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Ready-to-eat salads and berry fruits purchased in Italy contaminated by *Cryptosporidium* spp., *Giardia duodenalis*, and *Entamoeba histolytica*

A. Barlaam [a,b,*], A.R. Sannella [c], N. Ferrari [d,h], T.T. Temesgen [e,f], L. Rinaldi [g], G. Normanno [a], S.M. Cacciò [c], L.J. Robertson [e], A. Giangaspero [a]

[a] Department of Agriculture, Food, Natural Resources and Engineering (DAFNE), University of Foggia, Via Napoli 25, 71121 Foggia, Italy
[b] Faculty of Veterinary Medicine, University of Teramo, Località Piano d'Accio snc, 64100 Teramo, Italy
[c] Unit of Foodborne and Neglected Parasites, Department of Infectious Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
[d] Department of Veterinary Medicine, Università degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy
[e] Laboratory of Parasitology, Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oluf Thesens vei 22, 1433 Ås, Norway
[f] Now at: NABAS AS, Moer Allé 33, 1435 Ås, Norway
[g] Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR Via Delpino 1, 80137 Naples, Italy
[h] Coordinated Research Center "EpiSoMI", Università degli Studi di Milano, 20133 Milan, Italy

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ABSTRACT

Ready-to-eat (RTE) salads and berries are increasingly consumed in industrialized countries. These products can be contaminated by pathogenic parasites that have been responsible for foodborne outbreaks worldwide. In Italy, there are few data on contamination of RTE salads and berries with parasite transmission stages and this requires more-in-depth investigations. To estimate the prevalence of contamination with *Cryptosporidium* spp. and *Giardia duodenalis* in these fresh products, a total of 324 packages of local RTE mixed salads - belonging to three different industrial brands – and 324 packages of berries - blueberries from Peru, blackberries from Mexico, raspberries from Italy - were bought from supermarkets located in the Provinces of Bari and Foggia, Apulia, Italy. A pool size of nine packages was chosen and a total of 72 pools were processed in the whole year. After washing, the pellets were examined by microscopy (FLOTAC) and tested using conventional simplex PCR, targeting *Cryptosporidium* spp., *Giardia duodenalis*, and *Entamoeba* spp., and sequencing. Several *Cryptosporidium* species and *Giardia duodenalis* assemblages, some of which are of potential zoonotic relevance, as well as *Entamoeba* spp., were identified in both matrices. By microscopy, *Giardia*-like cysts in local raspberries and *Entamoeba*-like cysts in imported blueberries were detected. *Giardia duodenalis* (Assemblages A, B and E) and *Entamoeba histolytica* were molecularly confirmed with overall prevalences of 4.6% (95% C.I. 3.0–6.8) and 1% (95% C.I. 0.3–2.1), respectively. Molecular methods identified *Cryptosporidium ryanae*, *Cryptosporidium bovis*, *Cryptosporidium xiaoi*, and *Cryptosporidium ubiquitum* in both matrices, with a prevalence of 5.1% (95% C.I. 3.3–7.3). A distinct seasonality in prevalence was observed for *G. duodenalis*, with most positives occurring in spring, whereas *Cryptosporidium* showed no significant seasonal variations. These results highlight that inadequate management of fresh produce, both locally produced and imported, along the food chain may have the potential for consequences on human health.

1. Introduction

Over the past few decades, due to changes in culinary habits and the rising demand for healthy dietary options, ready-to-eat (RTE) salads and berry fruits have become increasingly popular among European consumers (Tefera et al., 2018; Trevisan et al., 2019). Despite representing healthy food choices, both these products could be contaminated with several foodborne pathogens, including parasites of faecal origin, during

* Corresponding author at: Faculty of Veterinary Medicine, University of Teramo, Località Piano d'Accio snc, 64100 Teramo, Italy.
E-mail addresses: abarlaam@unite.it, alessandra.barlaam@unifg.it (A. Barlaam), annarosa.sannella@iss.it (A.R. Sannella), nicola.ferrari@unimi.it (N. Ferrari), tamirat.temesgen@nabas.no (T.T. Temesgen), lrinaldi@unina.it (L. Rinaldi), giovanni.normanno@unifg.it (G. Normanno), simone.caccio@iss.it (S.M. Cacciò), lucy.robertson@nmbu.no (L.J. Robertson), annunziata.giangaspero@unifg.it (A. Giangaspero).

