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TITLE

Pathogenic parasites in ready-to-eat salads and berries sold on the Italian market

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"*Remember to look up at the stars and not down at your feet.*
Try to make sense of what you see and wonder about what makes the universe exist.
Be curious.
And however difficult life may seem, there is always something you can do and succeed at.
It matters that you don't just give up."

Stephen Hawking

To my mom,

who has inspired me my whole life

Content

ABSTRACT

Among fresh produce, in recent years, the consumption of ready-to-eat (RTE) salads and berries has significantly increased in industrialized countries due to a trend towards faster and healthier eating habits. These products can be contaminated along the production food chain by several microorganisms, including pathogenic parasites that have been responsible of foodborne outbreaks worldwide. In Italy, while data on contamination of RTE salads require more-in-depth investigations there is a complete lack of data on berries, mostly imported from countries where some of these parasitic infections are endemic.

The aim of this Ph.D. project was to investigate the occurrence of foodborne parasites as contaminants of imported and locally produced fresh produce sold on the Italian market.

From January to December 2019, 648 packages of three brands of RTE mixed salads and three berry-types were bought from supermarkets. To estimate the prevalence, for each fresh produce, a pool size of nine packages each month and 72 pools per one year were processed. After washing, the pellets were examined by microscopy (FLOTAC) and subjected to different molecular techniques (conventional PCR, simplex or multiplex *q*PCR) and sequencing.

By microscopy, *Cyclospora*-like oocysts and a taeniid egg were detected in a blueberry sample and in a RTE salad sample, respectively; *Entamoeba* and *Giardia* were also identified. *Cyclospora cayetanensis* and *Entamoeba histolytica* in imported blueberries and the Taeniid (*Echinococcus multilocularis*) in locally produced RTE salads were molecularly confirmed. *Cryptosporidium ryanae*, *Cryptosporidium bovis*, *Cryptosporidium xiaoi* and *Cryptosporidium ubiquitum* and *Giardia duodenalis* Assemblages A, B and E, most of them of zoonotic interest, were identified in both matrices. The overall prevalence of each parasite was 5.81% for *Cryptosporidium* spp., 4.63% for *G. duodenalis*, 0.15% for *C. cayetanensis*, 0.15% for *E. multilocularis. Entamoeba* spp. was also detected with a prevalence of 0.96%. *Toxoplasma gondii* was not found. *Cryptosporidium* spp. and *G. duodenalis* showed significant seasonal differences with higher values in winter and spring, respectively.

In this study, most of the parasite species were detected for the first time on fresh produce. The results obtained, beside opening a new epidemiological scenario in Italy, highlight the improper management of fresh produce, both locally produced or imported, along the food chain and the potential consequences of such contamination on human health.

Keywords: Prevalence, pathogenic parasites, ready-to-eat salads, berries, Italy.

1. INTRODUCTION

An increasing proportion of reported foodborne outbreaks has been linked to fresh produce, raising concerns that these foods may be an increasing source of foodborne infections (Sela-Saldinger and Manulis-Sasson, 2015). In fact, over the past few decades, the number of outbreaks linked to the consumption of fresh or minimally processed fruits and vegetables has grown in different regions of the world, including Europe, the USA and Canada (Callejón *et al*., 2015; Carstens *et al*., 2019; Johnson, 2019).

The demand for 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). Italy is the second largest producer of RTE salads in Europe, after France (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).

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 faecal origin (Robertson, 2018; Trevisan *et al*., 2019).

Several species of pathogenic protozoans and helminths 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, 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, 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 (Tefera *et al*., 2018).

In a European prioritisation of foodborne parasites (FBPs) 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) *E*. *multilocularis, T. 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 are *G. duodenalis, Toxocara* spp., *Taenia solium, Ascaris* spp., *E. histolytica, Fasciola* spp., and *C. 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).

Fresh produce remain the most often identified transmission vehicles for outbreaks associated with FBPs (Trevisan *et al*., 2019). Several studies have shown the presence of parasites in RTE packaged salads in different parts of the world (Dixon *et al*., 2013; McKerr *et al*., 2015), including Italy, where *C. cayetanensis* and zoonotic protozoans, such as, *Cryptosporidium parvum*, *G. duodenalis* and *T. gondii*, have been detected (Di Benedetto *et al*., 2007; Caradonna *et al*., 2017). Pre-cut salads have also been responsible for outbreaks of cryptosporidiosis in Europe (Åberg *et al*., 2015; McKerr *et al*., 2015). Berries have been found to be contaminated by *C. cayetanensis* and other zoonotic parasites (*T. gondii*, *E. multilocularis*, *G. duodenalis*, *Cryptosporidium* spp., *E. histolytica*) and have been responsible for *Cyclospora* outbreaks (Tefera *et al*., 2018; Temesgen *et al*., 2019a).

Although for protozoan detection in vegetable matrices, several microscopy techniques based on staining, sedimentation and/or flotation procedures are available, it has recently been demonstrated that the FLOTAC technique can be successfully applied for recovering FBPs of medical and veterinary concern in vegetables (do Nascimento Ramos *et al*., 2019).

Beside microscopy-based techniques, molecular techniques are important because of their sensitivity and specificity. Furthermore, these techniques can offer information about species and genotypes that can be used for source tracking. Among several molecular methods (conventional PCR, real-time PCR (*q*PCR), nested PCR (nPCR), magnetic-capture PCR, and loop-mediated isothermal amplification (LAMP) assays), *q*PCR is becoming the standard laboratory tool used for the detection of FBPs (Tefera *et al*., 2018).

Studies on RTE salads and berries are very few worldwide. As to Italy, previous findings revealed the presence of protozoan parasites in RTE salads (Di Benedetto *et al*., 2007; Caradonna *et al*., 2017) whereas for berries data are completely lacking.

In order to confirm and broaden the data on contamination of RTE salads in Italy and to fill the gaps in knowledge on contamination of berries, microscopy and molecular methods were employed for the detection of parasites in these food matrices in different laboratories.

1.1 AIMS

General aim:

The general aim of this Ph.D. study was:

- to investigate the presence of pathogenic parasites in fresh produce by microscopy and molecular tools.

Specific aims:

The specific aims are the following:

- to detect the presence and investigate the prevalence of *C. cayetanensis*, *G. duodenalis*, *Cryptosporidium* spp., *T. gondii*, *Echinococcus* spp. in RTE packaged mixed salads and, local and imported berries (blueberries, blackberries, and raspberries) by both microscopy (FLOTAC) and conventional and real time *q*PCR;
- to assess the health risks for consumers associated with the spread of these pathogens.

This research topic is unexplored and of great current interest; it is intensely debated among researchers and greatly encouraged by the international scientific community, due to the beneficial effects that the outcome of this project would have. In fact, the results of this project will increase the awareness on FBPs and help filling the knowledge gap in the epidemiology of these infections. In addition, the detection of the investigated protozoans would be the starting point for setting up a risk assessment procedure as the first step for the amendment of the current legislation (EC Reg. 2073/2005 and 1441/2007; Reg. UE 2017/625).

2. GENERAL PART

2.1 FRESH PRODUCE INDUSTRY

2.1.1 Ready-to-eat salads

The International Fresh-cut Produce Association (IFPA) defines “Fresh-cut produce" as any fresh fruit or vegetable or any combination thereof that has been physically altered from its original form, but remains in a fresh state. Regardless of the commodity, it has been trimmed, peeled, washed, cut and subsequently bagged or prepackaged to offer to the consumers high nutrition standards, convenience and value while maintaining freshness (IFPA, 2016).

Fresh-cut produce include any kind of fresh commodities and their mixtures in different cuts and packaging i.e., packaged salads. These products are also known by the term “minimally processed” and by the French term of “IV gamme” i.e., RTE vegetables or fruit, packaged and however fresh and natural without any additives (Sansavini and Ranalli, 2012). Within IV gamme products, RTE salads dominate the production of minimally processed foods.

Only fruit and vegetables of the best quality can resist the stress induced by the preparation (reviewed by Colelli and Elia, 2009). Temperature, storage time, relative humidity, and modified atmosphere packaging play an important role in the quality and the shelf-life of the final product (Legnani and Leoni 2004; reviewed by Colelli and Elia, 2009).

The production process of RTE salads is shown in **Figure 1**.

Figure 1. Production process of the RTE salads.

The first step in the production process of RTE salads is harvesting that can be performed manually or with operating machines. After this, the product is stored at a temperature of 4 °C in a controlled-humidity environment. The harvested product is then selected (either manually or automatically) based on the size, morphological characteristics, colour, and other aspects. All unwanted parts of the plant are discarded. After sorting, the salads are cut, shredded, and washed in a system that usually consists of three washing tanks: in the first tank (pre-washer) soil and any foreign bodies

that may be present are removed; in the other tanks, the product is washed and sanitized. The water used for washing salads is potable, used in sufficient quantity (from 5 to 10 liters/kg), adequately refrigerated (from 1 to 4°C), and constantly recycled with self-cleaning filter systems.

Chlorine is the most commonly used water disinfectant in the fresh produce industry. Chlorine is normally applied to process water as chlorine gas (Cl_2), sodium hypochlorite (NaOCl) solution or dry calcium hypochlorite ($CaCl_2O_2$). Sodium hypochlorite is the chlorine source most frequently used because it is affordable and can be handled without high expertise and with a minimal hazard (Suslow, 1997; Colelli and Elia, 2009). In water solution it is employed in concentrations variable between 50 and 200 ppm of chlorine (Parish *et al*., 2003; Soliva-Fortuny *et al*., 2006). However, the occurrence of chlorate residues in fresh produce chlorine-based disinfectants has been highlighted as a problem and the industry faces increasing pressure to find alternatives to chlorine which is currently banned as a wash for produce in some European countries, including Germany, the Netherlands, Switzerland and Belgium (Gil *et al*., 2016; https://ec.europa.eu/programmes/horizon2020/en/news/boost-efficient-safe-food-production).

Other disinfectant agents are hydrogen peroxide (H_2O_2), ozone (O_3), peracetic acid ($C_2H_4O_3$) and ultraviolet radiation (UV), however, their use is limited because some of them can only be used at high concentrations (H_2O_2; $C_2H_4O_3$) or can be dangerous for food operators (O_3) or require significant financial investments (UV) (Turatti, 2011).

After washing, the leaves reach a drying system the main purpose of which is to avoid high humidity levels inside the packages as this could promote a rapid deterioration of the product.

The dry product is then transported into the packing room where it is weighed, kept at 1-2°C (Turatti, 2011), and packaged in Modified Atmosphere Packaging (MAP) (Varoquaux and Mazollier, 2002). Finally, the product is sealed and labelled.

Fresh-cut produce have shown a positive trend in the market over the last two decades worldwide. An article by Cavaiuolo *et al*. (2015) reports that fresh-cut salads cover about the 50% of the market volume, fresh-cut fruits account for more than 10% of the share and other fresh-cut vegetables (e.g., crudités, soup mix, stir-fry vegetables) cover the remaining 40% worldwide.

In Europe, Italy is the second largest producer of fresh-cut produce (IsmeaMercati, 2016); in particular, RTE mixed salads cover about the 75% of the sales of fresh-cut vegetables, followed by rocket salads (9%) and other fresh cut vegetables (16%) (e.g., spinach, carrots, beets, mushrooms, cabbage, spices and vegetable soups) (IsmeaMercati, 2016) (**Figure 2**).

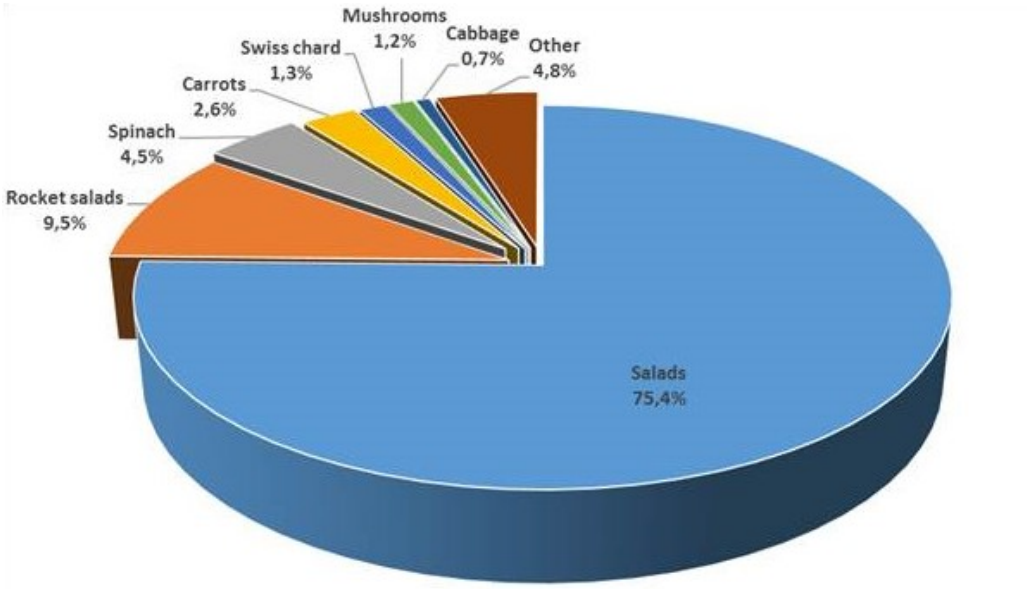


Figure 2. Sales of fresh cut vegetables in Italy in 2015 (% sales) (Source: IsmeaMercati, 2016).

In Italy, there are approximately 500 companies and 120 processing plants that produce RTE vegetables. While the processing plants are mainly located in the north of Italy (particularly in Lombardy) with the exception of Campania region, located in the south, the raw materials are mainly produced in Southern Italy (Baldi and Casati, 2011) (**Figure 3**).

Figure 3. Distribution of the fresh-cut companies in Italy (Source: Baldi and Casati, 2011).

2.1.2 Berries

Berries are defined as fruits derived from a variety of plants which are characterized by a high surface-weight ratio and the entire fruit, including seeds, can be consumed in a succulent form (Codex Alimentarius, 2000). Blueberries (*Vaccinium cyanococcus, Vaccinium corymbosum)*, blackberries (*Rubus)*, and raspberries (*Rubus idaeus*) are all members of the rose family and grow on perennial bushes.

All berries are rather small and have soft skins and this makes them vulnerable to damage; in fact, due to their fragility, berries do not undergo industrial washing, and are difficult to clean thoroughly prior to consumption (Ortega and Shields, 2015; Tefera *et al*., 2018).

A wide variety of food products are produced from berries for human consumption, and these vary in terms of physical and biological properties, and in the processes involved in production.

Berries can be harvested from the wild or cultivated. They can be grown on large-scale production sites or on smaller settings; in the latter situation, berries are more exposed to pathogens contamination due to less advanced infrastructure, and reduced ability to follow the principles of good agricultural practice (GAP) and good handling practice (GHP) (European Commission, 2006; Ganpat *et al*., 2014).

The production type can be outdoor or indoor. Berries grown outside are at greater risk of contamination from animals or the environment. Therefore, if production is outside, efforts should be made to ensure that the field is properly fenced, and/or other measures to prevent animal access to the field are implemented. The irrigation method also represents a crucial factor to consider: deep root irrigation systems are safer than the overhead methods of distribution, such as sprinkling, since they reduce the chance of contaminants reaching the edible parts of the plants. For plants grown outdoors there is also the chance of indirect contamination caused, among other things, by water during heavy rainfall or soil splashed onto the plants (reviewed by Tefera *et al*., 2018).

On production sites, identification of the potential sources of contamination is the first step for ensuring high quality produce for human consumption; the risk should be reduced by identifying critical control points throughout the production line and implementing a hazard analysis critical control points (HACCP) plan by installing proper tools for preventing contamination rather than controlling it after it has occurred (FDA, 1997). In fact, HACCP is a continuous process that describes appropriate ways to monitor production, including contamination; whenever contamination occurs, from farm-to fork, a planned corrective action should be taken in order to ensure safety of the product. Different types of berry production should be scrutinized separately for optimizing the

HACCP plan. In addition, should specific food products for human consumption be produced in the same facility, other than fresh berries, this shall absolutely be considered when establishing a HACCP regime. Other food safety management systems, such as, GAP, GHP and Good Manufacturing Practices (GMP) are highly recommended to ensure safety of the products.

European countries produce berries for a large part of the year. Poland is the main berry-producing country followed by Germany. Italy produces a variety of berries including blackberries, raspberries and blueberries (CBI, 2018) (**Figure 4**).

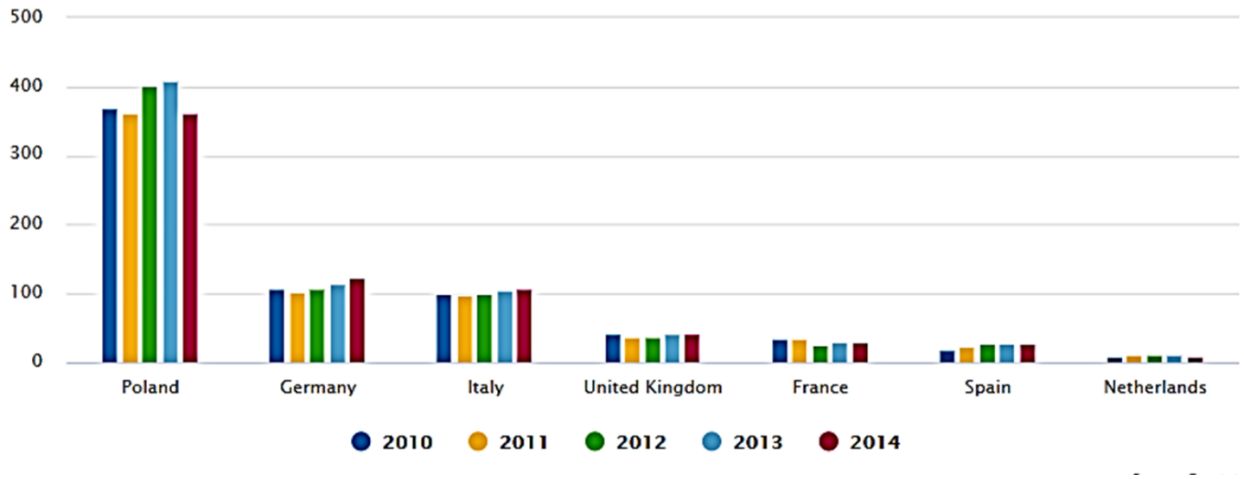


Figure 4. European production of fresh berries.

Nevertheless, to guarantee the provision of fresh berries all the year round, it also necessary to import berries into Europe; most berries are imported from other countries during the off-season (Kempler and Hall, 2013).

The total European import of fresh berries in 2016 was nearly 80 thousand tonnes, of which around 76 thousand tonnes were imported from developing countries (**Figure 5**). Most long-distance and off-season supplies come from Latin America (mostly Mexico, Chile, and Peru). Other countries, like Morocco and South Africa, are also rapidly increasing their export capacities. These countries have warm climates that ensure year-round production and, often, cheap labour costs (CBI, 2018).

Such countries, however, may be endemic for pathogens that are unusual or rare in the importing countries. As such countries may also have less developed infrastructure, there could be greater chances of contamination of the berries during production. For example, Morocco is endemic for cystic echinococcosis (Chebli *et al*., 2017) and *C. cayetanensis* is endemic in Southern and Central American countries (Almeria *et al*., 2019). In 1996 and 1997, cyclosporiasis outbreaks in North America were linked to eating Guatemalan raspberries. In the same period a study aimed at

describing the epidemiology of *Cyclospora* in Guatemala, explored potential environmental sources of contamination, including a raspberry farm cohort of 164 workers and 18 family members who submitted specimens from April 6 to May 29, 1997. Among these 182 raspberry farm workers and family members monitored, six (four farm workers and two family members) were positive for *C. cayetanensis* (Bern *et al*., 1999).

Many outbreaks of disease have been linked to produce imported from countries where HACCP implementation is suboptimal and the importance of these routines for ensuring product safety is underestimated (Dixon, 2016).

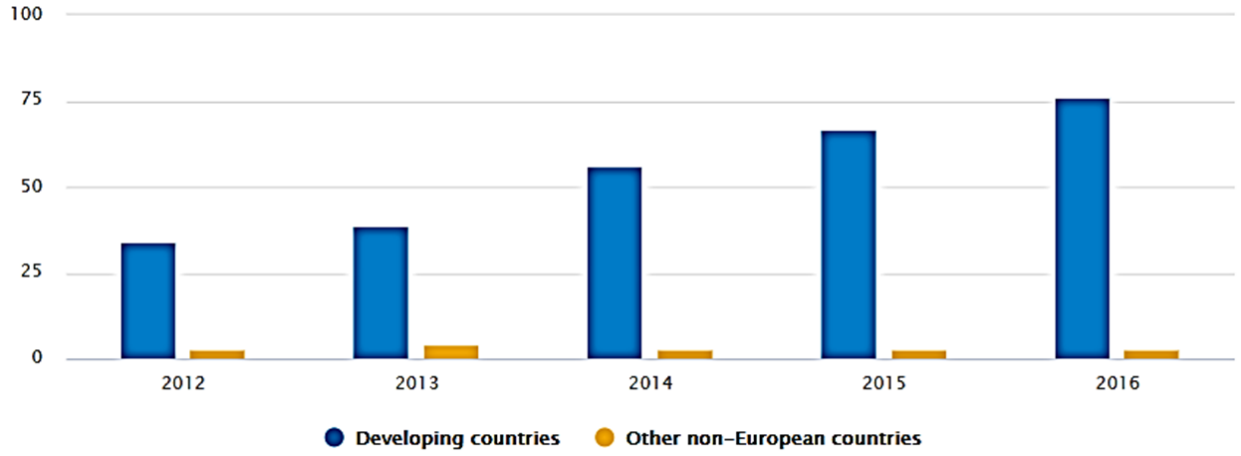


Figure 5. European import of fresh berries 2012-2016 (in 1000 tonnes).

The United Kingdom and Germany maintain the highest total import of fresh berries, The Netherlands is the third largest importer and France, Spain, Italy and Austria follow in 4th, 5th, 6th and 7th position, respectively (CBI, 2018) (**Figure 6**).

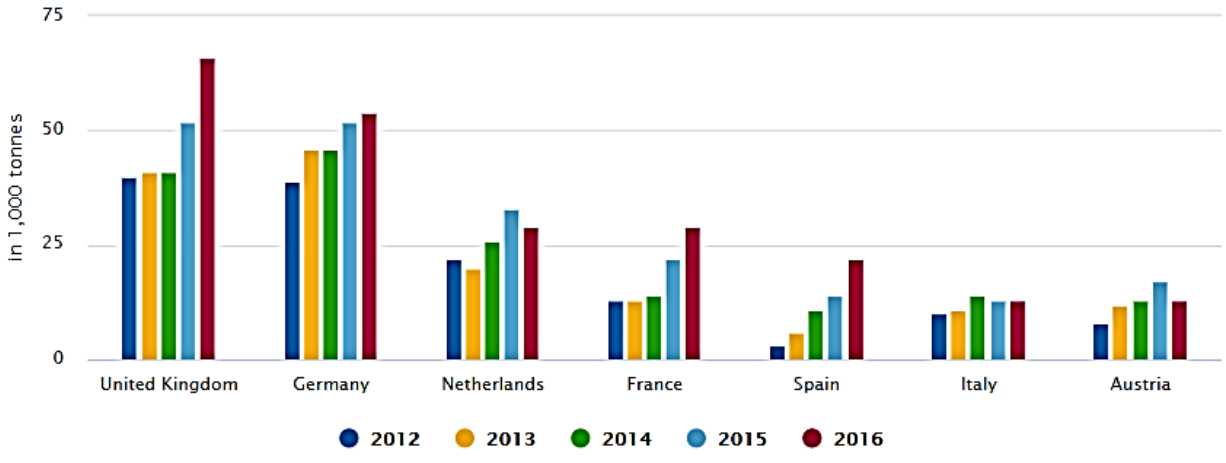


Figure 6. Main European importers of fresh berries 2012-2016 (in 1000 tonnes).

2.1 FOODBORNE PARASITES AND DRIVERS OF FOODBORNE PARASITIC DISEASES

Compared with bacterial and viral foodborne pathogens, FBPs have received less attention and represent a neglected pathogen group; however, they are increasingly being recognized as a public health problem.

Foodborne parasites are neglected for a variety of reasons (Robertson, 2018): *(i)* there is a perception that they are mostly associated with developing countries, conditions of poverty and inadequate sanitary conditions. However, this not always the case and FBPs are not just restricted to such places but spread all over the world, Europe included. Poor hygiene conditions and poverty settings certainly promote the spread of FBPs. However, nowadays, in such a globalised world with movement of people and animals and the internationalisation of commerce and globalised food supply (Robertson *et al*., 2014), it is impossible to make such a clear-cut distinction between developing and developed countries; *(ii)* most infections with FBPs do not manifest as acute diseases, but rather have a chronic course of the disease.

The most important FBPs and their main food vehicles are summarized in **Table 1**.

Parasites	Primary food category	Primary food vehicles	Secondary food vehicles
Anisakidae	Aquatic animals	Marine fish, crustaceans and cephalopods	
Ascaris spp.	Plants	Fresh produce	
Balantidium coli	Plants	Fresh produce	
Cryptosporidium spp.	Plants	Fresh produce, fruit juice, milk	
Cyclospora cayetanensis	Plants	Berries, fresh produce	
Diphyllobothriidae	Aquatic animals	Fish (freshwater and marine)	
Echinococcus granulosus	Plants	Fresh produce	
Echinococcus multi-locularis	Plants	Fresh produce	
Entamoeba histolytica (Older studies did not distinguish Entamoeba *histolytica* from *E. dispar.*)	Plants	Fresh produce	
Fasciola spp.	Plants	Fresh produce (aquatic plants)	
Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*)	Plants	Fresh produce	Molluscan shellfish
Heterophyidae	Aquatic animals	Fresh- and brackish-water fish	
Opisthorchiidae	Aquatic animals	Freshwater fish	
Paragonimus spp.	Aquatic animals	Freshwater crustacea	
Sarcocystis spp.	Land animals	Beef	Pork
Sparganosis – *Spirometra* spp.	Other	Frog, snake meat	
Taenia saginata	Land animals	Beef	
Taenia solium	Land animals	Pork	
	Plants (cysticercosis)	Fresh produce	
Toxocara spp.	Plants	Fresh produce	
Toxoplasma gondii	Land animals	Meat from small ruminants, pork, beef, game meat (red meat and organs)	Fresh produce, seafood, dairy products
Trichinella spiralis	Land animals	Pork	Horse, Game meat
Trichinella spp. (other than *Trichinella spiralis*)	Land animals	Game meat [5]	Pork
Trichuris trichiura	Plants	Fresh produce	
Trypanosoma cruzi	Plants	Fruit juices	

Table 1. Parasites and main food vehicles (Source: FAO/WHO, 2014).

