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TITOLO

**Emerging protozoan parasites and food safety:
investigation in '*ready to eat*' packaged salads by
microscopy and molecular tools**

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Il forte supererà un ostacolo; il saggio, l'intero percorso

(Cit. anonima)

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ABSTRACT

Background

Ready-to-eat (RTE) vegetables undergo minimum conservation treatments after harvesting in order to maintain their organoleptic and sensory characteristics, and are sold already cleaned, cut, washed and packed in a protected atmosphere.

Italy is Europe's second largest market for fresh-cut products after France. Vegetables may be contaminated in various ways along the food production chain i.e., during pre-harvesting process, during harvesting, transport and market processing

Since the majority of these products are eaten raw, to prevent the risk for human health, these products are covered by the EU and National laws, which defines the presence and microbiological limits only for *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. However, in addition to bacteria, several protozoan parasites contained in human/animal excreta can contaminate soil and vegetables. *Giardia duodenalis*, *Cryptosporidium* spp., *Toxoplasma gondii* and *Cyclospora cayetanensis* are the most important emerging parasitic protozoans, which commonly infect humans and animals, and numerous foodborne outbreaks associated with the consumption of fresh produce contaminated with these protozoan parasites have been reported worldwide.

Despite guidelines/regulations, which support the need for tracking, monitoring and surveillance of food products, studies investigating parasite contamination of RTE vegetables products are very limited worldwide.

The aim of this PhD research was to investigate by microscopy and molecular tools the occurrence and the prevalence of *G. duodenalis*, *Cryptosporidium* spp., *T. gondii* and *C. cayetanensis* oo/cysts in 'ready to eat' (RTE) salads on sale in Italy. A characterization of the isolates, quantification of the *T. gondii* and *C. cayetanensis* DNA isolates and an evaluation of the seasonal variation and prevalence variabilities between brands companies were also performed.

Methods

From March 2015 to February 2016, 648 packages of RTE salads produced by industrial and local branded companies were purchased from supermarkets and grocery shops, respectively. The sampling design was set in order to provide a 95% confidence level

with a precision of 0.6%. Nine individual packages (100g each) were collected for each brand per sampling month, and 72 pools were created and processed.

After concentration, the pellets were subjected to both microscopy (Lugol, Ziehl-Neelsen and IF) and molecular analyses (Nested and Semi-nested, *qPCR*, EndPoint PCR) according to the pathogen, and the isolates were sequenced.

The results were statistically analyzed through multiple testing and a combined prevalence as testing in parallel was estimated. Difference in prevalence between brands and sampling seasons was tested with a group regression model.

Results

A total of 864 slides were microscopically examined, and *Cryptosporidium* spp. and also *Blastocystis hominis* and *Dientamoeba fragilis* were detected using one or more microscopy techniques. *G. duodenalis* assemblage A, *Cryptosporidium parvum* and *Cryptosporidium ubiquitum*, *T. gondii* Type I and *C. cayetanensis* were identified using molecular tools. *B. hominis* and *D. fragilis* were also molecularly confirmed.

The overall prevalence of each protozoan species was 0.6% (95% C.I. 0.2-1.6%) for *G. duodenalis*, 0.8 (95% C.I. 0.3-1.8%) for *T. gondii*, 0.9% (95% C.I. 0.4-2.1) for *Cryptosporidium* spp., and 1.3% (95% C.I. 0.6-2.5%) for *C. cayetanensis* while the prevalence for *B. hominis* was 0.5% (95% C.I. 0.1-1.4%) and 0.2% (95% C.I. 0.0-0.9%) for *D. fragilis*.

In total, using both microscopy and molecular tools, 4.2% (95% C.I. 2.6-6.2%) of samples were found to be contaminated by at least 1 protozoan species, and 0.6% (95% C.I. 0.2-1.6%) presented coinfections of two species. The number of oocysts for *T. gondii* and *C. cayetanensis* in *qPCR* test-positive samples was predicted to range from 62 to 554 and 46 to 1.580 per g of vegetable product, respectively.

Discussion and Conclusions

The present PhD work represents the first large-scale study on the presence of protozoa in packaged salads in Europe. The results show that the prevalence of protozoan species in RTE salads is a cause for concern about human health in Europe, and in particular in Italy. Pending the inclusion of protozoan parasites in EU and Italian legislation to reduce the risks of RTE contamination and minimize their foodborne transmission, our results indicate the need for additional surveillance studies of possible sources of food

contamination. The sampling methods designed in this research and the results obtained can provide the direction for monitoring fresh produce in other areas, and for surveillance studies on products. In addition, they can provide the basis for food safety guidelines, based also on the HACCP system, in order to reduce the risk of RTE contamination and to minimize foodborne disease transmission.

1. INTRODUCTION

In recent years, the authorities responsible for food safety have become increasingly concerned about foodborne diseases, which not only significantly affect people's health and well-being, but they also have economic consequences for individuals, communities, businesses and countries. In industrialized countries, among other drivers (i.e., environment, climate, land use, trade), the risk of food-borne diseases transmission is also enhanced by the ongoing changes in dietary habits (Broglia and Kapel, 2011), involving a worldwide increase in consumer demand for ready foods, in particular for fresh vegetables/fruits, due to their health benefits.

'Ready-to-eat' (RTE) products are foods prepared to ease consumption, usually sealed in plastic film or packets as room temperature, shelf-stable or refrigerated/frozen food products. These food products do not require further processing for consumption, providing convenience to the modern consumer who needs healthy but quick to consume foods. This group of foods is included in the so called "IV gamme" products, i.e. all fresh foods, including the "ready to eat" vegetables, used for human consumption, packaged and ready to use, which after harvesting are subjected to minimum technological processes with the aim of maintaining their freshness by following good manufacturing practices: selection, sorting, cutting, washing, drying and packing in bags or in sealed containers, with possibly the use of a protective atmosphere.

Currently, the fresh-cut salads cover about the 50% of the market volume, fresh-cut fruits account for more than 10% and other fresh-cut vegetables (e.g. *crudit *, soup mix, stir-fry vegetables) cover the remaining 40% worldwide (Cavaiuolo *et al.*, 2015).

Italy is Europe's second largest market for fresh-cut produce after France. In the period 2010-2015, the Italian fresh cut salad market has registered + 9.9% increase; RTE salads account for approximately 75% of sales, and are mostly mixed salads (Confcooperative, 2016; IsmeaMercati, 2016). In Italy, approximately 500 companies and 120 processing plants are involved in the production of RTE vegetables. These companies are mainly located in Northern Italy, while the farms that provide the raw material are located mainly in Southern Italy (Casati and Baldi, 2011).

Vegetables may be contaminated by several pathogens in various ways and at any point before and along the food production chain, i.e., during pre-harvesting process, during

harvesting, transport and market processing (Francis *et al.*, 1999; Chaidez *et al.*, 2005; Johnston, 2005).

Since the majority of these products are eaten raw, to prevent the risk for human health, these products are covered by the EU and National laws (L.M 13.05.2011, No.77; EC Reg. 852 of 2004; EC Reg. 20703/2005 and 1441/2007; EC Reg. 209, 2013), which define the presence and microbiological limits for *Escherichia coli*, including some verocytotoxigenic *E. coli*, *Listeria monocytogenes* and *Salmonella* spp.

Despite these rules, the presence of bacteria on RTE products has been detected worldwide (Francis *et al.*, 1999; Legnani and Leoni, 2004; Kovacevic *et al.*, 2013; Jeddi *et al.*, 2014; Gurler *et al.*, 2015; Losio *et al.*, 2015).

However, in addition to bacteria, other pathogenic microorganisms can contaminate fresh vegetables, such as viruses (Hepatitis A virus, *Norovirus*, *Rotavirus*) and protozoan parasites, which through human and/or animal excreta - transported directly or by rainfall-initiated run-off from agricultural, suburban and urban wastewater discharges, irrigation water or infected food handlers - can contaminate vegetables.

Among protozoans *Giardia duodenalis*, *Cryptosporidium* spp., *Toxoplasma gondii* and *Cyclospora cayetanensis* are the most important and emerging parasitic protozoans and of zoonotic interest (Dubey, 2008; Fletcher *et al.*, 2012).

G. duodenalis and *Cryptosporidium* spp. are well-known causative agents of gastrointestinal disease in humans (particularly children) and animals worldwide (Putignani and Menichella, 2010; Feng and Xiao, 2011; Bouzid *et al.*, 2013). Infection occurs via the fecal-oral route by ingestion of *G. duodenalis* cysts and *Cryptosporidium* oocysts. Eight major genetic groups of *G. duodenalis* (assemblages) have been identified (A–H) to date, and assemblage A and, with a lesser extent, assemblage B are considered to be of the most zoonotic interest (Feng and Xiao, 2011). As to *Cryptosporidium*, of the 30 *Cryptosporidium* species recognized as valid, more than 20 species and genotypes have been identified in humans; however, the majority of human cryptosporidiosis is caused either by the zoonotic *Cryptosporidium parvum* or by the more anthroponotic *Cryptosporidium hominis* (Ryan *et al.*, 2016). Other species associated with human infections are *Cryptosporidium meleagridis*, *Cryptosporidium ubiquitum*, *Cryptosporidium cuniculus* (Ryan *et al.*, 2014).

T. gondii is an intracellular coccidian protozoan, and domestic and wild felids are the only hosts responsible for oocyst dissemination in the environment. Cats become infected after consuming intermediate host tissues harboring cysts, or after ingestion of sporulated oocysts. Humans become infected by ingesting raw or undercooked meat containing bradyzoites, or by ingesting sporulated oocysts through consumption of contaminated raw vegetables and drinking water or by direct contact with cat feces (Jones *et al.*, 2001). Toxoplasmosis is usually asymptomatic in immune-competent individuals, but may cause severe infections in immune-compromised patients and during pregnancy for fetuses and newborns (reviewed by Jones *et al.*, 2001; Barratt *et al.*, 2010). *T. gondii* has three clonal lineages that are widespread in North America and Europe (Sibley and Boothroyd, 1992; Howe and Sibley, 1995): Types I (highly pathogenic), II and III (less pathogenic but more likely to cause infection in immune-compromised patients) (Howe and Sibley, 1995; Khan *et al.*, 2005). Other genotypes and atypical strains are rare in Europe (Robert-Gangneux and Dardè, 2012).

C. cayetanensis is an obligate intracellular monoxenous coccidian parasite that infects the mucosal epithelium of the intestine or bile duct (Lainson, 2005), and the most frequently reported symptoms are diarrhea, nausea and abdominal pain. Humans are probably the only host for *C. cayetanensis* oocysts (Chacin-Bonilla, 2010), but since its zoonotic role is suspected, it remains to be determined (Chu *et al.*, 2004).

Giardia (cysts), *Cryptosporidium*, *Toxoplasma* and *Cyclospora* (oocysts) infective stages are highly resistant to routine chemical disinfectants or sanitizing water processing (Dawson, 2005; Jones and Dubey, 2010), which explains the reason of their wide diffusion in the environment (Jones and Dubey, 2010; Fletcher *et al.*, 2012) and in the food (Jones and Dubey, 2012; Fletcher *et al.*, 2012; Dixon *et al.*, 2013; Dixon, 2015).

Outbreaks of infections caused by protozoan parasites contained in contaminated fresh produce have been recorded worldwide (Putignani and Menichella, 2010; Ortega and Sanchez, 2010; Feng and Xiao, 2011; Dixon *et al.*, 2013; Kozak *et al.*, 2013), including Europe (Doller *et al.*, 2002; McKerr *et al.*, 2015; Aberg *et al.*, 2015).

In Italy, foodborne outbreaks associated with consumption of contaminated fresh vegetables have not been reported but the presence of these protozoans have been widely documented in humans (reviewed by Putignani and Menichella, 2010; Masucci *et al.*, 2011; Giangaspero *et al.*, 2015a) and in several animal species, both domestic and

wild animals (Berrilli *et al.*, 2011; Giangaspero *et al.*, 2007; Mancianti *et al.*, 2010, 2015; De Liberato *et al.*, 2015; Marangi *et al.*, 2015a), as well as in environmental samples, including vegetables (Mastrandrea and Micarelli, 1968; Briancesco and Bonadonna, 2005; Di Benedetto *et al.*, 2005, 2007; Lonigro *et al.*, 2006; Sacco *et al.*, 2006; Giangaspero *et al.*, 2015a, b; Marangi *et al.*, 2015b)

Despite FAO/WHO (2003) guidelines/regulations which support the need for tracking, monitoring and surveillance of food products, studies investigating parasite contamination of RTE products are limited to just a few reports from Canada (Dixon *et al.*, 2013; Lalonde and Gajadhar, 2016).

In this PhD work, we aimed to bridge a gap in knowledge about the safety of RTE salads and potential consumer health risks in Europe.

1.1 Aims

The general aim of this PhD study was:

- To investigate the occurrence and the prevalence of *G. duodenalis*, *Cryptosporidium* spp., *T. gondii* and *C. cayetanensis* in packaged salads ‘ready-to-eat’ mixed salad, sold by industrial and local branded companies and available in Italian food stores.

The specific aims were the following:

- To microscopically detect the presence of *G. duodenalis*, *Cryptosporidium* spp. and *C. cayetanensis* oo/cysts;
- To molecularly detect/confirm and genotype the isolates of *G. duodenalis*, *Cryptosporidium* spp., *T. gondii* and *C. cayetanensis*.
- To quantify the *T. gondii* and *C. cayetanensis* DNA isolates from the qPCR tested positive samples;
- To evaluate the seasonal variation and prevalence variabilities between the two kinds of brands, i.e., industrial vs. local brands.

2. GENERAL PART

2.1. Fresh-cut produce industry

Fresh-cut produce are low in calories and respond to the changing life-style and eating habits of the consumers. It is already known that fruits and vegetables are important components of a healthy diet because rich sources of vitamins and minerals, dietary fibers and a host of beneficial non-nutrient substances including plant sterols, flavonoids and other antioxidants (Slavin and Lloyd, 2012). In fact, the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) recommend the consumption of fruit and vegetable to decrease the risk of cardiovascular problems, diabetes and cancer (FAO, 2016).

International Fresh-cut Produce Association (IFPA) defines “Fresh-cut produce” 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 commodity, it has been trimmed, peeled, washed, cut and subsequently bagged or prepackaged to offer to the consumers the high nutrition, convenience and value while still maintaining freshness (IFPA, 2016).

Fresh-cut produce include any kind of fresh commodities and their mixtures in different cuts and packaging; packaged salads, baby carrots, stir-fry vegetable mixes, soup mixes and fresh-cut apples, pineapple, or melon are only just examples of this type of product. These products are also known with the term “minimally processed” and with the French term of “IV gamme”. From a commercial point of view the fruit and vegetable products are classified as “I gamme” i.e. fresh fruit and vegetable, “II gamme” i.e. vegetable preserves, “III gamme” i.e. frozen vegetables, “IV gamme” i.e. ready-to-eat vegetables or fruit, packaged and however fresh and natural without any additives, “V gamme” i.e. precooked vegetables, grilled, steamed without the addition of preservatives and condiments and not frozen (Sansavini and Ranalli, 2012).

The choice and quality of the material is important; only fruit and vegetables of the best quality in terms of development, physiological condition, appearance and integrity can withstand the minor induced stress during the preparation (reviewed by Colelli and Elia, 2009). However, fresh-cut produce are constituted by living cells and their shelf life is limited to few days or a week. Hence, the temperature, storage time, relative humidity

and modified atmosphere packaging can have effects on the quality and on the shelf life of the final produce (Legnani and Leoni 2004; reviewed by Colelli and Elia, 2009).

2.1.1. 'Ready-to-eat' salads and their productive process

Within the IV gamme products, 'ready-to-eat' (RTE) salads, dominate the production of minimally processed foods. RTE salads are mainly constitute by lettuces (*Lactuca* spp.), broad-leaved endive (*Cichorium intybus* L. cv *latifolium*), curly endive (*Cichorium intybus* L.) but also lamb's lettuce (*Valerianella locusta* L.), shredded carrots (*Daucus carota* L.) for the mixed salads.

The transformation processes of the ready to eat salads are aimed to reducing biological, physical, and chemical hazards associated with this type of products.

According to the current procedures, cultivars, weather conditions, irrigation practices, fertilizers and pest control programs, chilled storage throughout the entire cool chain, manufacturing and handling practices can affect the quality of the fresh produce (Nicola *et al.*, 2006; Colelli and Elia, 2009).