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each step of the food chain (Bouwknegt et al., 2018; Robertson, 2018). Contamination can occur during the pre-harvest period, when fruits and vegetables are irrigated using wastewater or fresh manure is used to fertilize the land or animals have access to the cultivated fields (EFSA, 2018). Fresh produce can also be contaminated during harvesting and in the post-harvest phase, during processing, packaging, transport, handling at the market, and preparation in the kitchen prior to consumption (Carstens et al., 2019; EFSA, 2018).

Some European countries, including Italy, produce berries for a large part of the year, but to ensure an adequate provision of soft fruits, these products are also imported into Europe from tropical and sub-tropical areas, such as Central and South American countries, where warm climates allow year-round production (CBI Ministry of Foreign Affairs, 2018). Such countries, however, may be endemic for parasites that are uncommon in Europe, and often lack developed infrastructures, such as suitable water treatments and sanitation facilities, and adequate hazard analysis and critical control point (HACCP) plans during production (Barlaam et al., 2021; Kempler and Hall, 2013; Tefera et al., 2018).

Among foodborne parasites, *Cryptosporidium* spp. and *Giardia duodenalis* are protozoa of significant public health importance. In fact, in a joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organisation (WHO) risk ranking, out of 24 foodborne parasites listed, *Cryptosporidium* spp. and *G. duodenalis* were ranked fifth and 11th, respectively (FAO/WHO, 2014). Also, in a more recent European prioritisation of foodborne parasites based on multicriteria analysis, both *Cryptosporidium* spp. and *G. duodenalis* were included in the top ten at a European level, and, in the chart referring to South-Western Europe, that includes Italy as well as other countries located in the same geographic area, *Cryptosporidium* spp. was listed and ranked 8th out of 10 (Bouwknegt et al., 2018).

Transmission of cryptosporidiosis and giardiasis occurs via the faecal-oral route through the ingestion of oocysts and cysts, respectively (Escobedo et al., 2014; Tzipori and Ward, 2002;). RTE salads and berries are eaten raw, however, *Cryptosporidium* oocysts and *Giardia* cysts are well known to be resistant to chemical disinfection with chlorine, which is the most widely used water disinfectant in the RTE salad industry (Colelli and Elia, 2009). Furthermore, even vigorous washing of berries has been shown to be ineffective at removing all the oocysts and cysts that stick on their surface, and may not be feasible for some berry types, such as raspberries, which are relatively fragile (Tefera et al., 2018; Temesgen et al., 2021). Another factor which facilitates foodborne transmission of *Cryptosporidium* spp. and *Giardia* is their low infectious dose (Ortega and Adam, 1997; Vanathy et al., 2017) and the ability of the oocysts and cysts to survive at refrigeration temperatures, employed for transporting and storing fresh produce (Hohweyer et al., 2016; Utaaker et al., 2017). Nevertheless, the European legislation and Italian health laws do not require fresh produce to be tested for the detection of protozoan parasites prior to being sold on the market.

Numerous foodborne outbreaks caused by the consumption of fresh produce contaminated with *Cryptosporidium* oocysts have been reported worldwide, including Europe (Åberg et al., 2015; Ethelberg et al., 2009; Insulander et al., 2008; McKerr et al., 2015) and salad items were among the most common foods implicated (Dixon et al., 2011, 2013; Zahedi and Ryan, 2020). Similarly, outbreaks of giardiasis have been associated with a variety of foods and, among them, fresh fruits and vegetables contaminated with *Giardia* cysts were the most common source of infection (Adam et al., 2016).

Epidemiological studies carried out in many different countries around the world, have shown the presence of *Cryptosporidium* spp. oocysts and/or *Giardia* cysts in fresh products including RTE salads (Caradonna et al., 2017; Di Benedetto et al., 2007; Dixon et al., 2013; Lalonde and Gajadhar, 2016). Also, berries were found to be contaminated with several zoonotic parasites including *Cryptosporidium* spp. and *G. duodenalis* (Tefera et al., 2018). Compared with other fresh produce, studies on RTE salads and berries are scarce, worldwide. Regarding Italy, previous findings revealed the presence of *Cryptosporidium* spp. and *G. duodenalis* in RTE salads (Caradonna et al., 2017; Di Benedetto et al., 2007), whereas data for berries are completely lacking. Therefore, in order to confirm and broaden the data on RTE salads and fill the gaps in knowledge on contamination of berries in Italy, the aim of this study was to detect the presence and investigate the prevalence of *Cryptosporidium* spp. and *G. duodenalis* in RTE packaged mixed salads and on local and imported berries using microscopy and molecular tools.