In a recent article by Robertson *et al*. (2020), FBPs are classified based on the food matrix by which they are transmitted:

- *FBPs transmitted by meat and fish*: Foods of animal origin are highly consumed in most European countries and represent a major vehicle for foodborne diseases (Da Silva Felicio *et al*., 2015). In 2012, *Trichinella*, *Toxoplasma*, and *Taenia solium* were ranked as being globally the most important FBPs in meat (FAO/WHO, 2014), and nematodes (in particular *Anisakis*) as being of greatest importance in fish worldwide. In a more recent risk-ranking restricted to Europe (Bouwknegt *et al*., 2018) (**Figure 7**), while in line with the previous one, *Toxoplasma* and *Trichinella* occupy the top positions among meatborne parasites and *Anisakis* is ranked as being of highest importance in fish, *Taenia solium* is considered to be of lower significance in Europe, reaching only the tenth position.

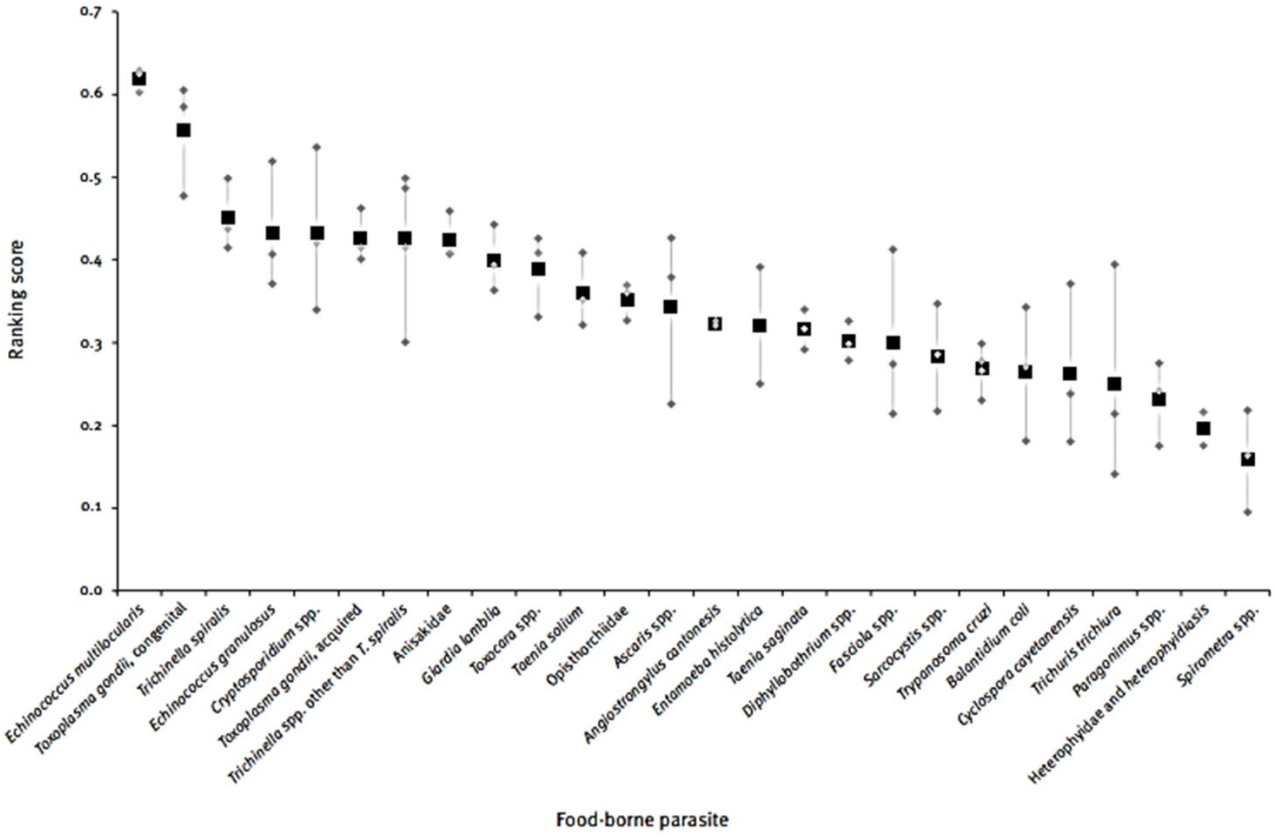


Figure 7. European ranking of FBPs based on the Euro-FBP criteria and weights, 2016 (Source: Bouwknegt *et al*., 2018).

- *FBP transmitted as contaminants of water, shellfish or fresh produce:* Many protozoan FBPs i.e., *Cryptosporidium* spp., *T. gondii* and *G. duodenalis* can be transmitted not only via food, but also, and perhaps more commonly, via water contaminated by oo/cysts released in the faeces of their definitive hosts. *Cryptosporidium* spp. and *G. duodenalis* are, in fact, the most commonly reported agents of waterborne outbreaks of disease (Efstratiou *et al*., 2017). Humans and animals can also

be infected by the consumption of contaminated raw fresh produce (fruits and vegetables). Contamination may occur directly by faeces (via animals, food handlers, surfaces, or equipment) or when such produce are irrigated or washed with contaminated water (Shapiro *et al*., 2019; Ryan *et al*., 2018, 2019).

A further transmission vehicle is bivalve molluscs, which concentrate contaminants in their organs, including protozoan oo/cysts (Robertson, 2007; Tedde *et al*., 2019). When they are consumed raw or lightly cooked, they may represent a vehicle of FBPs.

In a recent article by Trevisan *et al*., 2019, 19 drivers of foodborne parasitic diseases were identified, and those of greatest importance are discussed (**Figure 8**).

Figure 8. Top five drivers of foodborne parasitic diseases in Europe (Source: Trevisan *et al*., 2019).

1- Globalization of food supply. The broad distribution of foods has been made possible by improved transportation, particularly the cold chain. As a consequence, foodborne diseases are no longer geographically circumscribed and international and multistate outbreaks have become more common (Gould, *et al*., 2017; Lipcsei, *et al*., 2019) (e.g., outbreaks of cyclosporiasis in the USA associated with imported fresh produce (Hadjilouka and Tsaltas, 2020)).

2- Changing culinary habits and the role of human behaviour towards raw foods. Consumption of raw and undercooked foods has significantly increased, for example, eating sushi and sashimi has become extremely popular worldwide, leading to an increase of gastroallergic anisakiasis in consumers (Llarena-Reino *et al*., 2015). Travelling, strictly linked with trying new foods and *cuisines*, has increased consumers' exposure to unfamiliar risks and FBPs (Robertson *et al*., 2014). The increasing attention of consumers towards animal welfare and the expansion of the outdoors/organic farming systems has determined for the animals raised outdoors a greater possibility of infection with parasites such as *T. gondii*, *Trichinella*, *Taenia* spp. (Murrell, 2016; Devleesschauwer *et al*., 2017). Also, driven mostly by lack of time, more and more consumers opt for a ready meal, including RTE salads, which, as healthy as they may be, have also been found contaminated by protozoan parasites and responsible of outbreaks of disease (Caradonna *et al*., 2017; FDA, 2020).

3/4- Gaps in surveillance and control and lack of awareness from agencies. Surveillance of foodborne diseases is a fundamental component of food-safety systems (EFSA, 2018). For some FBPs, such as *Trichinella* and *Taenia* spp., there are EU-level regulations (Regulation (EU) 2015/1375; Regulation (EC) 854/2004) and official controls in place. However, for others such as *T. gondii* in meat or protozoan parasites in fresh produce there are not any. This is an issue that will need to be addressed and increased efforts will be needed to translate research results into policies.

5- *The role of water*. The increased employment of wastewater for irrigation due to extensive population growth and related increase in food requirements and new areas of cultivation, has led to greater possibilities for contamination of fresh produce. For instance, RTE-products are increasingly popular, and have been associated with outbreaks of FBPs (McKerr *et al*., 2015).

The FBPs investigated in the present survey, are discussed individually in the next section.

2.3 INVESTIGATED FOODBORNE PARASITES

2.3.1 *CYCLOSPORA CAYETANENSIS*

2.3.1.1 Classification and morphology

Cyclospora is a protozoan parasite that belongs to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, family Eimeriidae.

Nineteen species belonging to the genus *Cyclospora* are currently known. They infect reptiles, insectivores (Ortega and Sanchez, 2010) and primates (Lainson *et al*., 2005; Li *et al*., 2015), including humans (**Table 2**); these species cannot be microscopically distinguished as they all are morphologically highly similar.

***Cyclospora* species**	**Hosts**
C. papionis	Baboons
C. colobi	Colobus monkey
C. glomericola	Diplopods
C. cercopitheci	Green monkeys
C. cayetanensis	Humans
C. caryolitica	Insectivores
C. talpae	Insectivores
C. ashtabulensis	Insectivores
C. megacephali	Insectivores
C. parascalopi	Insectivores
C. macacae	Rhesus monkeys
C. angimurinensis	Rodents
C. viperae	Snakes
C. babaulti	Snakes
C. tropidonoti	Snakes
C. scinci	Snakes
C. zamenis	Snakes
C. niniae	Snakes
C. shneideri	Snakes

Table 2. Species of *Cyclospora* and their hosts (from Giangaspero and Gasser, 2019).

The first published report of *Cyclospora* infection in humans can be dated back to 1979 when it was described as a coccidian organism responsible for causing diarrhoea in two children and a woman in Papua New Guinea, but finally diagnosed as a coccidian belonging to the genus *Isospora* which,

in retrospect, was probably *C. cayetanensis* (Ashford *et al*., 1979). In subsequent reports, *Cyclospora* was described as a coccidian-like body (CLB), cyanobacterium-like body, blue-green alga, or large *Cryptosporidium* until 1993 (Ortega *et al*., 1993). In 1994 this organism was formally recognised as a new coccidian species capable of infecting humans and named *C. cayetanensis* (Ortega *et al*., 1994).

C. cayetanensis seems to be host specific and humans are so far the only confirmed hosts. Although it has been detected in the faeces of several animals, including ducks, chickens and other avian species, dogs, albino mice and non-human primates (reviewed by Almeria *et al*., 2019), attempts to establish experimental *C. cayetanensis* infection in laboratory animals (Eberhard *et al*., 2000) have not been successful, suggesting host specificity. In general, the presence of oocysts in stools from animals seems to be exclusively linked to their passage through the gastrointestinal tract with no evidence of infection (Ortega and Sherchand, 2015).

Cyclospora oocysts are rounded and measure 7.7-9.9 µm (avg. 8.6 µm); they have a colorless, thin (<1 µm) and bilayered wall. Unsporulated oocysts are undifferentiated spheres containing a morula (Long *et al*., 1991). When sporulated, the oocysts contain two ovoid structure called sporocysts (4 to 6.3 µm) inside each one of which there are two sporozoites (1.2 to 9 µm) folded in two (Ortega *et al*., 1993, 1994) (**Figure 9**).

Figure 9. *Cyclospora* oocysts. The images show unsporulated and sporulated oocysts (source: CDC, 2020).

2.3.1.2 Biology and life cycle

Cyclospora cayetanensis is an obligate intracellular parasite and is monoxenous, requiring a single human host to complete the entire life cycle (Ortega *et al*., 1997). The infection begins when a susceptible host ingests sporulated *C. cayetanensis* oocysts, potentially with contaminated food, water, or soil. In the upper gastrointestinal tract, the oocysts excyst and release the sporozoites that invade the enterocytes of the duodenum and the jejunum. Inside the enterocytes, the sporozoites transform into trophozoites and, after that, undergo asexual multiplication (merogony) into type I

and type II meronts, containing merozoites. The type I meronts contain eight to 12 merozoites, type II meronts contain four merozoites. While Type I merozoites remain in the asexual cycle, type II merozoites initiate the sexual (gametogony) phase and (male) microgametocytes or (female) macrogametocytes are formed. The microgametocytes fertilise the macrogametocytes to produce zygotes, which differentiate into unsporulated oocysts and, after being released into the lumen of the intestine, are excreted in the environment through the faeces. Sporulation occurs outside the host; the oocysts require 7 to 15 days to sporulate under ideal conditions (23 to 27°C) and become infectious to a susceptible host (Ortega and Sanchez, 2010). A sporulated oocyst contains two sporocysts, each with two sporozoites (reviewed by Giangaspero and Gasser, 2019 and Almeria *et al*., 2019) (**Figure 10**).

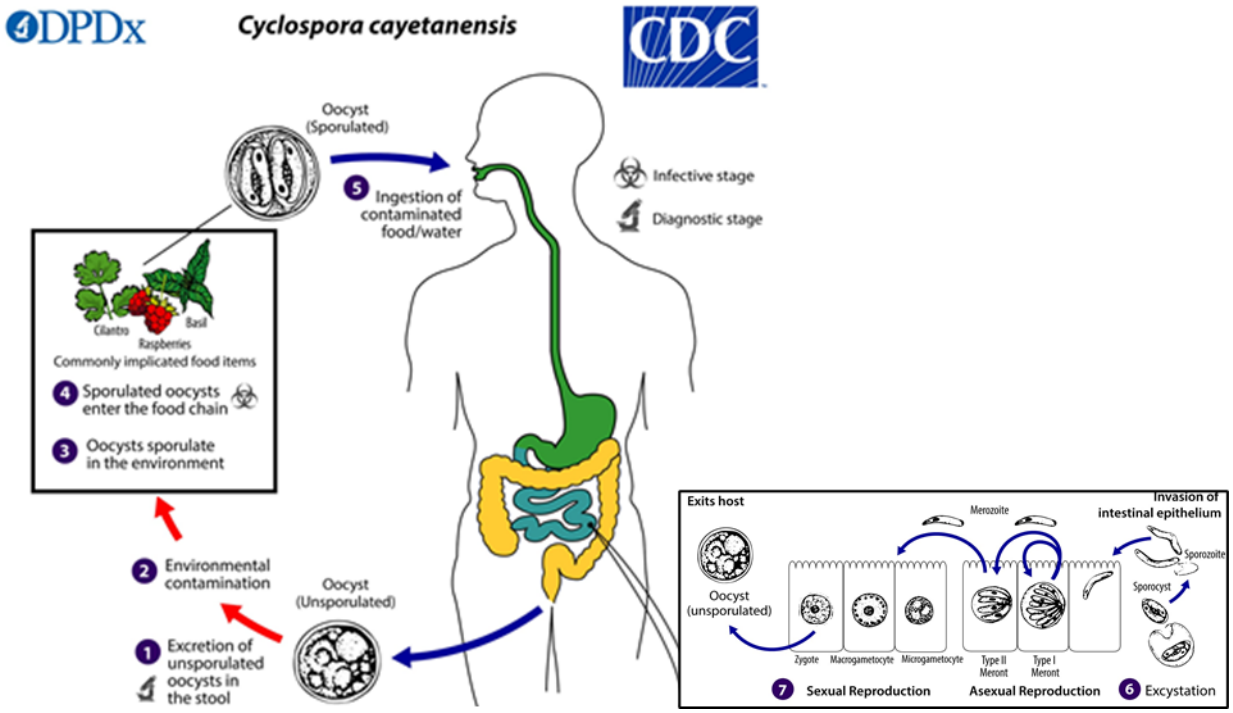


Figure 10. Life cycle of *C. cayetanensis* (source: CDC, 2018).

2.3.1.3 Pathogenesis and clinical symptoms

Cyclosporiasis affects the small intestine (Sun *et al*., 1996; Connor *et al*., 1999). Although *C. cayentanensis* pathogenesis and virulence factors are not yet fully understood, its asexual and sexual replications during merogony (schizogony) and gametogony, respectively, seem to be responsible for damaging and altering the architecture of the small intestine causing mucosal alterations, edema and infiltration by inflammatory cells, reactive hyperemia, vascular dilation, and congestion of capillaries in the upper intestinal tract (Ortega *et al*., 1997). These histopathological

changes result in the reduction of the intestine surface area, leading to a decreased uptake of electrolytes, water, and nutrients and to abundant and watery diarrhoea, often occurring in cyclical patterns alternating with constipation (Soave et a., 1998). In addition to diarrhoea, other gastrointestinal symptoms e.g., abdominal cramps, nausea and vomiting as well as low-grade fever, fatigue and weight loss are associated with this infection.

In immunocompetent patients the disease is mainly self-limiting and associated with mild symptoms. However, protracted or chronic diarrhoea has been reported in immunocompromised patients (Almeria *et al*., 2019) e.g., HIV-positive patients in which the average duration of diarrhoea is longer than in HIV-negative patients (199 days and 57.2 days, respectively) (Schubach *et al*., 1997; Sancak *et al*., 2006).

In endemic settings the infection is usually asymptomatic or mild among adults (Hussein *et al*., 2007; Gómez Martínez *et al*., 2016). The elderly and the young children are those most severely affected and show the most serious clinical symptoms; in the latter case, protracted diarrhoea can lead to severe dehydration and even death especially if cyclosporiasis is complicated by secondary viral, bacterial, or parasitic (e.g., *Cryptosporidium* and *Giardia*) infections and/or by malnutrition and malabsorption, which are quite common in underprivileged settings (Behera *et al*., 2008). Interestingly, the severity and duration of the infection tend to become milder after repeated episodes (Ortega and Sanchez, 2010). The median incubation period is around seven days (Almeria *et al*., 2019). Clinical symptoms can persist for a few days to a month or longer, if patients are not treated (Goldberg and Bishara, 2012).

Immunocompromised people such as individuals with HIV/AIDS (Pape *et al*., 1994) may also develop extraintestinal forms, including Guillain–Barre syndrome (Richardson *et al*., 1998) and reactive arthritis syndrome (formerly known as Reiter syndrome) (Connor *et al*., 2001). Further clinical manifestations of *C. cayetanensis* infection in HIV-positive patients are biliary disease, acalculous cholecystitis and cholangitis in AIDS patients (Sifuentes-Osornio *et al*., 1995; Connor *et al*., 2001; Zar *et al*., 2001).

2.3.1.4 Epidemiology

The infectious dose for *Cyclospora* is unknown but is presumed to be low (Sterling and Ortega, 1999). The factors affecting the survival of *Cyclospora* oocysts are summarized in **Table 3**.

Factors affecting survival of *Cyclospora* oocysts						
Environment	**Heat**	**pH**	**Freezing**	**Disinfectant**	**Drying**	**UV light**
Oocysts survive in water at 4 °C for 2 months and at 37 °C for 7 days (**Smith *et al.*, 1997**; **Ortega *et al.*, 1998**). Unsporulated *C. cayetanensis* oocysts are resistant to the most common pesticides (**Sathyanarayanan and Ortega, 2004**).	After heating at 60 °C for 1 h or 70 °C for 15 minutes oocysts cannot be induced to sporulate. Sporulation was not observed in basil leaves or water at 70 °C, and 100 °C for 15 min (**Sterling and Ortega, 1999**; **Sathyanarayanan and Ortega, 2006**).	No information.	Sporulation can be prevented after 2 days at -20 °C. Sporulation was not observed in basil leaves or water after exposure to -70 °C for 1 h (**Sathyanarayanan and Ortega, 2006**).	Oocysts are resistant to many disinfectants including chlorine at levels used in water treatment (**Soave *et al.*, 1998**). *Cyclospora* sporulation Is not affected by treatment with chlorine dioxide (**Ortega *et al.*, 2008**).	Oocysts are very sensitive to desiccation (after 15 min, the oocyst wall breaks) (**Long *et al.*, 1991**).	No information.

Table 3. Survival of *Cyclospora* oocysts. Adapted and modified from Dawson, 2005 and Gérard *et al*., 2019.

Humans and foodborne outbreaks caused by the consumption of fresh produce - C. cayetanensis infection among humans has been reported worldwide, in both developed and developing countries, but it is most common in tropical and subtropical areas - Central and South America, the Middle East, the Indian subcontinent and South East Asia - where cyclosporiasis is an endemic disease with a prevalence up to 13% in immunocompetent individuals (adults and/or children) and up to 36% in immunocompromised persons, mostly HIV/AIDS patients (Chacin-Bonilla, 2010).

In Latin America, the prevalence of cyclosporiasis in humans is low in Mexico (up to 3.3%) (de la Luz Galván-Ramírez *et al*., 2019) but reaches peaks of and 41.6% in Peru (Burnstein Alva, 2005), followed by Haiti with 36% (Raccurt *et al*., 2008), Venezuela with 24.2% (Cazorla *et al*., 2012) and Brazil with 10.8% (Borges *et al*., 2009), respectively.

Cyclospora infection in humans has also been registered in the Middle East e.g., Turkey with an overall prevalence of 17.4% (Turgay *et al*., 2012) and Saudi Arabia (≤52%) (Sanad *et al*., 2014) and in countries of the Indian subcontinent and South East Asia, including India, Nepal and China with prevalences of 22% (Dhanabal *et al*., 2014), 9.2% (Kimura *et al*., 2005) and 5.6% (Wang *et al*., 2002), respectively.

With regard to African countries, the highest prevalences have been recorded in Egypt (10%) (Nassef *et al*., 1998), South Africa (7.2%) (Samie *et al*., 2009), and Madagascar (16.5%) (Frickmann *et al*., 2015); in addition, an overall prevalence of 18% has been recorded in 14 sub-Saharan countries (Fletcher *et al*., 2011).

In developed and non-endemic countries, the prevalence does not exceed 3%. In the USA prevalences ranging from 0.3 to 0.5% (Wurtz, 1994; Ooi *et al*., 1995) were recorded in the 1990s,

however, there is evidence supporting that the incidence of infection has substantially increased since then. In Canada, a prevalence of 1.9% was recorded (Salehi *et al*., 2015).

In Europe, most cases of cyclosporiasis were related to traveller's diarrhoea, such as those registered in Germany, Spain and in Poland (Jelinek *et al*. 1997; Paschke *et al*., 2011; Ramírez-Olivencia *et al*., 2008*;* Bednarska *et al*., 2015), but single autoctonous cases 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).

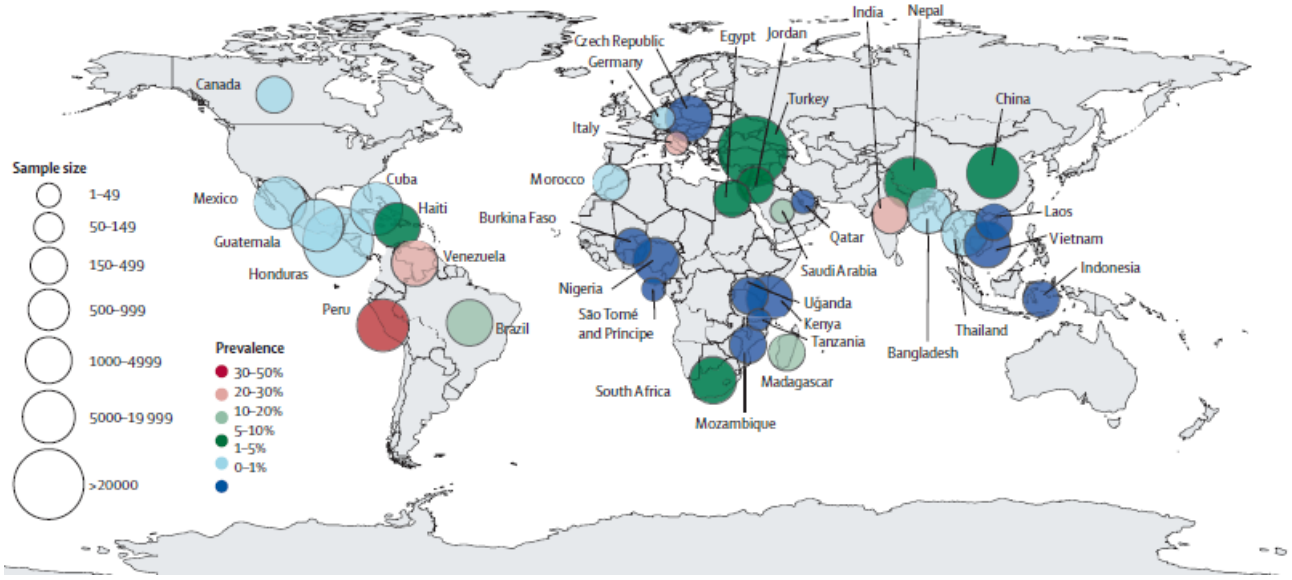


Figure 11. Estimated prevalence and distribution of *Cyclospora* infection in immunocompetent people (source: Giangaspero and Gasser, 2019)

Cyclosporiasis is accepted as often being a foodborne infection, and the main route of transmission of *C. cayetanensis* is represented by contaminated fresh produce, such as berries and leafy vegetables, which are eaten raw, without previous heating, and are difficult to wash properly before consumption (Ortega and Sanchez, 2010; Tefera *et al*., 2018).

Outbreaks of cyclosporiasis associated with contaminated fruits and vegetables have been reported worldwide e.g., Asia (i.e., Nepal and Indonesia) and South America (i.e., Mexico, Columbia, Guatemale and Perù) (Chacin-Bonilla, 2010).

In the United States, since the mid-1990s repeated outbreaks of cyclosporiasis associated with the consumption of various types of contaminated fresh produce, mainly imported from South American countries, such as raspberries and cilantro, have been reported (CDC, 2019).

Outbreaks of *C. cayetanensis* infections have also been registered in Europe. In Germany, a cyclosporiasis outbreak with 34 involved people was associated with the consumption of salads

prepared with fresh produce imported from France and Southern Italy (Döller *et al*., 2002). In Sweden, an outbreak of *C. cayetanensis* infection with 12 laboratory-confirmed cases was reported between May and June 2009. The incriminated food items were sugar snap peas imported from Guatemala (Insulander *et al*., 2010). In the United Kingdom, *C. cayetanensis* was identified in 79 travellers returning from the Riviera Maya region of Mexico between June and September 2015. The infections have been attributed to the consumption of fresh produce such as contaminated fruits (strawberries or raspberries), vegetables and herbs (coriander) as well as contaminated water and drinks (Nichols *et al*., 2015).