To ensure good results is essential to follow a few but fundamental criteria:

- Use of the high quality raw materials;
- Stringent levels of hygiene for both food and water used;
- Temperatures during the process, without ever interrupting the cold chain;
- Separation of process areas, avoiding the cross-contamination (Turatti, 2011)

The production process of the ready to eat salads is synthetically showed (**Figure 1**) and described below.

Figure 1. The production process of the 'ready to eat' (RTE) salads



Harvesting can be performed manually or with operating machines, especially for what concerns the mowing of baby leaf and the product, after harvesting, is transferred in a storage area.

After harvesting, the product has to be maintained in the storage area at a temperature of about 4 °C and in an environment with a controlled humidity and constantly monitored, to avoid a rapid perishability.

The first step in the process is the quality control of the raw materials. According to the good agricultural practice code (GAP, Good Agricultural Practices), the main criteria are the general appearance of the salads, including overall freshness, the absence of insects, physiological and microbial diseases, necrotic tissue, and compliance with regulations on pesticide residues and nitrate content.

Along the chain, the harvested product is manually selected by staff, place on counters or introduced in the line of processing by conveyor belts. In some cases, automatic systems can select the product according to the size, the morphological characteristics, the color or the component characteristics.

All unwanted parts of the plant, including most of the outer green leaves, yellowed or damaged leaves, core area, foreign materials are removed manually or mechanically (trimming). Generally, the blending of the different varieties of salads occurs manually on the trimming table or a sorting table. This operation involves the creation of a mixed salad of different types of salads.

During the production process of the 'ready to eat' salads waste materials are produced. These materials must be removed from the plant to avoid any cross-contamination. Inside the premises, equipment and machinery used for waste material must be clearly identified and never used for edible products. Moreover, they should be easy to wash and sanitize. Outside the premises, any reusable receptacle for waste material should be waterproof and easy to wash and sanitize.

After the operation of sorting, the salads are put on a conveyor belt and transported to a cutter machine, where the leaves will be cut. The blades should be sharpened and of the right thickness, in stainless steel and well sanitized with disinfectants. The staff should respect the good hygienic practices using appropriate individual protections for each operation to avoid any contamination.

After cutting, salad leaves are washed in washing tanks. Washing is essential in the production process of the ready to eat salads. In particular, it allows:

- the elimination of earthy debris and pesticides;
- the reduction of the amount of the bacterial load;
- the lowering of the temperature of the product, where the washing takes place in ice water;
- the elimination of the exudates and cellular juices, which could constitute nutrient resources for the proliferation of microorganisms.

An optimal wash system consists in three washing tanks where jets of water and air bubbles are blown, making more effective the removal of dirt from the surface of the product. In first tank (pre-washer), soil and any foreign bodies are removed, in the other tanks the product is washed and sanitized. The water used for washing of the salads is potable, in sufficient quantity (from 5 to 10 liters/kg), adequately refrigerated (from 1 to 4°C) and constantly recycled with self-cleaning filtering systems. Different chlorine-based disinfectants can be used for the sanitization of the washing waters as sodium hypochlorite (NaClO) or calcium (CaCl_2O_2), chlorine dioxide (ClO_2) or chlorine gas (Cl_2) are the most disinfectant agent used in the IV gamme industries. In water solution, the concentration of active chlorine is 50-200 ppm (Colelli and Elia, 2009); in Italy, active chlorine used during this step is 80-100 ppm for a contact time of 1-3 minutes.

Other disinfecting agents are hydrogen peroxide (H_2O_2), ozone (O_3), peracetic acid ($\text{C}_2\text{H}_4\text{O}_3$) and ultraviolet radiation (UV)¹ (Turatti, 2011).

After washing, the leaves arrive at the drying system by another conveyor belt. The main purpose is to prolong the shelf-life of the product in order to avoid the high amount of moisture, inside the packages (pouches or trays), which could promote the rapid deterioration of the product. Two methods are presently used for this operation: centrifugation systems and air tunnel systems.

In first system, the centrifugation cycle begins with a soft loading of the fragile leaves followed by a smooth acceleration and a careful discharge of the drained products. After the drying cycle, the basket rotates slowly and the product is discharged on the conveyor belt and transported to the next stage. The basket must have a completely

¹ The use of hydrogen peroxide (H_2O_2), ozone (O_3), peracetic acid ($\text{C}_2\text{H}_4\text{O}_3$) and ultraviolet radiation (UV) is limited because some of them can be used only at high concentrations (H_2O_2 ; $\text{C}_2\text{H}_4\text{O}_3$), or can be dangerous for food operators (O_3) or require financial investments (UV).

smooth inner surface without a central axle or other contact parts, which could directly damage the product.

By vibrating tables, the product is transported in the drying tunnel where the product is gently invested by a first ascending flow of dry air, which absorbs the water in contact with the surface of the salad leaves. A second dry airflow passes through countercurrent the product. At the end of the operation, the dried product is then cooled with cold air (2-4 ° C) until ready for the packaging phase.

In order to limit cross-contamination by airborne microorganisms, the airflow is filtered and disinfected with a UV tube (250–280 nm). The dry product is transported into the packing room. The packing room must be cleaned and refrigerated at 1–2°C and must be separated from the washing section (Turatti, 2011).

A weighing machine or an operator may be performed. In first case, the weight of plant tissues is transmitted to a computer that calculates the best combination to optimize the required weight. Weighed salad is released in a bag or in a tray and packaged in a modified atmosphere (Varoquaux and Mazollier, 2002).

It has been developed the Modified Atmosphere Packaging (MAP) to extend the “shelf-life” (the time interval during which the food maintains an acceptable safety and quality level, under certain storage conditions) and quality of these vegetables. MAP uses low oxygen and enriched CO₂ levels to preserve the quality of fresh-cut produce and their shelf life (5-7 days at 4-6°C) (Colelli and Elia, 2009).

Then, the product is sealed and labeled (day of production, place of production, storage conditions, expiry date and ingredients used) (<http://www.aiipa.it/prodotti-vegetali/prodotti-ortofrutticoli-di-iv-gamma/info-documenti/item/201-cosa-e-la-iv-gamma>).

2.1.2 ‘Ready to eat’ salads in Europe and in Italy

Fresh-cut produce showed a positive trend in the market over the last two decades worldwide. Currently, the 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. crudité, soup mix, stir-fry vegetables) cover the remaining 40% worldwide. (Cavauiolo *et al.*, 2015).

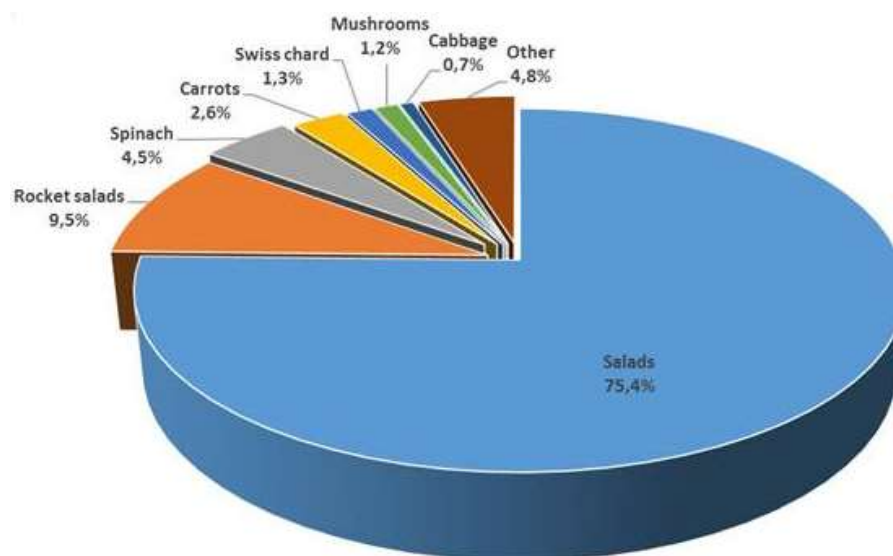
In Europe, Florette Group introduced ‘ready-to-eat’ salads in France in the early 1980s. It was the first production unit of fresh-cut vegetables in Europe, which subsequently

started various activities to export to other countries such as the United Kingdom, Italy, and Switzerland. Fresh-cut produce have been adapted to each country according to consumer preferences, production, distribution, and legislation (Rojas-Graü *et al.*, 2011). In period 2010 - 2015, in France, sales volume and sales value of the RTE salads have increased of + 9% and + 21.9% and in same period in Spain, sales volume and sales value have increased of + 12.8% and + 37.5%, respectively. In the German market, the RTE salads have been the products that have recorded the highest level of growth in terms of volume and value of sales, + 34.7% and + 47.6%, respectively.

In Europe, Italy is the second producer and RTE salads market has recorded an increase of + 9.9% and an increase of sales of + 10.1% (Confcooperative, 2016).

In particular, RTE mixed salads covers about the 75% of the sales of fresh-cut vegetables, followed by rocket salads (9%) and other fresh cut vegetables (e.g. spinach, carrots, beets, mushrooms, cabbage, spices and vegetable soups) with the remaining 16% (**Figure 2**) (IsmeaMercati, 2016).

Figure 2. Sales fresh cut vegetables in 2015 on Italian territory (% sales)



Source: elaborated by ISMEA on database Nielsen Market Track (IsmeaMercati, 2016)

In Italy, there are approximately 500 companies and 120 processing plants, involved in the production of RTE vegetables. These companies are located mainly in Lombardy (35%), Campania (26%) and Veneto (9%), while the companies that provide the raw material are located mainly in southern Italy (Casati and Baldi, 2011) (**Figure 3**).

Figure 3. Localization of the fresh-cut companies in Italy



Source: elaborated by DEPAAA on data from different producer organizations (Casati and Baldi, 2011)

According to the AOP UNOLOMBARDIA (the main association of producers of fourth range in Italy) estimated that the available area for the production of fresh-cut vegetables is about 6500 ha, mainly in greenhouse (in northern Italy) or in open air (in southern Italy) with 5-6 production cycles per year can be carried out (Baldi, 2007). In Italy, the leader companies in the field are *Bonduelle* srl (French company), and *La Linea Verde*, an Italian company in the province of Brescia, followed by *Coop*, *Conad* and *Jentu-Agronomia* (Angelini *et al.*, 2011) whereas in the Apulia, the area where this work has been carried out, the distributed brands are *OrtofrescoPulito*, *Agroama*, *Naturalissimi e Freschissimi* and *Terre e Gusti*.

2.1.3 European and National regulations

RTE products are regulated by the European - *EC Regulation No 852/2004* of 29 April 2004 and *No 2073/2005* of 15 November 2005, amended in *EC Regulation No 1441/2007* of 5 December 2007 – and by National Law *13 Maggio 2011 No 77*.

General criteria

Regulation No 852/2004 establishes the rules that the food business operators that carry out the primary production must take into account on the hygiene of foodstuffs, i.e.

- a) compliance with microbiological criteria for foodstuffs;
- b) procedures necessary to meet targets set to achieve the objectives of this Regulation;
- c) maintenance of the cold chain;
- d) sampling and analysis.

In particular, food business operators producing or harvesting plant products are required to take adequate measures, i.e.

- a) keep clean the facilities, equipment, containers, crates, vehicles and vessels;
- b) maintain a high degree of personal cleanliness, to wear clean and protective clothing and be in good health;
- c) ensure the hygiene during the production, the transport and storage;
- d) as far as possible, prevent animals and pests from causing contamination;
- e) store and handle wastes and hazardous substances so as to prevent contamination;
- f) take account of the analyses carried out on samples taken from plants or other samples that have importance to human health;
- g) use plant protection products and biocides correctly, as required by the relevant legislation.

In addition, during the production, potable water should be used to prevent contaminations.

According to the Regulation, in order to obtain a higher standard of food safety, food business operators must apply procedures based on the application of hazard analysis and critical control point (Hazard-Analysis and Critical Control Points) principles.

The HACCP principles are:

- i) identify any hazards that must be prevented, eliminated or reduced to acceptable levels;
 - ii) identify the critical control points at the step or steps at which control is essential to prevent or eliminate a hazard or to reduce it to acceptable levels;
 - iii) establish critical limits at critical control points which separate acceptability from unacceptability for the prevention, elimination or reduction of identified hazards;
 - iv) establish and implementing effective monitoring procedures at critical control points;
 - v) establish corrective actions when monitoring indicates that a critical control point is not under control;
 - vi) establish procedures for verification, which include supplementary tests, and procedures to confirm that the HACCP system is working effectively;
 - vii) establish documents and records commensurate with the nature and size of the food business to demonstrate the effective application of previously reported measures.
- When any modification is made in the product, process, or any step, the food operators shall review the procedure and make the necessary changes to it.

Microbiological criteria

Regulation (EC) No 2073/2005 establishes the microbiological criteria for foodstuffs and the implementing rules that must be respected by food operators.

This regulation specifies the detection methods that refer to specific International Organization for Standardization (ISO) standards for food samples.

For the RTE vegetables, the microbiological criteria include the detection of bacteria i.e. *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli*.

In particular:

- *L. monocytogenes*, must not exceed the limit of 100 cfu/g in products placed on the market and it must be absent (in 25 g of product) during the production processes.
- *Salmonella* spp. must be absent (in 25 g of product) in products placed on the market during their shelf-life.
- *E. coli* must be equal to m (minimum) = 100 cfu/g and not higher to M (maximum) = 1000 cfu/g during the production process.

The National Law *13 Maggio 2011 No 77* incorporate the European regulations and it establishes the marketing procedures on Italian territory. Products can be packaged individually or in mixture, in different weight and size containers and distributed by supermarkets and ipermarkets or by vending machines.

2.2. Foodborne pathogens

Vegetables may be contaminated by several pathogens (bacteria, virus, parasites) in various ways, along the productive chain, i.e., during crop production (e.g. human/animal excreta transported directly or by rainfall-initiated run-off from agricultural, suburban and urban wastewater discharges, irrigation with contaminated water, organic fertilizers, manure, soil, etc.) (Chaidez *et al.*, 2005; Amoros *et al.*, 2010; Jung *et al.*, 2014), during harvesting and processing (Francis *et al.*, 1999; Johnston, 2005; Jung *et al.*, 2014), directly by infected food handlers (Beuchat and Ryu, 1997). Practices in the food industries such as cutting, shredding and slicing processes of the leafy vegetables may increase the risk of contamination during preparation of fresh-cut salads (Zilelidou *et al.*, 2015). Furthermore, processing and storage conditions may influence the microbiological quality of minimally processed vegetables, favoring the development and the replication (this is the case of bacteria or viruses) (Legnani and Leoni, 2004) or the survival and infectivity (in the case of the protozoans).

2.2.1 Overview of bacterial and viral pathogens

Despite EU and National Laws regulate the presence of *Escherichia coli*, *Listeria* spp. and *Salmonella* spp. (see Chapter 2.1.2) these bacteria can be detected in fresh produce (Campos *et al.*, 2013), including RTE. Beside these pathogens, other bacteria, i.e. *Campylobacter* spp., *Yersinia enterocolitica*, and enteric viruses (*Norovirus* and *Rotavirus*) have been documented on ready-to-eat salads in several studies worldwide (Sagoo *et al.*, 2003; Mattison *et al.*, 2010; Althaus *et al.*, 2012; Campos *et al.*, 2013; Kovacevic *et al.*, 2013; Jeddi *et al.*, 2014; Gurler *et al.*, 2015; Losio *et al.*, 2015) and outbreaks associated with the consumption of contaminated leafy green vegetables and their ready to eat salads have been reported worldwide (Francis *et al.*, 1999; Legnani and Leoni, 2004; Mercanoglu-Taban and Halkman, 2011; Martínez-Vaz *et al.*, 2014).

2.2.2 Parasites

Several species of helminths and protozoans (reviewed by Dixon, 2015) can be detected in fresh produce.

