACGACTTCA Blasto-P: 6-FAM -TCGTGTAAATCTTACCATTTAGAGGA-BHQ1	118	95 °C for 10 min 1 cycle, 95 °C for 10 s, 58 °C for 8 s, 72 °C for 5 s 40 cycles	Stensvold et al. (2016)
Cryptosporidium COWP gene	COWP-F: CAAATTGATACCGTTTGTCCTTCTG COWP-R: GGCATGTCGATTCTAATTCAGCT COWP-P:6-FAM-TGCCATACATTGTTGTCCTGACAAATTGAAT-TAMRA	151	95 °C for 10 min 1 cycle, 95 °C for 10 s, 66 °C for 8 s, 72 °C for 6 s 40 cycles	Guy et al. (2003)
Cyclospora cayetanensis ITS-1 region	CyITS_TT-F: ATGTTTTAGCATGTGGTGTGGC CyITS_TT-R: GCAGCAACAACAACTCCTCATC CyITS_TT-P[b]: 6-FAM-TACATACTCATCCCATCCCTCGA-BHQ-1	141	95 °C for 10 min 1 cycle, 95 °C for 10 s, 58 °C for 8 s, 72 °C for 6 s 40 cycles	Temesgen et al. (2019)
Giardia β-giardin gene	P241-F: CATCCGCGAGGAGGTCAA P241-R: TCCAATCTGGGCATAAGATTTG P241-P:6-FAM-AAGTCCGCCGACAACATGTACCTAACGA-TAMRA	74	95 °C for 10 min 1 cycle, 95 °C for 10 s, 58 °C for 8 s, 72 °C for 6 s 40 cycles	Guy et al. (2003)
Entamoeba histolytica 18S rDNA gene	Ehd-239F: ATTGTCGTGGCATCCTAACTCA Ehd-88R: GCGGACGGCTCATTATAACA E. histo-TM: 6-FAM-TCATTGAATGAATTG GCCATT T-BHQ1	172	95 °C for 10 min 1 cycle, 95 °C for 10 s, 60 °C for 10 s, 72 °C for 7 s 40 cycles	Berglund et al. (2017)
Toxoplasma gondii 18S rDNA gene	Tox-9F: AGGAGAGATATCAGGACTGTAG Tox-11R: GCGTCGTCTCGTCTAGATCG Tox-TP1: 6-FAM-CCGGCTTGGCTGCTGCTTTTCCT-BHQ-1	162	95 °C for 10 min 1 cycle, 95 °C for 10 s, 58 °C for 8 s, 72 °C for 7 s 40 cycles	Opsteegh et al. (2010)
Vermamoeba vermiformis 18S rDNA gene	Hv1227F: TTACGAGGTCAGGACACTGT Hv1728R: GACCATCCGGAGTTCTCG	502	95 °C for 10 min 1 cycle, 95 °C for 10 s, 56 °C for 10 s, 72 °C for 25 s 40 cycles	Kuiper et al. (2006)

[a] qPCR conditions were optimized for LightCycler 2.0 platform in this study.
[b] Nucleic acid probe sequence was modified to be complementary to an internal segment of the synthetic template target ITS-1 DNA region control.

for the homogenization step at speed setting 6.5 m/s for 120 s. The final DNA was eluted in 70 µl of elution buffer.

2.5. Quantitative polymerase chain reaction (qPCR)

All samples were screened for the presence of *Acanthamoeba* spp., *Blastocystis* sp., *C. cayetanensis*, *E. histolytica*, *T. gondii* and *V. vermiformis*. Moreover, *Cryptosporidium* spp. and *Giardia* sp. presence was tested on IMS-IFA positive samples. The different qPCR and data analysis were carried out in a LightCycler 2.0 PCR system, using either LightCycler FastStart DNA Master SYBR Green I or LightCycler TaqMan Master (Roche, Barcelona, Spain) with the specific primers, probes and qPCR cycling conditions described in Table 1. Samples were considered positive for specific protozoan parasites and FLA if the cycle threshold (Ct) was less than or equal to 40. Samples with Ct values between 35 and 40 were considered positive but could not be quantified since, in these range, the variability is great, and quantification may be unreliable (Nolan et al., 2006).

DNA used as a positive control for qPCR reactions of *Acanthamoeba* spp., *Blastocystis* sp., *C. cayetanensis* and *V. vermiformis* were 180 bp, 118 bp, 141 bp and 502 bp fragments of double-stranded synthetic gBlocks synthesized by Bio Basic (Bio Basic Inc., Ontario, Canada), respectively. The sequence of *Acanthamoeba* spp. corresponded to a fragment of 18S rRNA gene (positions 234–413 of GenBank accession number KC164230). The sequence of *Blastocystis* sp. corresponded to a fragment of 18S rRNA gene (positions 1641–1759 of GenBank accession number AY244621). The sequence of *C. cayetanensis* corresponded to a fragment of the ITS-1 region (positions 58–198 of GenBank accession number AF301386). The sequence of *V. vermiformis* corresponded to a fragment of the 18S rRNA gene (positions 1203–1704 of GenBank accession number MK418871). Genomic DNA (gDNA) from *T. gondii* ATCC 50174D and *E. histolytica* ATCC 30459D were obtained from the American Type Culture Collection (ATCC). Standard curves for the quantification of each microorganism were generated using 10-fold serial dilutions of each gBlock or gDNA.