In Italy, foodborne outbreaks of *Cyclospora* infection associated with the consumption of contaminated fruits and/or vegetables have not been registered.

A recent review provides detailed information on the outbreaks associated with *C. cayetanensis* by country, year of occurrence, and possible sources of exposure (Almeria *et al*., 2019).

Fresh produce - Several surveillance studies have reported the presence of *C. cayetanensis* in fresh fruits and vegetables worldwide (reviewed by Almeria *et al*., 2019). In endemic areas, *C. cayetanensis* was reported in fresh produce in several Latin American countries such as Colombia (Ortiz Pineda *et al*., 2020), Peru (Burnstein Alva, 2005), Venezuela (Devera *et al*., 2006), and Costa Rica (Calvo *et al*., 2004) with prevalences ranging between 0.8% and 8% as well as in Nepal (Sherchand *et al*., 1999, 2010), Cambodia (Anh *et al*., 2007) and Egypt (Mossallam, 2010; El Said Said., 2012). The contaminated foods were mainly leafy vegetables (ranging from lettuce to water spinach) and berries such as strawberries and fresh strawberry juice.

In non-endemic industrialized countries, to date, no surveillance studies have been published in the USA. In Canada, *C. cayetanensis* was found in 9 (1.7%) out of 544 RTE packaged leafy greens (Dixon *et al*., 2013) and in 6 (0.51%) out of 1171 pre-packaged leafy greens (Lalonde and Gajadhar, 2016). As to Europe, *Cyclospora* was registered in Italy where *Cyclospora* DNA was detected in 6 (12.2%) out of 49 vegetable samples i.e., fennel, cucumber, celery, tomato, with the highest prevalence (18.7%) on fennel (Giangaspero *et al*., 2015a) and in RTE packaged salads sold on the Italian market with a prevalence of 1.3% (Caradonna *et al*., 2017).

A recent review gives detailed information on surveillance studies of *Cyclospora* spp. and/or *C. cayetanensis* oocysts detection in fresh produce worldwide (Almeria *et al*., 2019).

Location	% (No. Positive/Total Samples Analyzed)	Food Type
Cambodia	8.3 (3/36)	Water spinach
Costa Rica	8.0 (2/25)	Lettuce
Canada	1.7 (9/544)	Precut salads and leafy greens
Canada	0.5 (6/1171)	Arugula/baby arugula, baby spinach and spring mix
Egypt	16 (4/25)	Lettuce heads
Egypt	21.3 (64/300)	Rocket, lettuce, parsley, leek, green onion
Egypt	25.7 (9/35)	Fresh strawberry juice
Ethiopia	6.9 (25/360)	Vegetables and raw fruits (avocado, lettuce, cabbage, carrot, tomato, banana and mango) *
Ghana	5.1 (20/395)	Cabbage, green pepper, onion, tomato, lettuce
Italy	12.2 (6/49)	Fennel, cucumber, celery, tomato
Italy	1.3 (8/648)	Ready to eat-packaged salads
Korea	1.2 (5/404)	Winter grown cabbage, sprouts, blueberries, and cherry tomatoes
Nepal	Number not indicated	Cabbage, lettuce, mustard leaves
Nepal	Number not indicated	Lettuce, spinach, mustard and basil leaves
Peru	Two surveys: 1.8 (2/110) and 1.6 (1/62)	Lettuce, mint, black mint
Venezuela	5.9% (6/102)	Lettuce
Vietnam	8.4 (24/287)	Herbs (basil, coriander sativum and coriander, Vietnamese mint, marjoram, persicaria) and lettuce

* Unclear which commodity was contaminated by *Cyclospora* spp.

Table 4. Positive reports and surveillance studies of *Cyclospora* spp. and/or *C. cayetanensis* oocysts detection in fresh produce worldwide (see Almeria *et al*., 2019 and related references)

2.3.1.5 Detection methods in fresh produce

The detection of *C. cayetanensis* in fresh produce is challenging because of i) the low numbers of oocysts they are usually contaminated with, ii) the oocysts being trapped in pores of the food making them difficult to detach and difficult to detect, iii) the methods used for clinical samples not always being applicable for the detection of *Cyclospora* in fresh produce limiting, as a result, the options available.

The first step for the recovery of the *Cyclospora* oocysts is the washing aimed at detaching the parasite stages from the products' surface and concentrating them; for this purpose, among several solutions available (Shields *et al*., 2012; Chandra *et al*., 2014; Lalonde and Gajadhar, 2016), Alconox® is more efficient compared to others and significantly improves the recovery of parasitic protozoa from food (Shields *et al*., 2012).

The use of lectin-coated paramagnetic beads represents an option for the isolation of *Cyclospora* oocysts from fresh produce, however no significant differences in the recovery efficiency have been detected with or without this procedure (Robertson *et al*., 2000).

Antibody-specific coated beads could also be employed for the concentration of *C. cayetanensis* oocysts as used for the detection of *Cryptosporidium* spp., but the antibodies are not yet commercially available (Almeria *et al*., 2019).

Identification of *Cyclospora* oocysts in food matrices by microscopy is extremely challenging because the currently available methods, including acid-fast stain (Garcia *et al*., 2017), hot-safranin stain (Visvesvara *et al*., 1997; Maratim *et al*., 2002) and ultraviolet fluorescence microscopy (Ortega and Sterling, 1996; Varea *et al*., 1998), lack the required sensitivity for the detection of low concentration of oocysts.

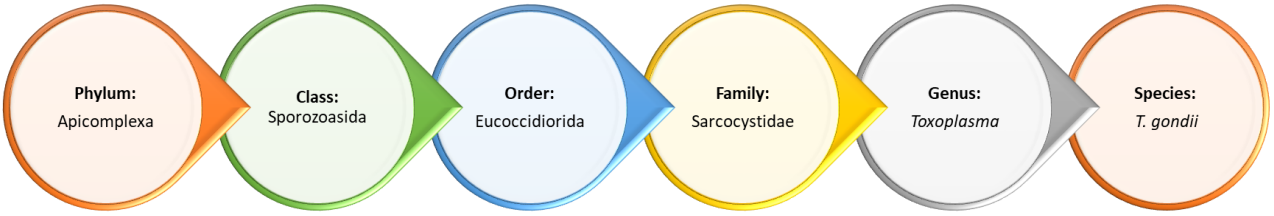
In recent years, several molecular techniques including conventional PCR, real-time PCR (*q*PCR), nested PCR (nPCR), magnetic-capture PCR, loop-mediated isothermal amplification (LAMP) assays, etc. have been used for the detection of FBPs in fresh produce.

Nested-PCR protocols based on the 18S rRNA gene (Steele *et al*., 2003; Dixon *et al*., 2013) have been described for *Cyclospora*. Among the *q*PCRs, a method for identification of protozoan oocysts, including *Cyclospora*, on leafy green vegetables and berry fruits based on 18S rDNA gene (Lalonde and Gajadhar, 2011, 2016) and one assay targeting a region within ITS-2 of *C. cayetanensis* (Giangaspero *et al*., 2015b) were developed. The BAM (FDA's Bacteriological Analytical Manual)-19 methods are the only standardized methods for detection of *C. cayetanensis* in fresh produce. The BAM-19a method (Orlandi *et al*., 2004) has recently been modified and replaced by the BAM-19b (Murphy *et al*., 2017). In addition, a new qPCR protocol for the molecular detection of *C. cayetanensis* as a contaminant of berry fruits targeting the internal transcribed spacer 1 (ITS-1) region was developed (Temesgen *et al*., 2019a) and eventually included in a multiplex *q*PCR for the simultaneous detection of *C. cayetanensis*, *T. gondii* and *E. multilocularis* (Temesgen *et al*., 2019b). With the above-mentioned methods, it is not possible to assess whether the parasites detected are viable and infectious. However, positive molecular results for parasite contamination should not be ignored and it should be assumed that at least some of the oocysts are viable and infectious (Dixon et al., 2013) and represent, therefore, a public health risk.

2.3.2 *TOXOPLASMA GONDII*

2.3.2.1 Classification and morphology

Toxoplasma gondii is a protozoan parasite that belongs to the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida, family Sarcocystidae (Uttah *et al*., 2013).



It is an obligate intracellular parasite that can infect all warm-blooded animals, including humans and birds (intermediate hosts). Felids are the only definitive hosts, but they can also serve as intermediate hosts (Frenkel *et al*., 1970).

Toxoplasma gondii was isolated for the first time from tissues of a hamster-like rodent (*Ctenodactylus gundi*) used as an animal model to study *Leishmania* in Tunis (Nicolle and Manceaux, 1908).

Toxoplasma gondii is the only species in the genus *Toxoplasma* and comprises three clonal lineages: (Howe and Sibley, 1995): Type I, highly pathogenic, and types II and III, relatively less pathogenic (Sibley and Boothroyd, 1992) mainly found in isolates from Europe and USA. Other atypical or exotic genotypes have largely been found in the other continents (Ajzenberg *et al*., 2004; Hassan *et al*., 2019) and are highly pathogenic, in fact, they have been reported to be virulent in mice (*Mus musculus* and *Mus m. castaneus*) (Hassan *et al*., 2019) and some of these have been associated with outbreaks of severe human toxoplasmosis (Carme *et al*., 2002; Carme *et al*., 2009).

Toxoplasma gondii has three infectious stages: the tachyzoites, the bradyzoites (in tissue cysts), and the sporozoites that are contained within the oocysts (Dubey, 1998a) (**Figure 12 a-c**).

Tachyzoites are arched shaped and measure 6 by 2 µm, with a pointed anterior end and a rounded posterior end whilst bradyzoites measure approximately 7 by 1.5 µm (Melhorn and Frenkel, 1980) and form tissue cysts. These cysts are spheroid in shape and can reach a size of 70 µm in diameter in the brain and, rarely, a diameter of 100 µm in the muscular cells (Dubey, 1993). The size of the cysts varies between 10 µm for the younger cysts, containing only two bradyzoites, and 100 µm for the older ones, containing from hundreds to thousands of bradyzoites (Robert-Gangneuxa and Dardé, 2012).

Unsporulated oocysts measure about 10-12 µm in diameter. After sporulation, they become subspherical, measure 11-14 x 9-11 µm and contain two ellipsoidal sporocysts, measuring from 6 to 8 µm, each containing four sporozoites (Dubey, 1998a).

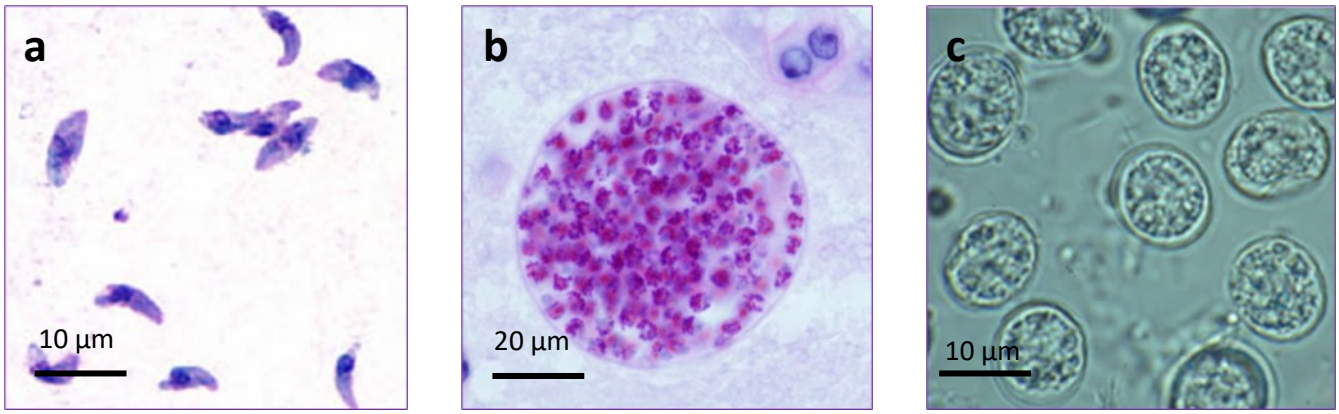


Figure 12 (a-c). The images show *T. gondii* tachyzoites, stained with Giemsa (a), *T. gondii* tissue cyst (individual bradyzoites can be seen within) (b) *T. gondii* oocysts in a faecal flotation (c) (Source: https://www.cdc.gov/dpdx/toxoplasmosis/index.html).

2.3.2.2 Biology and life cycle

In cats and wild felids both the extra-intestinal and the intestinal cycles can take place, whereas in the intermediate hosts (cattle, sheep, pigs, horses and other mammals, including humans, as well as birds) only the extra-intestinal development of *T. gondii* occurs. The life cycle of *T. gondii* is summarized in **Figure 13**.

Unsporulated oocysts are shed in the cat's faeces *(1)* and sporulate in the environment in 1-5 days depending upon aeration and temperature (*sporogony* phase) *(2)*. Intermediate hosts, cats included, become infected after ingesting sporulated oocysts, potentially with contaminated vegetables, soil, or water *(3)*.

EXTRA-INTESTINAL CYCLE: After the oocysts' ingestion, the sporozoites are liberated in the intestinal epithelium, where they differentiate into tachyzoites. Tachyzoites replicate rapidly by endodyogeny *(4-5)* inside the cells of the reticuloendothelial system and disseminate throughout the organism (acute phase). Tachyzoites multiply intracellularly for an undetermined period, convert from tachyzoites to bradyzoites and encyst *(6)*. Tissue cysts may remain throughout life in most hosts, predominantly in the brain or musculature (chronic phase) (Robert-Gangneuxa and Dardé, 2012).

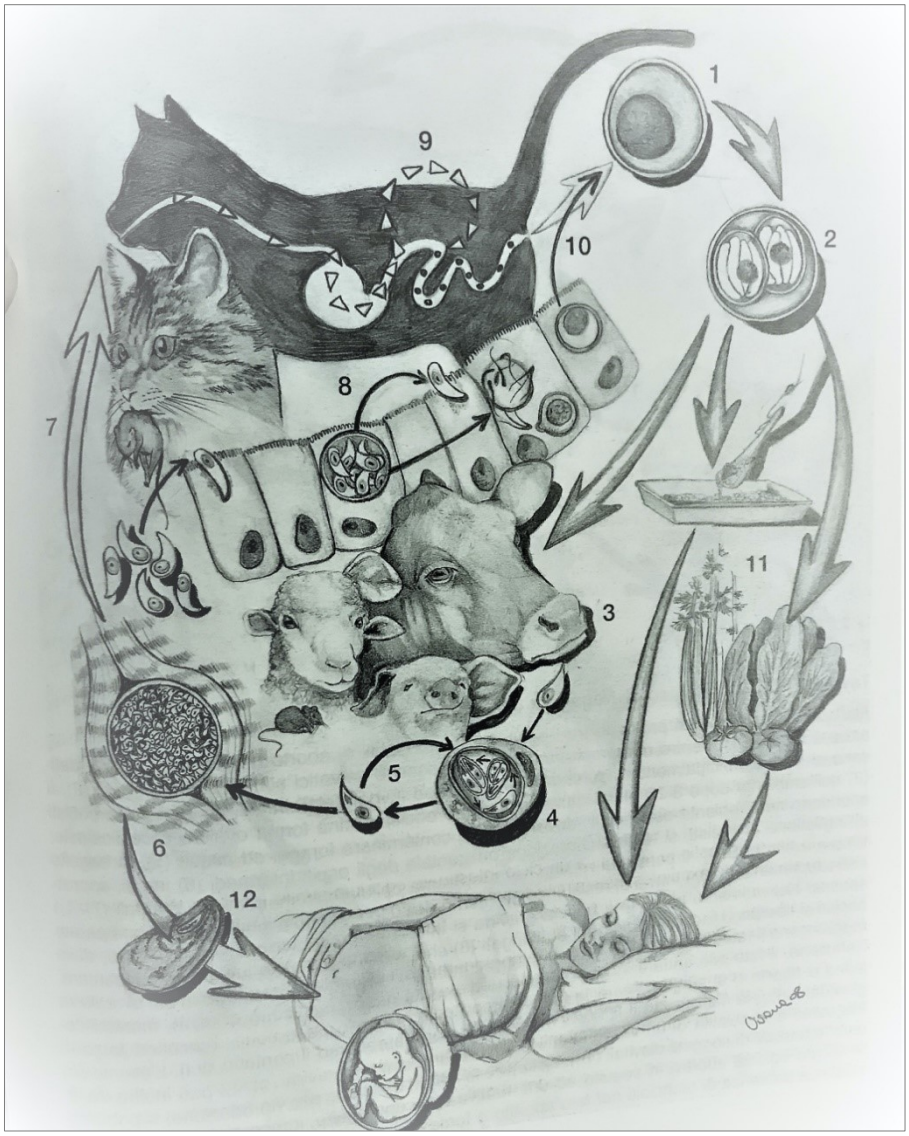


Figure 13. Life cycle of *T. gondii* (source: Taylor *et al*., 2010).

INTESTINAL CYCLE: Most definitive hosts become infected by ingesting intermediate hosts infected with tissue cysts. Upon the ingestion of these tissue cysts *(7)* through raw or undercooked meat, the cyst wall is dissolved by digestive enzymes and the bradyzoites are released in the stomach and intestine. Bradyzoites penetrate the epithelial cells of small intestine and give rise to schizonts and merozoites (asexual stages) that form male and female gamonts. After fertilization, the unsporulated (immature) oocyst is formed and passed in the faeces *(8-10)*. After exposure to air and moisture for 2 to 5 days, the oocyst sporulate *(1-2)* (Robert-Gangneuxa and Dardé, 2012).

Cats become infected:

- after ingesting intermediate hosts harbouring tissue cysts *(7)* (short prepatent period: 3-10 days);
- via tachyzoites, mainly by the ingestion of unpasteurised milk (long prepatent period: ≥13 days);

- by ingesting sporulated oocysts *(2-3)* (long prepatent period 20-48 days after ingestion) (Dubey, 1998a).

Successful infection of seronegative cats is revealed by the shedding of oocysts in their faeces. Fewer than 30% of these cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all of them shed oocysts after ingesting tissue cysts (Dubey and Frenkel, 1976; Dubey *et al*., 1996). Infected cats can shed more than 100 million oocysts in their faeces (Dubey and Frenkel, 1972; Jones and Dubey, 2010) for several weeks and once during their life.

Intermediate hosts, including livestock and wild game, may become infected after ingestion of sporulated oocysts in the environment *(3)* or by oral ingestion of bradyzoites/tissue cysts contained in muscles or primary offal (viscera) of previously infected intermediate hosts - route particularly relevant for omnivores and carnivores e.g., wild boars (*Sus scrofa*) and hunting dogs - or by ingesting tachyzoites.

Humans can become infected by several routes *(11-12)*. The main ones are listed below:

- eating raw or undercooked meat of animals harbouring tissue cysts (bradyzoites);
- eating raw vegetables and fruits or drinking water contaminated with sporulated oocysts or ingesting sporulated oocysts that cats have passed in their faeces, either in a cat litter box or in soil (gardening);
- blood transfusion or organ transplantation (tachyzoites);
- drinking unpasteurized milk from cows, sheep, goats (tachyzoites);
- transplacentally from mother to foetus (tachyzoites).

Given that, the bradyzoites are more resistant to digestion by gastric juices (pepsin–HCl) compared to the tachyzoites, that only survive for a short period of time (up to 2 h) in acid pepsin solutions (Dubey, 1998a), tissue cysts and oocysts are very important in the life cycle of *T. gondii*.

2.3.2.3 Pathogenesis and clinical signs

Toxoplasma gondii can invade many tissues and this process of dissemination is responsible for a variety of clinical manifestations in infected mammals, including humans (Akyar, 2011).

After the ingestion of oocysts or tissue cysts of *T. gondii*, it invades the small intestine and converts to the tachyzoite form, which replicates and disseminates rapidly to almost all tissues, including muscle, brain, eyes, liver, placenta and lungs. In this phase clinical manifestations of toxoplasmosis are caused by cell destruction due to multiplying tachyzoites. Necrosis in intestinal and mesenteric lymph nodes may occur and focal areas of necrosis may develop in many organs (Dubey *et al*., 1996).

Signs may persist for one to twelve weeks; a more severe disease occurs more rarely in immunocompetent individuals than in immunocompromised ones (Tenter *et al*., 2000).

The infection triggers an innate and an adaptive immune response and, therefore, the production of IFN-γ by NK and T cells, responsible for controlling the acute phase (Lieberman and Hunter, 2002). This immune pressure leads to a chronic, usually asymptomatic phase, where this organism persists as bradyzoites in cysts within the brain, the eyes and the muscles.

In the case of primary *T. gondii* infection during pregnancy, tachyzoites present in the blood may cross the placenta and infect the foetus (Jones *et al*. 2003; Montoya and Remington 2008). Congenital toxoplasmosis can have various effects on a growing foetus depending on the trimester during which the maternal infection is acquired (McAuley, 2014). The severity of clinical disease in congenitally infected infants is related inversely to the gestational age at the time of the infection (McAuley, 2014). Infection in the first trimester leads to more severe manifestations including miscarriage, stillbirth, or serious foetal damage, such as retinochoroiditis, endocranial calcification, hydrocephaly, and microcephaly (Jones *et al*., 2009). In the later stages of pregnancy, on the other hand, *T. gondii* infections are sub-clinical, even though retinochoroiditis and neurological disorders are sometimes found (Bossi and Bricaire, 2004). The characteristic triad of congenital toxoplasmosis (chorioretinitis, hydrocephalus, and cerebral calcifications) most commonly identifies the presence of active congenital disease (Hampton, 2015).

Despite affecting multiple tissues, the most common clinical manifestations of toxoplasmosis involve the brain and eye. Thus, even in adults, primary infection can present as chorioretinitis and, in immunocompromised patients with defects in T-cell function, such as during HIV infection or immunosuppressive therapy, the reactivation of cysts in the brain can lead to toxoplasmic encephalitis (TE) (Luft *et al*., 1983, 1984; Wong *et al*., 1984; Israelski *et al*., 1993). The clinical presentation often includes focal encephalitis with headache, confusion, motor weakness and fever and, if not treated, can progress to seizures and coma (Luft *et al*., 1983; Wong *et al*., 1984). Speech abnormalities and hemiparesis are the most common focal neurological findings (Luft *et al*., 1983). In addition, numerous studies have tried to link chronic toxoplasmosis with schizophrenia and bipolar disorders (Flegr, 2015; Chaudhury and Ramana, 2019); this hypothesis has yet to be confirmed.

Also, there are multiple instances of parasites that affect the nervous system of their hosts to alter behaviour and promote predation of intermediate hosts (Kavaliers and Colwell, 1995). Interestingly, behavioural studies using mice and rats indicated that chronic *T. gondii* infection results in a specific

switch from an aversion to cat urine to an attraction, presumably a change in behaviour that would lead to increased predation of infected rodents by cats (Berdoy *et al*., 2000; Vyas *et al*., 2007).
In addition, virulence experiments defined in mouse models after the intraperitoneal inoculation of a given number of tachyzoites have shown that Type I isolates are highly virulent, leading to the death of mice less than 10 days after the inoculation of <10 tachyzoites, while Type II or III strains are considered less virulent strains, allowing survival after the inoculation with >103 tachyzoites (Robert-Gangneux and Dardè, 2012).

2.3.2.4 Epidemiology

A factor to consider in the transmission of toxoplasmosis is the low infectious dose, although it has not yet been estimated (Dubey *et al*., 1996; Fayer *et al*., 2004). *Toxoplasma* oocysts are unsporulated when shed in the cat's faeces and sporulate and become infective in about 1-5 days depending on the environmental conditions. *Toxoplasma* oocysts can survive in the environment for long periods and are highly resistant to disinfectants, i.e., they are highly resistant to chlorine disinfection (Jones and Dubey, 2010). As to freezing conditions, oocysts can survive at -21°C for 28 days (Frenkel and Dubey, 1973) but are killed by temperatures above 60°C (Dubey, 1998b). Lindsay *et al*. (2008) inoculated raspberries with 5×10^4 oocysts of the *T. gondii* VEG strain exposed to high-pressure processing (HPP) 500, 400, 340, 300, 270, 250, 200, 100 MPa for 60 s and showed 340 MPa resulted in the oocysts becoming non-infectious for mice. *Toxoplasma* oocysts on raspberries irradiated with gamma irradiation with doses of ≥0.4 kGy (cesium-137) failed to cause infections in mice (Dubey *et al*., 1998). Ultraviolet radiation also will inactivate oocysts, depending on the dose (Wainwright *et al*., 2007; Dumètre *et al*., 2008).

Humans and foodborne outbreaks caused by the consumption of fresh produce - Toxoplasma gondii is a major pathogen of global public health concern. In humans, toxoplasmosis has been found worldwide and it is estimated that about one-third of the world's population is infected with latent toxoplasmosis, with the majority of cases being asymptomatic (Pereira *et al*. 2010).
The prevalence varies widely between different countries (from 10 to 80%) but also within the same country or even between different communities in the same region (Pappas *et al*., 2009). Low, moderate and high prevalences have been registered in different areas of the world (reviewed by Robert-Gangneux and Dardè, 2012):

- Low seroprevalence (from 10 to 30%) have been observed in North America, in South East Asia, in Northern Europe, and in Sahelian countries of Africa.

- Moderate seroprevalences (30 to 50%) have been found in countries of Central and Southern Europe.
- High seroprevalences have been found Latin America and in tropical African countries.