The presence of helminths in RTE salads has not been documented but helminths eggs and/or larvae have been reported in marketed raw vegetables mostly in Eastern countries, - related to poor sanitation and inadequate personal hygiene - such as in Ghana (*Strongyloides stercoralis*, *Trichuris trichiura* and *Enterobius vermicularis*) (Duedu *et al.*, 2014); Egypt (*Enterobius vermicularis*, *Hymenolepis nana*, *Hymenolepis diminuta* and *Ascaris lumbricoides*) (Eraky *et al.*, 2014); India (*Ascaris lumbricoides*) (Sunil *et al.*, 2014); Pakistan (*Ascaris* spp., *Trichostrongyloides* sp.) (ul-Haq *et al.*, 2014); Syria (*Enterobius vermicularis* and *Ascaris lumbricoides*) (Alhabbal, 2015); Sudan (*Ascaris lumbricoides*, *Strongyloides stercoralis* and *Trichuris trichiura*) (Mohamed *et al.*, 2016); Iran (*Ascaris lumbricoides*, *Trichuris trichiura*, *Toxocara* spp., *Trichostrongylus* spp., *Taenia* sp. and *Hymenolepis nana*) (Rostami *et al.*, 2016);

As to protozoans, the presence of *G. duodenalis*, *Cryptosporidium* spp., *T. gondii* and *C. cayetanensis* - which are considered by the scientific community the most important and emerging parasitic protozoans - in vegetables is discussed in detail in the present thesis (see Chapter 2.3).

Other protozoans which can contaminate fresh produce are *Entamoeba histolytica*, *Cystoisospora belli* (formerly *Isospora belli*) *Balantidium coli*, *Blastocystis hominis* but foodborne illness due to infection with these species is largely sporadic and few outbreaks have been documented (Dixon, 2015). *Entamoeba histolytica* has been recorded in raw vegetable in several countries (Dixon, 2015). *Cystoisospora belli* and/or *Balantidium coli* oocysts has been reported in raw vegetables in Kenya (Nyarango *et al.*, 2008) and in Iran (Ebrahimzadeh *et al.*, 2013; Nazemi *et al.*, 2012). *Blastocystis hominis* cysts have been reported on leafy vegetables, i.e., lettuce, cress, radish, green onion and leek, purchased from local markets in Saudia Arabia with a prevalence of the 12.6% (Al-Binali *et al.*, 2006) and 17.1% (Al-Megrin, 2010).

2.3 Investigated foodborne protozoan pathogens

2.3.1 *GIARDIA DUODENALIS*

2.3.1.1 Classification and Morphology

Giardia belongs to the Phylum Zoomastigophora, Class Zoomastigophorea, and Order Diplomonadida, Family Hexamitidae. *Giardia* is a flagellate protozoan observed by Van Leeuwenhoek in 1681 and then fully described by Lamb in 1859.

Species of the genus *Giardia* infect numerous hosts, ranging from mammals to amphibians and birds. Currently, seven *Giardia* species have been identified: *Giardia agilis* in amphibians, *Giardia ardeae* and *Giardia psittaci* in birds, *Giardia microti* in voles and muskrats, *Giardia muris* in rodents, *Giardia varani* in lizards and *Giardia duodenalis* (synonyms *G. intestinalis* and *G. lamblia*) in mammalians, including humans (**Table 1**).

G. duodenalis is the only *Giardia* species that causes human infection and it is now considered a multispecies complex. *G. duodenalis* consists of eight genetically distinct assemblages, designated A to H. Assemblages A and B are considered to have a broad range of hosts (humans and numerous species of mammals) and for this reason, they can be considered zoonotic. Assemblages C and D have been identified in dogs and wild canines, assemblage E in artiodactyls, assemblage F in cats, assemblage G in rodents and assemblage H in seals (reviewed by Feng and Xiao, 2011; Heyworth *et al.*, 2016) (**Table 1**).

Table 1. Established *Giardia* species and *G. duodenalis* assemblages (source: Feng and Xiao, 2010)

Species	Major host(s)
<i>G. agilis</i> Kunstler, 1882	Amphibians
<i>G. ardeae</i> Noller, 1920.....	Birds
<i>G. microti</i> Benson, 1908.....	Muskrats and voles
<i>G. muris</i> Benson, 1908.....	Rodents
<i>G. psittaci</i> Erlandsen and Bemrick, 1987.....	Birds
<i>G. varani</i> Lavier, 1923 ^a	Lizards
<i>G. duodenalis</i> Davaine, 1875.....	Mammals
Assemblage A (= <i>G. duodenalis</i> sensu stricto? ^{ab}).....	Humans, nonhuman primates, domestic and wild ruminants, alpacas, pigs, horses, domestic and wild canines, cats, ferrets, rodents, marsupials, other mammals
Assemblage B (= <i>G. enterica</i> ? ^{ab}).....	Humans, nonhuman primates, cattle, dogs, horses, rabbits, beavers, muskrats
Assemblage C (= <i>G. canis</i> ? ^{ab}).....	Domestic and wild canines
Assemblage D (= <i>G. canis</i> ? ^{ab}).....	Domestic and wild canines
Assemblage E (= <i>G. bovis</i> ? ^{ab}).....	Domestic ruminants, pigs
Assemblage F (= <i>G. cati</i> ? ^{ab}).....	Cats
Assemblage G (= <i>G. simondi</i> ? ^{ab}).....	Mice, rats
Assemblage H.....	Seals

^a To be supported by molecular biological characterizations.

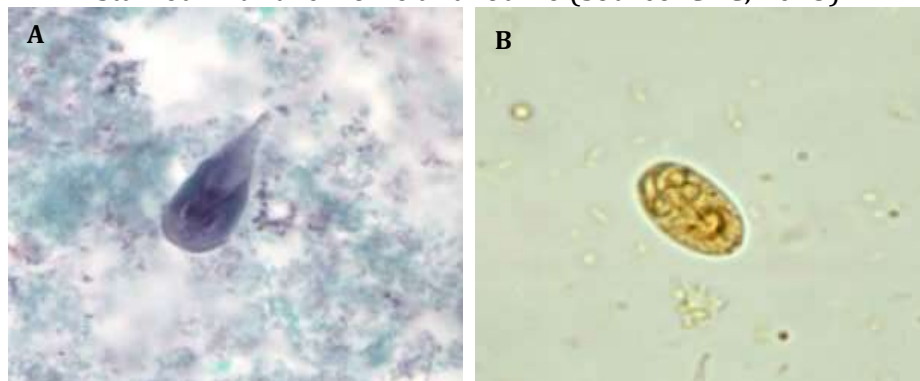
^b Species names recently proposed (180, 260, 261).

From a morphological point of view, *Giardia* has two forms: the “resistance form” represented by the cyst that allows the propagation of the parasite in the environment, and the “vegetative form” identified in the trophozoite, who lives in host intestinal lumen.

The cysts are approximately 8-12 μm long x 7-10 μm wide. A wall that is 0.3 to 0.5 μm thick and composed of an outer filamentous layer and an inner membranous layer with two membranes covers them. The outer portion of the cyst wall is covered by a web of 7 to 20nm filaments (Erlandsen *et al.*, 1989, 1990). Into the cytoplasm are present: 4 nuclei clustered in pairs at one end, axonemes that runs diagonally through the cyst, retracted flagella, basal bodies and part of the ventral disk, which will form the trophozoites after the excystation (Scaglia *et al.*, 1987).

The trophozoites are approximately 12 to 15 μm long and 5 to 9 μm wide. They are pear-shaped, rounded anterior end, posterior end pointed. The dorsal surface is convex while the ventral surface is concave. The ventral surface has a ventral disk or adhesive disk, which adheres to the surface of intestinal cell. The cytoskeleton includes a median body, four pairs of flagella (2 anterior, 2 posterior, 2 caudal, and 2 ventral), and the ventral disk (reviewed by Adam *et al.*, 2001) (**Figure 4a, b**).

Figure 4 (a, b). Trophozoite (A) and cyst (B) of *Giardia duodenalis* stained with trichrome and iodine (source: CDC, 2015)



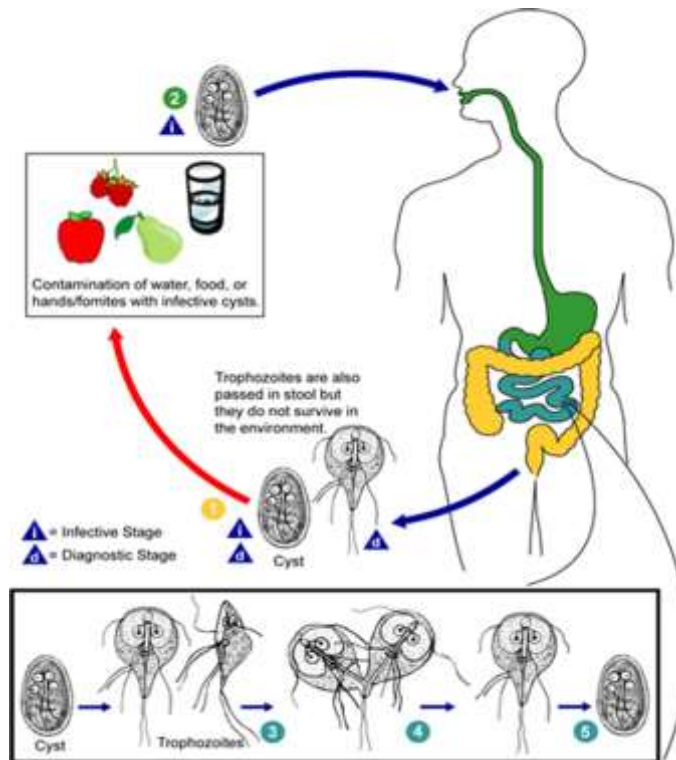
The ventral disk is a unique and important component and it appears as a concave structure with a maximum depth of 0.4 μm covering the entire ventral surface.

The trophozoites have two nuclei that are nearly identical in appearance and replicate approximately in the same time (Wiesehahn *et al.*, 1984).

2.3.1.2 Biology and Life Cycle

G. duodenalis has a direct life cycle (**Figure 5**) and the infective stage of the parasite, the cyst, encysts when released into the feces and is immediately infectious.

Figure 5. Life Cycle of *Giardia duodenalis* (source: CDC, 2015)



Cysts are the main responsible for transmission of giardiasis. However, in case of acute infections, the vegetative forms can be also eliminated with the feces and if soon ingested by a new subject or by the same host, they are able to reach the intestinal tract and colonize it (Thompson *et al.*, 1993).

After exposure to the acidic environment of the stomach (gastric acid, gastric and pancreatic enzymes), cysts excyst into trophozoites in the proximal small intestine (each cyst produced two trophozoites). The process of excystation is rapid, and it is completed within 10 minutes (Buchel *et al.*, 1987). The emerging trophozoite undergoes cytokinesis within 15 to 30 min after the onset of excystation, so that two trophozoites are formed from one cyst.

The trophozoites colonize the small intestine of their host, mostly in the mid-jejunum. Here, they replicate by binary fission and they adhere through their concave ventral surfaces (ventral adhesive disk) to the intestinal wall, where they obtain the necessary

nutrients (reviewed by Adam, 2001). After exposure to biliary secretions, trophozoites encyst in the jejunum and they pass in the feces. Once excreted, the cysts are immediately infective for a new host.

2.3.1.3 Epidemiology

Cysts can be ingested with contaminated water or food or through direct fecal-oral contact.

Due to the huge amount of data, prevalence recorded in humans, animals, water and food in the last 10 years are herewith synthesized and limited, except for vegetables, to the European scenario.

Humans - *G. duodenalis* has a global distribution. However, in developing countries, the infection rate of giardiasis in humans is very high, reaching up to 40%, whereas in developed countries the prevalence is lower (up to 7%) but in some specific areas it can be higher (Feng and Xiao 2011).

In Europe, studies report a prevalence of giardiasis in humans from 2.9 to 5.8% in the Nordic countries (i.e., Denmark, Finland, Norway and Sweden) (Horman *et al.*, 2004), 1.5% in Germany (Sagebiel *et al.*, 2009), 4.0% in Belgium (Geurden *et al.*, 2009), 1.3% in United Kingdom (Davies *et al.*, 2009), 3.7% in Portugal (Almeida *et al.*, 2006) and 5.4% in Spain (Manzardo *et al.*, 2008).

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; Giacometti *et al.* 2000). In non-immune-compromised human population, *G. duodenalis* was recorded with a prevalence from 0.94 to 4.66% (Giangaspero *et al.*, 2007). Later, 1.3% to 4.8% of prevalence have been registered in two different studies carried out in Northern (Guidetti *et al.*, 2010) and Central Italy (Masucci *et al.*, 2011) with the highest rate in children younger than 15 years of age (Masucci *et al.*, 2011).

Similarly to other countries, the zoonotic assemblage (assemblage A) was more common (54.2%) than assemblage B (32.5%) (Giangaspero *et al.*, 2007).

Animals- Giardiasis has been recorded in several animal species worldwide ranging from 1.5% to 57.8% in farm animals, from 2% to 100% in companion animals and from 1.4% to 100% in wild animals with higher prevalence of the assemblage A than B (Feng and Xiao 2011).

In Europe, data from the last 10 years are synthetized in this Table.

Animal species	Country	Prevalence	Assemblages	References
Cattle	Belgium	17.4%- 31.3%	A, E and A+ E	Geurden <i>et al.</i> , 2004, 2008a
	Denmark	43.6%	A, E	Maddox-Hyttel <i>et al.</i> , 2006; Langkjaer <i>et al.</i> , 2007
	Germany	38.0% to 72.4%	A, E	Jager <i>et al.</i> , 2005; Gillhuber <i>et al.</i> , 2013
	Norway	49.0%	A	Hamnes <i>et al.</i> , 2006; Robertson <i>et al.</i> , 2007
	Poland	2.2 to 14.0%	-	Bajer, 2008
	Portugal	9.0%	A, B, E	Mendonca <i>et al.</i> , 2007
	Spain	26.6 to 30.1%	E	Castro-Hermida <i>et al.</i> , 2006, 2007
	Italy	66.6%	A, B, E	Giangaspero <i>et al.</i> , 2007
Sheep	Belgium	25.5%	A, E	Geurden <i>et al.</i> , 2008b
	Norway	26.8%	A, E	Robertson <i>et al.</i> , 2010
	Poland	1.3%	A, B	Bajer, 2008
	Spain	19.2 to 42.0%	A, B	Castro-Hermida 2006, 2007; Gomez-Munoz <i>et al.</i> , 2009
	Italy	1.5%	A, B	Giangaspero <i>et al.</i> , 2007
Goat	Belgium	35.8%	A	Geurden <i>et al.</i> , 2008b
	Spain	13.0 to 42.2%	A, E	Castro-Hermida <i>et al.</i> , 2005, 2007; Ruiz <i>et al.</i> , 2008
	Italy	-	-	-
Dogs	Belgium	22.7%	A, B, C, D	Claerebout <i>et al.</i> , 2009
	Norway	8.2%	A, C	Hamnes <i>et al.</i> , 2007a
	Finland	5.3%	C, D	Rimhanen-Finne <i>et al.</i> , 2007
	Germany	2.3%	A, C, D	Epe <i>et al.</i> , 2004
	Netherlands	15.2%	A, C, D	van der Giessen <i>et al.</i> , 2006
	Poland	2.0 to 36.0%	A, C, D	Bajer, 2008, Solarczyk and Majewska, 2010
	United Kingdom	8.4 to 21.0%	A, C, D	Batchelor <i>et al.</i> , 2008; Upjohn <i>et al.</i> , 2010
	Spain	7.0% to 33%	A, B, C, D	Martinez-Moreno <i>et al.</i> , 2007; Dado <i>et al.</i> , 2012; Gil <i>et al.</i> , 2017
	Greece	4.3%	A, C, D	Papazahariadou <i>et al.</i> , 2007
	Italy	0.2% to 80%	A, C, D	Giangaspero <i>et al.</i> , 2007; Paoletti <i>et al.</i> , 2015
Cats	Netherlands	13.6%	A, D	Overgaauw <i>et al.</i> , 2009
	Germany	1.1%	A	Epe <i>et al.</i> , 2004
	United Kingdom	5.3%	A, F	Gow <i>et al.</i> , 2009
	Italy	5% to 15.8%	A, F	Giangaspero <i>et al.</i> , 2007; Papini <i>et al.</i> , 2007; Paoletti <i>et al.</i> , 2011
Marine animals	Island	54% and 20%	A and B	Lasek-Nesselquist <i>et al.</i> , 2008
Chamois	Italy	4.45%	A, E	De Liberato <i>et al.</i> , 2015
Fallow deer	Sweden	-	A	Lebbad <i>et al.</i> , 2010
	Italy	11.5%	A	Giangaspero <i>et al.</i> , 2007
Zoo parrots	Italy	18.7%	A	Papini <i>et al.</i> , 2012
Red fox	Norway	4.8%	A, B	Hamnes <i>et al.</i> , 2007b
Non-human primates	Sweden	-	B	Lebbad <i>et al.</i> , 2010
	Belgium	78.6%	B, A+B	Levecke <i>et al.</i> , 2009
	Italy	47.0%	B	Berrilli <i>et al.</i> , 2011

Water – *Giardia*, together with *Cryptosporidium*, is the most frequently reported waterborne protozoa worldwide (Baldursson and Karanis, 2011).