2.6. Statistical analysis

A chi-squared test was carried out using Excel software. The statistical significance level was set at a *p*-value below 0.05.

3. Results

The incidence of parasitic protozoa and FLA in the organic vegetables and strawberries in this study is reflected in Table 2 and Supplementary Table S1. The analysis revealed that, after performing the different specific qPCR analyses, 65.5% (72/110) of the tested samples were positive for *Acanthamoeba* spp. (Ct range 25.5–39.4, median 34.4), 1.8% (2/110) were positive for *Blastocystis* sp. (Ct range 37.7–37.7, median 37.7), 12.7% (14/110) were positive for *C. cayetanensis* (Ct range 36.8–39.9, median 39.1), 37.2% (41/110) were positive for *T. gondii* (Ct range 22.2–39.0, median 35.7) and, 17.3% (19/110) were positive for *V. vermiformis* (Ct range 19.2–33.9, median 27.3). However, none of the samples were positive for *E. histolytica*. By IMS-IFA method, 6.8% of the samples (4/59) were positive for *Cryptosporidium* spp., and 1.7% (1/59) were positive for *Giardia* sp. However, these results were not confirmed by qPCR technique, which retrieved no positive result in these IMS-IFA positive samples. *Giardia* cysts and *Cryptosporidium* oocysts were only detected in cabbage samples; thus, they were not detected in lettuce, spinach, nor in strawberry samples.

Both lettuce and spinach samples were contaminated with *Acanthamoeba* spp., *Blastocystis* sp., *T. gondii*, and *V. vermiformis*, and no *C. cayetanensis, E. histolytica, Cryptosporidium* spp. nor *Giardia* sp. were found in these two types of samples. Concerning cabbage samples, these were contaminated with *Acanthamoeba* spp., *C. cayetanensis*, *T. gondii*, *V. vermiformis*, *Cryptosporidium* spp., and *Giardia* sp., and no *Blastocystis* sp. nor *E. histolytica* were detected among these samples. Finally, strawberry samples were contaminated with *Acanthamoeba* spp., *C. cayetanensis* and, *T. gondii*, and no *Blastocystis* sp., *E. histolytica, V. vermiformis*, *Cryptosporidium* spp. nor *Giardia* sp. were found. As shown in Supplementary Table S1, in all types of samples there were samples contaminated with more than one protozoan.

The chi-squared test showed no significant differences in the positive rates of *Acanthamoeba* spp., *Blastocystis* spp., and *Giardia* sp.

Table 2
Detection and prevalence of parasitic protozoa and free-living amoebae (FLA) on organic leafy green vegetables and strawberries.

	qPCR						IMS-IFA	
Product	*Acanthamoeba* spp. (%)	*Blastocystis* sp. (%)	*Cyclospora cayetanensis* (%)	*Entamoeba histolytica* (%)	*Toxoplasma gondii* (%)	*Vermamoeba vermiformis* (%)	*Cryptosporidium* spp. (%)	*Giardia* sp. (%)
Lettuce	13/28 (46.4%)	1/28 (3.6%)	0/28 (0.0%)	0/28 (0.0%)	16/28 (57.1%)	10/28 (35.7%)	0/16 (0.0%)	0/16 (0.0%)
Spinach	20/30 (66.7%)	1/30 (3.3%)	0/30 (0.0%)	0/30 (0.0%)	14/30 (46.7%)	1/30 (3.3%)	0/14 (0%)	0/14 (0.0%)
Cabbage	24/30 (80.0%)	0/30 (0.0%)	11/30 (36.7%)	0/30 (0.0%)	4/30 (13.3%)	8/30 (26.7%)	4/19 (21.0%)	1/19 (5.2%)
Strawberry	15/22 (68.2%)	0/22 (0.0%)	3/22 (13.6%)	0/22 (0.0%)	7/22 (31.8%)	0/22 (0.0%)	0/10 (0.0%)	0/10 (0.0%)
Overall total	72/110 (65.5%)	2/110 (1.8%)	14/110 (12.7%)	0/110 (0.0%)	41/110 (37.2%)	19/110 (17.3%)	4/59 (6.8%)	1/59 (1.7%)

and the different types of fresh produce (lettuce, cabbage, spinach, and strawberry). However, it showed that there was a significant relationship ($p < 0.05$) between the presence of *C. cayetanensis*, *T. gondii*, *V. vermiformis*, and *Cryptosporidium* spp., and the type of fresh produce (Supplementary Table S2).