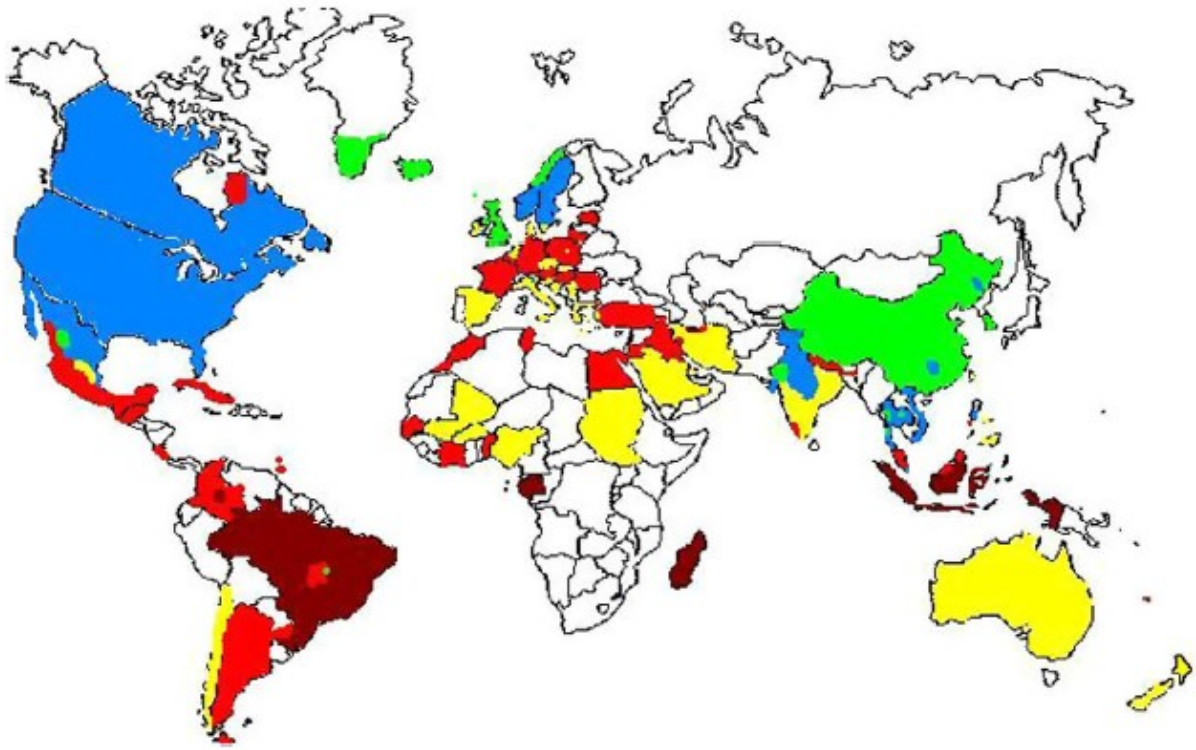


Figure 14. Global status of *T. gondii* seroprevalence. Dark red equals prevalence above 60%, light red equals 40-60%, yellow 20-40%, blue 10-20% and green equals prevalence <10%. (source: Pappas *et al*., 2009)

As to seroprevalence in females of reproductive age or pregnant women, serological screenings are not necessarily the rule and differ among countries according to the prevalence of *Toxoplasma* and health care policies. Most studies carried out in Latin American countries, Eastern and Central Europe, the Middle East, and parts of south-east Asia and Africa show significantly high seropositivity rates (Pappas *et al*., 2009). In the USA, the seroprevalence was estimated to be around 11% (Dubey and Jones, 2008).

As for Europe, studies in women of childbearing age or in pregnancy show that the seroprevalence ranges widely between 9.1% and 57.6% with peaks reported in Poland and in Romania (Pappas *et al*., 2009). The prevalence of congenitally acquired toxoplasmosis, reported as cases per 10000 live births, has been reported for France (2.9/10000) (Villena *et al*., 2010), Denmark (1.6/10000) (Röser *et al*., 2010). In Europe, the type II lineage is the predominant one followed by type I and occasionally type III. In France, type II represents more than 90% of isolates from both humans and animals. Type

III may be more frequently encountered in Southern Europe (reviewed by Robert-Gangneux and Dardè, 2012).

In Italy, seroprevalence studies conducted on pregnant women have shown various seroprevalences ranging between 17.4% and 34.4% (Pappas *et al*., 2009). A study conducted on pregnant women in a population from Palermo has estimated a prevalence of anti-Toxo IgG antibodies of 17.97% (152 on 846) (Puccio *et al*., 2014) and a recent serological study carried out to assess the prevalence of *T. gondii* in two populations of women of childbearing age in Siena (Tuscany, Central Italy) and Bari (Apulia, Southern Italy) between 2013 and 2017 and in a group of pregnant women in Bari in 2016-2017 has reported seroprevalences of 12.4% and 22.4% in Bari and Siena, respectively, and 13.8% among the pregnant women tested (Fanigliulo *et al*., 2020).

In an article by Pinto-Ferreira *et al*. (2019), covering the temporal progression of probable sources of infection and transmission routes for global human toxoplasmosis outbreaks, 33 articles covering 34 reports of outbreaks of acute toxoplasmosis worldwide between 1967 and 2018 were selected. As to the implicated sources of infection, between the 1960s and the 1990s, toxoplasmosis outbreaks were mainly caused by the ingestion of cysts in meat and meat derivatives, in the 1980s, by the consumption of milk contaminated with tachyzoites, in 2000, by the presence of oocysts in water, sand, and soil and, in 2010, by the consumption of raw fruits and vegetables contaminated with *Toxoplasma* oocysts (Pinto-Ferreira *et al*., 2019). The latter circumstance might reflect the increased consumption of healthy foods, e.g., fruits and vegetables, registered in the past 20 years (WHO, 2019). Among the different routes of transmission of *T. gondii,* consumption of raw vegetables and fruits contaminated with *Toxoplasma* oocysts represents an important one that may lead to foodborne outbreaks (Hussain *et al*., 2017).

The highest concentration of reported outbreaks occurred in Brazil; as to the ones linked to the ingestion of contaminated fresh produce, in 2009, 11 cases of acute toxoplasmosis in a factory in São Paulo, Brazil, were linked to the consumption of contaminated vegetables (Ekman *et al*., 2012) whereas, in 2013, an outbreak of toxoplasmosis involving 73 cases in the municipality of Ponta de Pedras, Brazil, was associated with the consumption of imported Açaí (Morais *et al*., 2016).

The geographic distribution of the reported *Toxoplasma* outbreaks and related causes is summarized in **Figure 15**.

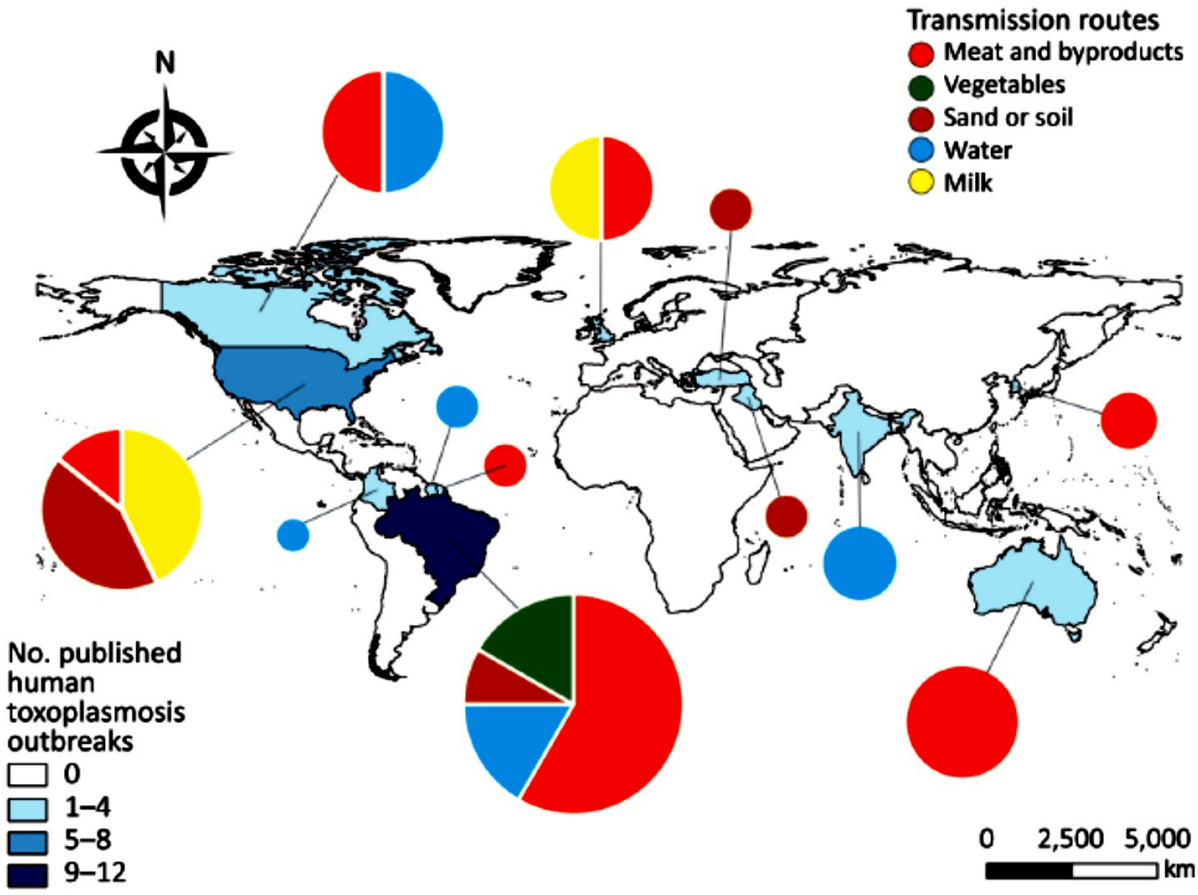


Figure 15. Geographic distribution of the 34 outbreaks of human toxoplasmosis reported worldwide and related transmission routes (indicated by color). Circle size corresponds to the number of outbreaks (Source: Pinto-Ferreira *et al*., 2019).

Fresh produce - *Toxoplasma gondii* has been recorded in fresh produce with various prevalences in several countries all over the world including Canada where *T. gondii* DNA has been identified in 3 (0.26%) samples of baby spinach out of 1171 samples of pre-packaged leafy green vegetables (Lalonde and Gajadhar, 2016), Saudi Arabia where 6.6% of the leafy vegetable examined were found contaminated by *T. gondii* (Al-Megrin, 2010), Pakistan where 1.9% of the vegetables were found positive for *T. gondii* (ul-Haq *et al*., 2014). Among South American countries, *T. gondii* was detected in Brazil in fresh vegetables (3.8%) (Marchioro *et al*., 2016) and in vegetables grown in organic gardens (Ferreira *et al*., 2018), and in Colombia where of the 120 strawberry samples analysed, 5% tested positive for *T. gondii* DNA (Ortiz Pineda *et al*., 2020).

This protozoan parasite has also been registered in fresh produce in European countries, such as Czech Republic (Slany *et al*., 2019) and Poland (Lass *et al*., 2012), with a positivity rate of 9.6% and 9.7%, respectively. More recently, the presence of this species was detected 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 Italy, *T. gondii* was detected in 0.8% of the RTE salad samples examined (Caradonna *et al*., 2017). The contamination of vegetables and fruits with *T. gondii* is summarized in **Table 5**.

Location	Detection method	Vegetable or fruit item	No. of samples tested	No. of positive samples (%)	*Toxoplasma gondii* genotypes identified
Brazil	PCR	Smooth lettuce	62	1 (0.6)	Toxo4-5 D (1)
		Crisp head lettuce	106	4 (3.7)	B22-23 D (4)
		Chicory	40	2 (5.0)	B22-23 D (1); Toxo4-5
		Rocket	7	1 (14.3)	B22-23 D (1)
		Parsley	5	1 (20.0)	B22-23 D (1)
Brazil	PCR	Vegetables	21	3 (14.3)	N/A (3)
China	Quantitative real-time PCR (qPCR)	Lettuce	71	5 (7.0)	Type I (4); Type II (1)
		Spinach	50	2 (4.0)	Type I (2)
		Pak choi	34	1 (2.9)	Type I (1)
		Chinese cabbage	26	0	
		Rape	22	1 (4.5)	Type II (1)
		Asparagus	18	0	
		Chrysanthemum coronarium	16	0	
		Endive	14	0	
		Chinese chives	11	0	
		Cabbage	9	0	
		Red cabbage	8	1 (12.5)	Type II (1)
Czech Republic	Triplex real time PCR	Carrots	93	7 (7.5)	
		Cucumbers	109	13 (11.9)	Type II (5)
		Salads	90	8 (8.9)	Type II (2)
Italy	qPCR	Ready-to-eat packaged salad	648	5 (0.8)	Type I (5)
Poland	qPCR	Strawberries	60	0	
		Radish	60	3 (5.0)	Type I (2); Type II (1)
		Carrot	46	9 (19.6)	Type I (3); Type II (1)
		Lettuce	50	9 (18.0)	Type I (1)
Total			1676	63 (3.8)	

Table 5. Contamination of vegetables and fruits with *T. gondii* (source: Junqiang *et al*., 2020 and related references).

2.3.2.5 Detection methods in vegetables

Toxoplasma gondii oocysts can be recovered from fresh produce by centrifugation (Al-Megrin, 2010; Lalonde and Gajadhar, 2016; Marchioro *et al*., 2016) or flocculation (Lass *et al*., 2012) of the washing solutions. Different washing solutions are employed for concentrating the oocysts *i*) physiological saline solution (Al-Megrin, 2010), *ii*) glycine buffer solution followed by flotation (Lalonde and Gajadhar, 2016), *iii*) Tween 80 solution (Marchioro *et al*., 2016), iv) Alconox®, a commercial laboratory detergent (Shields *et al*., 2012; Temesgen *et al*., 2019a). In the flocculation method described by Lass *et al*. (2012) different solutions such as calcium carbonate ($CaCO_3$) and sodium bicarbonate ($NaHCO_3$) are employed to obtain a pellet by precipitation. The concentration of the

oocysts may be performed by centrifugation (Lass *et al*., 2012; Lalonde and Gajadhar, 2016) or filtration followed by centrifugation (Al-Megrin, 2010; Marchioro *et al*., 2016).

After recovery from fresh produce, the oocysts can be identified by light microscopy adding a drop of lugol iodine (Al-Megrin, 2010). An immunomagnetic separation assay (IMS Toxo) targeting the cell wall of *T. gondii* oocysts has been established (Hohweyer *et al*., 2016), however, it is not commercially available.

Several molecular methods are available to detect *T. gondii* DNA: PCR-RFLP (Lass *et al*., 2012), conventional PCR (Marchioro *et al*., 2016), *q*PCR (Lalonde and Gajadhar, 2016), and a recently developed LAMP assay used to detect *T. gondii* in experimentally contaminated RTE baby lettuce (Lalle *et al*., 2018). In addition, a novel multiplex real-time *q*PCR for the detection of *T. gondii* as well as *E. multilocularis* and *C. cayetanensis* on berries has recently been developed. With this technique, a product of 162 bp from the 529 bp repeat of *T. gondii* is amplified (Opsteegh *et al*., 2010; Temesgen *et al*., 2019b).

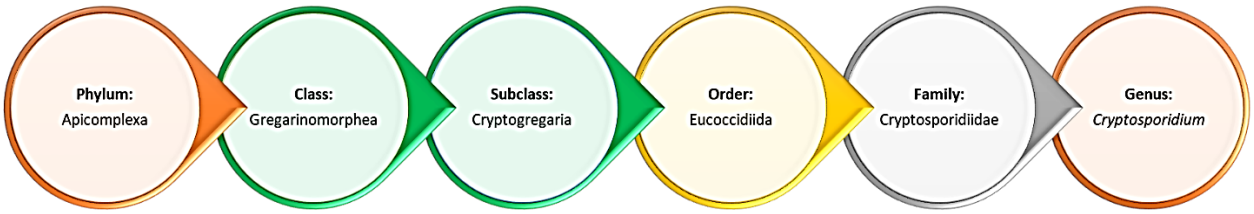
The genes mainly used for the detection of *T. gondii* are the B1 gene (Lass *et al*., 2012; Marchioro *et al*., 2016) and 18S rDNA (Lalonde and Gajadhar, 2016).

To date, there are no standard detection methods for *T. gondii* oocysts in fresh produce (EFSA, 2018).

2.3.3 *CRYPTOSPORIDIUM* SPP.

2.3.3.1 Classification and morphology

Cryptosporidium belongs to the Phylum Apicomplexa, class Gregarinomorphea, subclass Cryptogregaria, order Eucoccidiida, family Cryptosporidiidae.



Recent molecular studies indicate that *Cryptosporidium* is more closely related to the primitive apicomplexan gregarine parasites rather than to coccidians. The similarities between *Cryptosporidium* and gregarines have served as basis for the formal transfer of *Cryptosporidium* from subclass Coccidia, class Coccidiomorphea to a new subclass, Cryptogregaria, within class Gregarinomorphea. The genus *Cryptosporidium* is currently the only member of Cryptogregaria (Ryan *et al*., 2016).

Currently, a total of 42 *Cryptosporidium* species are recognized (Feng *et al*., 2018; Zahedi and Ryan., 2020) (**Table 6**).

Species name	Major host(s)
1. *C. ornithophilus*	Birds
2. *C. avium*	Birds
3. *C. galli*	Birds
4. *C. baileyi*	Birds
5. *C. meleagridis*	Birds and humans
6. *C. felis*	Cats
7. *C. ryanae*	Cattle
8. *C. bovis*	Cattle
9. *C. andersoni*	Cattle
10. *C. canis*	Dogs
11. *C. bollandi*	Fish
12. *C. huwi*	Fish
13. *C. molnari*	Fish
14. *C. wrairi*	Guinea pigs
15. *C. erinacei*	Hedgehogs, horses
16. *C. viatorum*	Humans
17. *C. hominis*	Humans
18. *C. varanii*	Lizards
19. *C. macropodum*	Marsupials
20. *C. fayeri*	Marsupials
21. *C. scrofarum*	Pigs
22. *C. suis*	Pigs
23. *C. proventriculi*	Psittaciformes birds
24. *C. cuniculus*	Rabbits
25. *C. testudinis*	Reptiles (mainly tortoise)
26. *C. ducismarci*	Reptiles (mainly tortoise)
27. *C. microti*	Rodents
28. *C. alticolis*	Rodents
29. *C. occultus*	Rodents
30. *C. homai*	Rodents
31. *C. proliferans*	Rodents
32. *C. tyzzeri*	Rodents
33. *C. muris*	Rodents
34. *C. apodemi*	Rodents (mainly mouse)
35. *C. ditrichi*	Rodents (mainly mouse)
36. *C. parvum*	Ruminants
37. *C. ubiquitum*	Ruminants, rodents, primates
38. *C. xiaoi*	Sheep and goats
39. *C. serpentis*	Snakes and lizards
40. *C. rubeyi*	Squirrels
41. *C. fragile*	Toads
42. *C. scophthalmi*	Turbot

Table 6. Recognised *Cryptosporidium* species (modified from **Zahedi and Ryan., 2020**)

Cryptosporidium species infect a wide variety of hosts, including humans who are mostly infected with *C. parvum, C. hominis,* and *C. meleagridis; however, C. parvum* and *C. hominis* are held responsible for about 90% of cryptosporidiosis cases (Xiao and Ryan, 2004).

The zoonotic *Cryptosporidium* species are summarized in **table 7**.

***Cryptosporidium* species**	**Zoonotic importance**
C. hominis	Main *Cryptosporidium* species infecting humans
C. parvum	Major (most important zoonotic species globally)
C. meleagridis	Major (of zoonotic importance is some regions, especially South America)
C. cuniculus	Responsible for several waterborne outbreaks and sporadic cases of cryptosporidiosis in the UK and has been identified in a human in Australia
C. muris	Numerous reports in humans
C. felis	Numerous reports in humans
C. canis	Numerous reports in humans
C. fayeri	Minor
C. andersoni	Minor
C. tyzerri	Occasionally reported in humans
C. bovis	Occasionally reported in humans
C. scrofarum	Occasionally reported in humans
C. suis	Occasionally reported in humans
C. erinacei	One report in humans
C. ubiquitum	Emerging human pathogen

Table 7. *Cryptosporidium* species and their zoonotic importance (modified from Zahedi *et al*., 2016).

Cryptosporidium oocysts can be spherical (4-6 µm in diameter) or oval (7 x 5 µm in diameter), depending on the species, and the oocyst wall presents an inner and an outer layer. Four sporozoites are present in each oocyst (**Figure 16**).

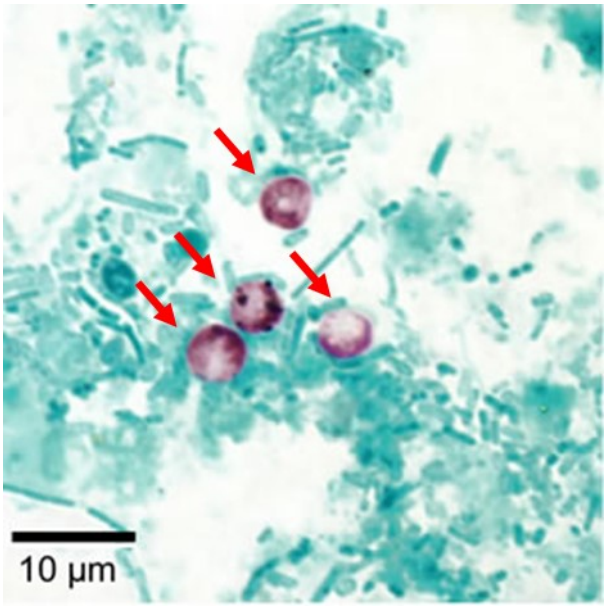


Figure 16. *Cryptosporidium* oocysts. The image shows *C. parvum* oocysts stained with modified acid-fast. Against a blue-green background, the oocysts stand out in a bright red stain. (source: https://www.cdc.gov/dpdx/cryptosporidiosis/index.html).

2.3.3.2 Biology and life cycle

The parasite completes its life cycle within a single host (monoxenous cycle), alternating asexual and sexual reproduction (Tzipori and Ward, 2002). The cycle begins when an appropriate host ingests the oocysts, which contain the infective stages of the parasite. Once ingested *(1)* the oocysts

undergo excystation in the intestinal lumen resulting in release of four motile sporozoites *(2)*, which adhere to and penetrate the intestinal epithelium *(3)*. The sporozoite, after adhering to an enterocyte, is encapsulated by the microvilli into a parasitophorous vacuole *(4)*. The parasitophorous vacuole is intracellular but extracytoplasmic. Inside the vacuole, the sporozoite develops into a trophozoite that undergoes asexual division, schizogony (merogony), to form eight merozoites *(6)*. After asexual reproduction, the sexual stage of development follows and the merozoites differentiate into gamonts *(7)* and gametocytes, either male or female sexual stages, called microgametocytes *(8)* or macrogametocytes *(9)*, respectively. The microgametes fertilize the macrogametocytes and the zygote *(10)* develops into an oocyst containing four sporozoites (sporulated oocyst) *(1)*. The resultant oocysts can be with thick-walled or thin-walled. The thick-walled oocysts (infective oocysts) are shed in the faeces, while the thin-walled oocysts are primarily involved in the process of autoinfection in the gut, increasing the infection level with the release of infective sporozoites (reviewed by Chalmers and Katzer, 2013). Cryptosporidiosis is transmitted primarily through the faecal-oral route, i.e., by the ingestion of viable oocysts of animal and/or human origin, emitted with faeces that contaminate food or water (Tzipori and Ward, 2002) and possibly other routes such as respiratory secretions (Sponseller *et al*., 2014).

The life cycle of *Cryptosporidium* spp. is summarized in **Figure 17**.

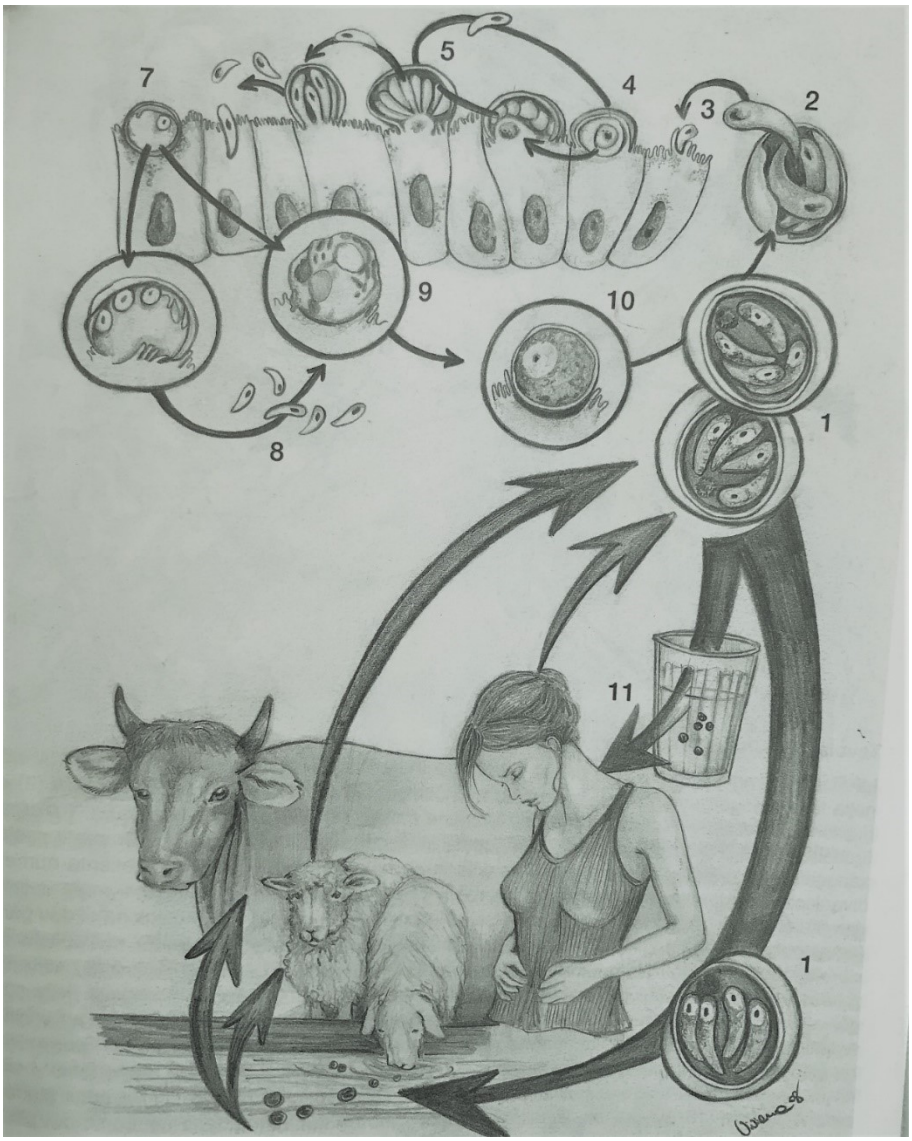


Figure 17. Life cycle of *Cryptosporidium* spp. (source: Taylor *et al*., 2010)

2.3.3.3 Pathogenesis and clinical signs

After invasion, *Cryptosporidium* does not penetrate deep into the tissue but is located in an intracellular/extracytoplasmic parasitophorous vacuole. Interaction between *Cryptosporidium* and the apical surface of the epithelial cell causes barrier disruption and cellular damage which are responsible for altering the function of the intestinal barrier, increasing its permeability, absorption, and secretion of fluid and electrolytes (Certad *et al*., 2017).

