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Contamination of fresh produce sold on the Italian market with *Cyclospora cayetanensis* and *Echinococcus multilocularis*

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ABSTRACT

To investigate the presence of *Cyclospora cayetanensis*, *Toxoplasma gondii* and *Echinococcus* spp. in fresh produce sold in Italy, 324 locally produced 'ready-to-eat' (RTE) mixed-salad packages belonging to three brands and 324 berries packages (blueberries and blackberries imported from Peru and Mexico, respectively, and raspberries grown in Italy) were purchased at retail. Nine individual packages from each of the six types of fresh produce were collected monthly for one year, and with the same produce pooled, this resulted in a total of 72 pools for the whole year. Using microscopy (FLOTAC), a *Cyclospora*-like oocyst was detected in a blueberry sample and a taeniid egg was detected in a RTE-salad sample. Molecular tools confirmed these to be *C. cayetanensis* and *Echinococcus multilocularis*, respectively. *Toxoplasma gondii* was not detected in any of the samples. This study shows for the first time in Europe that imported berries on the Italian market may be contaminated with *C. cayetanensis* and RTE salads grown in Italy with *E. multilocularis*. The results indicate a new epidemiological scenario and highlight that current management of fresh produce, locally produced or imported, does not ensure products are free from parasite contamination.

1. Introduction

The demand for ready-to-eat (RTE) salads and berry fruits in industrialized countries has increased significantly in recent years, partially due to a trend towards healthier eating habits (Broglia and Kapel, 2011; Eurostat, 2018; WHO, 2019) and partially due to improvements in the fresh-produce supply chain. In Europe, more than 65% of the population eats at least one portion of fresh produce per day, and this percentage reaches more than 75% in the UK, Portugal, Belgium, and Italy (Eurostat, 2018).

RTE salads combine the quality of fresh produce with simple preparation and represent a convenient source of vitamins and minerals (Legnani and Leoni, 2004; Sharma et al., 2014; Preti and Vinci, 2016). Berries contain bioactive compounds associated with decreased risk of cancer and cardiovascular diseases, and with beneficial effects on metabolic disorders (Skrovankova et al., 2015). Nevertheless, despite their health benefits, fresh produce can present a major food-safety challenge as they can be contaminated at various places along the production chain by a range of different microorganisms, including pathogenic parasites of fecal origin (Robertson, 2018; Trevisan et al., 2019).

Several species of pathogenic helminths and protozoans have been detected in fresh produce (Broglia and Kapel, 2011; Macori et al., 2018; Tefera et al., 2018). Furthermore, as these products are rarely heat treated prior to consumption, robust parasite transmission stages are usually not inactivated during food preparation. Contamination may occur during the pre-harvest phase, when untreated/raw sewage water is used for irrigation or animal waste is used as fertilizer or when infected animals have access to crops, as well as via insects (Steele and Odumeru, 2004; EFSA, 2018). Contamination can also occur during harvesting or in the post-harvest phase, e.g., packaging, transport,

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market and home processing (EFSA, 2018).

Although RTE salads are washed before packaging, the industrial washing technologies employed do not effectively inactivate parasites (Castro-Ibáñez et al., 2017; Caradonna et al., 2017) and, indeed, with industrial-level washing, a point contamination may spread throughout a production batch (Gil et al., 2009). Due to their fragility, berries do not undergo industrial washing, and are difficult to clean thoroughly prior to consumption. In addition, as many parasite transmission stages are sticky or can be trapped on the berries' surfaces, these may be particularly difficult to remove (Ortega and Shields, 2015; Tefera et al., 2018).

In a European prioritisation of foodborne parasites based on multi-criteria decision analysis, four parasites that can be transmitted by contaminated fruits and/or vegetables were ranked among the top-five. These are in order (from highest downwards): *Echinococcus multilocularis, Toxoplasma gondii, Echinococcus granulosus* and *Cryptosporidium* spp. (Bouwknegt et al., 2018). Other parasites that may be transmitted by contaminated fresh produce that were included in the ranking were *Giardia duodenalis, Toxocara* spp., *Taenia solium, Ascaris* spp., *Entamoeba histolytica, Fasciola* spp., and *Cyclospora cayetanensis* (Bouwknegt et al., 2018). Despite *C. cayetanensis* being prioritised relatively low in the ranking (at number 18 of 24 parasites), the numerous cyclosporiasis outbreaks in the USA associated with consumption of vegetables or berries imported from Southern and Central American countries, where this parasite is endemic, have also raised concerns for European consumers (Trevisan et al., 2019; Giangaspero and Gasser, 2019).