In Europe, recent studies reported the presence of *G. duodenalis* in water samples with a prevalence ranging from 87.5% to 100% in Spain (untreated and treated water) (Gómez-Couso *et al.*, 2005; Castro-Hermida *et al.*, 2008), of 38.3% in Portugal (raw and treated water) (Lobo *et al.*, 2009) and of 97.5% in Switzerland (surface water) (Wicki *et al.*, 2009).

As to Italy, the presence of *Giardia* has been recorded in surface water with a prevalence ranging from 33.3% to 100% (Briancesco and Bonadonna, 2005; Di Benedetto *et al.*,

2005; Vernile *et al.*, 2009), in groundwater from 11% to 14.3% (Di Benedetto *et al.*, 2005; Lonigro *et al.*, 2006) and in wastewater from 8.4% to 100% (Briancesco and Bonadonna, 2005; Di Benedetto *et al.*, 2005; Lonigro *et al.*, 2006; Marangi *et al.*, 2015b). Molecular typing of *Giardia* was performed in wastewater and in surface water and *G. duodenalis* assemblage A has been detected with a prevalence of 75% followed by assemblage B (5.35%).

Fresh produce - The presence of *Giardia* cysts in fresh produce has been documented worldwide with a prevalence ranging from 1.8% to 56% (Dixon, 2015). *Giardia* has been recently recorded mostly in Iran (Shahnazi and Jafari-Sabet, 2010; Ezatpour *et al.*, 2013), in Saudi Arabia (Al-Megrin *et al.*, 2010), in Egypt (Eraky *et al.*, 2014) and in Sudan (Mohamed *et al.*, 2016). Shahnazi and Jafari-Sabet (2010) and Ezatpour *et al.* (2010) reported a prevalence of the 1.8% (4 out of 218 vegetables samples) and 3.5% (19 out of 550 vegetables samples), respectively. In addition, Ezatpour *et al.* (2013) reported a high contamination on vegetables during the spring (5.8%) rather than in the winter (1.1%). In Saudi Arabia, out of 470 leafy vegetables samples, *G. duodenalis* has been found with a prevalence of the 31.6% (Al-Megrin *et al.*, 2010). In Egypt, out of 530 vegetables (lettuce, watercress, parsley, green onion and leek) *G. duodenalis* cysts has been detected with a prevalence of the 8.8% (Eraky *et al.*, 2014) in particular in lettuce (15.8%). In Sudan, out of 260 fresh vegetable samples, 59 (22.9%) were microscopically positive for *Giardia* cysts (Mohamed *et al.*, 2016).

As regard to RTE, the presence of *G. duodenalis* has been reported in Canada by Dixon *et al.* (2013). These authors recorded the presence of *G. duodenalis* in 10 (1.8%) out of 544 ready-to-eat packaged leafy greens; *G. duodenalis* assemblage A and B was 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 (Amoros *et al.*, 2010). In Norway, between August 1999 and January 2000, *Giardia* cysts have been found in 10 (2.1%) out of 475 samples between fruits and vegetables. Concentrations of detected parasites have been generally low (with an average of approximately 1-8 cysts per 100 g of produce). In Spain, *Giardia* cysts have been detected on salad products such as lettuces and Chinese cabbage. *Giardia* has been observed in 10 (52.6%) samples (with an average of 3-4 cysts and 6 oocysts per 50 g produce) out of 19 the salad products. A higher percentage of positive

samples have been observed for *Giardia* (61.5%) in lettuces than in Chinese cabbages (33.3%) (Amoros *et al.*, 2010).

In Italy, the presence of the *Giardia* cysts in vegetables is poorly documented; the first detection of *Giardia* in the vegetable is dated in 1968. *Giardia* cysts have been found on 48 (75%) out of 65 vegetables 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 ready to eat salads collected in Palermo by Di Benedetto *et al.* (2007); one sample out of 40 samples were found positive to *Giardia* cysts (12 cysts/50g of vegetables).

Significant aspects in the epidemiology of giardiasis is represented by their low infectious dose (10-100 cysts), by immediate infectivity of the cysts when excreted in the feces and by their resistance (Dawson, 2005).

Cysts remain infectious for months in cool, damp areas and rapidly accumulate in the environment. Studies report that infectivity of the cysts in soil can be reduced of 11% after 49 days at 4°C and after 7 days at 25°C, the cysts were not infective. In tap water, *Giardia* cysts were infectious for 56 days at 0°C to 4°C and 14 days at 20°C to 28°C. Similar results were obtained in lake water, with 56 days of survival at 0°C to 4°C or 6°C to 7°C and 28 days at 17°C to 20°C. Longer survival was noticed in river water, with 84 days of survival at 0°C to 4°C and 28 days at 20-28°C. In seawater, *Giardia* cysts could survive for over 65 days at 4°C (Erickson and Ortega, 2006). In addition, cysts are resistant to freezing (cysts killed at -18°C after 1h) and to chlorine but are sensitive to heat (heating of 71.7 ° C for 15s) (Dawson, 2005).

Due to these biological characteristics, outbreaks associated with consumption of fresh produce contaminated with *Giardia* have been documented in United States, from 1971 to 2011 (Adam *et al.*, 2016). Thirty-eight foodborne outbreaks (60.5%) have been documented; raw vegetables, a salad bar, unspecified vegetables and fresh fruit were implicated. In Italy, cases of giardiasis linked to fresh produce have not been documented.

2.3.1.4 Pathogenesis

The pathogenic mechanisms of *G. duodenalis* in producing chronic diarrhea and malabsorption are probably multifactorial and have still not been clearly defined (Buret, 2007).

Trophozoites perform a mechanical activity on the intestinal epithelium. Once excystation occurs, they use their flagella to reach the microvillus that cover the surface of the duodenum and jejunum. Here, they attach the enterocytes using the disk located on their ventral surface (Adam, 2001). The attachment process causes damage to the enterocytes and the rapid multiplication of trophozoites creates a physical barrier between the enterocytes and the intestinal lumen, interfering with nutrients absorption (Adam, 2001). In addition, this process damages the enterocytes and causes villus atrophy, crypt hyperplasia (Buret, 1992), and intestinal hyper permeability (Chin, 2002; Dagci, 2002).

Nutrients malabsorption characterizes also the pathophysiology of giardiasis; trophozoites use nutrients for 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 unable to survive in the small bowel in the absence of bile acids, and for this reason the trophozoites have to absorb bile acids by the host. It explains the fat malabsorption often seen in giardiasis patients (Vesny and Peterson, 1999).

Chronic giardiasis also results in malabsorption of lactose, vitamin B12, and fat-soluble vitamins, which can result in weight loss, nutritional deficiencies, and failure to thrive in children (Gardner and Hill, 2001).

G. duodenalis induce enterocytes apoptosis (Troeger *et al*, 2007). Intriguingly, this effect, and the resulting disruption of tight junctional integrity, can be inhibited with apical administration of epidermal growth factor (Buret *et al*, 2002). *Giardia* can also prevent the formation of epithelial nitric oxide, a compound known to inhibit giardial growth, by consuming local arginine, which effectively removes the substrate needed by enterocytes to produce nitric oxide (Eckmann *et al*, 2000). This mechanism may contribute to *Giardia*-induced enterocyte apoptosis, since the arginine deficiency in these cells is known to cause programmed cell death (Potoka *et al*, 2003).

However, symptoms during giardiasis may occur in the absence of overt villus atrophy or other signs of mucosal injury (Eckman *et al.*, 2001). In this patient, *G. duodenalis* causes malabsorption of glucose, sodium, and water, and reduced disaccharidase activity and maldigestion, due to a diffuse shortening of epithelial microvilli (Belosevic *et al.*, 1989; Buret *et al.*, 1992; Troeger *et al.*, 2007).

2.3.1.5 Clinical signs

The symptoms of giardiasis can be variable and generally show up about two weeks after exposure. Symptoms may be acute or chronic diarrhea associated with abdominal pain, flatulence, bloating, nausea, malabsorption, weight loss and vomiting. Fever is occasionally present at the beginning of the infection (Ortega and Adam, 1997).

Although giardiasis may resolve spontaneously, the illness frequently lasts for several weeks, and sometimes for months, if left untreated. Patients with chronic giardiasis have profound malaise, diffuse epigastric and abdominal discomfort. Usually infections are asymptomatic, but chronic diarrhea may occur in children (Maçin *et al.*, 2016) and low proportion of immunocompromised people (Angholi *et al.*, 2013).

2.3.1.6 Diagnosis in clinical samples

Diagnosis is usually based on the microscopic detection of *Giardia* cysts or trophozoites in a stool specimen. Stools may be examined directly as fresh smears by light microscopy, or preserved in formalin or polyvinyl alcohol and stained with iodine (Wolfe, 1990; Smith and Paget, 2007) and iron-haematoxylin (Garcia, 2007), Giemsa (Ament, 1972; Wolfe, 1990) or trichrome (Thornton *et al.*, 1983). Cysts can be concentrated using various methods, i.e., formalin-ether or formalin-ethyl acetate (Smith and Paget, 2007) or zinc sulphate solution (ZnSO₄) (Zajac *et al.*, 2002).

Motile trophozoites can be detected by direct microscopic examination of fresh samples (smears prepared immediately with warm [37°C] saline), while dead trophozoites can be detected in air-dried faecal smears stained, for instance, with Giemsa (Smith and Paget, 2007). Multiple faecal samples should be taken in a week and examined over a period of 1–2 weeks, because of the intermittent nature of excreted cysts (Smith and Paget, 2007; Garcia, 2009). Electron microscopy might be useful for the identification of some *Giardia* species (Adam, 2001), but is not applicable for routine use.

Other techniques are the direct fluorescence antibody (DFA) tests with a fluorescein isothiocyanate-conjugated anti-*Giardia* monoclonal antibody (Johnston *et al.*, 2003; Al *et al.*, 2006). There are also kits that can detect the presence of oo/cysts in feces samples, for example, Merifluor *Cryptosporidium/Giardia* kit (Meridian Diagnostics, Cincinnati, Ohio, USA). (Grigoriew *et al.*, 1994; Zimmerman and Needham, 1995).

PCR assays for *Giardia* have become more common in the last years. However, PCR amplification and sequences analysis are more frequently used for genotype/assemblage classification and are not routinely used for diagnosis (Cama and Mathison, 2015). Some genetic markers used to characterize and classify *G. duodenalis* include the small-subunit (SSU) rRNA gene (Cacciò *et al.*, 2008; Abe *et al.*, 2010; De Liberato *et al.*, 2015), glutamate dehydrogenase (*gdh*) gene (Cacciò *et al.*, 2008; Abe *et al.*, 2010), β -giardin (*bg*) gene (Lalle *et al.*, 2005; Cacciò *et al.*, 2008; Abe *et al.*, 2010), elongation factor 1 alpha (*ef1 α*) gene (Li *et al.*, 2013) and triosephosphate isomerase (*tpi*) gene (Sulaiman *et al.*, 2003; Cacciò *et al.*, 2008; Abe *et al.*, 2010).

Another diagnostic aspect to take into account is the infectivity of cysts, because only viable cysts are a concern for public health services. The detection methods described above cannot distinguish between viable and non-viable cysts. Despite several techniques have been developed to distinguish between viable and non-viable cysts, they are not completely reliable or are very expensive and complex. These are: inclusion or exclusion of fluorogenic vital dyes as 4',6'-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Robertson *et al.*, 2014), fluorescent in situ hybridization (FISH) assays, which use labelled complimentary oligonucleotide probes for the detection of rRNA (Lemos *et al.* 2005), infectivity methods i.e. mice models (Jenkins *et al.*, 1997) and cell culture (Saghaug *et al.*, 2015), and reverse transcriptase polymerase chain reaction (RT-PCR) assays (Nam and Lee, 2010).

2.3.1.7 Detection methods in vegetables

Methods for the detection of *G. duodenalis* in vegetable samples are based on concentration. Concentration methods use detergent solutions for the washing of the vegetables and for recovery of the cysts. Detergents used in recent studies are: *i)* saline buffer solutions (PBS) (Lonigro *et al.*, 2006; Dixon *et al.*, 2013); *ii)* physiological saline solutions (Robertson and Gjerde, 2000; Di Benedetto *et al.*, 2007; Al-Megrin *et al.*, 2010); *iii)* glycine buffer solutions (Cook *et al.*, 2007; Amoros *et al.*, 2010). The washing are performed into stomacher bags and in any case, the stomacher bags are subjected to movements of oscillation to allow the removal of cysts.

The concentration methods may provide different protocols for a better purification and recovery of the cysts: filtration (i.e. cellulose ester membrane) (Al-Megrin *et al.*, 2010); centrifugation (Dixon *et al.*, 2013); centrifugation followed by flotation (Lonigro *et al.*, 2006; Cook *et al.*, 2007) and immunomagnetic separation (Robertson and Gjerde, 2000; Cook *et al.*, 2007; Di Benedetto *et al.*, 2007; Amoros *et al.*, 2010; Ramirez-Martinez *et al.*, 2015;).

After recovery from vegetables, *Giardia* cysts can be identified by light microscopy adding a drop of Lugol's iodine solution (Al-Megrin *et al.*, 2010; Ramirez-Martinez *et al.*, 2015), by fluorescence microscopy using fluorescein isothiocyanate-conjugated anti-*Giardia* monoclonal antibodies (Robertson and Gjerde, 2000; Lonigro *et al.*, 2006; Cook *et al.*, 2007; Di Benedetto *et al.*, 2007; Amoros *et al.*, 2010; Dixon *et al.*, 2013; Ramirez-Martinez *et al.*, 2015) or fluorogenic dyes as 4',6'-diamidino-2-phenylindole (DAPI) (Robertson and Gjerde, 2000; Cook *et al.*, 2007; Amoros *et al.*, 2010).

Recently, various molecular methods have been developed for the identification of *Giardia* on vegetables such as nested-PCR (Dixon *et al.*, 2013), PCR (Ramirez-Martinez *et al.*, 2015) and PCR-RFLP (Tiyo *et al.*, 2016). These molecular techniques may provide information about the genotype or species of *Giardia*, and may identify the parasite in low quantities. Currently the used genes are *16S rRNA* gene (Dixon *et al.*, 2013), *b-giardin* gene (Ramirez-Martinez *et al.*, 2015) and *gdh* gene (Tiyo *et al.*, 2016).

2.3.2 CRYPTOSPORIDIUM SPP.

2.3.2.1 Classification and Morphology

Cryptosporidium belongs to the Phylum Apicomplexa, class Coccidiomorphea, order Eucoccidiida, family Cryptosporidiidae. However, 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 been supported by extensive microscopic, molecular, genomic and biochemical data, which have served as basis for the formal transfer of *Cryptosporidium* from subclass Coccidia, class Coccidiomorphea to a new subclass, Cryptogregarina, within class Gregarinomorphea. The genus *Cryptosporidium* is currently the only member of Cryptogregarina and is described as comprising epicellular parasites of vertebrates possessing a gregarine-like feeder organelle but lacking an apicoplast (reviewed by Ryan *et al.*, 2016)

Species of the genus *Cryptosporidium* infect birds, reptiles, amphibians and mammals including human (Šlapeta, 2013). Ernest Edward Tyzzer was the first person to recognize and describe *Cryptosporidium* in the gastric glands of tame variety of common mice (Tyzzer, 1907).

In the last decade, the number of named species has grown steadily, with approximately one new named species per year, and 10 named species during 2004-2013. Currently, 30 *Cryptosporidium* species are recognized (Šlapeta, 2013) (**Table 2**).

C. hominis and *C. parvum* are most commonly reported in human cryptosporidiosis. Other species, such as *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum* and *C. viatorum* have a zoonotic potential but are less common in humans (Xiao, 2010; Ryan *et al.*, 2014).