Clinical manifestations following infection occur after an incubation period of 2-14 days, and include diarrhoea, as well as abdominal cramps, nausea, vomiting, weight loss, and a low-grade fever (Hunter and Nichols, 2002).

The severity, persistence, and outcome of the infection depend on the immune status of the host (Petry *et al*., 2010):

- In immunocompetent subjects, the infection causes diarrhoea, which usually resolves in 1-3 weeks or runs in an asymptomatic form (Bouzid *et al*., 2013).
- In subjects with poor health or a weakened immune systems (HIV/AIDS, cancer, and transplant patients), *Cryptosporidium* infections are more common and often cause chronic, prolonged forms of disease (Florescu and Sandkovsky, 2016; Wang *et al*., 2018). Complications include biliary tract obstruction, sclerosing cholangitis, papillary stenosis, pancreatitis as well as respiratory tract disease (Hunter and Nichols, 2002; Florescu and Sandkovsky, 2016; Wang *et al*., 2018). Cryptosporidiosis represents one of the most important opportunistic infections for immunocompromised patients; it is, in fact, difficult to treat and can even result in death (Wang *et al*., 2018).

It has been reported that the *Cryptosporidium* infection can be associated with long term health implications i.e., the development of reactive arthropathies, painful, inflamed joints pain and lower back pain, and ocular inflammation (Hay *et al*., 1987; Shepherd *et al*., 1989; Hunter *et al*., 2004; Carter *et al*., 2020).

The Global Burden of Disease (GBD) study has estimated the morbidity and mortality (disease burden) that can be attributed to diarrhoeal disease caused by *Cryptosporidium* infection (GBD, 2015; GBD, 2016; Khalil *et al*., 2018). The disability-adjusted life-years (DALYs), which is the sum of years of life lost and years lived with disability, is used as a metric of disease burden (GBD, 2016).

2.3.3.4 Epidemiology

Cryptosporidium oocysts shed in faeces are infective on excretion and a low dose of 10-100 oocysts can transmit the infection (Vanathy *et al*., 2017a). Factors affecting the survival of *Cryptosporidium* oocysts in the environment are summarized in **Table 8**.

Factors affecting survival of *Cryptosporidium* oocysts						
Environment	**Heat**	**pH**	**Freezing**	**Disinfectant**	**Drying**	**UV light**
Oocysts remain infectious for prolonged periods in cool, damp environments e.g., more than 77 days at less than 10 °C (**Tamburrini and Pozio, 1999**; **World Health Organization, 2004**). One year has been reported in sea water (**Tamburrini and Pozio, 1999**). *Cryptosporidium* oocysts can survive for six months in tap water, river water and cow faeces (**Robertson *et al*., 1992**).	Commercial pasteurization (71.7 °C, 15 s) has been demonstrated to be effective in inactivating *Cryptosporidium* oocysts in apple cider (**Deng and Cliver, 2001**), whereas blanching at 96 °C for 3 min has been shown to inactivate 93% of *Cryptosporidium* oocysts inoculated onto green peppers (**Duhain *et al*., 2012**). No infectivity of oocysts treated at 71.7 °C for 5, 10 and 15 s in either water or milk (**Harp *et al*., 1996**). Heating to 64.2 °C for 2 min reduces to 0% in water or buffer (**Fayer, 1994**). Microwave heating is effective in inactivating 93% of *Cryptosporidium* oocysts inoculated onto green peppers and microwaved in a domestic microwave oven (2450 MHz; 850 W) for 5 min. Pretreatment with chlorine increases the efficacy of inactivation to 98% (**Duhain *et al*., 2012**).	Oocysts lose >85% viability at either 4 °C or 22 °C in orange juice at pH 3.9, or in carbonated beer or cola both at pH 3.9 (**Friedman *et al*., 1997**). Malic, citric and tartaric acids have been found to reduce infectivity by up to 88% (**Kniel *et al*., 2003**). Ethanol and low water activity also reduce infectivity (**Dawson *et al*., 2004**).	Freezing at -70 °C is enough to render oocysts non-infective. Over a 7-day trial, infectivity declined but viable oocysts remained from batches at -15 and -20 °C (**Fayer and Nerad, 1996**).	Very resistant to disinfectants; the oocysts are very resistant to chlorine, commonly used in water system disinfection (**Shrivastava *et al*., 2017**). **Ortega *et al*. (2008)** found that basil and lettuce exposed to relatively low concentrations of gaseous chlorine dioxide for 20 min (CT of 82 mg min/L) led to 2.6–3.31 log reduction in infectivity of *Cryptosporidium* oocysts. Exposure to hydrogen peroxide may inactivate *Cryptosporidium* oocysts (**Kniel *et al*., 2003**). **Slifko *et al*. (2000)** evaluated the effects of high-pressure processing at 550 MPa for 0, 30, 45, 60, 90, and 120 s on *C. parvum* oocysts suspended in apple and orange juice. HHP inactivated C. parvum oocysts by 3.4 log and 4.2 log after 30 s and 60 s of treatment (**Slifko *et al*., 2000**). Ozone is highly effective (**Casemore, 1995**).	Desiccation affects oocysts' viability dramatically: 95% died within 4 h at room temperature (**Deng and Cliver, 1999**). In another study, 10^6 oocysts died within 4 h when dried in air at 18-22 °C (**Robertson *et al*., 1992**).	UV light is effective at inactivating oocysts at current levels used in water treatment (**Hargy *et al*., 2000**).

Table 8. Survival of *Cryptosporidium* oocysts. Adapted and modified from Dawson, 2005 and Gérard *et al*., 2019.

Humans and foodborne outbreaks caused by the consumption of fresh produce - Protozoa of the genus *Cryptosporidium* have a global distribution (Putignani and Menichella, 2010). Although in recent years, the number of reported cases of cryptosporidiosis has increased worldwide, the frequency of infection is likely to be significantly higher than the number of reported cases

(Shrivastava *et al*., 2017). *Cryptosporidium parvum* and *C. hominis*, followed by *C. meleagridis*, are responsible for the majority of infections in humans worldwide. In addition, several other *Cryptosporidium* species have been reported in humans (Zahedi and Ryan, 2020).

In humans, *Cryptosporidium* infection is commonly found in children and immunocompromised individuals and the incidence of cryptosporidiosis is significantly lower in industrialized countries compared to developing countries in which the hygiene standards are generally poor (Shoultz *et al*., 2016; Gerace *et al*., 2019).

Among children from developing countries suffering from diarrhoea, cryptosporidiosis represents between 3% and 13% of the cases whereas in children from developed countries who experience diarrhoea, *Cryptosporidium*-related diarrhoea ranges between 1% and 4% (Shrivastava *et al*., 2017). Cryptosporidiosis in the case of human immunodeficiency virus is registered with the highest prevalence in India and in African countries where it reaches peaks of 56.5% (Vanathy *et al*., 2017b) and 73.6% (Tumwine *et al*., 2005) whereas in developed countries the reported prevalence ranges between 8% and 39% in Europe and between 3.5% and 11.9% in North America (Iqbal *et al*., 2012). The distribution of *Cryptosporidium* in different countries of the world between 1984-2013 is represented in **Figure 18**.

Cryptosporidium is a major cause of waterborne and foodborne outbreaks and to date more than 40 foodborne outbreaks have been documented. *Cryptosporidium parvum* was responsible for 96.5% of foodborne cryptosporidiosis outbreaks worldwide and unpasteurised milk, unpasteurised apple cider, and fresh produce, including salad items, onions, parsley, carrots, are among the most common food items implicated in foodborne outbreaks (Dixon *et al*., 2013; Zahedi and Ryan, 2020). In 2005, an outbreak of diarrhoea linked to the consumption of salad (peeled carrots, grated carrots, red pepper) affected a group of 99 company employees who consumed it at the company canteen near Copenhagen (Ethelberg *et al*., 2009). In 2008, a *C. parvum* outbreak in Sweden was linked to chanterelle sauce with fresh parsley added after the preparation of the sauce (Insulander *et al*., 2008), while in Finland a salad mixture was the suspected vehicle of a *C. parvum* outbreak (Pönka *et al*., 2009).

Outbreaks linked to RTE salads contaminated with *Cryptosporidium* spp. have been documented in Europe. *Cryptosporidium* spp. was reported in 648 cases across England and Scotland during 2012; *C. parvum* was confirmed in 182 cases (McKerr *et al*., 2015). In Finland, *C. parvum* was suspected as the causal agent of five (5.7%) of the 88 outbreaks notified during 2012 (Åberg *et al*., 2015).

The worldwide occurrence of foodborne outbreaks of cryptosporidiosis is represented in **figure 18** (round dots) (Shrivastava *et al*, 2017).

Outbreaks of cryptosporidiosis associated with different food matrices in the period 1984–2017 have been documented by Ahmed and Karanis (2018) and a recent review gives detailed information on the reported foodborne outbreaks of cryptosporidiosis for which molecular data is available in the last 10 years (Zahedi and Ryan, 2020).

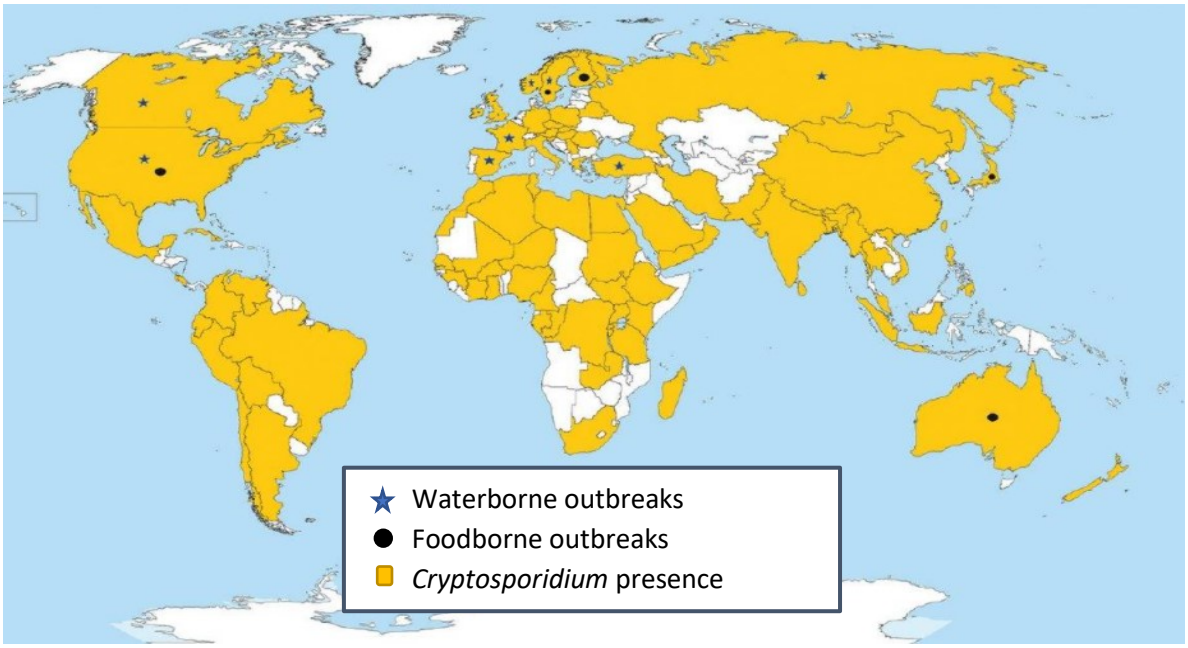


Figure 18. Distribution of *Cryptosporidium* infection in immunocompetent people: distribution of major cases of cryptosporidiosis reported in different countries of the world between 1984-2013. Waterborne outbreaks are represented with star symbol, round dots represent foodborne outbreaks, yellow color in the map represents the presence of cryptosporidiosis and white color represents no such reports are present (source: Shrivastava *et al*, 2017).

Fresh produce - Surveillance studies have reported the presence of *Cryptosporidium* in fresh produce worldwide (Dixon *et al*., 2013; Li *et al*., 2020). The contamination of vegetables and fruits with *Cryptosporidium* spp. has been documented in many countries and the average prevalence is estimated to be 6.0%. Among the *Cryptosporidium* species, *C. parvum, C. hominis*, and *C. ubiquitum* were detected in contaminated vegetable and fruit samples: *C. parvum* was detected in fresh vegetables in Ghana (Duedu *et al*., 2014), *C. parvum* and *C. ubiquitum* were isolated in RTE salads in Italy (Caradonna *et al*., 2017), *C. parvum* or *C. hominis* were found on fresh produce samples in Poland (Rzeżutka *et al*., 2010) and, recently, *C. parvum* was identified on the surfaces of vegetables and fruits in China (Li *et al*., 2019). Two recent reviews provide detailed information on the contamination of different food matrices, including vegetable and fruits, with *Cryptosporidium* and its geographical distribution (Ahmed and Karanis, 2018; Li *et al*., 2020).

2.3.3.5 Detection methods in fresh produce

Elution, extraction, isolation and detection of *Cryptosporidium* species (by non-molecular or molecular methods) are the usual steps for detecting *Cryptosporidium* in or on food items (Ahmed and Karanis, 2018).

Elution (i.e., extraction/wash) is the first step in any protocol for detecting *Cryptosporidium* oocysts that have been suspected in food and it is, in fact, crucial for the success of all the subsequent steps. Different buffers, including Alconox®, Glycine 1 M solution, 1 M sodium bicarbonate, PBS pH 7, deionized water, have been successfully used for recovering oocysts from fresh produce (Shields *et al*., 2012); Alconox® was superior to all the other reagents. Also, different methods were used to elute oocysts, i.e., stomaching, orbital shaking, rolling, rotating drum, sonication, kneading and pulsification, and, among those, stomaching is considered an effective method to extract oocysts from the surface of different food matrices including fresh produce (lettuce and raspberries) (reviewed by Ahmed and Karanis, 2018).

The most sensitive methods for the detection of *Cryptosporidium* in food products are based on the separation of the oocysts from the sample matrix, employing methods such as flotation or immunomagnetic separation (IMS), and detection by immunofluorescence microscopy (IFM) and molecular methods (EFSA, 2018). The ISO method (ISO 2016) currently represents the gold standard for the detection and enumeration of *Cryptosporidium* and *Giardia* in foodstuff and is based on IMS and IFA. However, with this method it is not possible to determine the species or genotypes/Assemblages of the *Cryptosporidium* oocysts and *Giardia* cysts; in order to do that, this method needs to be combined with molecular tools (Ahmed and Karanis, 2018; EFSA, 2018)

Different molecular approaches and PCR types have been tested over time in order to choose the best PCR condition for each food matrix (EFSA, 2018). Recently, various molecular methods have been developed for the identification of *Cryptosporidium* in fresh produce such as nested-PCR assays (Ryan at al., 2003; Ripabelli *et al*., 2004; Dixon *et al*., 2013) and *q*PCR assays (Lalonde and Gajadhar, 2011, 2016). Among the most common targets for *Cryptosporidium* species identification are: *cowp* gene (Ripabelli *et al*., 2004), *18S rDNA* gene (Ryan at al., 2003; Lalonde and Gajadhar, 2011; Dixon *et al*., 2013), and the gene encoding the glycoprotein 60 (gp60) protein is frequently used as a target for genotyping within the species.

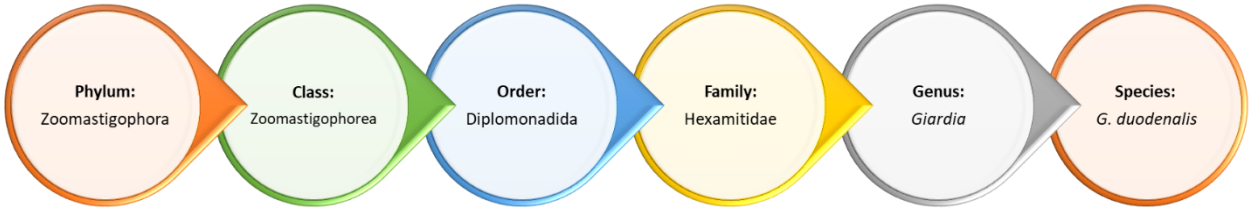
Additional molecular approaches for the detection of *Cryptosporidium* in food matrices are florescent *in situ* hybridization (FISH) and loop-mediated isothermal amplification (LAMP).

Detailed information on the methods and techniques for the detection of *Cryptosporidium* in food samples are available in literature (Ahmed and Karanis, 2018; EFSA, 2018).

2.3.4 *GIARDIA DUODENALIS*

2.3.4.1 Classification and morphology

Giardia is an intestinal protozoan flagellate parasite belonging to the Phylum Zoomastigophora, Class Zoomastigophorea, Order Diplomonadida and Family Hexamitidae.



Giardia was firstly observed by Van Leeuwenhoek in 1681 and fully described by Lambi in 1859 (Ortega and Adam, 1997).

Species of the genus *Giardia* infect numerous hosts, ranging from a wide range of mammals, including humans, to amphibians and birds. A total of eight *Giardia* species are currently recognised: *Giardia duodenalis* (syn. *Giardia intestinalis* and *Giardia lamblia*), which infects humans and animals, *Giardia agilis*, *Giardia ardeae*, *Giardia psittaci*, *Giardia muris*, *Giardia microti*, *Giardia peramelis* and *Giardia cricetidarum*, which infect non-human hosts including amphibians, birds, rodents and marsupials (Ryan and Zahedi, 2019).

The species *G. duodenalis* includes eight genotypes known as Assemblages designated A-H. Some authors have recently suggested these Assemblages may represent separate species, but their recognition has remained controversial (Thompson and Ash, 2019) (**Table 9**). Assemblages A and B have been reported in humans and a wide range of other mammalian hosts and are potentially zoonotic (Feng and Xiao, 2011). There are three recognized sub-Assemblages within Assemblage A (AI, AII, AIII), with sub-Assemblage AI mostly found in animals (e.g., cattle, water buffalo, cats, pigs, sheep) as well as in humans, sub-Assemblage AII predominantly reported in humans, and AIII mainly detected in wild ruminants (Sprong *et al*., 2009). In addition, numerous Assemblage B subtypes have been identified (Feng and Xiao, 2011; Xiao and Feng, 2017). The zoonotic potential of such sub-Assemblages needs to be further clarified. Other *G. duodenalis* Assemblages are host-adapted, with Assemblages C and D being found mainly in dogs and wild canines, Assemblage E in hoofed livestock and wildlife, Assemblage F in cats, Assemblage G in rodents, and Assemblage H in seals (Heyworth, 2016; Cacciò *et al*., 2018) (**Table 9**).

Species	Major host(s)
Assemblage A (=*G. duodenalis* sensu stricto?[b])	Humans, nonhuman primates, domestic and wild ruminants, alpacas, pigs, horses, domestic and wild canines, cats, ferrets, rodents, marsupials, other mammals
Assemblage B (=*G. enterica*?[b])	Humans, nonhuman primates, cattle, dogs, horses, rabbits, beavers, muskrats
Assemblage C (=*G. canis*?[b])	Domestic and wild canines
Assemblage D (=*G. canis*?[b])	Domestic and wild canines
Assemblage E (=*G. bovis*?[b])	Domestic ruminants, pigs
Assemblage F (=*G. cati*?[b])	Cats
Assemblage G (=*G. simondi*?[b])	Mice, rats
Assemblage H	Seals

Table 9. *Giardia duodenalis* Assemblages (source: **Feng and Xiao, 2011**).

Giardia has two morphological stages:

- the trophozoite or “vegetative form” is found in the intestinal lumen of the host. The trophozoites are approximately 12 to 15 µm long and 5 to 9 µm wide. They are pear-shaped with a rounded anterior end and a pointed posterior end. The dorsal surface is convex whereas the ventral one is concave. They have a ventral or adhesive disk, that can adhere to the intestinal cells. They have four pairs of flagella (2 anterior, 2 posterior, 2 caudal and 2 ventral) and two diploid nuclei (reviewed by Adam, 2001) (**Figure 19a**).

The cyst or “resistant form” oval in shape and measure approximately 8-12 µm in length x 7-10 µm in width. The cyst wall is 0.3 to 0.5 µm thick. Into the cytoplasm there are four nuclei clustered in pairs at one end and axonemes that run diagonally through the cyst (Scaglia *et al*., 1987) (**Figure 19b**).

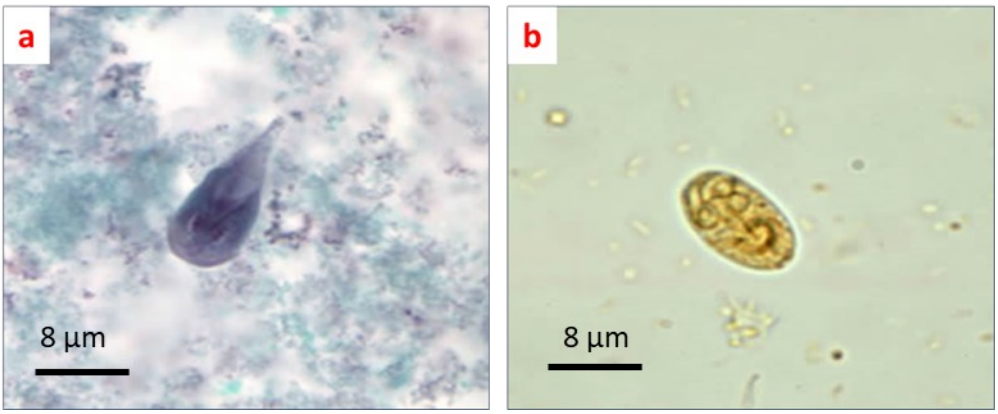


Figure 19. Trophozoite (a) and cyst (b) of *G*. *duodenalis* stained with trichrome and iodine (source: CDC, 2015)

2.3.4.2 Biology and life cycle

Giardia duodenalis has a direct life cycle and infection occurs when a susceptible host ingests the cysts, infective stages of the parasite *(1)*. After exposure to the acidic environment of the stomach, excystation occurs in the small intestine and each cyst releases two trophozoites *(2)*. The trophozoites remain in the lumen of the proximal small bowel (mostly in the mid-jejunum) and adhere to the intestinal mucosa by their disks *(3)* (reviewed by Adam, 2001). Trophozoites reproduce asexually by binary fission *(4)* in response to the presence of bile salts and slightly alkaline pH (Ankarklev *et al*., 2010). Encystation occurs as the parasites transit toward the colon, and the cysts are passed into the faeces *(4-5)*. The cysts are the resistant forms found in the faeces and they are immediately infectious when passed into the faeces. Transmission of giardiasis occurs through the faecal-oral route and it may be either direct (i.e., person-to-person, animal-to-animal or zoonotic) or indirect (i.e., waterborne or foodborne). Sexual transmission of giardiasis has also been documented (Escobedo *et al*., 2014).

The life cycle of *G*. *duodenalis* is summarized in **Figure 20**.

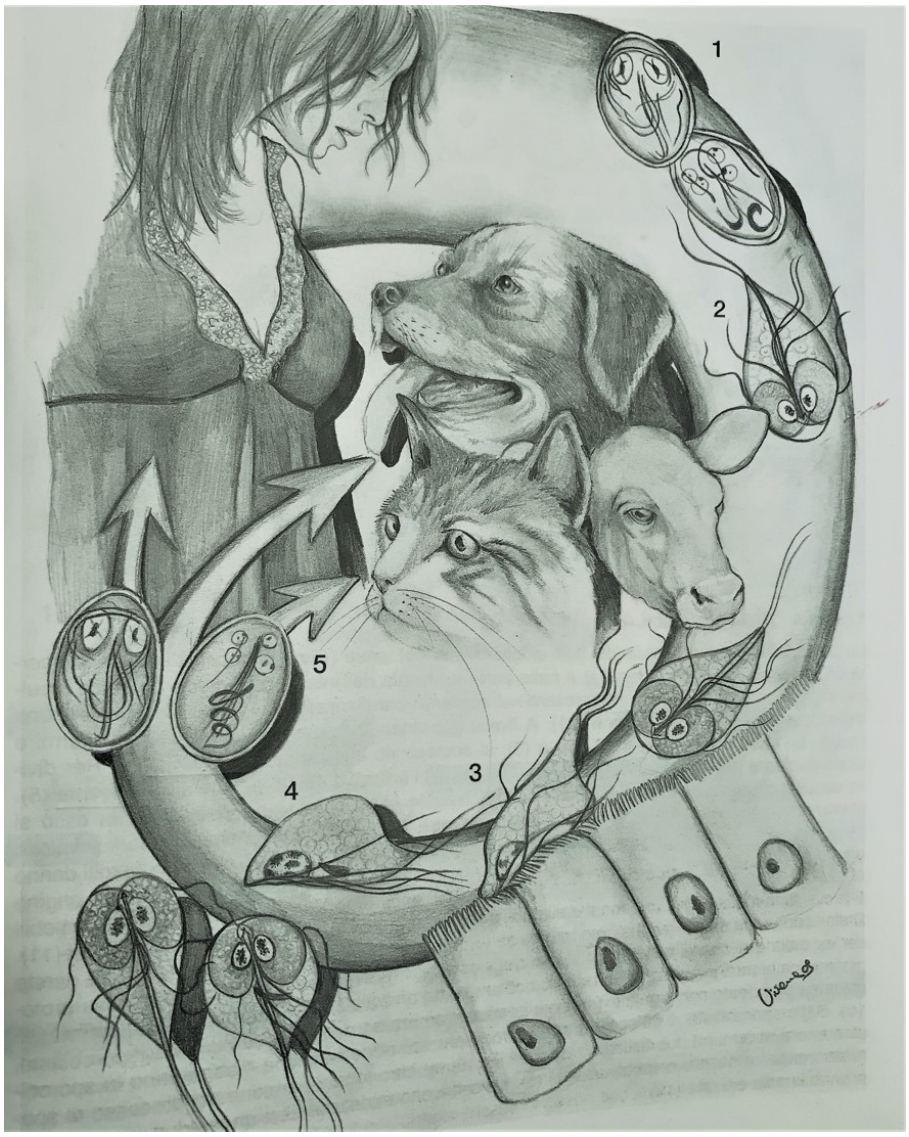


Figure 20. Life cycle of *G. duodenalis* (source: Taylor *et al*., 2010).