Italy is the second largest producer of RTE salads in Europe (IsmeaMercati, 2016; Eurofruit, 2020); there is also a local production of berries. However, the recent increase in the consumption of such products has led to a surge of imports from abroad, including Central and South American countries (CBI, 2018).

The objective of this study was to use a combination of complementary tools, based on microscopy and molecular methods, to investigate contamination of RTE salads and fresh berries (blueberries, blackberries, and raspberries) with parasites of public health importance. Here we present the results for three parasites that are the targets of a multiplex PCR approach used in this work, namely: *C. cayetanensis*, *T. gondii,* and *E. multilocularis*.

2. Materials and methods

2.1. Sample size estimation

A pooling design, based on testing homogenous groups of samples (pools) (Cowling et al., 1999), was used as a cost-efficient sampling strategy to keep the sample size manageable. The number of pools and the pool-size were established according to Worlund and Taylor (1983) based on the expected prevalence of contaminated RTE or berries batches, not lower than 1%, desired precision of 1.15% and desired level of confidence of 95% using the formula provided in equation (1) below.

A pool size of 9 packages (samples) per month was established for each of the six fresh product types, for a total of 54 samples and six pools per month, and 72 pools for the whole year (Table 1). This sampling protocol was aimed at providing the highest confidence of detecting contamination, even for parasites with low expected prevalence.

$$m = (Z^*(1-p)/(ek))^2 * ((1-p)^{-k}-1) \quad (1)$$

Where: m is the number of pools, p is the expected prevalence, k is the pool size, e is the desired precision and Z is the desired level of confidence.

2.2. Sampling plan

From January to December 2019, a total of 648 samples of fresh produce were purchased from supermarkets located in Foggia and Bari towns (Apulia region, southern Italy). The samples consisted of 324 RTE locally produced mixed-salad packages belonging to three brands (A, B, and C) and 324 berry packages. Two brands of RTE-salad (A and B) contained sugarloaf chicory, radicchio, rocket salad, and grated carrots, and the third brand (C) contained red cabbage, frisée salad, leek, rocket salad, and grated carrots. The berry packages contained either blueberries or blackberries, imported from Peru and Mexico, respectively, or raspberries grown in Italy.

Table 1
Sampling protocol.

Fresh produce	**No. samples/ month**	**No. pools/ month**	**Total no. samples/ year**	Total no. pools/ year
RTE MIXED SALADS (BRAND A)	9	1	108	12
RTE MIXED SALADS (BRAND B)	9	1	108	12
RTE MIXED SALADS (BRAND C)	9	1	108	12
SUBTOTAL	27	3	324	36
IMPORTED BLACKBERRIES (MEXICO)	9	1	108	12
IMPORTED BLUEBERRIES (PERU)	9	1	108	12
LOCAL RASPBERRIES (ITALY)	9	1	108	12
SUBTOTAL	27	3	324	36
TOTAL	54	6	648	72

Therefore, each month, per each fresh product, nine individual packages (not less than 100 g each) belonging to the same lot were bought and, after being mixed together, analyzed together as a single pool. After the purchase, the samples were transported to the laboratory in a cooler bag and stored in the refrigerator. The pooling and the washing procedures were carried out before the products' respective expiry dates.

2.3. Sample processing

Each month, the nine packages of each of the six produce types (three RTE-salads and 3 berries) were opened and the same produce types were mixed together by hand to create a pool of 900 g. These mixed pools were then divided into nine filter bags (BagPage®+ 400 ml filter bags, Interscience, Sant Nom, France), with 100 g of produce per bag, before further processing. The samples were processed according to the U.S. Food and Drugs Administration Bacteriological Analytical Manual (BAM) chapter 19b (Murphy et al., 2017), with slight modifications as follows.