2. Materials and methods

2.1. Sampling design

The sample size estimation and the sampling plan were as previously described by Barlaam et al. (2021). Briefly, from January to December 2019, 648 packages of fresh produce were bought from supermarkets located in the Provinces of Bari and Foggia, Apulia, Italy. Overall, 324 RTE mixed salads grown and produced in Italy, belonging to three industrial brands (A, B, and C), and 324 berry packages (blueberries imported from Peru, blackberries from Mexico and Italian raspberries) were collected. A total of 72 homogenous pools, each composed of nine packages of each product type, were analyzed (Table 1).

2.2. Fresh produce processing

One hundred g of each product type were weighed into filter bags (BagPage® + 400 ml filter bags, Interscience, Sant Nom, France), to which 200 ml of 1% Alconox (Alconox Inc., NY USA) was added. All the samples were washed according to Barlaam et al. (2021), and after repeated cycles of centrifugation, the resultant pellets were stored at 4 °C and at −20 °C pending microscopy and molecular analysis, respectively.

2.3. Microscopy analysis

At the Regional Center for Parasitosis Monitoring (CREMOPAR) at the University of Naples Federico II, Italy, all the 72 fresh produce pellets were microscopically examined using the FLOTAC double technique (Cringoli et al., 2010) in which zinc sulphate (specific gravity = 1.35) was used as the flotation solution. Magnifications of 100× and 400× were used to identify protozoan oocysts and cysts on a DM1000 LED microscope equipped with a ICC50 HD Camera (Leica).

2.4. Molecular investigation

2.4.1. DNA extraction

DNA was isolated from individual fresh produce pools using the DNeasy PowerSoil® extraction Kit (Qiagen, Italy) following the manufacturer's instructions, with slight modifications. Briefly, 250 μl of each eluate after being mixed with an appropriate volume of C1 solution, were subjected to bead-beating using a FastPrep-24 5G™ High Speed Homogeniser (MP Biomedicals, France) run in two cycles of 4 m/s for 60 s with 45 s pause. The final elution volume (50 μl) was stored at −20 °C until further analysis.

2.4.2. PCR protocols

2.4.2.1. Conventional nested PCR for Cryptosporidium spp.. At the Department of Infectious Diseases of the Istituto Superiore di Sanità, for the identification of *Cryptosporidium* spp., a nested PCR assay was used to amplify a fragment of the small subunit rRNA (*18S* rDNA) gene, as described elsewhere (Ryan et al., 2003). The primers 18SiCF2 (5′-GACATATCATTCAAGTTTCTGACC-3′) and 18SiCR2 (5′-CTGAAGGAGTAAGGAACAACC-3′) were used in the primary PCR to amplify a 763 bp fragment. For the nested reaction, the primers 18SiCF1 (5′-CCTATCAGCTTTAGACGGTAGG-3) and 18SiCR1 (5′-

Table 1
Prevalence of parasites in ready-to-eat mixed salads and berries sold on the Italian market investigated by microscopy and molecular tools.

Fresh products (Country of origin)	No. of pools	*Giardia duodenalis*			*Cryptosporidium* spp.			*Entamoeba* spp.		
		Microscopy FLOTAC	Nested PCR and sequencing	No. Total positive pools	Microscopy FLOTAC	Nested PCR and sequencing	No. Total positive pools	Microscopy FLOTAC	PCR and sequencing	No. Total positive pools
RTE salads - Brand A (Italy)	12	0	4 Ass. A, sub-Ass. AI (1) Ass. B (3)	4	0	4 *C. ryanae* (3) *C. bovis* (1)	4	0	0	0
RTE salads - Brand B (Italy)	12	0	6 Ass. A, sub-Ass. AI (1) Ass. B (4) Ass. E (1)	6	0	5 *C. ryanae* (3) *C. bovis* (1) *C. ubiquitum* (1)	5	1	0	1
RTE salads Brand C (Italy)	12	0	3 Ass. B (3)	3	0	6 *C. ryanae* (6)	6	1	0	1
RTE subtotal	36	0	13	13	0	15	15	2	0	2
Imported blueberries (Peru)	12	0	4 Ass. A, sub-Ass. AI (2) Ass. B (2)	4	0	4 *C. ryanae* (4)	4	1	1	1
Imported blackberries (Mexico)	12	0	4 Ass. B (4)	4	0	2 *C. ryanae* (1) *C. xiaoi* (1)	2	2	0	2
Local raspberries (Italy)	12	2	4 Ass. A, sub-Ass. AI (2) Ass. B (2)	4	0	6 *C. ryanae* (5) *C. xiaoi* (1)	6	1	0	1
Berries subtotal	36	2	12	12	0	12	12	4	1	4
Overall total (No)	72	2	25	25	0	27	27	6	1	6
Prevalence % (95% CI[a])		4.6% (3.0-6.8)			5.1% (3.3-7.3)			1.0% (0.3-2.1)		

Ass. = Assemblage; sub-Ass. = sub-Assemblage.
[a] The overall prevalence was calculated keeping into account group testing where all the 648 packages were tested in pool of 9 items for a total of 72 homogenous pools. 95% Confidence Interval.