2.3.4.3 Pathogenesis and clinical signs

Giardia duodenalis is a non-invasive parasite which colonizes the lumen and epithelial surface of the small intestine (Certad *et al*., 2017). The trophozoites covering the epithelial cells lining the duodenum and the jejunum with their ventral sucking disk are responsible for the shortening of microvilli, cells' apoptosis and barrier dysfunction, which contribute to diarrhoea (Certad *et al*., 2017; Allain and Buret, 2020). In addition, they create a physical barrier between the enterocytes and the intestinal lumen, interfering with nutrient absorption (Adam, 2001), and use the host's nutrients for their own sustenance and growth: glucose appears to be the primary energy source, but also amino acids such as alanine, arginine and aspartate (Adam, 2001). Animal models suggest that *Giardia* is incapable of surviving in the small bowel in the absence of bile acids.

Giardia duodenalis trophozoites have occasionally been identified in the stomach, distal small intestine, caecum, and pancreas (Halliez and Buret, 2013).

The symptoms of giardiasis set in following a 1-2 week incubation period. The main symptoms associated with the infection are diarrhoea, abdominal pain, flatulence, bloating, nausea, malabsorption, weight loss and vomiting (Certad *et al*., 2017; Allain and Buret, 2020). Fever is occasionally present at the beginning of the infection (Ortega and Adam, 1997).

An acute phase of giardiasis generally lasts for 1-3 weeks, after which it may resolve spontaneously or last for months. Patients with chronic giardiasis present the following symptoms: chronic diarrhoea, profound malaise, and diffuse epigastric and abdominal discomfort. Chronic giardiasis is also responsible for malabsorption of lactose, vitamin B12, and fat-soluble vitamins, which results in weight loss, nutritional deficiencies, and failure to thrive in children (Gardner and Hill, 2001).

Children and immunocompromised subjects are the most susceptible to symptoms (Thompson and Ash, 2016).

The post-infection phase can be associated with intestinal e.g., irritable bowel syndrome and functional dyspepsia and/or extra-intestinal complications (Leung *et al*., 2019; Allain and Buret, 2020). The causes of such extra-intestinal complications have not yet been established (Halliez and Buret, 2013).

2.3.4.4 Epidemiology

Giardia cysts are infectious when shed in the faeces and immediately infective when excreted (Dawson, 2005). The infectious dose is low: humans can be infected with as few as 10 cysts (Ortega and Adam, 1997). The factors affecting the survival of *Giardia* cysts are summarized in **Table 10**.

Factors affecting survival of *Giardia* cysts						
Environment	**Heat**	**pH**	**Freezing**	**Disinfectant**	**Drying**	**UV light**
Infective cysts survive >2 weeks in a cool moist environment. Vegetative trophozoites do not survive outside the host (**Casemore, 1995**; **Smith, 1993**; **Meyer and Jarroll, 1980**).	Heating regime of 71.7 °C for 15 s will destroy sufficient numbers of cysts (**WHO, 2004**). It has been shown that air-drying of *Giardia* cysts on lettuce results in viability abrogation within less than 24 h with almost 50% die-off of Giardia cysts recorded within the first 24 h (**Utaaker *et al.*, 2017**).	No information.	Cysts killed at -18 °C after 1 h (**Mahbubani *et al.*, 1991**).	Resistant to chlorine-routine. Chlorination of water usually has no effect (depends on time and temperature). Susceptible to phenol-based commercially available disinfectants (**Smith, 1993**; **Lee, 1992**). Undiluted vinegar resulted in complete inactivation of the cysts following incubation for 60 min at 21 °C corresponding to 5.7 log reduction (**Costa *et al.*, 2009**).	No information.	Work with *G. muris* infectivity suggest that UV doses of 2-3 mJ cm^{-2} can inactivate 4 log 10 of cysts (**Hayes *et al.*, 2003**). The sensitivity of *Giardia* to UV light has resulted in this becoming a standard treatment for drinking water.

Table 10. Survival of *Giardia* cysts. Adapted and modified from Dawson, 2005 and Gérard *et al*., 2019.

Humans and foodborne outbreaks caused by the consumption of fresh produce - Giardia duodenalis is one of the most common intestinal parasites in humans and has a global distribution with an estimated 200 million people infected (Certad *et al*., 2017). Prevalence of giardiasis is very high in developing countries, where it reaches peaks of 30%, whereas it ranges between 2% and 7% in developed countries (Dixon *et al*., 2011). High-risk groups include children, the elderly, travellers, and immunocompromised individuals (Cacciò *et al*., 2018; Leung *et al*., 2019).

In Europe, between 2013 and 2017 giardiasis cases were reported by 24 countries, with an overall rate of 5.8 per 100,000 population (**Table 11**). The highest number of confirmed cases was registered in the United Kingdom, followed by Germany and the majority (60.1%) of giardiasis cases were domestically acquired, with the exception of three Nordic countries (Iceland, Norway and Sweden), where 71%–83% of the cases were travel-associated.

Country	2013		2014		2015		2016		2017			
	Number	Rate	Number	Rate	Number	Rate	Number	Rate	Confirmed cases	Rate	ASR	Reported cases
Austria	.	.	.	.	.	.	.	.	.	.	.	.
Belgium	1 220	-	1 144	-	1270	11.3	1 998	17.7	1 996	17.6	17.5	1 996
Bulgaria	1 873	25.7	1 731	23.9	1245	17.3	1 367	19.1	788	11.1	12.5	788
Croatia	0	0.0	80	1.9	93	2.2	50	1.2	51	1.2	1.3	54
Cyprus	3	0.3	3	0.3	6	0.7	1	0.1	5	0.6	0.6	5
Czech Republic	46	0.4	42	0.4	33	0.3	45	0.4	28	0.3	0.3	28
Denmark	.	.	.	.	.	.	.	.	.	.	.	.
Estonia	195	14.8	221	16.8	181	13.8	187	14.2	161	12.2	12.3	161
Finland	336	6.2	287	5.3	259	4.7	282	5.1	278	5.1	5.3	278
France	.	.	.	.	.	.	.	.	.	.	.	.
Germany	4 107	5.1	4 013	5.0	3 583	4.4	3 479	4.2	3 329	4.0	4.2	3 338
Greece	.	.	.	.	.	.	.	.	.	.	.	.
Hungary	59	0.6	59	0.6	130	1.3	108	1.1	73	0.7	0.8	74
Iceland	20	6.2	22	6.8	25	7.6	19	5.7	26	7.7	7.3	26
Ireland	44	1.0	71	1.5	145	3.1	202	4.3	239	5.0	5.0	240
Italy	.	.	.	.	.	.	.	.	.	.	.	.
Latvia	37	1.8	73	3.6	184	9.3	76	3.9	49	2.5	2.5	49
Liechtenstein	.	.	.	.	.	.	.	.	.	.	.	.
Lithuania	13	0.4	13	0.4	9	0.3	10	0.3	9	0.3	0.3	9
Luxembourg	1	0.2	3	0.5	2	0.4	0	0.0	6	1.0	1.0	6
Malta	0	0.0	2	0.5	0	0.0	4	0.9	4	0.9	0.9	4
Netherlands	.	.	.	.	.	.	.	.	.	.	.	.
Norway	227	4.5	264	5.2	247	4.8	343	6.6	485	9.2	9.1	485
Poland	1 830	4.8	1 871	4.9	1 687	4.4	1 445	3.8	1 229	3.2	3.4	1 229
Portugal	-	-	-	-	26	0.3	30	0.3	45	0.4	0.5	45
Romania	328	-	796	-	959	-	892	-	1 060	-	-	1 060
Slovakia	180	3.3	166	3.1	228	4.2	284	5.2	190	3.5	3.5	190
Slovenia	42	2.0	38	1.8	30	1.5	54	2.6	64	3.1	3.3	64
Spain	885	-	1 487	-	1 627	-	2 069	-	2 953	-	-	2 953
Sweden	1 253	13.1	1 260	13.1	1 473	15.1	1 491	15.1	1 144	11.4	11.3	1 144
United Kingdom	3 840	6.0	3 628	5.6	4 536	7.0	4 723	7.2	5 225	7.9	8.1	5 225
EU/EEA	**16 539**	**5.5**	**17 274**	**5.4**	**17 978**	**5.5**	**19 159**	**5.8**	**19 437**	**5.5**	**5.6**	**19 451**

Source: Country reports.
ASR: age-standardised rate
.: no data reported
-: no rate calculated

Table 11. Distribution of confirmed giardiasis cases by country and year, EU/EEA, 2013–2017 (source: ECDC, 2017).

In Italy, before the introduction of the Highly Active Anti-Retroviral Therapy (HAART), *G. duodenalis* infected 6.15% of HIV-positive persons (Angarano *et al*. 1997; Brandonisio *et al*. 1999). In the immunocompetent population, *G. duodenalis* was recorded with prevalences ranging between 0.94% and 4.66% (Giangaspero *et al*., 2007) and between 1.3% and 4.8% in Northern Italy (Guidetti *et al*., 2010) and in the midlands (Masucci *et al*., 2011).

Similarly to other countries, potentially zoonotic Assemblage A was more common whereas Assemblage B and mixed Assemblages (A + B) were less represented (Giangaspero *et al*., 2007).

Giardia cysts are considerably less robust than *Cryptosporidium* oocysts. Therefore, although *Giardia* cysts may contaminate fresh produce, such as salad, if they are contaminated a considerable period prior to serving and they are exposed to ambient temperatures for some periods, the cysts may rupture and will be less likely to result in the consumers' infection. On the other hand, fresh

foods may act as a transmission vehicle for *Giardia* when they are contaminated during handling shortly before consumption (Utaaker *et al*., 2017).

Very few outbreaks of foodborne giardiasis have been identified and investigated (Ryan *et al*., 2019). Currently, only 38 foodborne outbreaks of giardiasis have been reported, all in the USA (Adam *et al*., 2016; Ryan *et al*., 2019). The food type or source of outbreak was frequently undetermined. However, a variety of foods have been implicated, with fresh produce including fresh vegetables (lettuce, onions, tomatoes, lettuce-based and mixed green salads) and fresh fruit contaminated with *Giardia* cysts (Adam *et al*. 2016) being the most common food type and infected food handlers the most common source.

In Italy, cases of giardiasis linked to fresh produce have not been documented.

The total number of giardiasis outbreaks is probably greatly underestimated. Under-reporting of giardiasis outbreaks is also likely due to the fact that many countries lack a system for reporting cases and outbreaks of foodborne diseases, and for countries that do, many surveillance systems do not include giardiasis.

Fresh produce - *Giardia* cysts have been detected on a variety of foods, including vegetables and fruits such as, leafy greens, herbs, berries, green onions, carrots, tomatoes, etc. (Dixon, 2020). The presence of *Giardia* cysts in fresh produce has been documented worldwide with a prevalence ranging from 1.8% to 56% (Dixon, 2015).

Giardia on fresh produce has been recorded in Iran (Shahnazi and Jafari-Sabet, 2010; Ezatpour *et al*., 2013), Saudi Arabia (Al-Megrin *et al*., 2010), in Egypt (Eraky *et al*., 2014), Sudan (Mohamed *et al*., 2016).

Giardia duodenalis has also been reported in RTE salads in Canada by Dixon *et al*. (2013). These authors recorded the presence of *G. duodenalis* in 10 (1.8%) out of 544 RTE packaged leafy greens; *G. duodenalis* Assemblages A and B were identified in 7 (1.2%) and 2 (0.4%) samples, respectively.

In Europe, *Giardia* cysts have been found on vegetables in Norway (Robertson and Gjerde, 2001) and in Spain (Amorós *et al*., 2010). In Norway, between August 1999 and January 2000, *Giardia* cysts have been found in 10 (2.1%) out of 475 samples of fruits and vegetables examined. In Spain, *Giardia* cysts have been detected on lettuce and Chinese cabbage (Amorós *et al*., 2010).

In Italy, the presence of the *Giardia* cysts in vegetables is poorly documented. The first detection of *Giardia* in vegetables is dated back to 1968 when *Giardia* cysts were found on 48 (75%) out of 65 vegetables samples collected from local markets in the city of Rome (Mastrandrea and Micarelli, 1968). Almost 40 years later, the presence of *Giardia* cysts has been documented in RTE salads by

Di Benedetto *et al*. (2007) and by Caradonna *et al*., (2017). The first study was carried out in Palermo and one sample out of 40 was found positive for *Giardia* whereas the latter was carried out in the Apulia region and *G. duodenalis* was molecularly detected with a prevalence of 0.633%.

A recent review gives detailed information on the contamination of vegetables and fruits with *G. duodenalis* and its geographical distribution (Li *et al*., 2020). The average prevalence is estimated to be 4.8%, and Assemblages A and B were the most detected (Li *et al*., 2020).

2.3.4.5 Detection methods in fresh produce

Testing foods for the presence of *Giardia* cysts is very challenging due to: *i*) the small numbers of cysts likely to be present on foods and associated difficulties in detecting them and *ii*) the inefficiencies in the methods available.

Elution, extraction, and concentration represent the first steps for detecting *Giardia* in or on food items (Ahmed and Karanis, 2018). Different elution methods for recovering parasites from contaminated vegetables or fruits are available.

The detection of *Giardia* on food has been improved by the use of immunomagnetic separation (IMS) methods to isolate cysts (Cook *et al*., 2007; Hohweyer *et al*., 2016). A standardised method for the detection and enumeration of *Giardia* cysts as well as *Cryptosporidium* oocysts on berry fruits and fresh leafy green vegetables based on IMS has become available in 2016 (ISO 2016); however, with this method it is not possible to determine the *Giardia* Assemblages and it would, therefore, need to be combined with molecular tools and sequencing if that is the objective.

Staining with Lugol's iodine is widely used for the detection of *G. duodenalis* cysts, with light microscopy used to view morphological features; this method is economical and rapid, however, it is labour intensive and lacks specificity and sensitivity (Soares and Tasca, 2016). An immunofluorescence assay is usually applied for the detection of *Giardia* cysts in food items and has both greater sensitivity and specificity (Ryan *et al*., 2018).

PCR detection is increasingly being used for the identification of *Giardia* on food as it offers improved sensitivity and specificity compared with microscopy and immunology based detection methods (Dixon *et al*., 2013; Ramirez-Martinez *et al*., 2015; Hohweyer *et al*., 2016) and allows the determination of the Assemblages through DNA sequencing and, therefore, may provide information for better understanding of the transmission patterns and the zoonotic potential of the isolates (Thompson and Ash, 2016).

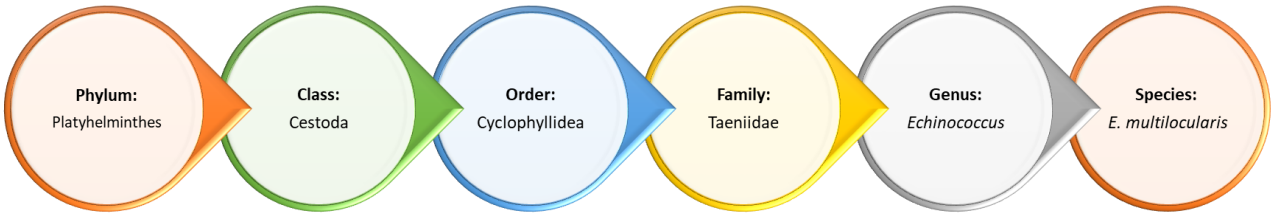
Recently, various molecular methods have been developed for the identification of *Giardia* on vegetables such as conventional PCR (Ramirez-Martinez *et al*., 2015), nested-PCR (Dixon *et al*., 2013) and PCR-RFLP (Tiyo *et al*., 2016).

Widely used genetic loci for the detection of *Giardia* include glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*), *ß-giardin* (*bg*), 16S rRNA gene and 18S rRNA (Feng and Xiao, 2011; Ryan and Cacciò, 2013; Dixon *et al*., 2013; Koehler *et al*., 2014).

2.3.5 *ECHINOCOCCUS MULTILOCULARIS*

2.3.5.1 Classification and morphology

Echinococcus multilocularis is a cestode (tapeworm) belonging to the Phylum Platyhelminthes, Class Cestoda, Order Cyclophyllidea and Family Taeniidae.



Eight recognised species belong to the genus *Echinococcus* and, among those, *E. multilocularis*, causing alveolar echinococcosis (AE) represents a significant public health concern (Deplazes *et al*., 2017).

An adult *E. multilocularis* measures 1.5 to 3.5 mm in length. It consists of a head, or scolex, a neck, and a segmented body, the strobila, formed by 3-5 proglottids. The last segment is the longest; however, it measures less than half of the whole cestode body. The rostellum has 2 rows of hooks (28 to 30) and four suckers. The gravid proglottid, found at the end of the strobila, contains the developed uterus, full of eggs. The gravid proglottid or the eggs are passed with the faeces into the environment (**Figure 21a**; **21b**).

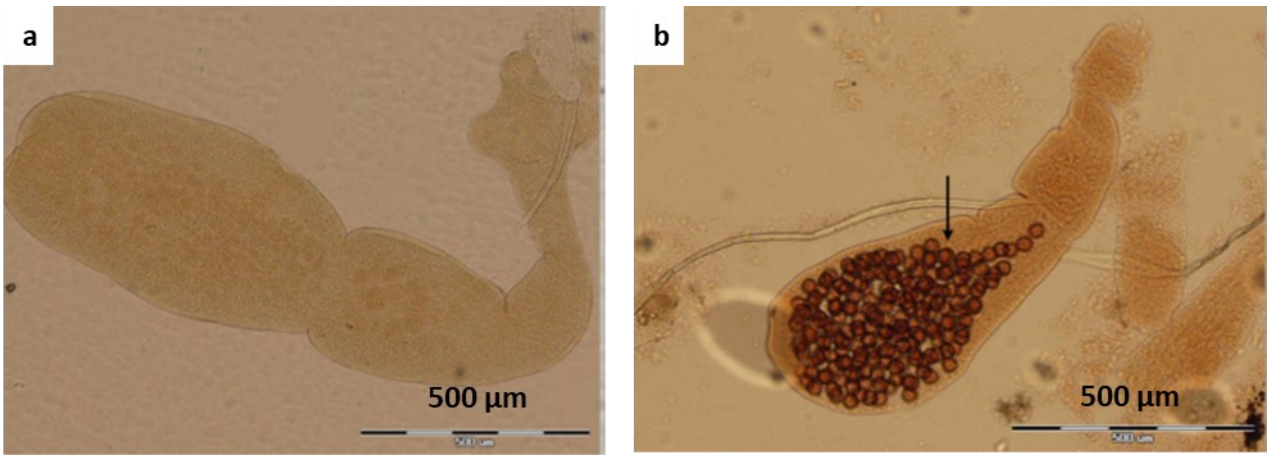


Figure 21. Photomicrographs of an adult *E. multilocularis* (a) and gravid proglottid (b). Scale-bars: 500 µm (Source: Heidari *et al*., 2019)

Echinococcus multilocularis eggs are indistinguishable from the eggs of *Taenia* spp. and other members of the family Taeniidae. The eggs measure 30-35 µm in diameter and are radially striated. The internal oncosphere contains 6 refractile hooks.

Figure 22. Egg of *Echinococcus* sp. in an unstained wet mount of concentrated stool from a dog. Image taken at 400x magnification. (Source: https://www.mcdinternational.org)

2.3.5.2 Biology and life cycle

Echinococcus multilocularis is commonly maintained in a wildlife life cycle where the definitive and the intermediate hosts are predators and preyed species, respectively. The main definitive hosts are foxes, particularly red foxes (*Vulpes vulpes*); however, wolves, raccoon dogs (*Nyctereutes procyonoides*), golden jackals (*Canis aureus*), dogs and, to some extent felid species, can also serve as competent definitive hosts (Romig *et al*., 2017). The intermediate hosts are lagomorphs and rodents and, among the latter order, the members of the subfamily Arvicolinae (voles and lemmings) are the ones most commonly involved. Humans, on the other hand, represent the aberrant intermediate hosts and become infected by ingesting eggs (Romig *et al*., 2017). Being dead-end hosts, humans do not contribute to the maintenance of the parasite lifecycle.

The adult stages of *E. multilocularis* are intestinal parasites of carnivores (definitive hosts) *(1)* that release eggs, each containing an infectious larva (oncosphere), with their faeces into the environment *(2)*. Intermediate hosts ingest egg-containing faeces, or ingest the eggs dispersed into the soil or onto the plants (Tamarozzi *et al*., 2020). Following ingestion, the oncospheres hatch in the small intestine, penetrate the intestinal wall *(3),* and migrate through the circulatory system into the liver or, rarely, disseminate into other organs, where they develop into the metacestode stage represented by a multi-chambered ("multilocular"), thin-walled (alveolar) cyst *(4)*. Inside these cysts, protoscolices are produced in large numbers *(5)*. Should infected organs of the intermediate host be consumed/ingested by a definitive host, the protoscolices may develop into adults in the definitive host's small intestine *(6).* After a few weeks, the adult worms, start producing eggs that will be eventually released into the environment with the faeces (Thompson *et al*., 2017).

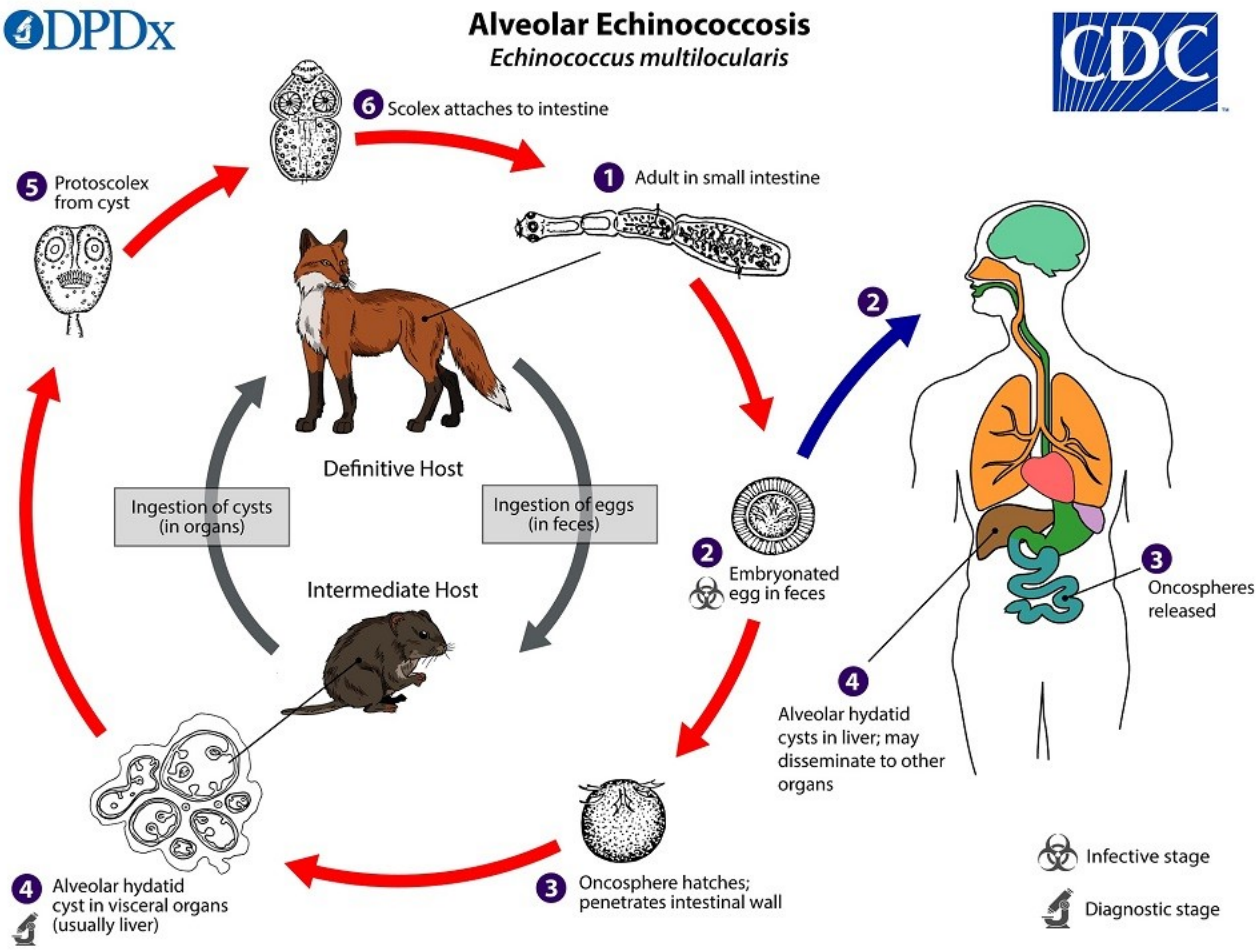


Figure 23. *Echinococcus multilocularis* life cycle
(Source: https://www.cdc.gov/parasites/echinococcosis/epi.html)

Humans can acquire the infection by ingesting viable eggs in many different ways (Tamarozzi *et al*., 2020):

- water- or foodborne transmission (vegetables/fruits/berries);
- hand-to-mouth transmission after contact with *E*. *multilocularis* eggs in the environment;
- birds and flies could be possible vectors (Lawson and Gemmell, 1990);
- direct contact with the definitive hosts when their coats is contaminated with *E*. *multilocularis* eggs i.e., after dogs have been rolling into their own or other dogs' faeces. Also, proglottids of *E. multilocularis* have been found and documented in the perianal region of a naturally infected dog (Deplazes *et al*., 2004). Dog ownership is one of the most important risk factors for humans to be infected with *E. multilocularis* (Alvarez Rojas *et al*., 2018).