To each filter bag was added 200 ml of 1% Alconox (Alconox Inc., NY USA), which was then sealed with a clip, placed on a rocker platform (Vibramax 100, Heidolf), and agitated for 30 min at room temperature at 150 rpm. The wash solution from each bag was then transferred into four 50 ml tubes for concentration by repeated cycles of centrifugation, removal of supernatant, and combination of pellets. The resultant pellets were transferred into a single 2 ml microfuge tube and centrifuged at 14,000×*g* for 4 min, and all but approximately 500 μl of the supernatant removed by aspiration. Thus, for each type of pooled sample, nine pellets that were representative of the whole pool were obtained (Supplementary Figure 1). Of these, two were stored in the refrigerator at 4 °C for analysis by microscopy and seven were stored at −20 °C pending DNA extraction.

As well as different approaches being used for investigation of contamination, these were also conducted in different laboratories on samples from the same pools, providing interlaboratory strength to the results obtained.

2.4. Microscopy analysis

The FLOTAC double technique was used for the analysis by microscopy (Cringoli et al., 2010) and was carried out at the Regional Center for Parasitosis Monitoring (CREMOPAR) at the University of Naples Federico II, Italy. In this approach, to 6 ml of saturated zinc sulfate solution (specific gravity = 1.35), one aliquot of the concentrated pooled washings (approx. 500 μl) was added. The mixture was homogenized by shaking on a vortex mixer, and then transferred to one of the FLOTAC chambers. Once both chambers had been filled with two different samples, the FLOTAC apparatus was centrifuged for 5 min at 120×*g* and examined by light microscopy at 100× and 400× magnification on a DM1000 LED microscope equipped with a ICC50 HD Camera (Leica). For each sample, suspected findings were enumerated, measured using a calibrated graticule, and photographed.

2.5. Molecular investigation

2.5.1. DNA extraction

DNA was isolated from 72 aliquots (one aliquot per each pool) using DNeasy PowerSoil extraction Kit (DNeasy PowerSoil®, Qiagen, Italy). The manufacturer's instructions were followed with slight modifications. After thawing, the 500 μl sample for DNA extraction was centrifuged and the supernatant removed. The pellet was resuspended to a volume of 250 μl that was added to a bead-beating tube with the appropriate volume of lysis buffer (C1 solution) and subjected to 2 cycles of bead-beating (FastPrep 24G, MP Biomedicals, France) of 4 m/s for 60 s with 45 s pause between cycles. The lysate was then centrifuged at 10,000×*g* for 1 min at room temperature, and 500 μl of the supernatant was used for the next steps of the protocol in which spin columns were used to capture the DNA on silica membranes. Finally, the DNA was eluted from the membrane with 50 μl of elution buffer (C6 solution). Samples were stored at −20 °C pending further molecular testing.

2.5.2. PCR protocols

2.5.2.1. Simplex qPCR for Cyclospora cayetanensis (Assay 1). At the Department of Infectious Diseases of the Istituto Superiore di Sanità (Rome, Italy), a real-time PCR assay targeting the multicopy 18S ribosomal RNA gene was used for detection of *Cyclospora* (Murphy et al., 2017). The assay uses an internal amplification control (IAC), which is a synthetic 200 bp ultramer DNA sequence (Deer et al., 2010), to monitor for potential matrix-derived inhibition of the reaction. The assay also provides a traceable synthetic positive control that allows identifying false positives, possibly originating from laboratory contamination. This positive control (HMgBlock135m) is a 998 bp double-stranded synthetic gBlocks® Gene Fragment that corresponds to the sequence at positions 203–1200 of the *C. cayetanensis* 18S rRNA gene but contains traceable mutations (T885A and C886G) within the amplicon generated by the real-time PCR.

The PCR conditions were as described by Murphy et al. (2017). In brief, primers Cyclo250F (5′-TAGTAACCGAACGGATCGCATT-3′) and Cyclo350RN (5′-AATGCCACGGTAGGCCAATA-3′) were used in combination with the probe Cyclo281T (5′-CCGGCGATAGATCATTCAA GTTTCTGACC-3′) labeled with 5′ FAM reporter dye and double quenched with an internal ZEN quencher and a 3′ Iowa Black® FQ quencher.

Amplification of the IAC target was achieved using the primers dd-IAC-F (5′-CTAACCTTCGTGATGAGCAATCG-3′), dd-IAC-R (5′-GAT-CAGCTACGTGAGGTCCTAC-3′), and the probe dd-IAC-Cy5 (5′-AGC-TAGTCGATGCACTCCAGTCCTCCT-3′), labeled with 5′ Cy5 reporter dye and 3′ Iowa Black® RQ-Sp quencher.