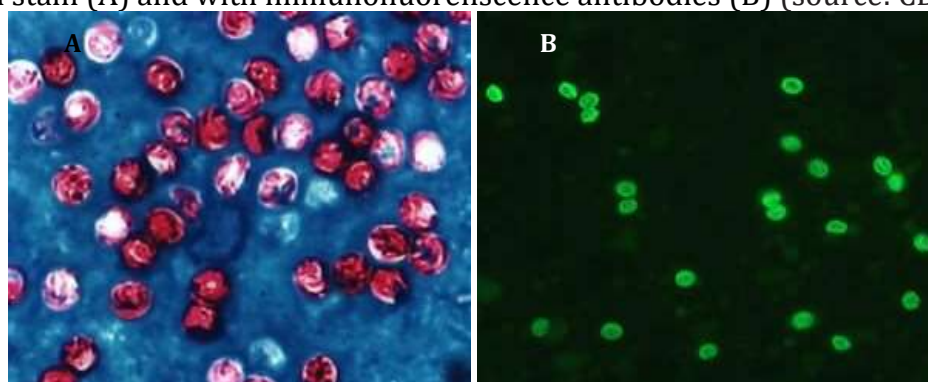
Table 2. Some valid species of *Cryptosporidium* (source: Ryan *et al.*, 2014)

Species name	Author(s)	Type hosts	Major host	Reports in humans
<i>C. muris</i>	Tyzzer (1907, 1910)	Tame mice	Rodents	Numerous reports (cf. Feng <i>et al.</i> 2011b)
<i>C. wrairi</i>	Vetterling <i>et al.</i> (1971)	<i>Cavia porcellus</i> (Guinea pig)	Guinea pigs	None reported
<i>C. felis</i>	Iseki (1979)	<i>Felis catus</i> (Cat)		Many reports (cf. Lucio-Forster <i>et al.</i> 2010)
<i>C. serpentis</i>	Levine (1980)	<i>Elaphe guttata</i> , <i>E. subocularis</i> , <i>Sanzinia madagascarensis</i> (Snakes)	Snakes and lizards	None reported
<i>C. meleagridis</i>	Slavin (1955)	<i>Meleagris gallopavo</i> (Turkey)	Birds and humans	Commonly reported in humans
<i>C. parvum</i>	Re: Upton and Current (1985) Tyzzer (1912)*	<i>Bos taurus</i> (Cattle)	Ruminants	Commonly reported in humans
<i>C. baileyi</i>	Current <i>et al.</i> (1986)	<i>Gallus gallus</i> (Chicken)	Birds	None reported
<i>C. varanii</i>	Pavlašek <i>et al.</i> (1995)	<i>Varamus prasimus</i> (Emerald Monitor)	Lizards	None reported
<i>C. andersoni</i>	Lindsay <i>et al.</i> (2000)	<i>Bos taurus</i> (Cattle)	Cattle	Leoni <i>et al.</i> (2006); Morse <i>et al.</i> (2007); Waldron <i>et al.</i> (2011); Agholi <i>et al.</i> (2013); Liu <i>et al.</i> (2014)
<i>C. canis</i>	Fayer <i>et al.</i> (2001)	<i>Canis familiaris</i> (Dog)	Dogs	Many reports (cf. Lucio-Forster <i>et al.</i> 2010)
<i>C. molnari</i>	Alvarez-Pellitero and Sijja-Bobadilla (2002)	<i>Sparus aurata</i> and <i>Dicentrarchus labrax</i> (Fish)	Fish	None reported
<i>C. hominis</i>	Morgan-Ryan <i>et al.</i> (2002)	<i>Homo sapiens</i> (Human)	Humans	Most common species in humans
<i>C. galli</i>	Re: Ryan <i>et al.</i> (2003c) Pavlašek (1999)*	Spermetidae, Frangillidae, <i>Gallus gallus</i> , <i>Tetrao urogallus</i> , <i>Pimicola enucleator</i> (Birds)	Birds	None reported
<i>C. suis</i>	Ryan <i>et al.</i> (2004)	<i>Sus scrofa</i> (Pig)	Pigs	Xiao <i>et al.</i> (2002a); Leoni <i>et al.</i> (2006); Cama <i>et al.</i> (2007); Wang <i>et al.</i> (2013)
<i>C. bovis</i>	Fayer <i>et al.</i> (2005)	<i>Bos taurus</i> (Cattle)	Cattle	Khan <i>et al.</i> (2010); Ng <i>et al.</i> (2012); Helmy <i>et al.</i> (2013)
<i>C. fayeri</i>	Ryan <i>et al.</i> (2008)	<i>Macropus rufus</i> (Kangaroo)	Marsupials	Waldron <i>et al.</i> (2010)
<i>C. fragile</i>	Jirku <i>et al.</i> (2008)	<i>Duttaphrym melanostictus</i> (Toad)	Toads	None reported
<i>C. macropodum</i>	Power and Ryan (2008)	<i>Macropus giganteus</i> (Kangaroo)	Marsupial	None reported
<i>C. ryanae</i>	Fayer <i>et al.</i> (2008)	<i>Bos taurus</i> (Cattle)	Cattle	None reported
<i>C. xiaoi</i>	Fayer <i>et al.</i> (2010)	<i>Ovis aries</i> (Sheep)	Sheep and goats	Adamu <i>et al.</i> (2014)
<i>C. ubiquitum</i>	Fayer <i>et al.</i> (2010)	<i>Bos taurus</i> (Cattle)	Ruminants, rodents, primates	Commonly reported (cf. Fayer <i>et al.</i> 2010; Elwin <i>et al.</i> 2012a)
<i>C. cuniculus</i>	Re: Robinson <i>et al.</i> (2010) Inman and Takeuchi (1979)*	<i>Oryctolagus cuniculus</i> (European rabbit)	Rabbits	Chalmers <i>et al.</i> (2009b); Anon (2010); Molloy <i>et al.</i> (2010); Chalmers <i>et al.</i> (2011a)
<i>C. tyzzeri</i>	Re: Ren <i>et al.</i> (2012) Tyzzer (1912) (<i>C. parvum</i>)*	<i>Mus musculus</i> (Mouse)	Rodents	Raskova <i>et al.</i> (2013)
<i>C. viatorium</i>	Elwin <i>et al.</i> (2012b)	<i>Homo sapiens</i> (Human)	Humans	Elwin <i>et al.</i> (2012b); Insulander <i>et al.</i> (2013)
<i>C. scrofarum</i>	Kvác <i>et al.</i> (2013b)	<i>Sus scrofa</i> (Pig)	Pigs	Kvác <i>et al.</i> (2009a); Kvác <i>et al.</i> (2009b)
<i>C. erinacei</i>	Kvác <i>et al.</i> (2014b)	European hedgehog (<i>Erinaceus europaeus</i>)	Hedgehogs and horses	Kvác <i>et al.</i> (2014a)

Re, redescription.
* Initial description.

Cryptosporidium oocysts can be spherical (4-6 μm in diameter) or oval (7 x 5 μm in diameter), according to the species (Chalmers and Katzer, 2013) (Figure 6).

Figure 6. *Cryptosporidium* oocysts. The images show oocysts stained with Ziehl-Neelsen stain (A) and with immunofluorescence antibodies (B) (source: CDC, 2015)



The oocyst wall of *Cryptosporidium* spp. is similar to that of the other coccidia, in fact presents inner and outer layers, except for the presence of a suture on one end, which is not present in others. The oocysts wall of *C. parvum* is a trilaminar structure with an average thickness of about 49nm (Harris and Petry, 1999). The wall is continuous except at one pole where it is interrupted by a single seam or suture that extends one-third to one-half the way around the periphery (Fayer *et al.*, 1997). Trophozoites contain a

prominent nucleolus within a single nucleus surrounded by cytoplasm and a well-developed attachment/feeder organelle for the fusion with host cell (Fayer *et al.*, 1997). Immature microgamonts resemble to merontes but contain small, compact nuclei. Microgametes are rod-shaped ($1.4 \times 0.5\mu\text{m}$ for *C. parvum*), with a flattened anterior end. Macrogamonts of *C. parvum* are approximately 4 to 6 μm , spherical to ovoid, have a large central nucleus with a prominent nucleolus, lipid bodies, amylopectin granules, and unique wall-forming bodies in the cytoplasm. The fertilized macrogamonts, or zygote, develops into an oocyst with either a thin or a thick wall (Fayer *et al.*, 1997).

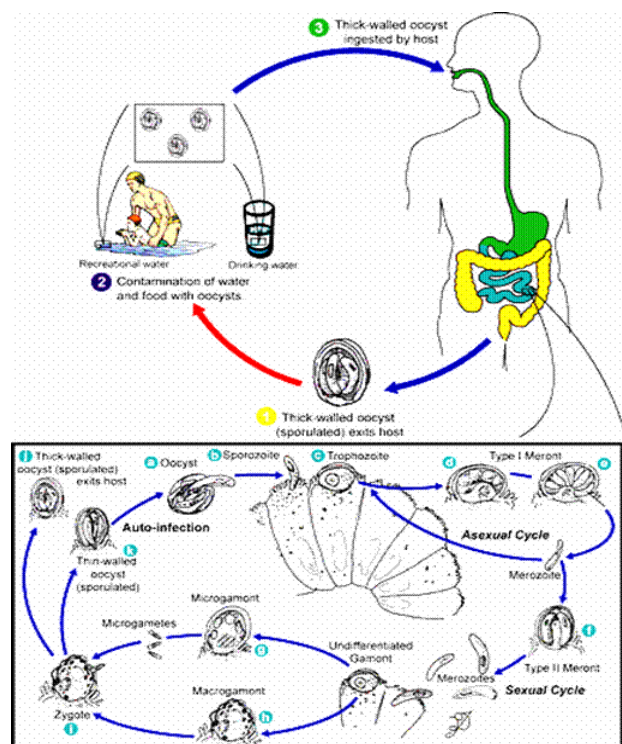
2.3.2.2 Biology and Life Cycle

Cryptosporidium spp. has a biological cycle monoxenous i.e., all development stages (asexual and sexual) occur within the same host.

Transmission of the parasite occurs via the faecal-oral route, by ingestion of contaminated water or food, or by human-to human and animal-to-human transmission (Xiao, 2010).

The infective cycle begins when an appropriate host ingests the oocysts, the infective stages of the parasite (O'Haraa and Chen, 2011) (**Figure 7**).

Figure 7. Life Cycle of *Cryptosporidium* spp. (source: CDC, 2015)



The exposure to stomach acids and bile salts promotes the excystation of the oocysts, resulting in release of four motile sporozoites into the small intestine, which adhere and penetrate the intestinal epithelium. Through a mechanism of host cell-parasite attachment and subsequent invasion, a sporozoite is encapsulated into a parasite-modified host membrane (McDonald *et al.*, 1994), the parasitophorous vacuole. The parasitophorous vacuole of *Cryptosporidium* is unique in that it remains extracytoplasmic on top of epithelial cells. During internalization, a unique highly invaginated membrane, the feeder and/or adherence organelle, is formed between the parasite and host cytoplasm (Perkins *et al.*, 1999; Zapata *et al.*, 2002). Subsequently the trophozoite undergoes asexual reproduction, for schizogony (multiple division) where each daughter cell is surrounded by its own membrane. Two developmentally distinct types of meronts are formed: Type-I and Type-II meronts (Current and Rees, 1986). Both Type-I and Type-II produce merozoites morphologically similar to sporozoites. Eight merozoites are released from Type-I meronts when mature; these can invade neighboring enterocytes, increasing the infection level, forming other Type-I meronts, or can continue the development with the formation of Type-II meronts. Type-II meronts after cell division, produce four Type-II merozoites, each capable of infecting another enterocyte, but through unknown mechanisms are developmentally programmed to differentiate into gametocytes to complete the sexual stage of development. Type-II merozoites produce either male or female sexual stages i.e. microgametocytes or macrogametocytes. The microgametocytes can develop up to sixteen non-flagellated microgametes. The macrogametocytes develop uninucleate macrogamonts. The microgametes, through an unknown mechanism, fertilize a uninucleate macrogamont and the resultant diploid zygote undergoes a process similar to meiosis (sporogony) with developing of four haploid sporozoites within an oocyst (sporulated oocyst). The resultant oocysts can be with thick-walled or thin-walled. The thick-walled oocysts (infective oocysts) are shed in the feces, 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 (Current and Rees, 1986).

2.3.2.3 Epidemiology

Humans - Protozoa of the genus *Cryptosporidium* have a global distribution (Putignani and Menichella, 2010). Previous studies have shown that *C. parvum* and *C. hominis* are responsible for >90 % of human cases of cryptosporidiosis in most areas, with the balance attributable to *C. meleagridis*, *C. canis* and *C. felis*, especially in developing countries (Xiao and Feng, 2008). Cryptosporidiosis occurs more frequently in infants and children than in adults, in developed and developing countries (Putignani and Menichella, 2010; Xiao, 2010).

Due to the huge amount of data, prevalence recorded in humans, animals, water and food are herewith synthetized and limited, except for vegetables, to the European scenario.

In the European Nordic countries (e.g. Denmark, Finland, Norway and Sweden), the prevalence of *Cryptosporidium* spp. was estimated of 0.99% in the asymptomatic (i.e. no gastroenteric symptoms) and 2.91% in the symptomatic population (Horman *et al.*, 2004). In United Kingdom, the prevalence of *Cryptosporidium* spp. was of the 1.3% and *C. hominis* has been found (Davies *et al.*, 2009).

C. hominis and *C. parvum* have been documented in Portugal (Alves *et al.*, 2006), Spain (Llorente *et al.*, 2007; Davies *et al.*, 2009), Poland (Bajer *et al.*, 2008), Belgium (Geurden *et al.*, 2009), United Kingdom (Chalmers *et al.*, 2011), Ireland (Zintl *et al.*, 2009) and recently, cases of cryptosporidiosis have been reported in 21 European countries with a prevalence of 1.96%, where the highest case rate was observed in Ireland (9.04%) followed by United Kingdom (5.76%), and Sweden (4.03%) (ECDC, 2013). Recently, human cases of cryptosporidiosis have been attributed to *C. ubiquitum* in Spain (Cieloszyk *et al.*, 2012) and in United Kingdom (Elwin *et al.*, 2012).

In Italy, the prevalence of *Cryptosporidium* spp. in human was estimated of 0.84-11% with higher prevalence for *C. parvum* (90%) (Giangaspero *et al.*, 2007). In immunocompetent patients, independently of age, the prevalence of cryptosporidiosis was usually lower than giardiasis, with a range from 1.27% (Moretti *et al.*, 1988) to 5.4% (Magi *et al.*, 2006). A high prevalence (8.33–21%) was registered in HIV+ patients (Brandonisio *et al.*, 1999); a case of pulmonary cryptosporidiosis was described in an AIDS patient (Palmieri *et al.*, 2005). Recently in Italy, *Cryptosporidium* spp. has been identified 14 human isolates, 9 were *C. parvum* and 5 were *C. hominis* (Drumo *et al.*, 2012).

Animals - Molecular studies have identified a wide range of *Cryptosporidium* spp. in animals worldwide (Ryan *et al.*, 2014) and 152 mammalian hosts emerged to be infected with *C. parvum* (Fayer *et al.*, 2000). Recently, *C. ubiquitum* (another zoonotic species that infects humans) has been reviewed in rodents, carnivores, herbivores (Li *et al.*, 2014). In Europe, several studies indicate that the *Cryptosporidium* is common in foals and adult horses, with a prevalence of 6.4% in the UK (Sturdee *et al.*, 2003), 9.4% in Poland and higher prevalence of *Cryptosporidium* infection in pigs has been reported in Spain (21.9%) (Ramirez *et al.*, 2004).

In Italy, infection rates of cryptosporidiosis in farm animals (cattle, sheep, pigs, horse and poultry) ranged from 8% to 58.3% and in companion animals (dogs) were 3.3% with higher prevalence of *C. parvum* than *C. hominis* in both farm and companion animals (Giangaspero *et al.*, 2007). In recent studies, *Cryptosporidium* oocysts have been found in 7.7% of the birds and in 18.7% of zoo parrots (Papini *et al.*, 2012).

Water - The ubiquitous presence of *Cryptosporidium* spp. in the aquatic environment is explained by the extremely high number hosts that can excrete oocysts, and the remarkable stability of oocysts (Šlapeta, 2013). Thus, water represents an important vehicle of infection for the population, and waterborne cryptosporidiosis are a serious public health concern, particularly for populations at risk of severe infection (pregnant women, children and immunocompromised) (Rochelle and Di Giovanni, 2014). Furthermore, the World Health Organization has categorized *Cryptosporidium* as a reference pathogen for the assessment of drinking water quality (WHO, 2009).