TCTAAGAATTTCACCTCTGACTG-3′) were used to amplify a fragment of ~587 bp. Reactions were performed in a final volume of 50 μl containing 25 μl of 2× GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 μl of each primer (10 pmol/μl), 5 μl of extracted DNA and 18 μl of nuclease free water. Genomic DNA extracted from commercially available *C. parvum* oocysts and DNA-free water were included in each experiment as positive and a negative controls, respectively. Reactions were performed on a Perkin Elmer 9700 apparatus (Life Technologies, Carlsbad, USA). PCR conditions, for both primary and secondary amplification, were as follows: after an initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min were performed, followed by a final extension step at 72 °C for 7 min. Aliquots of PCR reactions (10 μl) were loaded on 1.5% agarose gel stained with ethidium bromide and visualized under a UV transilluminator.

2.4.2.2. Conventional nested PCR for Giardia duodenalis. At the Department of Infectious Diseases of the Istituto Superiore di Sanità, for the identification of *G. duodenalis*, a nested-PCR assay was used to amplify a fragment of the beta-giardin gene, using a previously described protocol (Cacciò et al., 2002; Lalle et al., 2005). The primers G7 (5′-AAGCCCGACGACCTCACCCGCAGTGC-3′) and G759 (5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′) were used in primary PCR to amplify a 753 bp fragment. For the nested reaction, the primers βGiarF (5′-GAACGAGATCGAGGTCCG-3′) and βGiarR (5′-CTCGACGAGCTTCGTGTT-3′) were used to amplify a 511 bp fragment. Reactions were performed in a final volume of 50 μl containing 25 μl of 2× GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 μl of each primer (10 pmol/μl), 5 μl of extracted DNA and 18 μl of sterile water. Reactions were performed on a Perkin Elmer 9700 apparatus (Life Technologies, Carlsbad, USA). PCR conditions, for both primary and secondary amplification, were as follows: after an initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min were performed, followed by a final extension step at 72 °C for 7 min. Genomic DNA extracted from commercially available *G. duodenalis* cysts was used as a positive control and DNA-free water as a negative control. Aliquots of PCR reactions (10 μl) were loaded on 1.5% agarose gel stained with ethidium bromide, and visualized under a UV transilluminator.

2.4.2.3. Conventional PCR for Entamoeba spp.. At the Department of Agriculture, Food, Natural Resources and Engineering (DAFNE) at the University of Foggia, a protocol of conventional PCR was used to amplify the 18S rRNA genus-specific fragment of *Entamoeba* spp., ranging from 622 to 667 bp, depending on the *Entamoeba* species. The primers JVC (5-GTTGATCCTGCCAGTATTATATG-3) and DSPR2 (5-CACTATTGGAGCTGGAATTAC-3) were used according to Santos et al., 2010. Amplification was performed in a total reaction volume of 25 μl containing 12.5 μl of DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 1 μl of each primer, 8.5 μl of water, and 2 μl of genomic DNA. The PCR protocol was as follows: 2 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 2 min with a final extension step of 72 °C for 5 min. A positive control and a negative control (PCR grade water) were included in each experiment. The PCR products were run on 2% agarose gel.

2.5. Sequencing

Amplicons from samples positive for *Cryptosporidium* spp. and *Giardia duodenalis* were purified using the QiaQuick PCR purification kit spin columns (Qiagen, Hilden, Germany). Purified amplicons were sequenced on both strands by a commercial company (BMR Genomics, Padua, Italy). Sequence data were edited and assembled using the SeqMan 7.1 software package (DNASTAR, Madison, WI, USA). BLAST

searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank database were used to identify *Cryptosporidium* at the species level and *Giardia* Assemblages.

Purification and sequencing of samples positive for *Entamoeba* spp. were performed by Eurofins MWG Operon (Ebersberg, Germany). The sequences generated were edited and aligned manually using Geneious version 2020.0.5 (https://www.geneious.com) and compared with the sequences available in GenBank using Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Representative sequences were submitted to GenBank.