4. Discussion

Protozoa contamination can arise from different sources, such as environmental, animal, or human origins. Some protozoa could only have a certain origin, e.g. *T. gondii* is only found in cats and wild felids, while *C. cayetanensis* is only found in humans. Cysts and oocysts have the capacity to remain viable for an extended period in the environment due to their structure, which protects them in exogenous stages (Marques et al., 2022). Moreover, outdoor vegetable cultivation allows the access of animals (both domestic and wild), which can defecate in the fields and, therefore, be a source of contamination (Maffei et al., 2016). The use of manure as fertiliser could be an additional source of parasitic contamination, as well as farm workers and irrigation water (Tefera et al., 2018; Nasser, 2022).

Badri et al. (2022) analysed the global prevalence of intestinal protozoan parasite contamination in vegetables and fruits. Their analysis revealed that the most prevalent protozoan parasites in vegetables and fruits were *Cryptosporidium* spp. (11%, 7%–15%) and *E. histolytica* (9%, 4%–14%), respectively (Badri et al., 2022). However, in the present study, the most prevalent protozoa in organic fresh produce were *Acanthamoeba* spp. and *T. gondii*.

T. gondii, a worldwide zoonotic parasite (Lopes et al., 2021), was detected in 37.2% (41/110) of the organic vegetables. From these, one lettuce and one spinach sample had 336 and 360 oocysts per 50 g, respectively (Supplementary Table S1). In a previous study carried out by Lilly and Webster (2021) they detected *T. gondii* in 16% of organic vegetables. Moreover, Ferreira et al. (2018) also detected *T. gondii* in 12.9% of the analysed organic vegetables. However, Pinto-Ferreira et al. (2020) did not detect *T. gondii* in any organic lettuce.

In this study, the overall presence of *T. gondii* in organic vegetables was relatively high compared to rates obtained from conventionally grown vegetables. A significant association of *T. gondii* infections with the consumption of contaminated raw vegetables was observed in previous studies (Ekman et al., 2012). In a study carried out in Poland, vegetables from shops and home gardens presented a contamination rate by *T. gondii* of 9.7% (Lass et al., 2012); in a study from China, the prevalence *T. gondii* DNA was detected in 3.6% of the samples (Lass et al., 2019), whereas in a study from Morocco these data increased to 29.6% in lettuce, coriander, and parsley (Berrouch et al., 2022). *T. gondii* was not detected in organic strawberries, although the nature of the berries may assist in the attachment of parasites. It has been shown that *T. gondii* oocysts can attach to and remain infective after inoculation on both blueberries and raspberries (Kniel et al., 2002). *T. gondii* oocysts are remarkably stable (Torrey and Yolken, 2013). A single oocyst of *Toxoplasma* is sufficient to cause infection in susceptible hosts (Torrey and Yolken, 2013). Currently there are no standardized methods for its detection in the food industry (Marín-García et al., 2022).

Cyclospora cayetanensis is another important protist parasite, usually transmitted via food, that causes human gastrointestinal cyclosporiasis (Giangaspero and Gasser, 2019). *Cyclospora cayetanensis* oocysts were detected in 12.7% (14/110) of the samples, which included three strawberry and eleven cabbage samples, but at very low concentrations (Ct >35) (Supplementary Table S1). The contamination of vegetables and fruits with *C. cayetanensis* oocysts has been documented in many countries (Li et al., 2020). The average prevalence in vegetables and fruits of *C. cayetanensis* contamination is calculated as 3.9% (Li et al., 2020). In the current study, its overall prevalence was 12.7%; however, it greatly differed depending on the type of organic vegetable. *Cyclospora cayetanensis* has received relatively little attention in Europe as a food pathogen, despite its reported significance in other industrialized countries in North America, where the vast majority of cyclosporiasis outbreaks have been recorded (Mansfield and Gajadhar, 2004; Giangaspero et al., 2015). There are few data for *Cyclospora* contamination on vegetables from Europe, although several outbreaks have been reported. For example, in 2000 there was an outbreak in Germany due to the consumption of salad whose ingredients originated from other European countries. In 2009, there was another outbreak in Sweden probably due to sugar snap pea consumption cultivated out of Europe (Döller et al., 2002; Insulander et al., 2010). Regarding surveillance studies on fresh produce, in Italy, *Cyclospora* was found in vegetables (mainly fennel) collected from farms of the Apulia region at a prevalence of 12% (Giangaspero et al., 2015), which are similar values to our study. Moreover, Temesgen et al. (2022) found the overall occurrence of *C. cayetanensis* on berries sold in the Norwegian market to be 6.6%.

Other protozoa, such as *Blastocystis* sp., which are among the most common intestinal protists of humans and animals (Parfrey et al., 2011), were present at low relative abundances (<2%), being only detected in one lettuce and one spinach organic samples and in non-quantifiable levels. The multi-layering and roughness of vegetable stems could create micro-niches, encouraging *Blastocystis* attachment (Jinatham et al., 2023). *Blastocystis* cysts are mainly transmitted by contaminated food and water as well as by close contact with animals (Clark et al., 2013).