Amplification was performed in a final reaction volume of 20 μl, using the QuantiFast Multiplex PCR Master Mix. All reaction mixtures contained the primers/probe to detect 10^4 copies per μl of the synIAC target. In each *q*PCR experiment, a negative control (no template control, NTC) and three positive controls (the HMgBlock135m, used at 5 × 10^2 copies/μl, and DNA extracted from a human stool sample positive for *Cyclospora* and from a vegetable matrix spiked with 200 oocysts) were used. Isolated DNA from each of the pools (i.e., 36 from RTE mixed salads and 36 from berries) was tested in triplicate using this assay.

2.5.2.2. Multiplex qPCR for Cyclospora cayetanensis, Toxoplasma gondii, and Echinococcus multilocularis (Assay 2). A multiplex real-time PCR assay for the simultaneous detection of *C. cayetanensis*, *T. gondii* and *E. multilocularis* was performed on one aliquot of all pools according to the protocol of Temesgen et al. (2019a) at the Parasitology Laboratory, at the Norwegian University of Life Sciences (Oslo, Norway).

In this multiplex, the primers, CyITS1_TT-F (ATGTTTTAG-CATGTGGTGTGGC) and CyITS1_TT-R (GCAGCAACAACAACTCCTCATC), and probe, CyITS1_TT-P (HEX-TACATACCCGTCCCAACCCTCGA-MGBEQ), were used for the detection of *C. cayetanensis* to amplify a product of 141 bp from the ITS-1 region (Temesgen et al., 2019b). A product of 162 bp from the 529 bp repeat of *T. gondii* was amplified with the primers Tox-9F (AGGA-GAGATATCAGGACTGTAG), Tox-11R (GCGTCGTCTC GTCTAGATCG) and probe Tox-TP1 (Cy5-CCGGCTTGGCTGCTTTTCCT-MGBEQ) (Opsteegh et al., 2010). A forward primer, EmMGB_F (GTGCTGCTYATAAGAGTT TTTG), a reverse primer, EmMGB_R (CTATTAAGTCCTAAACAATACCATA), and an EmMGB_P probe (FAM-ACAACAATATTCCTATCAATGT-MGBEQ) were used to amplify a product of 77 base pairs (bp) from the 12 S rRNA region of *E. multilocularis* (Isaksson et al., 2014).

The *q*PCR was performed using the Stratagene Mx3005P *q*PCR System (Agilent Technologies, Germany) in a final volume of 20 μl, using 10 μl of 2 × KiCqStart Probe *q*PCR ReadyMix low ROX (Sigma Aldrich), and 2 μl of DNA template. Primers and probes were added in appropriate concentrations, according to Temesgen et al. (2019a). Cycling conditions were as follows: 95 °C for 3 min (initial denaturation), followed by 45 cycles at 95 °C for 15 s (denaturation) and 45 cycles at 60 °C for 30 s (combined annealing and extension). ROX was used as a reference dye for normalization of the fluorescent signal. Each sample was analyzed in triplicate and C*q* values were analyzed after each run.

To confirm that the PCR for *E. multilocularis* did not also amplify *E. granulosus* DNA, we tested DNA isolated from *E. granulosus* eggs with the same *q*PCR assay. Two samples of DNA derived from *E. granulosus sensu stricto* genotypes G1 and G3 that had been isolated from naturally infected dogs in the Campania region of southern Italy (provided by Prof. Laura Rinaldi, University of Naples Federico II, Naples, Italy) were tested in triplicate.

2.5.2.3. Conventional nested PCR for Cyclospora (Assay 3). At the Department of Infectious Diseases of the Istituto Superiore di Sanità, a conventional nested PCR assay targeting the 18S rRNA gene (Yoder et al., 1996) was also used for detection and characterisation of *Cyclospora*. In the primary PCR amplification, the primers F1E (5′-TACCCAATGAAAACAGTTT′3) and R2B (5′- CAGGAGAAGCCA AGGTAGG-3′) were used to amplify a 636 bp DNA fragment. In the secondary PCR amplification, the primers CC719 (5′-GTAGCCT TCCGCGCTTCG′3) and CRP999 (5′- CGTCTTCAAACCCCCTACTGTCG 3′) were used to amplify an internal 298 bp DNA fragment.