2.3.5.3 Pathogenesis and clinical signs

Echinococcus multilocularis causes AE, a chronic and severe disease characterized by slow growing, destructive parasitic tumors in the liver and, occasionally, other organs. After ingestion of viable eggs, the oncospheres reach the liver where they establish a germinal layer (GL), from which protoscolices will be eventually produced, and form a cyst (Gottstein *et al*., 2017). After 7-10 days a laminated layer, that protects the parasite from host immunity, is formed. The parasites generate additional cysts that typically remain attached to the parent one, but occasionally detach from it and form a "multilocular" metacestode (reviewed by Woolsey and Miller, 2020).

In the early stages, the infection is usually asymptomatic and the only symptoms that the patient may show are abdominal pain and biliary obstruction. It takes years before lesions and related symptoms become evident (Kern *et al*., 2017). The incubation period is estimated to be 10-15 years in most cases (Ammann and Eckert, 1996).

Abdominal pain, jaundice and cholestasis are the main symptoms associated with AE which is often misdiagnosed as liver cancer (Kern *et al*., 2017).

It has been reported that the severity of the disease mainly depends on the location of the lesions in the liver (Bresson-Hadni *et al*., 2000): if the parasite is located centrally, the infection typically manifests with milder symptoms, whereas lesions situated close to the hepatic veins or inferior vena cava can result in a Budd-Chiari-like presentation; in addition, in the latter scenario, there is a higher risk of metastasis and lesions spread to different organs (i.e., lungs, spleen, brain, and heart). Untreated infections have a high fatality rate. Death is mainly associated with liver failure, septic shock, biliary cirrhosis leading to gastrointestinal bleeding or cerebral AE (Kern *et al*., 2017).

2.3.5.4 Epidemiology

In large parts of Central Europe, included Italy, *E. multilocularis* is present in red fox populations with average prevalence ranging between 0.1% and 50%. Besides foxes, domestic dogs with patent infections have been found in several areas and may play an important role for parasite transmission to humans. Cats, with low worm burdens and low egg excretion, are probably of negligible zoonotic significance in the maintenance of the *E. multilocularis* life cycle (reviewed by Deplazes *et al*., 2017).

The infectious dose for *E. multilocularis* in humans is unknown; it has been reported that 200-500 eggs of *E. multilocularis* collected from faeces of infected carnivores were needed to ensure the establishment of the parasite in rodents (Hildreth & Granholm 2003; Matsumoto *et al*., 2010); however, the hosts' susceptibility to the infection has not yet been clarified and varies significantly, even between different strains of mice (Hildreth & Granholm, 2003).

Factors affecting the survival of *E. multilocularis* eggs are summarized in **Table 12**.

Factors affecting survival of *E. multilocularis* eggs						
Environment	**Heat**	**pH**	**Freezing**	**Disinfectant**	**Drying**	**UV light**
Echinococcus multilocularis eggs are able to survive temperatures of 4 °C for 478 days (**Veit *et al.*, 1995**). They are more resistant to elevated temperatures when suspended in water than in environments with 70% relative humidity (**Federer *et al.*, 2015**). *E. multilocularis* eggs remained viable in their natural environment in Germany for 240 days in autumn/winter and for 78 days in summer (**Veit *et al.*, 1995**).	*Echinococcus multilocularis* eggs can survive temperatures of 65°C for 2 h, but they are killed after 3 h at 65°C at or at 70°C, 75°C and 80°C for 30 min, 15 min or 7.5 min, respectively (**Federer *et al.*, 2015**). *Echinococcus multilocularis* eggs appear to lose infectivity at temperatures even as low as 43 °C in low humidity conditions (**Veit *et al.*, 1995**).	No information.	*Echinococcus* eggs remain viable at temperatures below zero for long periods of time (-18°C for 240 days). They were found to be still infective to voles after storage for 54 days at -27°C (**Schiller, 1955**). Very low temperatures (-70°C to -80°C for 96 and 48 h, respectively), inactivate the eggs (**Eckert *et al.*, 2001**). EFSA recommends deep freezing at -80 °C for a minimum of 24 h in order to inactivate potentially contaminant *Echinococcus* eggs (**EFSA, 2018**);	*Echinococcus. multilocularis* eggs are sensitive to higher temperatures and also to desiccation in in vitro conditions (**Laws, 1968**; **Veit *et al.*, 1995**)	No information.	No information.

Table 12. Survival of *E*. *multilocularis* (Source: EFSA, 2018 and Gérard *et al*., 2019).

Humans and foodborne outbreaks caused by the consumption of fresh produce

The true distribution of *E. multilocularis* is likely underdiagnosed and underestimated due to: *(I)* limited knowledge of the parasite, *(II)* misdiagnosis with other similar diseases (e.g., liver cancer) *(III)* limited availability of advanced diagnostic techniques (Vuitton *et al*., 2015).

Echinococcus multilocularis is restricted to the Northern hemisphere, with no known endemic areas further south than Tibet and the Sichuan province in China (Deplazes *et al*., 2017; Feng *et al*., 2015). *Echinococcus multilocularis* is widespread in several countries across northern Asia including Russia, the Japanese island of Hokkaido, central Asia (particularly Kyrgyzstan) (Paternoster *et al*., 2020) and western China (Torgerson *et al*., 2010). China is estimated to be responsible for over 90% of AE cases worldwide (Torgerson *et al*., 2010). In some districts of Siberia, numerous human AE cases have been registered, whereas only sporadic cases have been reported from northern India or Pakistan. Based on data on human AE, most parts of the Russian Federation are considered endemic for *E. multilocularis* (Bessonov, 1998).

Central Europe has been a core endemic area for AE since the end of the 19th century (Eckert and Thompson, 2017). In fact, between 1982 and 2000, 599 AE cases (42% in France, 24% in Germany, 21% in Switzerland, 13% in other countries) were registered in this area (Kern *et al*., 2003). An expansion of the Central European endemic area has been observed to the north, west and to the east (Gottstein *et al*., 2015). The emergence of AE has been documented in the Baltic region (Marcinkute *et al*., 2015) with an increasing number of human AE cases whereas Southern Europe is regarded as free even though studies focusing on *E. multilocularis* are lacking in most areas. As of the end of 2015, no autochthonous AE cases have been described in Western and Northern Europe, however, single imported cases in humans were reported from Denmark, United Kingdom, and Sweden (Wahlström *et al*., 2015). During the past three decades, the implementation of oral rabies vaccination (ORV) programmes in 24 countries has led to the elimination of fox-mediated rabies from vast areas of Western and Central Europe (Freuling *et al*., 2013). This successful campaign has led to an increase of the red fox population in many parts of Europe and, as a consequence, to the increase of *E. multilocularis* circulation and distribution (Takumi *et al*., 2008). In addition, the red fox population is spreading in Europe in all habitats, including urban areas (reviewed by Deplazes *et al*., 2017).

A recent review by Deplazes *et al*., 2017 gives detailed information on the global distribution of alveolar echinococcosis. The geographical distribution of *E. multilocularis* and AE is summarized in **Figure 24**.

Figure 24. Geographical distribution of *E. multilocularis* and alveolar echinococcosis (Source: Rodriguez-Morales *et al*., 2015).

The global burden of disease of AE is estimated to be 18,200 cases per annum, resulting in approximately 666,000 DALYs (37 DALYs per case) (Torgerson *et al*., 2010). However, 91% of cases and 95% of the DALYs were estimated to be in China. Thus, there are approximately 1600 cases of AE per annum in Europe, Russia and central Asia resulting in 33,000 DALYs or 21 DALYs per case.

As to foodborne outbreaks caused by the consumption of fresh produce, 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).

It is similarly impossible to assess whether *E. multilocularis* infections occur following the foodborne route and what the source of infection is due to the long incubation period, which varies between 5 and 15 years (Eckert and Deplazes, 2004). However, a systematic review and meta-analysis by Torgerson *et al*. (2020) shows that the foodborne transmission is a relevant route of infection and empirical evidence shows that contaminated food and contaminated environment would, between them, account for 41% of the pathways of transmission.

Fresh produce

The presence of taeniid eggs and *Echinococcus* DNA has been documented in food and it has been demonstrated that fresh produce represents a potential transmission vehicle for taeniid eggs (Malkamäki *et al*., 2019).

Contamination of vegetables by taeniid eggs has been identified microscopy, mainly in Asia and Africa, with contamination rates ranging between 0.9 and 18.3% (Alvarez Rojas *et al*., 2018). One study in Poland used PCR to assess the presence of *E. multilocularis* DNA in 103 samples of fruits, vegetables, and mushrooms and 23.3 % of samples were found to be positive (Lass *et al*. 2015). This publication opened a debate in which the finding of positive raspberries collected from plants at some distance from the ground raised some questions (Robertson *et al*., 2016). However, such contamination could not be excluded, as it has been reported that flies can transport such eggs (Lawson and Gemmell, 1990). In addition, an investigation into the presence of cestode eggs in the wash water of fresh produce (vegetables, fruit) used as feed for primates kept at a zoo was carried out in Switzerland and taeniid-DNA was detected (Federer *et al*., 2016).

2.3.5.5 Detection methods in fresh produce

There is no standardised method for the isolation of *Echinococcus* eggs present on fresh produce (EFSA, 2018). However, methods for the isolation of taeniid eggs from faecal samples (Conraths and Deplazes, 2015) can be applied for food samples.

After a washing stage that can be performed using different detergents such as SDS (1%) and/or Tween 80 (0.1%), the parasites eluted in the washwater can be concentrated by flotation, sedimentation, and/or sieving steps, and then detected by microscopy. As all taeniid eggs have very similar morphologies, microscopy-based detection of taeniid eggs should be accompanied by molecular tools in order to identify them at the species level. The sensitivity of PCR amplification is high and the limit of detection is described to be as low as one egg. This is not unexpected, as taeniid eggs contain between 18 and 56 cells (Alvarez Rojas *et al*., 2018), and a single taeniid egg contains around 7,000 mitochondrial targets (Trachsel *et al*., 2007).

Various methods have been developed for the detection of *Echinococcus* from fresh produce. These include a nested PCR based on the mitochondrial 12S rRNA gene (Lass *et al*., 2015; 2017), a real-time PCR method targeting a 77 base pair region of the 12 s gene in the mitochondrial genome of *E. multilocularis* (Isaksson *et al*., 2014), and a novel multiplex real-time PCR for the detection of *E. multilocularis,* as well as *T. gondii*, and *C. cayetanensis* on berries (Temesgen *et al*., 2019b).

3. EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 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 per month was established for each fresh product, for a total of 54 samples and 6 pools per month and 72 pools for the whole year (**Table 13**). This sampling protocol was aimed at providing the highest confidence of detecting contamination, even for parasites with low expected prevalence.

Equation 1

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

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.

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**

Table 13. Sampling protocol.

3.1.2 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 berries 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.

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, analysed 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.

3.1.3 Sample processing

Each month, the nine packages of each of the six produce types (three RTE-salads and three 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®+ 400ml 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 minutes 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 minutes, 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 (**Figure 25**). 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.

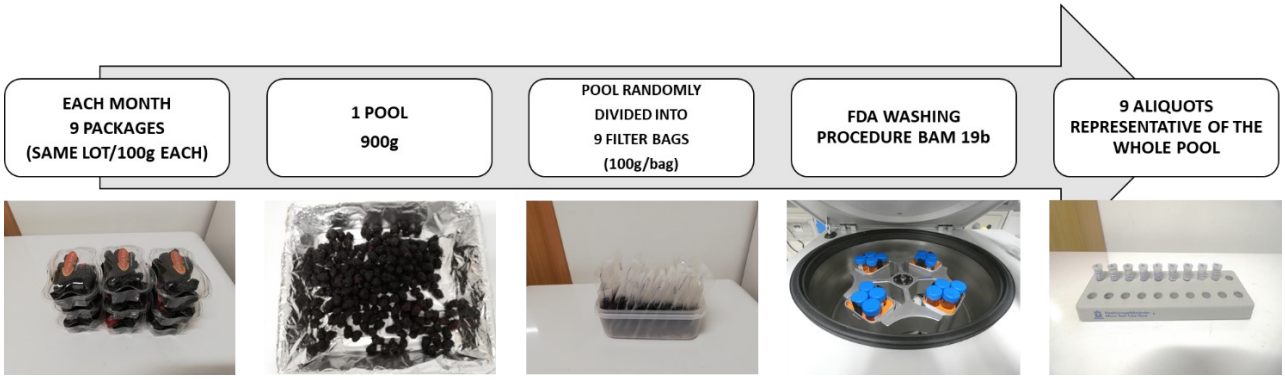


Figure 25. Flowchart of sample processing: blackberries

3.1.4 Microscopy investigation

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 sulphate 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. The microscopy investigation steps are summarized in **figure 26**.

Figure 26. Microscopy investigation using the FLOTAC apparatus

3.1.5 Molecular investigation

3.1.5.1 DNA isolation

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.
The complete DNeasy PowerSoil Kit protocol has been included in **Annex 1**.

3.1.5.2 Real-time PCR and quantitative analysis (*q*PCR)

- Simplex *q*PCR 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'-CCGGCGATAGATCATTCAAGTTTCTGACC-3') labelled 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’-GATCAGCTACGTGAGGTCCTAC-3’), and the probe dd-IAC-Cy5 (5’-AGCTAGTCGATGCACTCCAGTCCTCCT-3'), labelled 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 1E4 µl copies 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.

- Multiplex *q*PCR 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*. (2019b) at the Parasitology Laboratory, at the Norwegian University of Life Sciences (Oslo, Norway).

In this multiplex, the primers, CyITS1_TT-F (ATGTTTTAGCATGTGGTGTGGC) 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*., 2019a). A product of 162 bp from the 529 bp repeat of *T. gondii* was amplified with the primers Tox-9F (AGGAGAGATATCAGGACTGTAG), Tox-11R (GCGTCGTCTC GTCTAGATCG) and probe Tox-TP1 (Cy5-CCGGCTTGGCTGCTTTTCCT-MGBEQ) (Opsteegh *et al*., 2010). A forward primer, EmMGB_F (GTGCTGCTYATAAGAGTTTTTG), 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*. (2019b). 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 analysed in triplicate and C*q* values were analysed after each run.

To investigate whether the PCR for *E. multilocularis* might also amplify *E. granulosus* DNA, we tested DNA isolated from *E. granulosus* eggs kindly provided by Prof. Laura Rinaldi, University of Naples Federico II, Naples, Italy, using the same *q*PCR assay.

3.1.5.3 Conventional PCR and DNA sequencing

- 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'- CAGGAGAAGCCAAGGTAGG-3') were used to amplify a 636 bp DNA fragment. In the secondary PCR amplification, the primers CC719 (5'-GTAGCCTTCCGCGCTTCG'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 sec and extension at 72°C for 90 sec 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 sec and extension at 70°C for 90 sec.

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.

- 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 (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'-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 2x 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. Negative and positive controls were included in each experiment.

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 sec 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. Positive PCR products were purified using spin columns (QiaQuick PCR purification kit, Qiagen, Hilden, Germany) and bidirectional sequencing of the PCR products was performed 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).

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

- 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 (Lalle *et al*., 2005). The primers BGFor71 (5'-CCCGACGACCTCACCCGCAGTCG-3') and BGRev794 (5'-GCCGCCCTGGATCTTCGAGACGA-3') were used in primary PCR to amplify a 763 bp fragment. For the nested reaction, the primers BGintFor (5'-GAACGAGATCGAGGTCCG-3') and BGintRev (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 2x 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. Negative and positive controls were included in each experiment.

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 sec 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. Positive PCR products were purified using spin columns (QiaQuick PCR purification kit, Qiagen, Hilden, Germany) and bidirectional sequencing of the PCR products was performed 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). BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank database were used to identify *Giardia* Assemblages and genotypes.

- PCR amplification of *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 sec, 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 positive control was kindly provided by Prof. Federica Berrilli, Tor Vergata University of Rome, Italy. The PCR products were run on 2% agarose gel.

Purification and sequencing of PCR products with the above-described primers (in both forward and reverse directions) 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 database using Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.1.6. 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 $p < 0.05$.

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).

4. RESULTS

4.1 MICROSCOPY INVESTIGATION

A total of 72 pools were examined and the following parasites were identified:

- a *Cyclospora*-like oocyst (8 µm in diameter) in one pool of blueberries (**Figure 27a**);
- a taeniid egg (32 µm in diameter) in one RTE-salad pool (brand A) (**Figure 27b**);
- *Giardia*-like cysts (12 µm x 8 µm) in two pools of berries (raspberries) (**Figure 27c**);

During the microscopy investigation, an additional parasite, not included in the original aims, was detected:

- *Entamoeba*-like cysts (15 µm) in six pools: two were RTE salad pools (brand B and C) and four out of six were berries (two pools of blackberries, one pool of blueberries and one pool of raspberries) (**Figure 27d**);

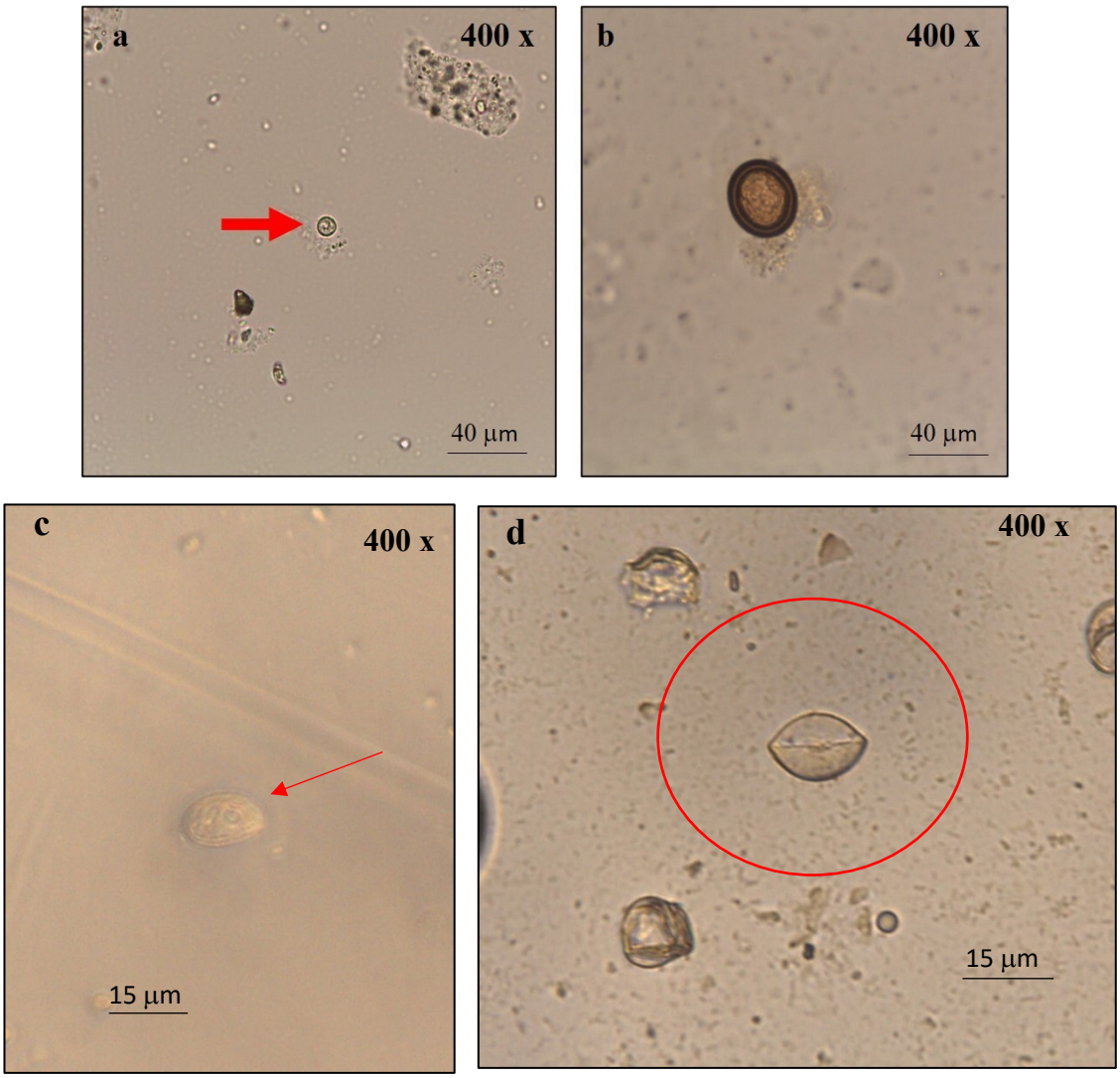


Figure 27. *Cyclospora*-like oocyst (a), Taeniid egg (b), *Giardia*-like cyst (c) and *Entamoeba*-like cyst (d) detected by FLOTAC technique.

4.2 MOLECULAR INVESTIGATION AND SEQUENCING

Cyclospora cayetanensis: *C. cayetanensis* DNA was detected in the same pool of blueberries imported from Peru in which a *Cyclospora*-like oocyst was identified. 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 (**Figure 28**). 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).

***Echinococcus* spp.**: *E. multilocularis* DNA was detected in the same pool of RTE salad in which a taeniid egg was identified. For the *E. multilocularis* positive sample, only one of the triplicate repeats provided a positive result with a C*q* value of 38.1 (**Figure 28**).

DNA from *E. granulosus* in the same PCR set up gave a negative result.

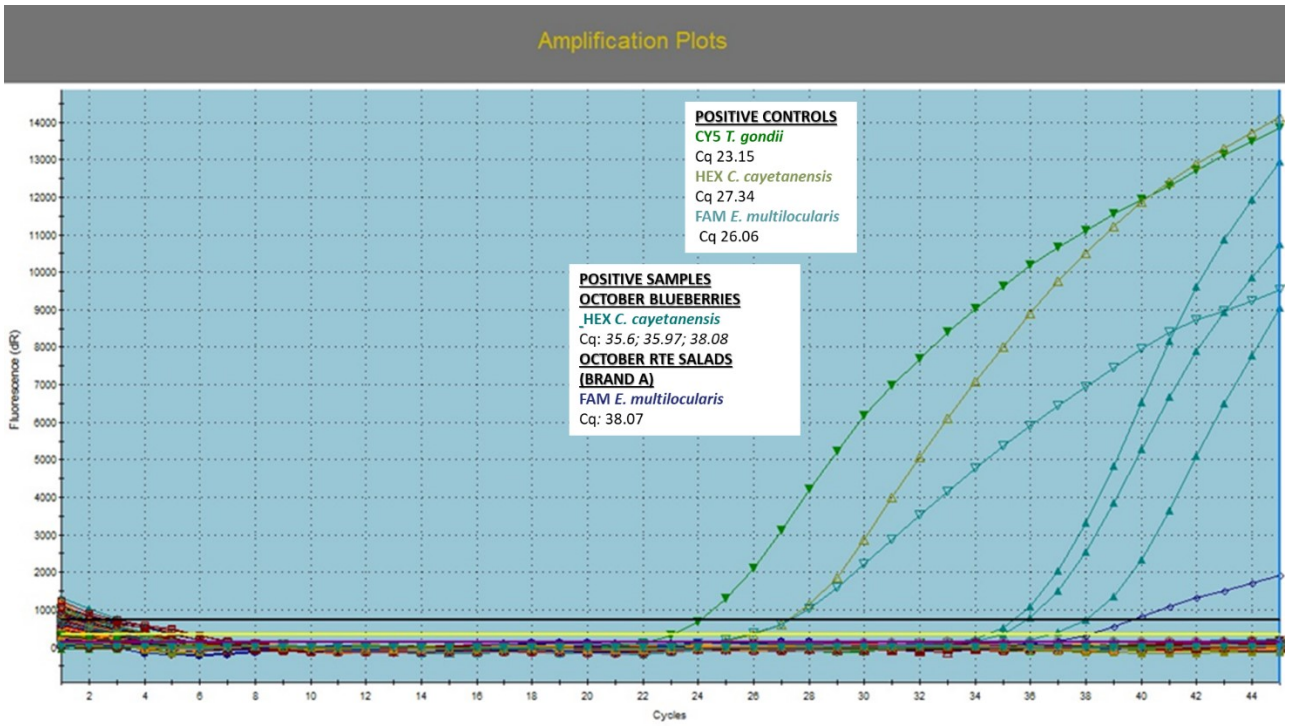


Figure 28. Multiplex *q*PCR (Assay 2) amplification chart. Pools tested: from 43 to 60.

Toxoplasma gondii: *T. gondii* DNA was not detected in any of the samples.

***Cryptosporidium* spp.**: *Cryptosporidium* was detected in 30 pools, 14 of which were berries (six pools of blueberries, six pools of raspberries and two pools of blackberries) and 16 RTE salads (four pools belonging to brand A, six pools belonging to brand B and six pools belonging to brand C). Sequencing confirmed the presence of the following species: *C. ryanae* in 22 pools, *C. bovis* in three pools, *C. xiaoi* in two pools, *C. ubiquitum* in one pool; also, two pools with mixed sequences were identified.

Giardia duodenalis: *G. duodenalis* was detected in 25 pools: 12 pools of berries (four pools of blueberries, four pools of raspberries, in two of which, *Giardia* cyst like objects had been identified, and four pools of blackberries) and 13 pools of RTE salad (five pools belonging to brand A, five pools belonging to brand B and three pools belonging to brand C).
With sequencing and comparisons with sequences already in GenBank, seven pools were assigned to Assemblage A, 17 to Assemblage B and one to Assemblage E.

***Entamoeba* spp.**: *Entamoeba* DNA was molecularly confirmed in the same pool of blueberries imported from Peru in which the *Entamoeba*-like cyst was identified. The sample gave clear bands that matched with the estimated PCR product sizes on the agarose gel and the molecular BLAST analysis showed that the sequences matched with *E. histolytica.*

4.3 PREVALENCE DATA

The observed prevalence of the inspected parasites is shown in **Table 14**.

Parasite	N° pools analysed	N° positive pools	Prevalence	95% C.I.
T. gondii	72	0	0.000%	0.00-0.00
C. cayetanensis	72	1	0.155%	0.003-0.862
E. multilocularis	72	1	0.155%	0.003-0.862
Entamoeba spp.	72	6	0.962%	0.351-2.083
G. duodenalis	72	25	4.629%	2.986-6.784
Cryptosporidium spp.	72	30	5.813%	3.909-8.242

Table 14. Observed prevalence of parasites combining microscopy and molecular results.