In the present work, *Giardia* cysts and *Cryptosporidium* oocysts were detected in four cabbage samples (Supplementary Table S1). Thus, neither *Cryptosporidium* oocysts nor *Giardia* cysts were detected in lettuce, spinach and strawberry samples. Low levels of *Cryptosporidium* oocysts (1 oocyst) and *Giardia* cysts (1 cyst) were found in our positive samples by IMS and IFA method. Despite the high sensitivity of qPCR technique, qPCR detection was negative for *Cryptosporidium* oocyst and *Giardia* cyst in the five IMS-IFA positive samples. In this work, no amplification controls were used for qPCR analysis, which could have an impact on results of these and the other analysed protozoa. However, all positive and negative controls yielded adequate results.

The International Standard Method (ISO), published in 2016 for the detection of *Cryptosporidium* and *Giardia* in leafy green vegetables and berry fruits, must be considered an advantage to standardize methods. However, some authors (Chalmers et al., 2020) consider these methods to present some deficiencies as they are time-consuming, require expensive reagents, and the recovery

efficiencies are variable. Also, other studies showed that depending on the type of vegetables analysed and the elution buffers utilized can change the final recovery of cysts and oocysts. (Utaaker et al., 2017).

Several modifications to this standard method for recovering these parasites to increase recovery efficiency as well as lower costs have been published by Utaaker et al. (2015). On spinach leaves, Razakandrainibe et al. (2020) also reported that the recovery of *Cryptosporidium* from saponin-rich leaves, such as spinach (*Spinacia oleraceae*), can be increased by using an alkaline solution instead of the acidic 1 M glycine buffer recommended in ISO 18744:2016. In the present study, an alkaline detergent elution buffer at pH 8 has been employed; however, *Cryptosporidium* oocysts counts on spinach leaves remained zero. Despite the immunofluorescence microscopy technique's inconveniences for *Cryptosporidium* and *Giardia* detection, it has been shown more sensitive than light microscopy (Chalmers et al., 2020).

Fruit and vegetable contamination with *Cryptosporidium* spp. and *Giardia* have been documented in numerous countries, with an estimated average prevalence of 6.0% and 4.8%, respectively (Li et al., 2020). Several studies on the presence of protozoan parasites in ready-to-eat salads and berry fruits in Italy reported a prevalence of 0.9% for *Cryptosporidium* and 0.6% for *G. duodenalis* (Giangaspero et al., 2015) and 5.1% for *Cryptosporidium* spp. and 4.6% for *G. duodenalis* (Barlaam et al., 2022). Similar prevalence values have been reported in a survey on fresh produce in India, with 6% of the samples contaminated with *Cryptosporidium* oocysts and 5% with *Giardia* cysts (Utaaker et al., 2017). Higher levels of *G. duodenalis* (41.5%) and *Cryptosporidium* spp. (20%) in organic vegetables were recently detected by Trelis et al. (2022) in a survey of the presence of *G. duodenalis* and *Cryptosporidium* spp. in different organic leafy green vegetables in Valencia (Spain). The high level of reported contamination, compared with other similar studies, could be because outermost leaves were used for analyses. In contrast, in other studies, as in the present work, outermost leaves were discarded, as recommended by Cook et al. (2006).

One of the reasons for the differences shown in *Cryptosporidium* and *Giardia* data is related to the fact that many studies from different countries utilize detection methods different from the standard. Therefore, the obtained data are not comparable, challenging the interpretation of results and the accurate contamination levels, which may be underestimated (Utaaker et al., 2017).

A recent review of the prevalence of *Cryptosporidium* spp., *G. duodenalis* and *T. gondii* concluded that the variation in parasitic prevalence in food could be due to different factors such as the geographical location, the size of analysed samples and the methods used for parasite detection (Berrouch et al., 2020).

Regarding FLA, the number of *Acanthamoeba* and *Vermamoeba* cells are shown in Supplementary Table S1. Since FLA are ubiquitous protozoa, it is not rare that these, especially *Acanthamoeba,* were found in many organic samples. *Acanthamoeba* was detected with more than one cell per 50 g in all types of organic samples, but *Vermamoeba* was only detected with more than one cell per 50 g on leafy greens (lettuces, spinaches, and cabbages), not being even detected in non-quantifiable levels. The greatest levels of quantification were found in cabbage for *Acanthamoeba* spp. (3412 cells/50 g) and in spinach for *V. vermiformis* (1781 cells/50 g). Other studies have described the presence of FLA in conventionally grown lettuce samples (Moreno-Mesonero et al., 2020) and aromatic vegetables (Fatemi et al., 2022). The source of contamination of vegetables with FLA could be soil and irrigation water used in farming. FLA are considered an emerging group of opportunistic pathogens (Lorenzo-Morales et al., 2015) since they represent a health risk not only because they can cause several diseases in humans and other animals, but also because they can act as vehicles for potentially pathogenic bacteria (Siddiqui and Khan, 2012). Fatemi et al. (2022) pointed out that more studies are needed to better understand the implications of the gastrointestinal tract via oral ingestion as a possible route to exposure to FLA.