PCR reactions were performed on a Perkin Elmer 9700 apparatus (Life Technologies, Carlsbad, USA), using 25 μl of 2X HotStarTaq® Master Mix (Qiagen) with additional $MgCl_2$ for a final concentration of 2.0 mM, 10 pmol of each primer, 5.0 μl of DNA, and nuclease-free water up to a final volume of 50 μl. Negative and positive controls were included in each experiment.

PCR conditions for the primary amplification were as follows: initial denaturation at 94 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 90 s followed by a final extension step at 72 °C for 10 min.

PCR conditions for the secondary amplification were as follows: an

initial denaturation at 94 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 70 °C for 90 s.

Aliquots of nested PCR reactions (10 μl) were loaded on a 1.5% agarose gel stained with ethidium bromide. PCR products were purified using spin columns (QiaQuick PCR purification kit, Qiagen, Milan, Italy) and sequenced on both strands by a commercial company (BMR Genomics, Padua, Italy). Sequence chromatograms were edited and assembled using the SeqMan 7.1 software package (DNASTAR, Madison, WI, USA). Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to compare the sequences against the GenBank database.

3. Results

3.1. Microscopy results

Among the 72 fresh produce pools examined by microscopy, a single *Cyclospora*-like oocyst (8 μm in diameter) and a single taeniid egg (32 μm in diameter) were detected in one pool of blueberries imported from Peru and in one RTE-salad pool belonging to brand A, respectively (Fig. 1). None of the samples contained parasitic forms that were similar in morphology to *Toxoplasma* oocysts.

3.2. Molecular analysis results

Among the DNA extracted from the 72 fresh pools, only those that had been found positive by microscopy were found to be positive by PCR, with *C. cayetanensis* DNA detected in the same pool of blueberries imported from Peru in which a *Cyclospora*-like oocyst was identified, and *E. multilocularis* DNA detected in the same pool of RTE-salad in which a taeniid egg was identified. *T. gondii* DNA was not detected in any of the samples.

Both real-time PCR assays used detected *C. cayetanensis* DNA, with quantitation cycle (C*q*) of 27.5, 27.5 and 27.7 for the triplicate repeats for Assay 1 and C*q* for the triplicate repeats of 35.6, 36.0 and 38.1 for Assay 2. In addition, the nested PCR yielded the expected 298 bp amplification product, and sequencing confirmed the presence of *C. cayetanensis* (>99% identity with sequences available in GenBank, e.g., XR_003297358).

For the *E. multilocularis* positive sample, only one of the triplicate repeats provided a positive result with a C*q* value of 38.1. DNA from *E. granulosus* in the same PCR set up gave a negative result.

The results of the analytical methods, microscopy and molecular tools, were in agreement with each other, with most of the 72 sample pools negative for these parasites, but two sample pools (2.8%) indicating contamination. The results are summarized in Table 2.

4. Discussion

The main finding of this study was the occurrence of contamination of fresh produce on the Italian market with pathogenic parasites of considerable public health significance, specifically *C. cayetanensis* in blueberries imported from an endemic country (Peru) and *E. multilocularis* in RTE salads that had been cultivated and processed in Italy. The significance of these two positive findings is discussed individually below.

Cyclospora cayetanensis infection among humans has been reported worldwide, in both low and high-income countries. Endemic areas are mainly considered to be Central and South American countries, where the prevalence of cyclosporiasis ranges from 3.3% in Mexico (de la Luz Galván-Ramírez et al., 2019) to 41.6% in Peru (Burnstein Alva, 2005), as well as several countries in the Middle East, the Indian subcontinent, and South East Asia (Almeria et al., 2019).

In Europe, most reported cases of cyclosporiasis are related to traveller's diarrhea such as those registered in Spain, Netherlands, and the UK (Ramírez-Olivencia et al., 2008; Blans et al., 2005; Nichols et al., 2015), associated with travel to Cuba, Indonesia, and Mexico, respectively. In addition, sporadic autochthonous cases (i.e., without any travel history outside the country) of cyclosporiasis have been registered in the Czech Republic (Jelinkova et al., 2011) as well as in Italy (Scaglia et al., 1994; Maggi et al., 1995; Masucci et al., 2008, 2011).