Cryptosporidium as well as *Giardia* are the most frequently reported waterborne protozoa worldwide (Baldursson and Karanis, 2011).

In Europe, recent studies have been reported the presence of *C. parvum* and *hominis* in untreated (87.5%) and treated water samples (75.0%) in Spain (Castro-Hermida *et al.*, 2008) and in Portugal *C. parvum* and *C. hominis* in raw and treated water with a prevalence of 78.9% and 13.2% (Lobo *et al.*, 2009). In Belgium, *C. parvum* and *C. hominis* have been reported with a prevalence of 41– 44 % (Ehsan *et al.*, 2015). In Poland *C. parvum* has been detected in all water samples collected from 36 natural water bodies (Adamska, 2015).

In Italy, the presence of *Cryptosporidium* was recorded in surface water with a prevalence ranging from 10% to 100% (Brandonisio *et al.*, 2004; Di Benedetto *et al.*,

2005; Sacco *et al.*, 2006), in groundwater of 7.1% (Di Benedetto *et al.*, 2005) and in wastewater from 10% to 100% (Briancesco and Bonadonna, 2005; Di Benedetto *et al.*, 2005; Lonigro *et al.*, 2006; Marangi *et al.*, 2015b). Molecular typing of *Cryptosporidium* was performed in wastewater and in surface water and only *C. parvum* has been detected.

Fresh produce - Surveillance studies have reported the presence of *Cryptosporidium* in fresh produce worldwide (Dixon *et al.*, 2015). Studies in Costa Rica and Peru (Monge and Arias 1996; Ortega *et al.*, 1997a) have shown contamination of numerous raw vegetables, including basil, cabbage, celery, cilantro, green onions, leeks, lettuce, parsley, and yerba buena. In a recent study in Costa Rica, *Cryptosporidium* spp. was detected on lettuce, parsley, cilantro and blackberries collected from local markets (Calvo *et al.*, 2004). Recently, study on *Cryptosporidium* contamination in vegetable farms was carried out in Teheran, Iran (Ranjbar-Bahadori *et al.*, 2013). A total of 496 samples from 115 vegetable farms were collected. The study has revealed that 6.6% of samples were contaminated with *Cryptosporidium* species and when wastewater were used to irrigate vegetable farms, the contamination rate was of 33.3% (Ranjbar-Bahadori *et al.*, 2013).

Food contamination with *Cryptosporidium*, however, is not limited to developing countries. In Norway, a search for parasites in fruits and vegetables was undertaken in the period from 1999 to 2001 (Robertson and Gjerde 2001); out of 475 samples tested, 29 (6%) have been found positive for *Cryptosporidium* oocysts and *Giardia* cysts, of which 19 (4%) only for *Cryptosporidium* (lettuce and mung bean sprouts). Concentration of detected parasite has been generally low (with an average of approximately 1-6 oocysts per 100 g of produce). (Robertson and Gjerde 2001). In Poland, *Cryptosporidium* oocysts have been found in 6 (3.6%) out of the 128 vegetables samples between leek, celery, red and Peking cabbage (range 1-47 oocysts per vegetables) (Rzezutka *et al.*, 2010). In Spain, *Cryptosporidium* oocysts have been detected on salad products such as lettuces and Chinese cabbage. *Cryptosporidium* has been observed in 12 (63,1%) samples (with an average of 6 oocyst per 50 g produce) out of 19 the salad products. A higher percentage of positive samples have been observed for *Cryptosporidium* (76.9%) in lettuces than in Chinese cabbages (33.3%) (Amoros *et al.*, 2010).

C. parvum was found in pre-packaged leafy green vegetables (Lalonde and Gajadhar, 2016) and “ready-to-eat” leafy greens (Dixon *et al.*, 2013) in Canada; Lalonde and

Gajadhar (2016) reported *C. parvum* on 2 (0.17%) samples out of 1171 samples whereas Dixon *et al.* (2013) reported the presence of *Cryptosporidium* in 32 (5.9%) out of the 544 ready-to-eat packaged leafy greens; *C. parvum* was identified in 29 samples.

The factors that affect the epidemiology of *Cryptosporidium* are: *i*) the ability of the oocysts to be transmitted to humans by contact with infected persons (e.g., from young children to caregivers) (DuPont *et al.*, 1995; Roy *et al.*, 2004) and animals (Miron and Kenes, 1991; Sayers *et al.*, 1996) or mainly by ingestion contaminated water and/or food (Cacciò and Putignani, 2014; Dixon, 2015); *ii*) low infectious dose (10 or fewer oocysts) and immediate infectivity of the cysts when excreted in feces (Dawson, 2005); *iii*) high resistance in cool and damp conditions for long periods (Tamburrini and Pozio, 1999). *Cryptosporidium* oocysts can survive for 6 months in different environments (tap water, river water, seawater and cow faeces) (Robertson *et al.*, 1992). Robertson *et al.*, (1999) has also reported that snap-freezing of the oocysts and desiccation (between 18 and 20°C) for longer periods (8 h) determined the death of the 100%; slow freezing, however, is less effective at killing oocysts. Oocysts are very resistant to disinfectants; routine chlorination of water has no effect. Ozone is highly effective (Peeters *et al.*, 1989).

Outbreaks of cryptosporidiosis have been associated with contaminated water, in particular with contaminated drinking water, treated recreational water and untreated water supply (Baldursson and Karanis, 2011). *Cryptosporidium* spp. was the etiological agent in 60.3% (120 cases) of the outbreaks, following *G. duodenalis* in 35.1% (70 cases) and other protozoa in 4.5% (9 cases). In this review, the source of infection in 32.7% (65 cases) of described outbreaks of *Cryptosporidium* spp. have been caused through recreational waters. Geographically, the outbreaks seem to be concentrated in the USA, Canada, Australia and Europe, especially in the UK and Ireland (Baldursson and Karanis, 2011). Recently, two waterborne outbreaks of *Cryptosporidium* have been described also in Sweden involving 27000 persons and then 20000 persons. One suspected source was sewage water from a few households who discharged the waters directly into a stream, from which the drinking water was obtained (Guzman-Herrador *et al.*, 2015). Twelve waterborne outbreak of cryptosporidiosis have been described in the South East of Ireland in late Spring 2012; eleven cases have been linked to *C. parvum* and the public water supply was the only common risk factor identified (Mahon and Doyle, 2017).

In Italy, cryptosporidiosis is not a notifiable disease and only one study summarizes waterborne outbreaks of *Cryptosporidium* in different geographical areas (Brandonisio, 2006).

Outbreaks have been linked to the consumption of contaminated vegetables (Dixon, 2015). Outbreaks of cryptosporidiosis have been associated with oocyst-contaminated apple cider, unpasteurized milk, prepared foods such as chicken salad, and fresh produce, including onions, parsleys and carrots in US (Dixon *et al.*, 2011). In 2005 an outbreak of diarrhea, affecting a group of 99 company employees, was described near Copenhagen (Ethelberg *et al.*, 2009). All people were ill and 13 were positive for *C. hominis*. Disease was associated with food from the canteen. Three separate ingredients were found to be likely sources: peeled whole carrots served in a bowl of water, grated carrots, and red peppers. The likely source of infection was an infected food-handler, who may have contaminated food served at the buffet (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 for a *C. parvum* outbreak (Ponka *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 a causal agent in 5 (5.7%) of 88 outbreaks notified in during 2012 (Aberg *et al.*, 2015).

Recently, the role of the food handlers has been investigated in Venezuela, where cryptosporidiosis is an important public health problem (Freites-Martinez *et al.*, 2009). Despite a basic investigation approach, 14 out of 119 fecal samples from food workers were found positive for *Cryptosporidium* spp. in association with other protozoa (i.e. *Endolimax nana*, *Blastocystis hominis*, *Entamoeba coli*, *Giardia* spp., and *E. histolytica/Entamoeba dispar*).

In Italy, foodborne outbreaks of *Cryptosporidium* have not been recorded.

2.3.2.4 Pathogenesis

The pathogenic mechanisms by which *Cryptosporidium* causes diarrhea, malabsorption and indisposition are usually multifactorial, involving a complex interplay between the parasite and the host. Various host factors, including age, sex, and the status of the immune system, influence the outcome of the host-parasite interaction (Okhuysen and Chappell, 2002). *Cryptosporidium* does not normally cause a systemic infection or penetrate deep tissue; rather, the parasite establishes itself in a membrane-bound compartment on the apical surface of the intestinal epithelium.

Invasion of host cells is restricted to the luminal border of the enterocytes and leads to displacement of the microvillous border and loss of the surface epithelium, causing changes in the villous architecture, with villous atrophy, blunting and crypt cell hyperplasia, and mononuclear cell infiltration in the *lamina propria* (Meisel *et al.*, 1976; Farthing, 2000). The enterocyte malfunction has been demonstrated in a pig model by decreased Na⁺ absorption caused by villous blunting and increased Cl⁻ secretion due to crypt cell hyperplasia (Argenzio *et al.*, 1990). These results in overall enhanced secretion. Disruption and changes in the microvillous border may also lead to the loss of membrane-bound digestive enzymes, reduction in the absorptive surface and uptake of fluids, electrolytes and nutrients (Adams *et al.*, 1994; Griffiths *et al.*, 1994). This damage causes significant abnormalities in the absorptive and in the secretory functions of the gut, resulting in diarrhea.

It has also been suggested that Crohn's disease and ulcerative colitis might be reactivated by *Cryptosporidium* infection (Manthey *et al.*, 1997), in that loss of intestinal barrier function, attributable to *Cryptosporidium*, is similar to changes seen in a variety of intestinal disorders, including the inflammatory bowel diseases (IBD) Crohn's disease and ulcerative colitis (Fiocchi, 1998).

2.3.2.5 Clinical signs

Cryptosporidiosis characteristically results in watery diarrhea that may sometimes be profuse and prolonged (Chalmers and Davies, 2010). Diarrhea and abdominal pain are generally the symptoms. The extent of spread and the sites involved determine whether the infection is clinical or subclinical as well as the overall intensity of the disease.

Other acute symptoms are abdominal pain, nausea or vomiting, pyrexia, anorexia, malaise, fatigue and weight loss (Fayer and Ungar, 1986).

The severity of a *Cryptosporidium* infection can vary from an asymptomatic shedding of oocysts to a severe and life-threatening disease. Immunocompetent individuals experience a transient self-limiting illness (up to 2 to 3 weeks). The duration of shedding of oocysts, from the onset of symptoms to the first negative stool specimen, is between 11 - 28 days in adults and 9 - 50 days in children (Shepherd *et al.*, 1988). Some patients experience chronic diarrhea of a month or longer and oocysts may continue to be shed for a mean period of 7 days (range 1–15 days) after symptoms have ceased, exceptionally for up to 2 months (Jokipii and Jokipii, 1986). However, for immunocompromised patients, cryptosporidiosis can be a critical illness with persistent symptoms, chronic watery diarrhea lasting for more than 2 months, leading to dehydration (Desai *et al.*, 2012). In addition, *Cryptosporidium* infection can cause atypical manifestations in immunocompromised patients, such as a typical gastrointestinal disease, biliary tract disease, respiratory tract disease, and pancreatitis (Manabe *et al.*, 1998; Hunter and Nichols, 2002).

2.3.2.6 Diagnosis in clinical samples

Traditionally, the identification of *Cryptosporidium* oocysts was based on microscopic examination. The iodine-saline wet mount is used in routine screening procedure for *Cryptosporidium* detection but it is not permanent (Coppola, 2010). Faeces from patients with acute cryptosporidiosis do not usually require concentration for detect oocysts. Although the numbers of oocysts excreted can fluctuate during the course of the infection, for this reason concentration and staining of the oocysts have used to enhance the sensitivity of the light microscopy. The techniques for the concentration and purification of oocysts from fecal samples include formalin-ethyl-acetate concentration (Weber *et al.*, 1992; Clavel *et al.*, 1996) and formalin-ether (Tahvildar-Biderouni and Salehi, 2014); sucrose density (Robertson *et al.*, 1992; Bukhari and Smith, 1995; Jenkins *et al.*, 1997) and zinc sulfate (Bukhari and Smith, 1995) flotation; and Percol discontinuous density gradient centrifugation (Waldman *et al.*, 1986).

The techniques for the permanent staining of *Cryptosporidium* oocysts include modified Ziehl Neelsen technique (Casemore, 1991; Tahvildar-Biderouni and Salehi, 2014); Kinyoun's acid-fast staining technique (Parghi *et al.*, 2014) and the iron-hematoxylin staining technique (Ferreira *et al.*, 2001).

Immunological methods have been used widely in the diagnosis of cryptosporidiosis, as these assays are thought to be more sensitive than conventional staining and more effective in cases where oocysts numbers are low. Immunological-based techniques include enzyme-linked immunosorbent assays (ELISA) (Parghi *et al.*, 2014; Jafari *et al.*, 2015), reverse passive haemagglutination (RPH) (Farrington *et al.*, 1994) and solid-phase qualitative immunochromatographic assays (Garcia and Shimizu, 2000). Direct fluorescent-antibody (DFA) assay are used with relatively high specificities (96 to 100%) and sensitivities (98.5 to 100%) for the detection of *Cryptosporidium* oocysts in clinical and environmental samples (Graczyk *et al.*, 1996; Garcia and Shimizu, 1997). There are also kits that can detect the presence of oo/cysts in feces samples for example Merifluor *Cryptosporidium/Giardia* kit (Meridian Diagnostics, Cincinnati, Ohio, USA) (Grigoriev *et al.*, 1994; Graczyk *et al.*, 1996; Garcia and Shimizu, 1997).

Molecular methods have been developed for the detection and differentiation of *Cryptosporidium* spp. at the species/genotype and subtype levels (Samie *et al.*, 2006). These methods include nested PCR-RFLP (Samie *et al.*, 2006), Nested-PCR (Tahvildar-Biderouni and Salehi, 2014), real-time PCR (Samie *et al.*, 2006; Hadfield *et al.*, 2011) and multiplex real-time PCR (Stark, *et al.* 2011). The most used genes for the detection of the protozoan are the oocyst wall protein (*cowp*) gene, the heat shock protein (*hsp70*) gene, the glycoprotein 60 (*gp60*) gene and the small subunit (*ssu*) rRNA gene (Xiao, 2010).

Techniques have been developed to distinguish between viable and non-viable; however, they cannot be either considered completely reliable or are very expensive and/or complex. These are: inclusion or exclusion in fluorogenic vital dyes as 4',6'-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Campbell *et al.*, 1992; Jenkins *et al.*, 1997; Robertson *et al.*, 2014), fluorescent in situ hybridization (FISH) assay, which use labelled complimentary oligonucleotide probes for the detection of rRNA (Lemos *et al.* 2005), infectivity methods i.e. mice models (Jenkins *et al.*, 1997) or cell culture (Najdrowski *et al.*, 2007) and reverse transcriptase polymerase chain reaction (RT-PCR) assays (Jenkins *et al.*, 2000; Nam and Lee, 2010).

2.3.2.7 Detection methods in vegetables

Methods for the detection of *Cryptosporidium* spp. in vegetables samples are mainly based on concentration of the washing solutions used for recovery of the cysts. Washing solutions used to recover *Cryptosporidium* oocysts are mainly saline buffer solutions (Robertson and Gjerde, 2000; Ripabelli *et al.*, 2004; Lonigro *et al.*, 2006; Dixon *et al.*, 2013; Ranjbar-Bahadori *et al.*, 2013) and glycine buffer solutions (Cook *et al.*, 2007; Amoros *et al.*, 2010; Dixon *et al.*, 2013; Lalonde and Gajadhar, 2016).

The concentration of the oocysts may be performed by centrifugation (Dixon *et al.*, 2013; Ranjbar-Bahadori *et al.*, 2013) or centrifugation followed by immunomagnetic separation (Robertson and Gjerde, 2000; Cook *et al.*, 2007; Di Benedetto *et al.*, 2007; Amoros *et al.*, 2010), or followed by flotation (Lonigro *et al.*, 2006), and filtration followed by immunomagnetic separation (Ripabelli *et al.*, 2004).