2.6. Statistical analyses

Analyses were conducted in R 3.6.3 (R Development Core Team R, 2020) with "binGroup" library for group testing and the threshold for statistical significance set as $p < 0.05$ (Bilder et al., 2010). Prevalences and 95% confidence intervals were estimated with the bgtCI function using the Clopper-Pearson method based on a single binomial proportion estimated from a binomial group testing trial (Schaarschmidt, 2007). For pathogens with a prevalence above 2%, the statistical difference of prevalence between seasons and product (RTE salad vs berries) was tested through group testing regression, considering the contamination status of the pool (positive/negative) as the response variable and pool identity as a grouping factor (Vansteelandt et al., 2000).

3. Results

3.1. Microscopy results

Out of the 72 fresh produce pools examined by microscopy, none of the samples contained parasitic forms that were similar in morphology to *Cryptosporidium* spp. oocysts. However, *Giardia*-like cysts (12 μm × 8 μm) were identified in two pools of Italian raspberries and *Entamoeba*-like cysts (15 μm) were identified in four pools of berries and in two RTE salad pools (Table 1, Fig. 1).

3.2. Molecular results

Cryptosporidium was detected in 27 pools, 15 of which were RTE salads and 12 berries. Sequencing confirmed the presence of the following species: *Cryptosporidium ryanae*, *Cryptosporidium bovis*, *Cryptosporidium xiaoi* and *Cryptosporidium ubiquitum*. Of the 27 pools positive for *Cryptosporidium*, 16 matched with *C. ryanae* with 100% homology (Gen-Bank accession numbers MT374189.1, KY711520.1), two had 100% homology with *C. bovis* (GenBank accession numbers KC618605.1, MH028031.1), and finally one showed 100% homology with *C. ubiquitum* (GenBank accession number MN876847.1). Six pools showing a 99% homology with *C. ryanae* and two pools with a 99% homology with *C. xiaoi* were deposited in GenBank under accession numbers OK510366, OK510367, OK510368, OK510369, OK510370, OK510371 (*C. ryanae*), OK510372, OK510373 (*C. xiaoi*).

Giardia duodenalis was detected in 25 pools: 13 pools of RTE salad and 12 pools of berries, in two of which *Giardia* cyst-like objects had already been identified by microscopy. Sequencing the pools positive for *Giardia* and comparing them with the sequences available in GenBank, six were assigned to Assemblage A (sub-Assemblage AI), 18 to Assemblage B, and one to Assemblage E. Specifically, six had 100% homology with Assemblage A, sub-Assemblage AI (GenBank accession number MK610392.1, MK610391.1) and 10 had 100% homology with sequences of Assemblage B (GenBank accession number MN044602.1, KX469053.1, MK982544.1). Of the other positive pools, eight had 99% homology with Assemblage B sequences and one had 99% homology with Assemblage E. These sequences were deposited in GenBank under the following accession numbers: OK539673, OK539674, OK539675, OK539676, OK539677, OK539678, OK539679, OK539680 (Assemblage B), OK539681 (Assemblage E).

Entamoeba DNA was molecularly confirmed in one of the pools in which an *Entamoeba*-like cyst had been identified by microscopy (imported blueberries from Peru). This pool gave a clear band that matched with the estimated PCR product size. Sequencing and BLAST analysis revealed 100% homology with *Entamoeba histolytica*; however, it should be noted that a highly conserved gene was targeted, and the sequence for *E. histolytica* only has two nucleotides of difference from that of *E. nutalli*. The sequence was deposited in GenBank under the following accession number: OK576922. The other five pools in which *Entamoeba*-like cysts were observed by microscopy could not be confirmed by the molecular assay, as the weak products generated yielded non-interpretable sequence data.

Most pools testing positive in the present study were contaminated with just one parasite, but eleven pools were contaminated by two protozoa species. Specifically, eight pools were found to contain both *Cryptosporidium* spp. and *G. duodenalis*, two pools contained *G. duodenalis* and *Entamoeba* spp. and one pool *Cryptosporidium* spp. and *Entamoeba* spp. (**Supplementary Data**). Among the 46 positive pools, 13 (28%) were imported fresh produce whereas 33 (72%) were grown in