Outbreaks of cyclosporiasis have mostly been reported in North America, with the majority linked to imported food, e.g., berries (mostly raspberries), and leafy vegetables and herbs (cilantro, basil, and mixed salad) (reviewed by Giangaspero and Gasser, 2019). In Europe, outbreaks of cyclosporiasis have been registered in Germany in 2000 in association with the consumption of salad, green leafy herbs, locally produced and grown and imported from France and Southern Italy (Döller et al., 2002), and in Sweden (Insulander et al., 2010), where sugar snap peas imported from Guatemala were the suspected vehicle of infection.

Surveys on contamination of fresh produce with *Cyclospora* are relatively limited; although *Cyclospora* oocysts have been identified on vegetables in several countries (see Almeria et al., 2019) including Costa Rica (Calvo et al., 2004), Peru (Burnstein Alva, 2005), and Venezuela (Devera et al., 2006), surveys of contamination of berries with *C. cayetanensis* are even more scanty. Our finding of *C. cayetanensis* contamination of berries (1.4%) overlaps with data obtained in a similar

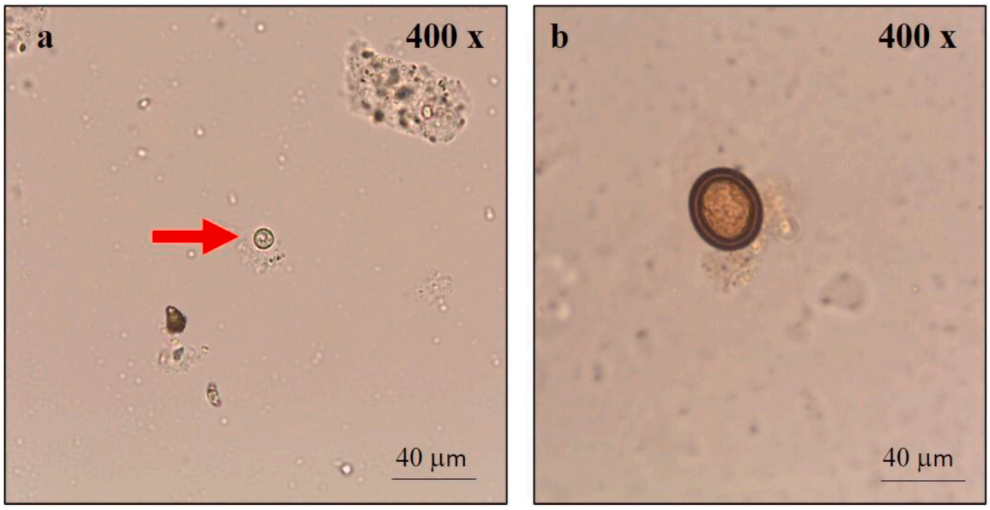

Fig. 1. *Cyclospora*-like oocyst (a) and taeniid egg (b) detected by FLOTAC technique.

Table 2
Detection of parasites in fresh produce (ready-to-eat mixed salads and berries) sold at retail in Italy and investigated by microscopy and molecular assays.

	No. of pools	*Cyclospora cayetanensis*					*Echinococcus multilocularis*			*Toxoplasma gondii*			Combined values
		Microscopy FLOTAC	*q*PCR (Assay 1)	Multiplex *q*PCR (Assay 2)	Conventional nested PCR	No. Total positive pools	Microscopy FLOTAC	Multiplex *q*PCR	No. Total positive pools	Microscopy FLOTAC	Multiplex *q*PCR	No. Total positive pools	No. Positive pools (%)
RTE mixed salads (Brand A)	12	0	0	0	0	0	1	1	1	0	0	0	1/12 (8.3%)
RTE mixed salads (Brand B)	12	0	0	0	0	0	0	0	0	0	0	0	0
RTE mixed salads (Brand C)	12	0	0	0	0	0	0	0	0	0	0	0	0
RTE subtotal	**36**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**1/36 (2.8%)**
Imported blueberries (Peru)	12	1	1	1	1	1	0	0	0	0	0	0	1/12 (8.3%)
Imported blackberries (Mexico)	12	0	0	0	0	0	0	0	0	0	0	0	0
Local raspberries (Italy)	12	0	0	0	0	0	0	0	0	0	0	0	0
Berries subtotal	**36**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**1/36 (2.8%)**
OVERALL TOTAL (No)	**72**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**2/72 (2.8%)**
OVERALL TOTAL (%)		**1/72 (1.4%)**					**1/72 (1.4%)**			**0**			