Cryptosporidium spp. and *G. duodenalis* showed significant seasonal differences (**table 15** and **16**) with higher values in winter and spring, respectively.

	Deviance	Degrees of freedom	p value
Cryptosporidium spp.			
Season*	8.06	3	0.044
Product	0.22	1	0.639
G. duodenalis			
Season*	10.86	3	0.012
Product	0.06	1	0.806

Table 15. Group testing regression results of comparison between season and products
* Winter: December, January and February; Spring: March, April and May; Summer: June, July and August; Autumn: September, October and November.

	Spring*	**Summer***	**Autumn***	**Winter***
Cryptosporidium spp.	6.3% (2.6-12.3)	6.3% (2.6-12.3)	2.0 % (0.4-5.7)	9.9% (4.7-17.7)
G. duodenalis	8.6% (4.0-15.7)	2.0 % (0.4-5.7)	2.0 % (0.4-5.7)	7.4% (3.2-13.9)

Table 16. Seasonal prevalence observed in fresh salads and berries

* Winter: December, January and February; Spring: March, April and May; Summer: June, July and August; Autumn: September, October and November.

All the results are summarized in **Table 17**.

	No. of pools	*Cyclospora cayetanensis*		*Echinococcus multilocularis*		*Entamoeba* spp.		*Giardia duodenalis*		*Cryptosporidium* spp.	
		Microscopy FLOTAC	Molecular assays	Microscopy FLOTAC	Molecular assays	Microscopy FLOTAC	Molecular assays	Microscopy FLOTAC	Molecular assays	Microscopy FLOTAC	Molecular assays
RTE mixed salads (Brand A)	12	0	0	1	1	0	0	0	5	0	4
RTE mixed salads (Brand B)	12	0	0	0	0	1	0	0	5	0	6
RTE mixed salads (Brand C)	12	0	0	0	0	1	0	0	3	0	6
RTE subtotal	**36**	**0**	**0**	**1**	**1**	**2**	**0**	**0**	**13**	**0**	**16**
Imported blueberries (Peru)	12	1	1	0	0	1	1	0	4	0	6
Imported blackberries (Mexico)	12	0	0	0	0	2	0	0	4	0	2
Local raspberries (Italy)	12	0	0	0	0	1	0	2	4	0	6
Berries subtotal	**36**	**1**	**1**	**0**	**0**	**4**	**0**	**0**	**12**	**0**	**14**
OVERALL TOTAL (No)	**72**	**1**		**1**		**6**		**25**		**30**	
PREVALENCE (95% C.I.)		**0.155% (0.003-0.862)**		**0.155% (0.003-0.862)**		**0.962% (0.351-2.083)**		**4.629% (2.986-6.784)**		**5.813% (3.909-8.242)**	

Table 17. Detection of parasites in fresh produce (RTE mixed salads and berries) sold at retail in Italy and investigated by microscopy and molecular assays

5. DISCUSSIONS

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.

In particular, *Cyclospora cayetanensis*, *Echinococcus multilocularis*, *Giardia duodenalis* (Assemblages A, B and E), and several *Cryptosporidium* species, i.e., *C. ryanae*, *C. bovis*, *C. xiaoi*, and *C. ubiquitum* have been detected. The prevalences for these parasite contaminants ranged from 0.15% (*C. cayetanensis* and *E. multilocularis*) to 5.81% (*Cryptosporidium* spp.). Moreover, *Entamoeba histolytica* was detected with a prevalence of 0.96%.

Microscopy and molecular tools identified *C. cayetanensis* in blueberries imported from an endemic country (Peru). 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 survey in Korea (non-endemic country) where, among 44 blueberry samples analysed 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 analysed 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 analysed for parasite contamination by microscopy following lectin-magnetic separation (Robertson and Gjerde, 2001). *Cyclospora* oocysts were not detected on any of the samples examined. However, more recent information derived from an ongoing survey of berries on the Norwegian market, using molecular tools for analysis, has indicated that just under 1% were contaminated with *Cyclospora* DNA. *Cyclospora* contamination was detected on berries imported from Portugal, Morocco, Belgium, and The Netherlands (Temesgen, 2020).

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*., under review). 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 indeed 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, domestic dogs or cats can play a role in the synanthropic cycle of *E. multilocularis* (Eckert and Deplazes, 2004). As to Europe, only imported cases were reported from Denmark, United Kingdom, and Sweden in humans (Wahlström *et al*., 2015) and none (neither autochthonous nor imported) were registered in Italy. Interestingly, 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 taeniid eggs (Federer *et al*., 2016).

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 to south (Faunalia, 2020), suggesting the possibility that they could have brought this parasite with them. Also, given the high number of stray dogs, particularly in Southern Italy (Infodata.ilsole24ore, 2019), their potential role in the dissemination of *E. multilocularis* eggs 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 real time *q*PCR in Portugal and Spain, in both RTE salads and berries with very

high proportions of sample 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 analysed compared with other European surveys, or, potentially, to the limit of detection of the multiplex *q*PCR assay (Temesgen *et al*., 2019b), 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 it was higher (e.g., 100 oocysts; Lass *et al*., 2012), arguing against this as a reason for the lack of detection in our study. 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*. Similarly, this assay has been used in an ongoing study of berries on the Norwegian market, and currently available data suggest a contamination rate of around 3%, with contamination detected on berries imported from Chile, Zimbabwe, Morocco as well as on berries coming from European countries i.e., Norway, Poland, Portugal, Belgium, and the Netherlands (Temesgen, 2020).

In our study *Cryptosporidium* spp. was detected with an overall prevalence of 5.81% in line with the contamination of vegetables and fruits with *Cryptosporidium* spp. documented in many countries accounting for 6.0% (reviewed by Li *et al*., 2020). Four species were identified using molecular tools: *C. ryanae*, *C. bovis*, *C. xiaoi,* and *C. ubiquitum*. *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, at least five *Cryptosporidium* species, including *C. bovis*, *C. ryanae*, *C. ubiquitum* and *C. hominis*, have been identified in cattle, but *C. parvum* was the most frequently detected species (Grana *et al*. 2006; Duranti *et al*. 2009; Merildi *et al*. 2009; Imre and Dărăbus 2011; Di Piazza *et al*. 2013). *Cryptosporidium parvum* was, in fact, the only species identified in three different studies carried out in bovine farms in central Italy (Grana *et al*., 2006; Duranti *et al*., 2009; Mangili *et al*., 2009). *C*. *parvum* and *C. hominis* were found in cattle from Tuscany (Central Italy) by Merildi *et al*., (2009). In a cross-sectional study carried out to determine the prevalence of *Cryptosporidium* in calves of Palermo area (Sicily) a total of 149 calves' faecal samples were collected and, of the 12 samples from which DNA amplification and genetic sequencing was obtained, seven matched

with *C. bovis*, four matched with *C. ryanae* and, finally, one sample showed 100% homology with *C. ubiquitum* (Di Piazza *et al*., 2013).

The available epidemiological studies show that the prevalence of *Cryptosporidium* spp. in Italian cattle farms ranges between 5 and 11.4% and increases significantly when only diarrhoeic calves are taken into account (50–62.1%) (reviewed by Diaz *et al*., 2018).

Sheep can be infected with several *Cryptosporidium* species of which, *C. parvum*, *C. xiaoi* and *C. ubiquitum* are the most common (reviewed by Dessì *et al*., 2020). In Italy, little is known of the presence of *Cryptosporidium* spp. in sheep and the only data available are limited to two surveys. The first survey was carried out in Abruzzo, Central Italy, where 17.45% of the lambs tested were found positive for *Cryptosporidium* and, after the molecular investigation, showed 100% identity with *C. parvum* (Paoletti *et al*., 2009). A second survey carried out very recently aimed at evaluating the prevalence of *Cryptosporidium* spp. in sheep farms in Italy (Dessì *et al*., 2020). A total of 915 faecal samples, from 61 sheep farms, were collected and genotype analyses showed the presence of two *Cryptosporidium* species: *C. parvum* and *C. ubiquitum*.

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 oocysts infectious to humans.

Among the four species we detected, while *C. ubiquitum* and *C. bovis* have been occasionally reported in humans, only one report in humans is available for *C. xiaoi* (Adamu *et al*., 2014) and none for *C. ryanae*. Cases of zoonotic cryptosporidiosis due to *C. ubiquitum* (Fayer *et al*., 2010; Li *et al*., 2014) have been registered in the UK (Elwin *et al*., 2012), and in Spain (Cieloszyk *et al*., 2012), whereas human cases caused by *C. bovis* have been reported in Australia (Ng *et al*., 2012) and in Egypt (Helmy *et al*., 2013).

So far, among the *Cryptosporidium* species, *C. parvum*, *C. hominis*, and *C. ubiquitum* are the only ones previously reported from contaminated vegetable and fruit samples (Rzezutka *et al*., 2010; Duedu *et al*., 2014; Caradonna *et al*., 2017; Li *et al*., 2019) and our survey represents the first detection of *C. ryanae, C. bovis* and *C. xiaoi* in fresh produce. Our detection of *C. ubiquitum i*n RTE salads is in line with the results of another Italian survey (Caradonna *et al*., 2017). However, the detection of *C. parvum* would have been more likely as this parasite DNA has already been found in RTE salads in Italy (Caradonna *et al*., 2017) and Canada (Dixon *et al*., 2013; Lalonde and Gajadhar,

2016), and is known to be shed in enormous numbers from infected ruminants, particularly calves and lambs.

Given that all the *Cryptosporidium* species detected are mainly found in livestock, it is plausible to assume that the contamination of the fresh produce occurred in the pre-harvest phase when contaminated water could have been used for irrigation or animal waste was used as fertilizer, or infected animals could have directly contaminated these products.

Our finding of *G. duodenalis* in fresh produce with a prevalence of 4.63% is in line with the average prevalence of contamination of vegetables and fruits with *G. duodenalis* reported in literature from many countries and estimated to be 4.8% (reviewed by Li *et al*., 2020). The presence of *Giardia* in the fresh produce samples investigated here is indicative of faecal contamination of human and/or animal origin. In the present study, genotypes with zoonotic potential i.e., A, B and, to a lesser extent, E were detected. In congruence with our results, zoonotic Assemblages A and B are the ones most commonly reported in literature in contaminated vegetable and fruit samples (Colli *et al*., 2015; Tiyo *et al*., 2016; Caradonna *et al*., 2017; Rafael *et al*., 2017). Although *Giardia* of Assemblage B, has occasionally been reported in cattle, sheep, horses, dogs, cats, and rabbits, Assemblage A is more frequently found in livestock and companion animals, and both are found in wild animals (reviewed by Feng and Xiao, 2011). Because these Assemblages are found in humans and numerous species of mammals, they are both considered to have a broad host specificity and can be transmitted zoonotically (Cacciò and Ryan, 2008; Xiao and Fayer, 2008).

The *G. duodenalis* genotype identified most frequently in this study was Assemblage B followed by Assemblage A and Assemblage E. Assemblage B is much more commonly reported in humans than livestock, suggesting a possible human source of contamination, whereas Assemblage A, more commonly found in livestock, could suggest faecal contamination of animal origin.

One RTE salad sample from the present study, positive for *G. duodenalis*, matched with Assemblage E. Although Assemblage E is mainly found in cattle, bovines, goats, sheep and swine, recent studies have reported the presence of this genotype in human stools (Fantinatti *et al*., 2016; Abdel-moein *et al*., 2016) indicating its zoonotic potential. However, given the strong association between Assemblage E and livestock, we might speculate that such contamination was caused by the use of contaminated manure to fertilize the crops or by the use of contaminated irrigation water; also, the passage of livestock on crop may have led to contamination of soil and water, taking also into account that vegetable crops are often cultivated open-air.

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 significantly associated with the consumption of contaminated fresh produce (Anuar *et al*., 2012; Gabre *et al*., 2016; Sitotaw *et al*., 2019; Azim *et al*., 2018). *Entamoeba histolytica* is a protozoan parasite of humans responsible for human amebiasis associated with a variety of symptoms: from no symptoms to severe fulminating intestinal and/or life-threatening extraintestinal disease worldwide (especially in developing countries). *Entamoeba histolytica* is considered an important cause of prolonged traveller's diarrhoea (Cui *et al*., 2019; Li *et al*., 2020). The detection of *Entamoeba*-like cysts by microscopy in a blueberries sample from Peru corroborates this molecular finding and, once again, indicates the contamination of fresh produce by multiple parasites of faecal origin and the risk for consumers linked to the consumption of fresh produce imported from third countries.

A significant statistical association between the detection of *Cryptosporidium* spp. and *G. duodenalis* in fresh produce and the season emerged. Significant seasonal differences with higher values of detection in winter and spring were detected. However, seasonality calculated in the present study did not take into account the origin of the different fresh produce and related climate differences between the different countries from where some of the contamination originated; therefore, this aspect shall be further investigated, and no firm conclusions can be drawn.

Isolation and detection of parasites in fresh produce is very challenging (Lass *et al*., 2012; Dixon *et al*., 2013; Giangaspero *et al*., 2015a; Caradonna *et al*., 2017), particularly for detection of multiple species. The combination of microscopy and molecular assays employed in this study 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 microscopic tool mainly employed for quali-quantitative copromicroscopic diagnosis of parasites in animals and humans (Cringoli *et al*., 2010), but, more recently, successfully tested for the detection of gastrointestinal parasites in smooth lettuces (do Nascimento Ramos *et al*., 2019).
Although, in the present study, the molecular assays confirmed most of the microscopy results, the efficiency of the FLOTAC technique for the detection and identification of parasites in fresh produce requires specific and more in-depth investigations using both naturally and experimentally contaminated samples.

Regarding molecular tools, the DNA extraction kit employed in this study, DNeasy PowerSoil Kit, currently represents the method of choice for extracting DNA of coccidian oocysts from berry matrices and subsequent detection by *q*PCR (Temesgen *et al*., 2020).

In the literature, several PCR protocols have been described for the isolation and detection of different parasites from fresh produce; however, none of them is considered the "gold standard" method, due to differences in the properties of the various food matrices and parasites of concern. In this study, for each pathogen we used the molecular protocols that seemed to be the most efficient presently and according to our experience.

As to *Cyclospora*, among the two *q*PCR assays employed for its detection in our fresh produce, while for Assay 1 there is a potential for cross-reactivity between *C. cayetanensis* and other coccidia (i. e., *T. gondii*, *Eimeria mitis*, *Cystoisospora canis*, and *C. parvum*), Assay 2 is more specific (Temesgen *et al*., 2019b). However, the two techniques have provided consistent results and therefore we have no reason to believe that cross-reactivity between *C. cayetanensis* and related coccidia occurred during Assay 1.

All the parasites considered in the present study survive refrigeration temperatures, which are the ones employed for transporting and storing fresh produce, and chlorine concentrations used in the water for RTE salads disinfection (80-100mg/L) in the food industry (Sathyanarayanan and Ortega, 2006; Hohweyer *et al*., 2016). Therefore, although viability and infectivity could not be determined, it should be assumed that the detection of pathogenic parasites in fresh produce represents a risk to public health (Dixon *et al*., 2013).

As far as we are aware, none of the lots of the fresh produce 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 the protozoan parasites we have detected are not routinely investigated in people with gastrointestinal disorders in most European countries, including Italy (Giangaspero *et al*., 2015a). As to *E. multilocularis*, determining whether the RTE salad pool contaminated with it resulted in human infection is similarly impossible; the incubation period for AE varies approximately between 5 and 15 years (Eckert and Deplazes, 2004).

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 and Mexico that were contaminated with pathogenic parasites 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 this study, we were unable to assess:

i) at which point "from farm to fork" (pre-harvest, harvest or post-harvest phase) the contamination occurred;

ii) which of the different vegetables included in the RTE mixed salad packages analysed were contaminated or whether only one type of vegetable or more than one type within the package was contaminated;

iii) the number of oocysts or eggs in the positive samples. It should be kept in mind that, given the structure of our survey, such assessment would have only been possible for those parasites tested with *q*PCR, *C. cayetanenis* and *E. multilocularis*, whereas for the parasites tested with conventional PCR, *Cryptosporidium* spp., *G. duodenalis*, *Entamoeba* spp., such assessment would have not been possible as this technique provides only presence/absence results.

iv) whether the parasites detected were viable; although viability assays are available for some of these parasites (e.g., Rousseau *et al*, 2018) such assessments are generally unsuitable for low numbers of parasite transmission stages and it would have been difficult, if not impossible, to conduct such analyses;

iv) whether any of the contaminated batches included in our study were associated with reported illness or outbreaks of disease.

6. CONCLUSIONS

The results of this survey demonstrate that the contamination of fresh produce by pathogenic parasites is a cause for concern in Europe and, in particular, in Italy.

The potential for transmission of pathogenic parasites from contaminated RTE salads and berries sold on the Italian market and the detection of DNA from these parasites on fresh produce indicates that faecal contamination occurred at some point along the agri-food production chain. Therefore, appropriate management of the pre- and post-harvest phases should remain an essential concern in order to reduce the likelihood of contamination of fresh produce and, thus, infection of consumers. Such management may include treating and monitoring the quality of water before irrigation, minimizing animal access to crops, reducing the use of manure as fertilizer, promoting personal hygiene and food safety education for food handlers as well as consumers, implementing appropriate hazard analysis and critical control point (HACCP) plans to reduce the risk of contamination.

It is well known that the establishment of risk-based criteria for controlling parasites in fresh produce requires very complex procedures. In the meantime, it is crucial to accrue data, not only on the occurrence of parasites on fresh produce (and, it should be noted, that recently developed, standardized analytical methods, like those used in the present study, may help in obtaining unambiguous and comparable results), but also on the spread and impact of foodborne pathogenic parasites in Europe by broadening the algorithm for implementing clinical diagnostic tests in suspected patients.

Large scale surveys and the detection of pathogenic parasites represent the starting point for setting up a risk assessment procedure as the first step for the amendment of the current legislation. In fact, our results further highlight the need to integrate the microbiological criteria required by EU Law; in fact, monitoring only certain bacteria in vegetables, does not rule out faecal contamination by pathogenic parasites and does not guarantee food safety for consumers.

In order to prevent parasitic contamination of fresh produce, it is essential that the importance of applying the One Health approach is emphasised. This translates into cooperation among different professions (agronomists, veterinarians, public health authorities, politicians, food business operators) to establish a comprehensive and integrated prevention process that leads to the development of strategies and methods for minimizing contamination along the food-chain.

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8. ANNEX 1: ISOLATION OF DNA WITH DNEASY POWERSOIL KIT

The protocol is used for the isolation of genomic DNA from the concentrated RTE salads and berry washes.

Note: If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.

1. To the **PowerBead Tube**, add 250 µl of the sample and gently vortex to mix.
2. Add 60 µL of **Solution C1** and vortex briefly.
3. Secure **PowerBead Tubes** and **vortex briefly.**
4. Subject the tubes to 2 cycles of bead-beating of 4 m/s for 60 s with 45 s pause between cycles.
5. Centrifuge tubes at 10,000 x g for 30 seconds.
6. Transfer the supernatant (about 500 µL) to a clean 2 ml collection tube.
7. Add 250 µL of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
8. Centrifuge the tubes at room temperature for 1 minute at 10,000 × g.
9. Avoiding the pellet, transfer up to 600 µL of supernatant to a clean 2 ml Collection Tube.
10. Add 200 µL of **Solution C3** and vortex briefly. **Incubate at 4°C for 5 minutes**
11. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
12. Avoiding the pellet, transfer up to 750 µl of supernatant into a clean 2 ml Collection Tube.
13. Shake to mix **Solution C4** before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds.
14. Load 675 µl onto MB Spin column and centrifuge at 10,000 x g for 1 minute.
15. Repeat step 13 until all of the sample has been processed.
16. Add 500 µL of **Solution C5** and centrifuge at 10,000 x g for 30 seconds.
17. Discard the flow through. Centrifuge again at 10,000 x g for 1 minute.
18. Carefully place MB spin column in a clean 2 ml Collection Tube. Avoid splashing any Solution C5 onto the Spin column.
19. Add 50 µL of **Solution C6** to the center of the white filter membrane.
20. Centrifuge at 10,000 x g for 30 seconds and discard the Spin column. The DNA is now ready and can be stored at 4°C for imminent use or stored at -20°C for later use.

9. ANNEX 2: EXPERIENCE

This thesis shows the most important results obtained during my Ph.D. However, during this three-year period, I had the opportunity to take part in other projects and collaborate with several Italian and foreign institutions.

The **main activities not related to the Ph.D. project** are listed below:

- Antimicrobial resistance in the food chain.
  University of Foggia.

- Evaluation of the prevalence of vector-borne diseases, i.e., *Bartonella* spp., *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Leishmania* spp, in cats in Central and Southern Italy.
  In collaboration with the University of Teramo.

- Microscopy and molecular investigation on *Lipoptena* (Diptera: Hippoboscidae) circulating in wild ungulates in Northern Italy and elaboration of an updated taxonomic key for the identification of *Lipoptena* spp. of Europe.
  University of Foggia.

- Laboratory activities finalized at comparing two UNEX-based DNA extraction methods for the molecular detection of *C. cayetanensis*, *T. gondii*, and *C. parvum* as contaminants of berries.
  In collaboration with the Norwegian University of Life Sciences (NMBU).

- Molecular and microscopical investigation on *Megaselia* spp. larvae collected from a child.
  In collaboration with Bambino Gesù Children's Hospital, Rome.

- Molecular and microscopical investigation on a *Dermanyssus gallinae* specimen isolated from a dog and specimens collected from a poultry farm.
  In collaboration with the University of Torino.

Publications:

- Cafiero, M. A., **Barlaam, A.**, Camarda, A., Radeski, M., Mul, M., Sparagano, O., & Giangaspero, A. (2019). *Dermanyssus gallinae* attacks humans. Mind the gap! *Avian Pathology*, *48*(sup1), S22-S34. https://doi.org/10.1080/03079457.2019.1633010

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- **Barlaam, A.**, Temesgen, T., Tysnes K., Rinaldi, L., Ferrari, N., Sannella, A., Normanno, G., Cacciò, S., Robertson, L. Giangaspero, A. Contamination of fresh produce sold on the Italian market with *Cyclospora cayetanensis* and *Echinococcus multilocularis*. Under review.

Research stays:

- **From the 4th to the 8th of September 2018**: Faculty of Veterinary Medicine, Teramo. Training on laboratory techniques applicable to the VBDs project.

- **From the 25th to the 27th of July 2018** & **from the 15th to the 18th of July 2019**: Short stay at Istituto Superiore di Sanità (ISS) to carry out research experiments in molecular biology related with the Ph.D thesis. Istituto Superiore di Sanità, Roma.

- **From the 20th to the 23rd of May 2019** & **from the 25th to the 26th July 2019**: Microscopic observation of fresh produce samples using the FLOTAC technique. Cremopar, University of Naples Federico II.

- **From the 1st to the 18th of February 2019 & from the 14th of August 2019 to the 31st of January 2020**: 6-months research stay at the Norwegian University of Life Sciences (NMBU), Oslo, Norway.

Participation to International and National Congress:

- **15th of March 2018**: "*La prospettiva* One Health *nella diagnostica infettivologica*". Bambino Gesù Children's Hospital, Rome.

- **From the 26th to the 28th of March 2018**: **"**One Health overview of PRM infestation and treatment – interdisciplinary approach" - Workshop - Working Group 2. Faculty of Veterinary Medicine – Skopje.

Speaker at workshops/conferences:

- **22nd of November 2018**: Workshop: "Nuovi rischi infettivi e parassitari nella catena alimentare". Department of Sciences of Agriculture, Food and Environment, University of Foggia. Oral presentation: "Contaminazione da protozoi negli alimenti vegetali, nell'ottica One Health".

- **From the 12th to the 14th of February 2019**: COST Action: FA1408. Euro-FBP: what next? INIAV, Oeiras, Portugal. Poster Presentation: "*Cyclospora* in Italy: current situation and perspectives".

- **From the 17th to the 21st of June 2019**: 1st Joint Meeting of Agriculture-Oriented PhD Programs at UniCT, UniFG, and UniUD. Higher education for sustainable food production. Department of Agriculture, Food and Environment, University of Catania. Oral presentation: "Investigation of *Cyclospora cayetanensis* in fresh produce in Italy".

- **From the 24th to the 26st of June 2019**: 7th International *Giardia* and *Cryptosporidium* conference. Rouen, France. Short talk: "Investigation on *Cyclospora cayetanensis* in fresh produce in Italy".

- **From the 14th to the 16th of September 2020**: 2nd Joint Meeting of Agriculture-oriented PhD Programs (UniCT, UniFG and UniUD). Department of the Sciences of Agriculture, Food and Environment (SAFE), University of Foggia. Oral presentation: "Investigation on pathogenic parasites in fresh produce sold on the Italian market".

- **22nd of September 2020**: Spring Parasitology. SOIPA (Società Italiana di Parassitologia). ONLINE_Microsoft Teams. Oral presentation: "Investigation on pathogenic parasites in fresh produce sold on the Italian market".

I had the opportunity to take part in different **teaching activities**:

- **From January 2018 to present**: Practical training for undergraduate students on microscopy and parasites identification in the context of the courses “Parasitic diseases of livestock” and “Anthropozoonoses” (Prof. Annunziata Giangaspero). University of Foggia.

- **From February 2020 to present**: Teaching assistant in the context of the courses “Parasitic diseases of livestock” and “Anthropozoonoses” (Prof. Annunziata Giangaspero). University of Foggia.

I successfully completed an **English language course** and passed the exam for the Cambridge English Qualification:

From November to June 2019: CAE (C1) Advanced English Preparation Course. University Language Centre, Università di Foggia. Cambridge English: C1 Advanced (CAE). Score: 195/210.

10. ACKNOWLEDGMENTS

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Mom and dad, you got me here. Thank you for having always supported me along my journey in life.

Mattia, my nephew, whom I love so much, for reminding me with his smile how beautiful life is, even in the darkest of times.

Last but not least, my biggest thanks go **to my big crazy family and all my friends**, I feel lucky to be surrounded by such an amazing crew. Thank you all!

Ph.D. Student: Alessandra Barlaam

Tutor: Prof. Annunziata Giangaspero

Giangaspero Annunziata 09.01.2021 11:21:20 UTC