After recovery from vegetables, *Cryptosporidium* oocysts can be identified by staining with modified Ziehl-Neelsen's acid-fast (Ripabelli *et al.*, 2004; Ranjbar-Bahadori *et al.*, 2013), by fluorescence microscopy using fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* monoclonal antibodies (Robertson and Gjerde, 2000; Cook *et al.*, 2007; Di Benedetto *et al.*, 2007; Amoros *et al.*, 2010; Dixon *et al.*, 2013) or fluorogenic dyes as 4',6'-diamidino-2-phenylindole (DAPI) (Robertson and Gjerde, 2000; Cook *et al.*, 2007; Amoros *et al.*, 2010).

Recently, various molecular methods have been developed for the identification of *Cryptosporidium* on vegetables such as nested-PCR assay (Ripabelli *et al.*, 2004; Dixon *et al.*, 2013) and qPCR assay (Lalonde and Gajadhar, 2011). Currently the used genes for genotyping of *Cryptosporidium* spp. are *cowp* gene (Ripabelli *et al.*, 2004) and *18S rDNA* gene (Lalonde and Gajadhar, 2011; Dixon *et al.*, 2013).

As for *Giardia*, no viability tests are reported for *Cryptosporidium* spp. on vegetables.

2.3.3 *TOXOPLASMA GONDII*

2.3.3.1 Classification and Morphology

Toxoplasma gondii belongs to the phylum Apicomplexa, Class Sporozoasida, Order Euccidiorida, Family Sarcocystidae) (Uttah *et al.*, 2013). It is an obligate intracellular parasite that can infect humans as well as all warm-blooded animals, including mammals and birds. Felids are the definitive hosts and warm-blooded animals are intermediate hosts (Frenkel *et al.*, 1970).

The parasite has been found, for the first time, in tissues of a hamster-like rodent, the gundi, *Ctenodactylus gundi*, by Nicolle and Manceaux (1908).

T. gondii is the only single species in the genus *Toxoplasma*. Early studies on the parasite strains from North America and Europe have identified a limited genetic diversity, which were classified into three genetic types (Howe and Sibley, 1995): Types I (highly pathogenic), II and III (less pathogenic but more likely to cause infection in immune-compromised patients) (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Khan *et al.*, 2005). Other genotypes and atypical strains are rare in Europe (reviewed by Robert-Gangneux and Dardè, 2012).

T. gondii has three infectious stages: the tachyzoites (in groups or clones), the bradyzoites (in tissue cysts), and the sporozoites (in oocysts) (Dubey *et al.*, 1998) (**Figure 8 a-d**).

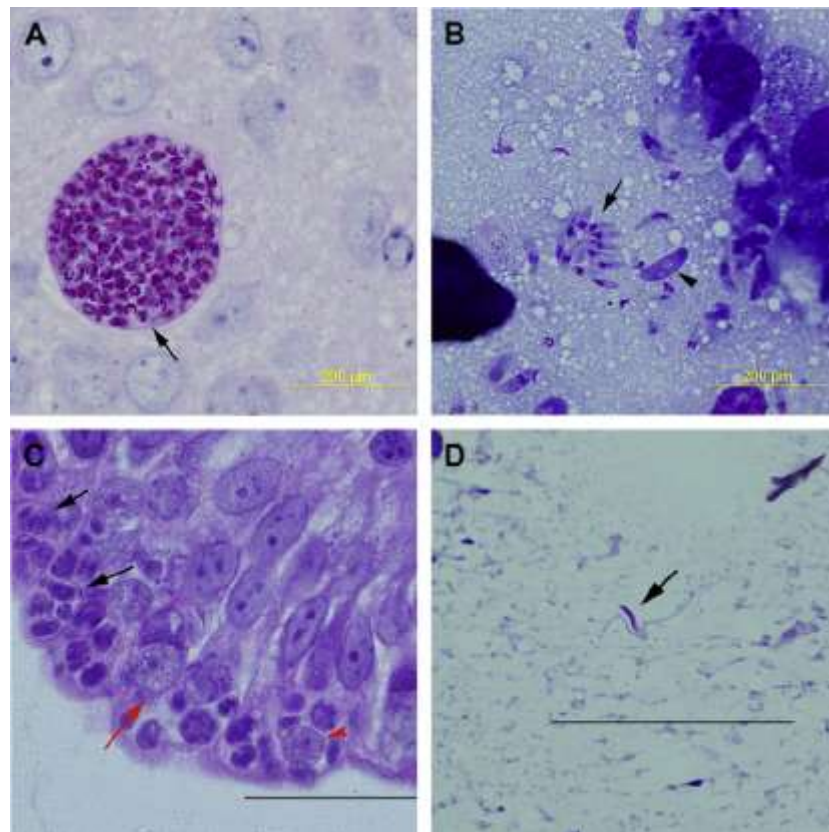
Tachyzoites are often crescent shaped, approximately 2 by 6 mm, with a pointed anterior (conoidal) end and a rounded posterior end. They are limited by a complex membrane, called the pellicle, closely associated with a cytoskeleton involved in the structural integrity and motility of the cell (Roos *et al.*, 1999). As for other members of the phylum Apicomplexa, they concentrate in their apical part a specialized cytoskeletal structure (the conoid, involved in cell invasion) and numerous secretory organelles (rhoptries [ROPs], dense granules, amylopectin granules and micronemes). Rhoptries are excretory structures, each consisting of an anterior narrow neck up to 2.5 mm long that extends into the interior of the conoid, and a saclike, often labyrinthine posterior end (up to 1 mm long). In addition, they have a secretory function associated with host cell penetration, secreting their contents through the plasmalemma just above the conoid to the exterior (Nichols, 1983) and contain a proteolytic enzyme (Saffer *et al.*, 1992). Micronemes are rod-like structures, which occur mostly at the anterior end of the parasite (Dubey *et al.*, 1998), and the micropore is a cytosome-like structure formed by

the invagination of the outer membrane of the pellicle (Nichols *et al.*, 1994). The nucleus is usually situated toward the central area of the cell and contains clumps of chromatin and a centrally located nucleolus (Dubey *et al.*, 1998).

Bradyzoites result from the conversion of tachyzoites into a slow-dividing stage and form tissue cysts. These cysts are more or less spheroid in brain cells and rarely reach a diameter of 70 μm , whereas in muscular cells are elongated and may be 100 μm long (Dubey, 1993). They vary in size from 10 μm for the younger cysts, containing only two bradyzoites, to up to 100 μm for the older ones, containing hundreds or thousands of densely packed bradyzoites. Although tissue cysts may develop in visceral organs, including the lungs, liver, and kidneys, they are more prevalent in the neural and muscular tissues, including the brain, eyes, and skeletal and cardiac muscles (Dubey, 1988). The tissue cyst wall consists of a limiting membrane presenting numerous invaginations and an underlying layer of electron-dense granular material (Ferguson, 2004). It is elastic and thin (0.5 μm thick), and it encloses hundreds of crescent-shaped bradyzoites, each approximately 7 by 1.5 μm in size (Melhorn and Frenkel, 1980). Bradyzoites differ structurally only slightly from tachyzoites and are more slender than tachyzoites. They have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located (Dubey *et al.*, 1998).

Unsporulated oocysts are subspherical to spherical and are 10 by 12 μm in diameter. Under light microscopy, the oocyst wall consists of two colorless layers. Polar granules are absent, and the sporont almost fills the oocyst. Each oocyst contains two ellipsoidal sporocysts measuring from 6 to 8 μm (Dubey *et al.*, 1998). Ultrastructurally, the sporozoite is similar to the tachyzoite, except that there is an abundance of micronemes, rhoptries, and amylopectin granules in the former (Speer *et al.*, 1998).

Figure 8 (a-d). The images show tissue cyst in the brain containing bradyzoites and the thin tissue cyst wall (arrow) (A) and schizonts containing small merozoite (arrow) and the larger merozoite (arrowhead) stained with Giemsa stain (B), asexual stages (black arrows), a developing macrogamont (red arrowhead), and oocyst (red arrow) stained with Hematoxylin and eosin stain (C), a single microgamete (arrow) stained with Giemsa stain (D). (source: https://www.researchgate.net/figure/40025721_fig9_Fig-9-Life-cycle-stages-of-Toxoplasma-gondii-from-cats-A-Tissue-cyst-in-the-brain)



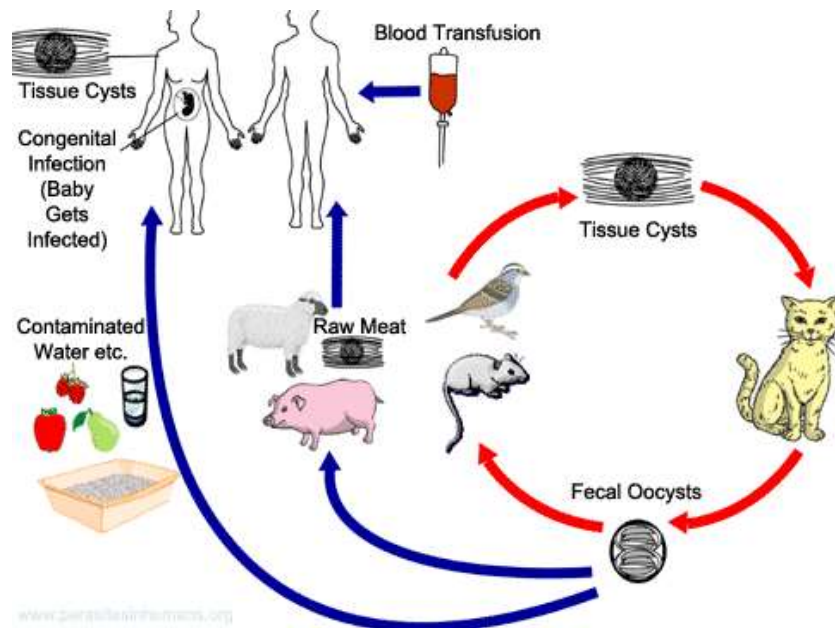
2.3.3.2 Biology and Life cycle

As mentioned above, *T. gondii* have three infective stages: a rapidly dividing invasive tachyzoite, a slowly dividing bradyzoite in tissue cysts, and an environmental stage, the sporozoite, protected inside an oocyst.

T. gondii is a tissue-cyst-forming coccidium and it is unique among this group because it can be transmitted between intermediate and definitive hosts (sexual cycle) and between intermediate hosts via carnivorous (asexual cycle) or even between definitive hosts. Sexual and asexual cycles and dynamic transmissions in a given environment vary according to physical and structural characteristics of both host populations (intermediate and definitive). Sexual reproduction occurs only in felids (domestic and wild cats) (Robert-Gangneux and Dardé, 2012).

The life cycle (**Figure 9**) begins with ingestion of tissue cysts tissues by definitive host (domestic cats and other felines), the proteolytic enzymes in the stomach and small intestine destroy the tissue cyst wall. The released bradyzoites penetrate the epithelial cells of the small intestine, form a parasitophorous vacuole and initiate the development of numerous generations of *T. gondii*. Five morphologically distinct types (i.e. types A–E) of *T. gondii* develop in intestinal epithelial cells before gametogony begins (Dubey *et al.*, 1972). These forms multiply and the nucleus divides two or more times without cytoplasmic division (Ferguson *et al.*, 1974). With the last nuclear division, the formation of the merozoites is initiated near the center of the schizont. The merozoites eventually move towards the periphery of the schizont and the plasmalemma invaginates around each merozoite, forming the plasmalemma of the merozoite. Then, the merozoites separate from the schizont at their posterior ends. This first step is followed by sexual development and the sexual cycle starts 2 days after tissue cysts were ingested by the cat. After the formation of the zygote, oocysts are liberated by the disruption of the cell and excreted as unsporulated forms in cat feces (Robert-Gangneux and Dardé, 2012). Sporulation occurs outside the cat within 1 to 5 days of excretion depending upon aeration and temperature. It implies a meiotic reduction and morphological changes leading to the formation of a sporulated oocyst with two sporocysts, each containing four haploid sporozoites (Ferguson *et al.*, 1979a,b,c). Prepatent periods (time to the shedding of oocysts after initial infection) are 3 to 10 days after ingesting tissue cysts, ≥ 18 days after ingesting oocysts and ≥ 13 days after ingesting tachyzoites (Dubey, 1998). Fewer than 30% of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts (Dubey and Frenkel, 1976 Dubey, 1996). Infected cats can shed more than 100 million oocysts in their feces (Dubey and Frenkel, 1972; Jones and Dubey, 2010) for several weeks and once during their life.

Figure 9. Life cycle of *Toxoplasma gondii* (source: CDC, 2015)



Within intermediate hosts, the parasite undergoes only asexual development. After oocyst ingestion, sporozoites are liberated. They penetrate in the intestinal epithelium, where they differentiate into tachyzoites. Tachyzoites rapidly replicate by endodyogeny inside any kind of cell (neutrophils, lymphocytes, capillary and lymphatic endothelial cells, macrophages, fibroblasts and occasionally eosinophils and mast cells) and disseminate throughout the organism. As result of the conversion from tachyzoite to bradyzoite, tissue cysts may remain throughout life in most hosts, predominantly in the brain or musculature (Robert-Gangneuxa and Dardé, 2012). Upon the ingestion of these tissue cysts by an intermediate host through raw or under cooked meat, cysts are destroyed in the digestive tract, causing the release of bradyzoites. The bradyzoites infect the intestinal epithelium of the new host and differentiate in tachyzoite for dissemination throughout the body (Robert-Gangneuxa and Dardé, 2012). Given that, the bradyzoites are more resistant to digestion by gastric juices (pepsin-HCl) respect to the tachyzoites that are destroyed immediately (Jacobs *et al.*, 1960), the tissue cysts are very important in the life cycle of *T. gondii*.

2.3.3.3 Epidemiology

Toxoplasmosis is one of the most common infections in humans and warm-blooded animals, with a worldwide distribution. In humans, toxoplasmosis has been found in all parts of the world and it is estimated about one-third of the world's population is infected with latent toxoplasmosis with the majority of cases being asymptomatic (Pereira *et al.* 2010).

Humans - It is generally assumed that approximately 25 to 30% of the world's human population is infected by *Toxoplasma*. Actually, the prevalence varies widely between countries (from 10 to 80%) and often within a given country or between different communities in the same region. 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 prevalence (30 to 50%) have been found in countries of Central and Southern Europe, and high prevalence have been found Latin America and in tropical African countries (reviewed by Robert-Gangneux and Dardè, 2012). High prevalence of toxoplasmosis seroprevalence in females of reproductive age or pregnant were reported in Latin America, parts of Eastern/Central Europe, the Middle East, and parts of south-east Asia and Africa (Pappas *et al.*, 2009). In the US, seroprevalence was estimated of 10.8-11% (Dubey and Jones, 2008), a slight drop from the preceding decade.

In Europe, toxoplasmosis seroprevalence was estimated of 9.1-57.6% with peaks reported in Poland and in Romania (Pappas *et al.* 2009). Recently, incidence or prevalence at birth of congenitally acquired toxoplasmosis, reported as cases per 10000 live births, have been reported for France (2.9/10000) (Villena *et al.* 2010), Denmark (1.6/10000) (Roser *et al.* 2010). In Europe, the population structure of *T. gondii* is markedly clonal, with a predominance of strains belonging to the type II lineage followed by type I and occasionally type III. In France, type II strains represent 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 was estimated to be of 17.4-34.4% (Pappas *et al.* 2009) and a recently study conducted on pregnant women in a population from Palermo has estimated a prevalence of anti-Toxo IgG antibodies of the 17.97% (152 on 846) (Puccio *et al.*, 2014).

Animals -*Toxoplasma* infection has been described for more than 350 host species, mammals and birds, with the vast majority of them living in a wild environment (Robert-Gangneux and Dardè, 2012). Seroprevalence showed a higher seropositive in wild felids (in zoo and free-ranging) (up to 100%) compared with domestic cats (up to 80%) (reviewed by Jones and Dubey, 2010).

Seroprevalence of *T. gondii* infection in swine is up to 70%, in sheep and/or lamb is near to 100%, in goat ranging from 3.7% to 81.8% and in cattle is up to 83.3%, worldwide. In Europe, seroprevalence of *T. gondii* infections in swine and in sheep ranging from 0.4% to 36% and 20.5% to 89%, respectively (reviewed by Guo *et al.*, 2015). Rates of seroprevalence reported for goats vary from 4 to 77%, while in horses is generally lower (Robert-Gangneux and Dardè, 2012).

Special interest has recently been focused on marine mammals. A variety of marine mammals (sea otters, dolphins, seals, and walruses) has been found to be infected, with prevalence ranging from 47 to 100%. These marine mammals serve as sentinels of environmental contamination by oocysts via freshwater runoff into the marine ecosystem (Conrad, 2005).