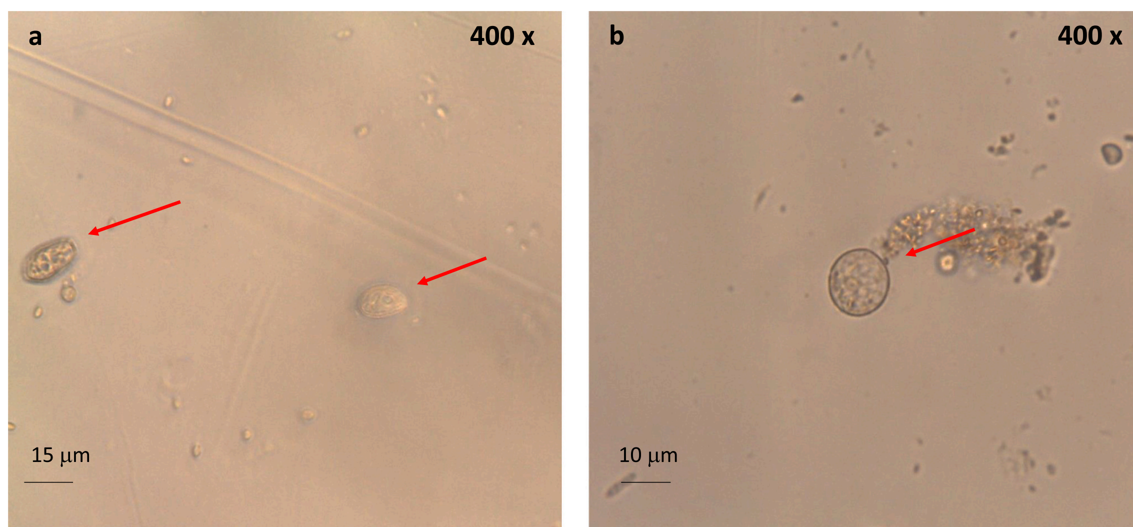

Fig. 1. *Giardia*-like cyst (a) and *Entamoeba*-like cyst (b) detected by FLOTAC technique.

Italy. In imported products, the most prevalent protozoan species was *G. duodenalis* (4.4%, 95% C.I. 1.9–8.6%), followed by *Cryptosporidium* spp. (3.1%, 95% C.I. 1.1–6.7%) and *E. histolytica* (1.5%, 95% C.I. 0.3–4.2%). In the Italian fresh products, the prevalence varied between the values of 0.7% (95% C.I. 0.1–2%) for *E. histolytica* and 4.7% (95% C.I. 2.7–7.5%) for *G. duodenalis* and the highest value of 6.1% (95% C.I. 3.8–9.4%) for *Cryptosporidium* spp.

All results are summarized in Table 1 and Supplementary Data.

3.3. Prevalence data and seasonality

Prevalence was estimated on the basis of both microscopy data and molecular data. A sample was considered positive if it had been considered positive by at least one of these methods. The most prevalent species was *Cryptosporidium* spp. (5.1%, 95% C.I. 3.3–7.3) followed by *G. duodenalis* (4.6%, 95% C.I. 3.0–6.8%). *Entamoeba* spp. (1.0%, 95% C.I. 0.3–2.1) was also detected (Table 1).

Giardia duodenalis showed seasonal differences (Dev = 10.9, d.f. = 3, $p = 0.012$), with highest values in spring (8.6%, 95% C.I. 4.0–15.7) and lowest in summer and autumn (2.0%, 95% C.I. 0.4–5.7). No significant seasonal variations were observed for *Cryptosporidium* spp. At the same time, no significant difference in contamination between salads and berries was observed for both *Cryptosporidium* spp. and *G. duodenalis*.

4. Discussion

The main result of the present study is that fresh produce sold on the Italian market throughout 2019, were found to be contaminated with parasites, specifically several *Cryptosporidium* species, i.e., *C. ryanae*, *C. bovis*, *C. xiaoi* and *C. ubiquitum* and *G. duodenalis* - Assemblages A (sub-Assemblage AI), B, and E - based on their detection by both microscopy and PCR. Moreover, *Entamoeba histolytica*, which was not included in the original aims of the study, was detected with a prevalence of 1%. Although inhibition of DNA amplification in fresh produce matrices is a known problem (Schrader et al., 2012), we did not have the resources to test for this, and thus these occurrence percentages should be considered as conservative estimates.