survey in Korea (non-endemic country) where, among 44 blueberry samples analyzed coming from the US, Chile, and locally produced, the one imported from Chile was found to be contaminated by *C. cayetanensis* DNA (Sim et al., 2017). Other records refer to strawberries; one article from Egypt reports that 25.7% (9/35) of the strawberry juice samples analyzed by microscopy were found positive for *C. cayetanensis* (Mossallam, 2010), and a more recent study of strawberries from retail markets in Colombia, reported that one of 120 (0.83%) samples was contaminated with *C. cayetanensis* DNA (Ortiz Pineda et al., 2020). Information on contamination of berries on the European market with *Cyclospora* is, to the best of our knowledge, limited to a study from Norway where samples of raspberries and strawberries, and various vegetables, were analyzed for parasite contamination by microscopy following lectin-magnetic separation (Robertson and Gjerde, 2001). *Cyclospora* oocysts were not detected on any of the samples examined.

The detection of *E. multilocularis* DNA on RTE-salads grown and processed in south of Italy was an unexpected finding from this study. Indeed, *E. granulosus* would have been more likely as this parasite is endemic in the Mediterranean countries (Tamarozzi et al., 2020; Cringoli et al., 2021). However, the lack of cross-reactivity of the primers used in the PCR (Assay 2) with *E. granulosus* DNA supports that the amplified DNA was derived from *E. multilocularis*. Furthermore, the independent detection of a taeniid egg by microscopy in the same sample further corroborates this finding and argues against in-house contamination as a possible source of false positive results.

Echinococcus multilocularis causes alveolar echinococcosis (AE) in humans as dead-end hosts, with mainly foxes, and potentially wolves and shepherd dogs, as definitive hosts (Massolo et al., 2018). However, in some areas, domestic dogs or cats can play a role in the synanthropic cycle of *E. multilocularis* (Eckert and Deplazes, 2004). Although no cases of human infection with *Echinococcus* spp., have been linked to contaminated fresh produce, several cases of AE in zoo primates in Switzerland seem to have been caused by consumption of fresh vegetables contaminated with *E. multilocularis* eggs (Federer et al., 2016). Furthermore, in another study from Switzerland, *E. multilocularis* was detected in two out of 157 lettuce samples tested using a one-way isolation test kit followed by genetic identification (PCR and sequencing) (Guggisberg et al., 2020). Given that Switzerland is considered an endemic area for this parasite, but nevertheless only a low number of positive samples was detected may reflect method sensitivity, but it is difficult to conclude on this with little available data.

Echinococcus multilocularis is widely distributed around the Northern hemisphere, including central and northern Europe. However, recent evidence indicates a likely expansion of *E. multilocularis* southward, even into areas where it was previously absent (EFSA, 2018). *E. multilocularis* has been reported in red foxes (*Vulpes vulpes*) in the North Eastern Alps in the Trentino-Alto Adige Region in Italy (Di Cerbo et al., 2008) and, more recently, in shepherd dogs and wolves in the South Western Italian Alps (Massolo et al., 2018). In the last 40 years, red foxes in Italy have spread southwards (Faunalia, 2020), suggesting the possibility that, due to their potentially long-range dispersion, they could have brought *E. multilocularis* with them, being responsible for the spread of this parasite in the southern part of the country. In addition, given the high number of stray dogs, particularly in Southern Italy (Infodata.ilsole24ore, 2019), their potential role in disseminating *E. multilocularis* eggs and maintaining the lifecycle locally should also be considered.

The lack of positive results obtained for *T. gondii* in the investigated matrices was unexpected, considering that this parasite has been documented in fresh produce in various European countries, such as Czech Republic (Slany et al., 2019) and Poland (Lass et al., 2012), with contamination rates of 9.6% and 9.7%, respectively. More recently, the presence of this species was detected by conventional PCR and *q*PCR in Portugal and Spain, in both RTE salads and berries, with very high proportions of samples being positive (35.3% and 42.9%, respectively) (Marques et al., 2020). In a previous study from Italy, *T. gondii* DNA was

detected in 0.8% of 648 RTE salad samples examined by *q*PCR (Caradonna et al., 2017).