5. Conclusion

The findings of this study showed that organic leafy greens and strawberries can provide a source of parasitic protozoa and FLA. It is noteworthy that only DNA was detected, consequently, the viability and infectivity of the protozoan parasites found in this study is not demonstrated. Although there was a previous study done in Spain in which the presence of *Cryptosporidium* spp. and *Giardia* sp. had been determined in organic leafy green vegetables (Trelis et al., 2022), to our knowledge, this is the first report in Spain where the presence of these two and other important protozoan parasites and FLA such as *Toxoplasma*, *Cyclospora*, *Blastocystis*, *Acanthamoeba* and *Vermamoeba* were detected in organic leafy green vegetables and strawberries using qPCR.

Results of the current research evidenced the importance of investigating commercialized organic fresh produce to establish tougher quality control strategies.

Funding

This research was funded by the Ministerio de Ciencia e Innovación, Spain, grant number PID2019-105691RB-I00.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2023.e00200.

References

Badri, M., Olfatifar, M., Karim, M.R., Modirian, E., Houshmand, E., Abdoli, A., Nikoonejad, A., Sotoodeh, S., Zargar, A., Samimi, R., Hashemipour, S., Mahmoudi, R., Harandi, M.F., Hajialilo, E., Piri, H., Bijani, P., Eslahi, A.V., 2022. Global prevalence of intestinal protozoan contamination in vegetables and fruits: a systematic review and meta-analysis. Food Control 133 (Part B), 108656. https://doi.org/10.1016/j.foodcont.2021.108656.

Balczun, C., Scheid, P.L., 2017. Free-living amoebae as hosts for and vectors of intracellular microorganisms with public health significance. Viruses 9, 65. https://doi.org/10.3390/v9040065.

Barlaam, A., Sannella, A.R., Ferrari, N., Temesgen, T.T., Rinaldi, L., Normanno, G., Cacciò, S.M., Robertson, L.J., Giangaspero, A., 2022. Ready-to-eat salads and berry fruits purchased in Italy contaminated by *Cryptosporidium* spp., *Giardia duodenalis*, and *Entamoeba histolytica*. Int. J. Food Microbiol. 370, 109634 https://doi.org/10.1016/j.ijfoodmicro.2022.109634.

Berglund, B., Dienus, O., Sokolova, E., Berglind, E., Matussek, A., Pettersson, T., Lindgren, P.E., 2017. Occurrence and removal efficiency of parasitic protozoa in Swedish wastewater treatment plants. Sci. Total Environ. 598, 821–827. https://doi.org/10.1016/j.scitotenv.2017.04.015.

Berrouch, S., Escotte-Binet, S., Harrak, R., Huguenin, A., Flori, P., Favennec, L., Villena, I., Hafid, J., 2020. Detection methods and prevalence of transmission stages of T*oxoplasma gondii*, *Giardia duodenalis* and *Cryptosporidium* spp. in fresh vegetables: a review. Parasitology 147, 516–532. https://doi.org/10.1017/S0031182020000086.

Berrouch, S., Escotte-Binet, S., Madline, A., Aubert, D., Nast, E., La Carbona, S., Hoummadi, L., Hafid, J., Villena, I., 2022. Protozoan parasites and leafy greens in Marrakech: study of occurrence using a molecular method. Acta Parasitol. 67, 546–554. https://doi.org/10.1007/s11686-021-00488-z.

CAECV, 2021. Informe del Sector Ecológico de la Comunitat Valenciana. Comité de Agricultura Ecológica de la Comunitat Valenciana, Generalitat Valenciana.

Caradonna, T., Marangi, M., Del Chierico, F., Ferrari, N., Reddel, S., Bracaglia, G., Normanno, G., Putignani, L., Giangaspero, A., 2017. Detection and prevalence of protozoan parasites in ready-to-eat packaged salads on sale in Italy. Food Microbiol. 67, 67–75. https://doi.org/10.1016/j.fm.2017.06.006.

Chalmers, R.M., Robertson, L.J., Dorny, P., Jordan, S., Kärssinfg, 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.

Clark, C.G., van der Giezen, M., Alfellani, M.A., Stensvold, C.R., 2013. Recent developments in *Blastocystis* research. Adv. Parasitol. 21, 1–32. https://doi.org/10.1016/B978-0-12-407706-5.00001-0.

Cook, N., Paton, C.A., Wilkinson, N., Nichols, R.A.B., Barker, K., Smith, H.V., 2006. Towards standard methods for the detection of *Cryptosporidium parvum* on lettuce and raspberries. Part 2: validation. Int. J. Food Microbiol. 109, 222–228. https://doi.org/10.1016/j.ijfoodmicro.2005.12.014.

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.

Döller, P.C., Dietrich, K., Filipp, N., Brockmann, S., Dreweck, C., Vonthein, R., Wagner-Wiening, C., Wiedenmann, A., 2002. Cyclosporiasis outbreak in Germany associated with the consumption of salad. Emerg. Infect. Dis. 8, 992–994. https://doi.org/10.3201/eid0809.010517.