Cryptosporidium bovis and *C. ryanae* are among the main species infecting cattle, whereas *C. ubiquitum* and *C. xiaoi* are the predominant species in sheep and goats (Zahedi and Ryan, 2020). In Italy, *C. parvum*, *C. ryanae*, *C. bovis*, *C. ubiquitum* and *C. hominis* have been reported in cattle (Di Piazza et al., 2013; Duranti et al., 2009; Grana et al., 2006; Imre and Dărăbuș, 2011; Merildi et al., 2009), while little is known on the presence of *Cryptosporidium* spp. in sheep and the only data available are limited to two surveys that have shown the presence of *C. parvum* and *C. ubiquitum* (Dessì et al., 2020; Paoletti et al., 2009). The latter species was also recently detected in alpine wild ruminants (Trogu et al., 2021). Despite the lack of data on the spread of *Cryptosporidium* infection in sheep in Italy, lambs have been identified as a source of zoonotic transmission to humans. In fact, Cacciò et al. (2013) reported for the first time the transmission of cryptosporidiosis in Italy involving lambs as the source of the oocysts infecting a toddler.

Among the four *Cryptosporidium* species detected in the present study, *C. bovis* and *C. ubiquitum* have been occasionally reported in humans (Fayer et al., 2010; Li et al., 2014; Ryan et al., 2016), one study reports the presence of *C. xiaoi* in two HIV/AIDS patients' stools in Ethiopia (Adamu et al., 2014), and human infection with *C. ryanae* has not been reported, suggesting that the public health significance of these species is relatively unimportant. *Cryptosporidium parvum*, *C. hominis* and *C. ubiquitum* are the only *Cryptosporidium* species previously reported from contaminated fresh produce (Caradonna et al., 2017; Duedu et al., 2014; Li et al., 2019; Rzezutka et al., 2014; Temesgen et al., 2022) and the present survey represents the first detection of *C. ryanae*, *C. bovis* and *C. xiaoi* in these matrices. The detection of *C. ubiquitum* in RTE salads is in line with the results of another Italian survey (Caradonna et al., 2017), but the lack of detection of *C. parvum* is quite unexpected, given the ubiquity of this species in Europe and that DNA of this parasite has previously been detected in RTE salads in Canada (Dixon et al., 2013; Lalonde and Gajadhar, 2016) and, more recently, in Italy (Caradonna et al., 2017). It is noteworthy that in no batch did we detect more than one species of *Cryptosporidium*. This may have been the actual distribution, or could reflect that amplification of DNA from a predominant species masked the presence of a species occurring at a lower concentration.

The presence of *Giardia* in the investigated fresh produce is indicative of faecal contamination of human and/or animal origin. In the present study, Assemblages with zoonotic potential i.e., A, B and, to a lesser extent, E, were detected. Although farm animals are mainly infected with the host-specific assemblage E, Assemblage A is increasingly being detected in the bovine population and, the sub-Assemblage AI, identified in the present study, is the most common (Ryan and Cacciò, 2013). The zoonotic transmission of this sub-Assemblage between cattle and humans has been reported and may occur not only through direct contact with the animals but also indirectly via contaminated water and fresh produce (Budu-Amoako et al., 2012; Khan et al., 2011; Rafael et al., 2017). Assemblage B is not commonly found in farm animals (Feng and Xiao, 2011), but has been reported in sheep in several European countries including Italy where an outbreak of giardiasis in lambs was attributed to this Assemblage (Aloisio et al., 2006). In congruence with our results, Assemblages A and B are the ones most commonly reported in literature in contaminated vegetable and fruit samples (reviewed by Li et al., 2020). One RTE salad sample from the present study matched with Assemblage E, which is mainly found in hoofed animals and predominates in cattle. Recent studies have reported the presence of this genotype in human stools (Abdel-Moein and Saeed, 2016; Fantinatti et al., 2016; Zahedi et al., 2017) indicating that this Assemblage may potentially be zoonotic.

Given the *Cryptosporidium* species and *Giardia* Assemblages and sub-Assemblages detected in the present survey, it is plausible to assume that the contamination of the fresh produce in question may have occurred in the pre-harvest phase, either directly via infected animals accessing the crops or indirectly via contaminated water used for irrigation or untreated manure used as fertilizer.

The detection of an additional protozoan species i.e., *Entamoeba histolytica* cannot be considered as completely unexpected, since this pathogen has been frequently found in raw vegetables and fruits worldwide (reviewed by Li et al., 2020) and the infection is associated with the consumption of contaminated fresh produce (Anuar et al., 2012; Azim et al., 2018; Gabre and Shakir, 2016; Sitotaw et al., 2019). *Entamoeba histolytica* is a protozoan parasite of humans responsible for human amebiasis associated with a variety of symptoms which vary from no or mild symptoms to severe fulminating intestinal and/or life-threatening extraintestinal diseases worldwide, especially in developing countries (Chou and Austin, 2021). *Entamoeba histolytica* is considered an important cause of prolonged traveller's diarrhoea (Cui et al., 2019; Li et al., 2020). However, contamination with this parasite must have occurred following contamination with human faeces; it is commonly associated with food handlers (Ben Ayed et al., 2008; Yimam et al., 2020), and this could be the route of contamination here also.