The lack of detection of *T. gondii* contamination in the berries and RTE salad samples investigated here could be due to the limited number of pools analyzed compared with other European surveys, or, potentially, to the limit of detection of the multiplex *q*PCR assay (Temesgen et al., 2019a), which is estimated to be 10 oocysts per 30 g of berries for *Toxoplasma*. However, the limit of detection of the methods used in the other European surveys, in which *Toxoplasma* DNA was detected was either not specified or was higher (e.g., 100 oocysts; Lass et al., 2012), arguing against this as a reason for the lack of detection in our study. The absence of *Toxoplasma* oocysts in the samples observed by microscopy, also supports that this is a real absence of the parasite, rather than reflecting low sensitivity of the molecular method used. Furthermore, it should be noted that the survey of strawberries from Colombia also used the same assay (Ortiz Pineda et al., 2020), and reported that 5% (6 samples) were positive, indicating that this assay is appropriate for investigating contamination of fresh produce with *Toxoplasma*.

As far as we are aware, none of the lots of the imported berries that were found to be contaminated in the present study were associated with reported illnesses or outbreaks of disease. However, it is possible that such infections might have gone undetected, as *Cyclospora* is not routinely investigated in people with gastrointestinal disorders in most European countries, including Italy (Giangaspero et al., 2015). Determining whether the RTE salad pool contaminated with *E. multilocularis* resulted in human infection is similarly impossible; the incubation period for AE varies approximately between 5 and 15 years (Eckert and Deplazes, 2004). All the parasites considered in the present study survive refrigeration temperatures, which are those employed for transporting and storing fresh produce, and chlorine concentrations used in the water for RTE salads disinfection (80–100 mg/L) in the food industry (Sathyanarayanan and Ortega, 2006; Hohweyer et al., 2016). Therefore, although in the present study viability and infectivity could not be determined, and no attempt was made to enumerate the number of oocysts/eggs in the positive samples, it should be assumed that the detection of pathogenic parasites in fresh produce represents a risk to public health.

The global trade of food is partially responsible for the spread of infectious diseases across wide geographical scales, compromising food safety and human health (Robertson et al., 2014). In this study, berries imported from Peru that were apparently contaminated with *Cyclospora* represent a matter of concern. The Regulation (EU) 2017/625 of the European Parliament and of the Council on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products states that: "*...food and feed imported into the Union comply with the relevant requirements of the Union's food law or with requirements considered to be at least equivalent thereto*". However, no routine or regular monitoring of berries is set by the current European Union legal framework, which does not include any microbiological criteria applicable for fresh, uncut, and unprocessed fruits. However, the Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs applies to RTE packaged salads, although they are only routinely tested for *Escherichia coli*, *Salmonella* spp., and *Listeria monocytogenes*.

This gap in the European legislation is complicated by the fact that berries are difficult to wash prior to consumption without affecting their quality; moreover, parasites resist the normal disinfection strategies (mostly chlorination) used in the fresh-produce processing industry (Tefera et al., 2018; Gérard et al., 2019). In addition, *Cyclospora* oocysts adhere firmly to berries due to specific adhesins (Ortega and Shields, 2015) and *Echinococcus* spp. eggs are also known to be sticky (Tefera et al., 2018).

5. Conclusions

In conclusion, the detection of *C. cayetanensis* and *E. multilocularis* calls for closer monitoring of fresh produce. The recent increase in the consumption of berries in Europe and related import from foreign countries, many of which endemic for *Cyclospora*, indicates the need of implementing import restrictions from endemic countries in order to ensure safety of these products. The detection of *E. multilocularis* by both microscopy and molecular methods in RTE salads grown and packaged in the south of Italy was both unexpected and a cause for concern. However, pending large-scale, comprehensive investigations to assess the epidemiology of this taeniid species in the south of Italy, minimizing animal access to crops is recommended, bearing in mind the extensive spread of foxes and the high number of stray dogs, particularly in Southern Italy.

Declarations of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103792.

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