Ekman, C.C., Chiossi, M.F., Meireles, L.R., Andrade Júnior, H.F., Figueiredo, W.M., Marciano, M.A., Luna, E.J., 2012. Case-control study of an outbreak of acute toxoplasmosis in an industrial plant in the state of São Paulo, Brazil. Rev. Inst. Med. Trop. Sao Paulo. 54, 239–244. https://doi.org/10.1590/s0036-46652012000500001.

FAO/WHO, 2014. Multicriteria-Based Ranking for Risk Management of Food-Borne Parasites. Rome. available at. http://www.who.int/iris/handle/10665/112672. Accessed date: 16 February 2022.

Fatemi, M., Niyyati, M., Rouhani, S., Karamati, S.A., Mirjalali, H., Karanis, P., 2022. Contamination of fresh vegetables in municipal stores with pathogenic *Acanthamoeba* genotypes; a public health concern. Int. J. Environ. Health Res. 21, 1–12. https://doi.org/10.1080/09603123.2022.2067328.

FDA BAM, 2022. Chapter 19: Molecular Detection of *Cyclospora cayetanensis* in Fresh Produce using Real-time PCR. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-19b-molecular-detection-cyclospora-cayetanensis-fresh-produce-using-real-time-pcr.

Ferreira, F.P., Caldart, E.T., Freire, R.L., Mitsuka-Breganó, R., Freitas, F.M., Miura, A.C., Mareze, M., Martins, F.D.C., Urbano, M.R., Seifert, A.L., Navarro, I.T., 2018. The effect of water source and soil supplementation on parasite contamination in organic vegetable gardens. Rev. Bras. Parasitol. Vet. 27, 327–337. https://doi.org/10.1590/S1984-296120180050.

Giangaspero, A., Gasser, R.B., 2019. Human cyclosporiasis. Lancet Infect. Dis. 19, e226–e236. https://doi.org/10.1016/S1473-3099(18)30789-8.

Giangaspero, A., Marangi, M., Koehler, A.V., Papini, R., Normanno, G., Lacasella, V., Lonigro, A., Gasser, R.B., 2015. Molecular detection of *Cyclospora* in water, soil, vegetables and humans in southern Italy signals a need for improved monitoring by health authorities. Int. J. Food Microbiol. 15, 95–100. https://doi.org/10.1016/j.ijfoodmicro.2015.07.002.

Guy, R.A., Payment, P., Krull, J., Horgen, P.A., 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl. Environ. Microbiol. 69, 5178–5185. https://doi.org/10.1128/AEM.69.9.5178-5185.2003.

IFST, Institute of Food Science and Technology, UK, 1999. Organic food. Int. Food Saf. News 8, 2–6.

Insulander, M., Svenungsson, B., Lebbad, M., Karlsson, L., de Jong, B., 2010. A foodborne outbreak of *Cyclospora* infection in Stockholm, Sweden. Foodborne Pathog. Dis. 7, 1585–1587. https://doi.org/10.1089/fpd.2010.0628.

ISO 18744, 2016. Microbiology of the food chain - Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits.

Jinatham, V., Wandee, T., Nonebudsri, C., Popluechai, S., Tsaousis, A.D., Gentekaki, E., 2023. *Blastocystis* subtypes in raw vegetables from street markets in northern Thailand. Parasitol. Res. https://doi.org/10.1007/s00436-023-07781-y.

Kim, M.J., Ki, H.C., Kim, S., Chai, J., Seo, M., Oh, C.S., Shin, D.H., 2014. Parasitic infection patterns correlated with urban–rural recycling of night soil in Korea and other East Asian countries: the archaeological and historical evidence. Korean Stud. 38, 51–74. https://www.muse.jhu.edu/article/594899.

Kniel, K.E., Lindsay, D.S., Sumner, S.S., Hackney, C.R., Pierson, M.D., Dubey, J.P., 2002. Examination of attachment and survival of *Toxoplasma gondii* oocysts on raspberries and blueberries. J. Parasitol. 88, 790–793. https://doi.org/10.1645/0022-3395(2002)088[0790:EOAASO]2.0.CO;2.

Kuiper, M.W., Valster, R.M., Wullings, B.A., Boonstra, H., Smidt, H., van der Kooij, D., 2006. Quantitative detection of the free-living Amoeba *Hartmannella vermiformis* in surface water by using real-time PCR. Appl. Environ. Microbiol. 72, 5750–5756. https://doi.org/10.1128/AEM.00085-06.

Lass, A., Pietkiewicz, H., Szostakowska, B., Myjak, P., 2012. The first detection of *Toxoplasma gondii* DNA in environmental fruits and vegetable samples. Eur. J. Clin. Microbiol. Infect. Dis. 31, 1101–1108. https://doi.org/10.1007/s10096-011-1414-8.

Lass, A., Ma, L., Kontogeorgos, I., Zhang, X., Li, X., Karanis, P., 2019. First molecular detection of *Toxoplasma gondii* in vegetable samples in China using qualitative, quantitative Real-time PCR and multilocus genotyping. Sci. Rep. 9, 17581. https://doi.org/10.1038/s41598-019-54073-6.