A significant seasonal difference in the investigated fresh produce with a higher detection of *G. duodenalis* in fresh products sold on the Italian market in spring was recorded. However, due to the lack of surveillance for these infections in Italy, it was not possible to link these findings with any illness events in the population. In addition, given the different origin of the berries tested in the present study, inference of climatic effects is limited; however, these results support a heterogeneous human infection risk between seasons.

Isolation and detection of parasites in fresh produce is very challenging (Caradonna et al., 2017; Dixon et al., 2013), particularly for detection of multiple species. The combination of microscopy and molecular assays allowed us to provide more robust evidence on the presence of pathogenic parasites in both RTE salads and berries sold on the

Italian market. In this study we have used the FLOTAC technique, which is a microscopy-based tool, mainly employed for quantitative copromicroscopic diagnosis of parasites in animals and humans (Cringoli et al., 2010). However, it has more recently been used successfully for the detection of gastrointestinal parasites in smooth lettuces (do Nascimento Ramos et al., 2019) and for investigating the presence of *Cyclospora cayetanensis*, *Toxoplasma gondii* and *Echinococcus* spp. in fresh produce (Barlaam et al., 2021). Although, in the present study, the employed molecular assays confirmed some microscopy results, the efficiency of the FLOTAC technique for the detection and identification of parasites in fresh produce, as shown by contrasting results of *Cryptospidium* spp., requires specific and more in-depth investigations using both naturally and experimentally contaminated samples. In particular, FLOTAC may not be an appropriate microscopy tool for the detection of *Cryptosporidium* oocysts in fresh produce, where only low numbers of the particularly small oocysts (3–5 μm diameter) may occur. The standard ISO Method (ISO 18744), that describes the use of immunofluorescent antibody testing as the detection technique, would appear more suited for examining fresh produce (leafy vegetables and berries) for contamination with *Cryptosporidium*, due to its greater sensitivity (ISO, 2016).

In the present study, no attempt was made to assess whether the parasites detected were viable and infective and this could be considered a limitation of the study; however, although viability assays are available for *Cryptosporidium* oocysts and *G. duodenalis* cysts (e.g., Rousseau et al., 2018), such methods are generally unsuitable for low numbers of parasite transmission stages.

5. Conclusions

The results of this survey demonstrate that the contamination of fresh produce by pathogenic parasites is a cause for concern in Europe and, specifically, in Italy. The detection of DNA from these parasites on fresh produce indicate that faecal contamination occurred at some point along the agri-food production chain, resulting in the potential for transmission of pathogenic parasites from contaminated RTE salads and berries sold on the Italian market. This also highlights a potential risk linked to the consumption of fresh produce imported from developing countries.

Our results further highlight the need to integrate the microbiological criteria required by the EU Law; in fact, monitoring only certain bacteria in fresh produce does not rule out faecal contamination by pathogenic parasites, which are more robust and resistant to commonly used sanitizers, and does not guarantee food safety for consumers. Therefore, it is of vital importance to keep gathering data, not only on the occurrence of parasites on fresh produce, but also on the spread and impact of foodborne pathogenic parasites in Europe by implementing clinical diagnostic tests in suspected patients and making efforts to identify the source of infection.

In order to prevent parasitic contamination of fresh produce, it is essential that appropriate HACCP plans are implemented along the food-chain and the importance of the One Health approach is emphasised.

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CRediT authorship contribution statement

Alessandra Barlaam (AB): investigation - carried out sampling and experiments, project administration, writing - original draft, writing - review & editing; Simone Marco Cacciò (SMC): methodology, investigation - carried out experiments, supervision, writing - review & editing; Anna Rosa Sannella (ARS) methodology, investigation - carried out experiments; Nicola Ferrari (NF): data curation and statistical analysis, writing - review & editing; Tamirat Tefera Temesgen (TTT): methodology, investigation - carried out experiments, writing - review & editing; Laura Rinaldi (LR): methodology, microscopy investigation, writing - review & editing; Giovanni Normanno (GN): writing - review & editing; Lucy Robertson (LR): conceptualization, supervision, writing - review & editing; Annunziata Giangaspero (AG): conceptualization, funding acquisition, supervision, writing - review & editing.

Declaration of competing interest

None.

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