Li, J., Wang, Z., Karim, M.R., Zhang, L., 2020. Detection of human intestinal protozoan parasites in vegetables and fruits: a review. Parasit. Vectors 13, 380. https://doi.org/10.1186/s13071-020-04255-3.

Lilly, E.L., Webster, N.J., 2021. Detection of *Toxoplasma gondii* oocysts on organic and conventionally grown produce. Food Microbiol. 99, 103798 https://doi.org/10.1016/j.fm.2021.103798.

Lopes, C., Brandão, R., Lopes, A.F., Sargo, R., Casero, M., Nunes, C., Silva, F., Dubey, J.P., Cardoso, L., Lopes, A.P., 2021. Prevalence of antibodies to *Toxoplasma gondii* in different wild bird species admitted to rehabilitation centres in Portugal. Pathogens 10, 1144. https://doi.org/10.3390/pathogens10091144.

Lorenzo-Morales, J., Khan, N.A., Walochnik, J., 2015. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. Parasite 22, 10. https://doi.org/10.1051/parasite/2015010.

Maffei, D.F., Batalha, E.Y., Landgraf, M., Schaffner, D.W., Franco, B.D., 2016. Microbiology of organic and conventionally grown fresh produce. Braz. J. Microbiol. 47S, 99–105. https://doi.org/10.1016/j.bjm.2016.10.006.

Mansfield, L.S., Gajadhar, A.A., 2004. *Cyclospora cayetanensis*, a food- and waterborne coccidian parasite. Vet. Parasitol. 126, 73–90. https://doi.org/10.1016/j.vetpar.2004.09.011.

Marchioro, A.A., Tiyo, B.T., Colli, C.M., de Souza, C.Z., Garcia, J.L., Gomes, M.L., Falavigna-Guilherme, A.L., 2016. First detection of *Toxoplasma gondii* DNA in the fresh leafs of vegetables in South America. Vector Borne Zoonotic Dis 16, 624–626. https://doi.org/10.1089/vbz.2015.1937.

Marín-García, P.J., Planas, N., Llobat, L., 2022. *Toxoplasma gondii* in foods: prevalence, control, and safety. Foods 11, 2542. https://doi.org/10.3390/foods11162542.
Marques, C.S., Sousa, S., Castro, A., Ferreira, V., Teixeira, P., da Costa, J.M.C., 2022. Protozoa as the "underdogs" for microbiological quality evaluation of fresh vegetables. Appl. Sci. 12, 7145. https://doi.org/10.3390/app12147145.
Martín-Escolano, R., Ng, G.C., Tan, K.S.W., Stensvold, C.R., Gentekaki, E., Tsaousis, A.D., 2023. Resistance of *Blastocystis* to chlorine and hydrogen peroxide. Parasitol. Res. 122, 167–176. https://doi.org/10.1007/s00436-022-07713-2.
Moreno-Mesonero, L., Hortelano, I., Moreno, Y., Ferrús, M.A., 2020. Evidence of viable *Helicobacter pylori* and other bacteria of public health interest associated with free-living amoebae in lettuce samples by next generation sequencing and other molecular techniques. Int. J. Food Microbiol. 318, 108477 https://doi.org/10.1016/j.ijfoodmicro.2019.108477.
Nasser, A.M., 2022. Transmission of *Cryptosporidium* by fresh vegetables. J. Food Prot. 1 https://doi.org/10.4315/JFP-22-152.
Nolan, T., Hands, R.E., Bustin, S.A., 2006. Quantification of mRNA using real-time RT-PCR. Nat. Protoc. 1, 1559–1582. https://doi.org/10.1038/nprot.2006.236.
Oliveira, M., Usall, J., Viñas, I., Anguera, M., Gatius, F., Abadias, M., 2010. Microbiological quality of fresh lettuce from organic and conventional production. Food Microbiol. 27, 679–684. https://doi.org/10.1016/j.fm.2010.03.008.
Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kiljstra, A., van der Giessen, J., 2010. Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. Int. J. Food Microbiol. 139, 193–201. https://doi.org/10.1016/j.ijfoodmicro.2010.02.027.
Parfrey, L.W., Walters, W.A., Knight, R., 2011. Microbial eukaryotes in the human microbiome: ecology evolution, and future directions. Front. Microbiol. 2, 153. https://doi.org/10.3389/fmicb.2011.00153.
Pazoki, H., Niyyati, M., Javanmard, E., Lasjerdi, Z., Spotin, A., Mirjalali, H., Behravan, M.R., 2020. Isolation and phylogenetic analysis of free-living amoebae (*Acanthamoeba*, *Naegleria*, and *Vermamoeba*) in the farmland soils and recreational places in Iran. Acta Parasitol. 65, 36–43. https://doi.org/10.2478/s11686-019-00126-9.
Pinto-Ferreira, F., Reis, J.B., Paschoal, A.T.P., Balbino, L.S., Bertão-Santos, A., Lucas, J.I., Kakimori, M.T.A., Navarro, I.T., Mitsuka-Breganó, R., 2020. Molecular diagnosis of the curly lettuce parasitic contamination from hydroponic cultivation from supermarkets. Rev. Bras. Parasitol. Vet. 29, e015820 https://doi.org/10.1590/S1984-29612020095.
Qvarnstrom, Y., Visvesvara, G.S., Sriram, R., da Silva, A.J., 2006. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. J. Clin. Microbiol. 44, 3589–3595. https://doi.org/10.1128/JCM.00875-06.
Rayamajhee, B., Subedi, D., Peguda, H.K., Willcox, M.D., Henriquez, F.L., Carnt, N., 2021. A systematic review of intracellular microorganisms within *Acanthamoeba* to understand potential impact for infection. Pathogens 10, 225. https://doi.org/10.3390/pathogens10020225.
Razakandrainibe, R., Kubina, S., Costa, D., Robinson, G., La Carbona, S., Aubert, D., David, A., Gargala, G., Villena, I., Favennec, L., Chalmers, R.M., 2020. Evaluation of a modified method for the detection of *Cryptosporidium* oocysts on spinach leaves. Food Waterborne Parasitol. 21, e00097 https://doi.org/10.1016/j.fawpar.2020.e00097.
Reyes-Batlle, M., Díaz, F.J., Sifaoui, I., Rodríguez-Expósito, R., Rizo-Liendo, A., Piñero, J.E., Lorenzo-Morales, J., 2021. Free living amoebae isolation in irrigation waters and soils of an insular arid agroecosystem. Sci. Total Environ. 753, 141833 https://doi.org/10.1016/j.scitotenv.2020.141833.
SCF/CS/FMH/SURF, 2002. Risk Profile on the Microbiological Contamination of Fruits and Vegetables Eaten Raw. Report of the Scientific Committee on Food of European Commission.
Siddiqui, R., Khan, N.A., 2012. Biology and pathogenesis of *Acanthamoeba*. Parasit. Vectors 5, 6. https://doi.org/10.1186/1756-3305-5-6.
Siddiqui, R., Makhlouf, Z., Khan, N.A., 2021. The increasing importance of *Vermamoeba vermiformis*. J. Eukaryot. Microbiol. 68, e12857 https://doi.org/10.1111/jeu.12857.
Sousa-Ramos, D., Reyes-Batlle, M., Bellini, N.K., Rodríguez-Expósito, R.L., Martín-Real, C., Piñero, J.E., Lorenzo-Morales, J., 2022. Pathogenic free-living amoebae from water sources in Cape Verde. Parasitol. Res. 121, 2399–2404. https://doi.org/10.1007/s00436-022-07563-y.
Stensvold, C.R., Ahmed, U.N., Andersen, L.O., Nielsen, H.V., 2016. Development and evaluation of a genus-specific, probe-based, internal-process-controlled real-time PCR assay for sensitive and specific detection of *Blastocystis* spp. J. Clin. Microbiol. 50, 1847–1851. https://doi.org/10.1128/JCM.00007-12.
Tefera, T., Tysnes, K.R., Utaaker, K.S., Robertson, L.J., 2018. Parasite contamination of berries: risk, occurrence, and approaches for mitigation. Food Waterborne Parasitol. 10, 23–38. https://doi.org/10.1016/j.fawpar.2018.04.002.
Temesgen, T.T., Tysnes, K.R., Robertson, L., 2019. A new protocol for molecular detection of *Cyclospora cayetanensis* as contaminants of berry fruits. Front. Microbiol. 10, 1939. https://doi.org/10.3389/fmicb.2019.01939.
Temesgen, T.T., Stigum, V.M., Robertson, L., 2022. Surveillance of berries sold on the Norwegian market for parasite contamination using molecular methods. Food Microbiol. 104, 103980 https://doi.org/10.1016/j.fm.2022.103980.
Torrey, E.F., Yolken, R.H., 2013. *Toxoplasma* oocysts as a public health problem. Trends Parasitol. 29, 380–384. https://doi.org/10.1016/j.pt.2013.06.001.
Trelis, M., Sáez-Durán, S., Puchades, P., Castro, N., Miquel, A., Gozalbo, M., Fuentes, M.V., 2022. Survey of the occurrence of *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts in green leafy vegetables marketed in the city of Valencia (Spain). Int. J. Food Microbiol. 379, 109847 https://doi.org/10.1016/j.ijfoodmicro.2022.109847.
Utaaker, K.S., Huang, Q., Robertson, L.J., 2015. A reduced-cost approach for analyzing fresh produce for contamination with *Cryptosporidium* oocysts and/or *Giardia* cysts. Food Res. Int. 77, 326–332. https://doi.org/10.1016/j.foodres.2015.05.010.
Utaaker, K.S., Kumar, A., Joshi, H., Chaudhary, S., Robertson, L.J., 2017. Checking the detail in retail: occurrence of *Cryptosporidium* and *Giardia* on vegetables sold across different counters in Chandigarh, India. Int. J. Food Microbiol. 263, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2017.09.020.
Visvesvara, G.S., Moura, H., Schuster, F.L., 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol. Med. Microbiol. 50, 1–26. https://doi.org/10.1111/j.1574-695X.2007.00232.x.