In Italy, recent studies have documented a high prevalence of both *T. gondii* antibodies and (*Toxoplasma* Type I) oocysts in colony cats (Mancianti *et al.*, 2010; 2015) and several contributions have been published on the positivity to *T. gondii* of intermediate hosts to but few investigated the lineages, except the most recent ones carried out in free-range and industrial pigs in which the presence of *T. gondii* Type I, Type II and Type I/II (Bacci *et al.*, 2015; Papini *et al.*, 2017) lineages was demonstrated.

Water - Twelve surveillance studies reported the presence of *T. gondii* in different kinds of water such as surface waters, wastewaters, drinking waters and recreational water from Asia, Latin American and Europa with a prevalence ranging from 5% to 56.2%. In particular, *T. gondii* was detected in waste and drinking water, with a prevalence rate up to 50%. In Europe, *T. gondii* has been recorded in various water sources only in Bulgaria, Greece, France, Germany and Poland with a prevalence ranging from 5% to 44.4% (reviewed by Plutzer and Karanis, 2016). In Italy, surveillance studies that report the presence of *T. gondii* have not been registered. Contact with soil has been identified as a strong risk factor in a European multicenter case-control study, and a percentage of the 6-17% of primary infections in humans were attributed to this risk factor (Cook *et al.*,

2000). Contaminated water and soil may act as vehicles for the transfer of oocysts to vegetables and fruit for human consumption, although there are few data available to confirm this.

Fresh produce -*T. gondii* has been recorded with a prevalence of the 6.6% out of 470 leafy vegetables in Saudi Arabia (Al-Megrin *et al.*, 2010). In Pakistan, *T. gondii* has been observed on 3 (1.9%) (lettuce, coriander and beet) out of 500 samples between leafy vegetables, roots and fruits (ul-Haq *et al.*, 2014). In South America, out of 238 samples of vegetables, the prevalence of *T. gondii* DNA has been of the 0.6% (1/62) in smooth lettuce, 3.7% (4/106) in crisp head lettuce, 5% (2/40) in chicory, 14.3% (1/7) in rocket and 20% (1/5) in parsley (Marchioro *et al.*, 2016).

In Europe (i.e. Poland), one study report the presence of *T. gondii* DNA in 21 (9.7%) out of 216 fruits and vegetables. Among the 21 tested positive samples, six were *T. gondii* Type I and two were Type II (Lass *et al.*, 2012).

In Canada, *T. gondii* DNA has been identified in 3 (0.26%) samples of baby spinach out of 1171 samples of pre-packaged leafy green vegetables or bulk leafy greens from domestic (24.25%) and imported (75.75%) sources between April 2014 and March 2015 (Lalonde and Gajadhar, 2016).

Epidemiological studies have shown that toxoplasmosis can be transmitted to humans by three principal routes. First, humans can eat raw or inadequately cooked infected meat (especially pork, mutton, and wild game meat) or uncooked foods that have come in contact with infected meat. Second, humans can inadvertently ingest oocysts that cats have passed in their feces, either in a cat litter box or in soil (e.g., soil from gardening or unwashed fruits or vegetables) (Jones *et al.*, 2011). Third, a woman can transmit the infection to her unborn fetus because the parasite can cross the placenta and infect the fetus (congenital transmission) (Hampton, 2015). The oocysts play an important role in the transmission of *T. gondii*. They can infect a wide range of intermediate hosts, virtually all warm-blooded animals, from mammals to birds, when ingested with food or water (Jones and Dubey, 2010, 2012).

Oocysts can survive in the environment for long periods and are highly resistant to disinfectants and freezing (Jones and Dubey, 2010); for example, oocysts can survive at -21°C for 28 days (Frenkel and Dubey, 1973) but are killed by temperatures above 60°C

(Dubey, 1998b). Ultraviolet rays also will inactivate oocysts, depending on the dose (Wainwright *et al.*, 2007; Dumètre *et al.*, 2008). Another 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).

Waterborne outbreaks of toxoplasmosis in humans were reported in many areas of the world. Up to the year 2010, least 525 water-associated outbreaks of protozoan disease have been reported worldwide and *T. gondii* has been the etiological agent in 10 reported waterborne outbreaks (1.9%). The reporting countries were Brazil, Panama, Canada, French Guinea and India (Karanis *et al.*, 2007; Baldursson and Karinis, 2011).

Foodborne infection of toxoplasmosis in human has been attributed to ingestion of raw or undercooked ground beef, lamb, pork, venison (reviewed by Jones and Dubey, 2012) and unpasteurized goat's milk contaminated (Sacks *et al.*, 1982).

Despite the presence of *T. gondii* in fruits and vegetables, confirmed outbreaks associated with their consumption have not been documented.

2.3.3.4 Pathogenesis

When the organism is ingested, bradyzoites from the tissue cysts or sporozoites released from oocysts penetrate the intestinal epithelial cells and multiply in the intestine. Attachment to the host cell membrane is a prerequisite for invasion. It requires the calcium-dependent secretion of adhesins from micronemes, such as the microneme protein MIC2, which recognize host cell receptors and promote the reorientation and attachment of the parasite. Cell invasion relies on a complex interaction between the host cell surface and the parasite, promoted by actin-myosin interactions and dynamic rearrangements of the parasite cytoskeleton (Carruthers and Boothroyd, 2007). Attachment to the host requires also the distribution over the entire surface of the parasite of an apical membrane antigen (AMA1), also secreted by micronemes, and the secretion of rhoptry (ROP) neck proteins (RONs) inserted into the host cell membrane (Dubremetz, 2007).

The pathogenic role of *Toxoplasma* is represented by the ability of the parasite to spread both locally to mesenteric lymph nodes and to distant organs by invading the lymphatics and blood. Necrosis in intestinal and mesenteric lymph nodes may occur before those other organs may become severely damaged and focal areas of necrosis may develop in many organs (Dubey, 1996). *T. gondii* does not produce a toxin but the necrosis is

caused by intracellular multiplication of tachyzoites (Dubey, 1996). 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 avirulent strains, allowing survival after the inoculation with >10³ tachyzoites (Robert-Gangneux and Dardè, 2012).

2.3.3.5 Clinical signs

The infection presents with a wide range of clinical manifestations in infected mammals, including humans (Akyar, 2011). When symptoms develop, they are nonspecific and include malaise, fever, sore throat, and myalgia. Clinical manifestations of toxoplasmosis are caused by cell destruction due to multiplying tachyzoites, which most commonly affect the brain, liver, lungs, skeletal muscles and eyes. Oocyst-induced infection may be more severe than that induced by ingestion of tissue cysts. Signs may persist for one to twelve weeks but more severe disease is very rare in immunocompetent individuals (Tenter *et al.*, 2000).

Infection may be associated with other diseases such as HIV/AIDS in humans or immunosuppressive therapy in any species (Akyar, 2011).

Toxoplasmic encephalitis is the most common clinical presentation of toxoplasmosis among persons with AIDS and it is usually the result of reactivation of latent tissue cysts (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, stupor and coma (Luft *et al.*, 1983; Wong *et al.*, 1984). Speech abnormalities and hemiparesis are the most common focal neurological findings (Luft *et al.*, 1993).

Prenatal infection is the direct consequence of a primary infection of the mother during pregnancy. Congenital transmission occurs during acute toxoplasmosis in a seronegative mother when tachyzoites present in the blood may cross the placenta and infect the fetus (Jones, Lopez *et al.* 2003; Montoya and Remington 2008). Congenital toxoplasmosis has varying effects on the growing fetus. Various clinical manifestations involve prematurity to perinatal death. Presentation of congenital infection may result in severe impairment particularly on the developing fetus' brain and eyes; ocular toxoplasmosis is a consequence of prenatal infection. The clinical picture presents

necrotizing retinitis with variations of the lesion in size, number, and aspect. Lesions can be either monolateral or bilateral. More rarely, but not less serious, are the manifestations of anterior uveitis, and inflammation of sclera and papilla (Hall *et al.*, 2009).

The characteristic triad of congenital toxoplasmosis (chorioretinitis, hydrocephalus, and cerebral calcifications) most commonly identifies the presence of active congenital disease (Hampton *et al.*, 2015). The major fetal implications of congenital toxoplasmosis occur early in pregnancy, generally in the first trimester. Grave implications of first trimester transmission of toxoplasmosis include miscarriage, stillbirth, or serious fetal damage, such as retinochoroiditis, endocranial calcification, hydrocephaly, and microcephaly (Jones *et al.*, 2009b). 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).

2.3.3.6 Diagnosis in clinical samples

In feline feces, oocysts of *T. gondii* can be detected by flotation but this finding has limited clinical significance because the presence of oocysts is not correlated with development of disease in the cat. *Toxoplasma* oocysts are morphologically indistinguishable from those of *Hammondia* sp. and *Besnoitia* sp., which may also be present in feline feces.

Tissue cysts can be stained, helping to distinguish the parasites from host cells. Giemsa and Haematoxylin and Eosin (HE) staining are commonly used for this purpose (Dubey and Carpenter, 1993; Gordon *et al.*, 1993; da Silva *et al.*, 2010). Periodic acid schiff (PAS) can stain amylopectin granules in bradyzoites (da Silva *et al.*, 2010). These methods are relatively time consuming and require considerable skill to obtain reliable detection results.

Serological assays are the first diagnostic tests used in detection of current and past infection (Liu *et al.*, 2015).

Determining when *T. gondii* infection occurred in a pregnant woman is important because infection before conception poses little risk for transmission of infection to the fetus; however, infection after conception does pose such risk. The presence of elevated levels of *Toxoplasma*-specific IgG antibodies indicates infection, but does not distinguish between an infection acquired recently and one acquired in the distant past. The

presence of elevated levels of *Toxoplasma*-specific IgG antibodies indicates infection, but does not distinguish between an infection acquired recently and one acquired in the distant past. In acute infection, IgG and IgM antibodies generally rise within 1 to 2 weeks of infection. Detection of *Toxoplasma*-specific IgM antibodies are used as an aid in determining the time of infection, but IgM antibodies persist for up to 18 months post-infection. A negative IgM with a positive IgG result indicates infection at least 1 year previously. A positive IgM result may indicate more recent infection or may be a false-positive reaction (Jones *et al.*, 2001). A variety of serological tests, such as dye test (DT), modified agglutination test (MAT), enzyme-linked immunosorbent assays (ELISA), immunosorbent agglutination assay (ISAGA), indirect fluorescent antibody test (IFAT) and indirect haemagglutination assays (IHA), have been developed to detect different antibody classes or antigens (Liu *et al.*, 2015).

Molecular methods are used in addition to conventional serological methods for the diagnosis of toxoplasmosis. Conventional methods are usually not misleading, but are limited in prenatal cases or in immunocompromised patients. Several multicopies targeting genes are usually used for the detection of *T. gondii* in biological samples, including the B1 gene, the 529 bp repeat element and the internal transcribed spacer (ITS-1) or 18S rDNA sequences (Liu *et al.*, 2015).

Real-time PCR can detect low concentrations of target DNA and quantify copies of specific template DNA. The amplification product is measured during each cycle using probes or intercalating dyes, and can be quantified using the standards at known concentration. Real-time PCR has been successfully used to detect *T. gondii* DNA in human amniotic fluid, cerebrospinal fluid, blood and aqueous humor (Dworkin *et al.*, 2002; Kompalic-Cristo *et al.*, 2007; Kasper *et al.*, 2009; Nogui *et al.*, 2009). The real-time PCR assay with the B1 gene is considered as the best-performing technique for diagnosis of congenital toxoplasmosis, compared with conventional PCR and nested-PCR (Teixeira *et al.*, 2013). Genotyping methods based on molecular technologies including microsatellite analysis, multilocus sequence typing, PCR-RFLP, RAPD-PCR, and high-resolution melting (HRM) analysis, have been developed (reviewed by Liu *et al.*, 2015). In addition, serotyping methods based on polymorphic polypeptides have been developed from the *T. gondii* antigens SAG2A, GRA3, GRA6, and GRA7. These can accurately recognize the Type I, II, and III in mice (Kong *et al.*, 2003).

Techniques have been developed for determining the viability of *Toxoplasma* oocysts; however, they either cannot be considered completely reliable or are very expensive and/or complex. These are such as fluorogenic substrate i.e. fluorescein-3',6'-diacetate (Ziegler and Buchholz, 1975), reverse transcriptase polymerase chain reaction (RT-PCR) (Cultrera *et al.*, 2002) and infectivity methods using mice models (Gomez-Samblas *et al.*, 2016).

2.3.3.7 Detection methods in vegetables

The most recent concentration methods of the oocysts of *Toxoplasma* in vegetables are based on the concentration (Al-Megrin *et al.*, 2010; Lalonde and Gajadhar, 2016; Marchioro *et al.*, 2016) and flocculation (Lass *et al.*, 2012) of the washing solutions used for recovery of the oocysts. Concentration methods use different washing solutions to elute of the oocysts i.e. i) physiological saline solution (Al-Megrin *et al.*, 2010), ii) glycine buffer solution followed by a flotation (Lalonde and Gajadhar, 2016) and iii) Tween 80 solution (Marchioro *et al.*, 2016), whereas flocculation method described by Lass *et al.* (2012) provide different solutions such CaCO₃ solution, NaHCO₃ solution and NH₃O₃S solution in order 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 *et al.*, 2010; Marchioro *et al.*, 2016)

After recovery from vegetables, the *Toxoplasma* oocysts can be identified by light microscopy adding a drop of Lugol iodine (Al-Megrin *et al.*, 2010) or by various molecular methods i.e. PCR-RFLP assay (Lass *et al.*, 2012), qPCR assay (Lalonde and Gajadhar, 2016) and PCR assay (Marchioro *et al.*, 2016). The genes used for the detection of *Toxoplasma gondii* are *B1* gene (Lass *et al.*, 2012; Marchioro *et al.*, 2016) and 18S rDNA (Lalonde and Gajadhar, 2016).

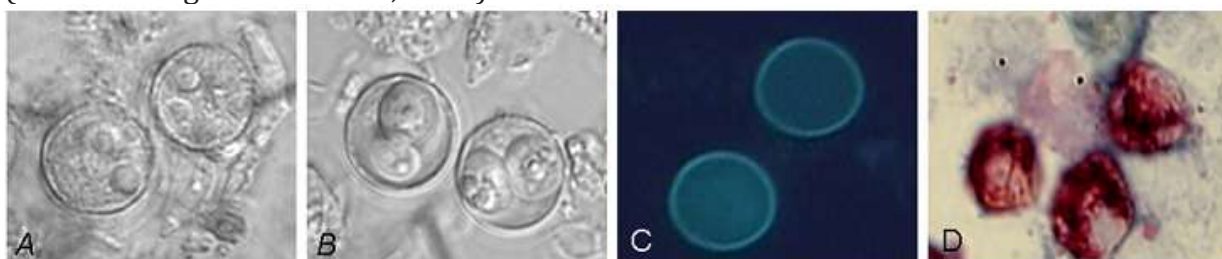
2.3.4 *CYCLOSPORA CAYETANENSIS*

2.3.4.1 Classification and Morphology

C. cayetanensis belongs to the phylum Apicomplexa, subclass Coccidiasina, order Eucoccidiorida, family Eimeriidae (Ortega *et al.*, 1994). The first published report of *Cyclospora* infection in humans was described in two children and a woman in Papua New Guinea by Ashford (1979). Eighteen species of *Cyclospora* have been described in several animal species. *Cyclospora viperae*, *C. glomericola*, *C. babaulti*, *C. tropidonoti*, *C. anglomurinensis*, *C. caryolytica*, *C. talpae*, *C. ashtabulensis*, *C. megacephali*, *C. parascalopi*, *C. niniae*, *C. scinci*, and *C. zamenis* in vipers, moles, myriapodes, and rodents (Lainson, 2005), *C. cercopithecii* in green monkeys, *C. colobi* in colobus monkeys, and *C. papionis* in baboons (Eberhard *et al.*, 1999). These species cannot be differentiated by light microscopy, as they are morphologically similar. *C. cayetanensis* is the only species described in humans (Ortega *et al.*, 1993, 1994).

Cyclospora oocysts are small and spheroidal in shape with a diameter of 7.7–9.9 µm. It appears as non-refractive spheres that contain a cluster of refractive membrane-bound globules enclosed within a membrane (morula) (Long *et al.*, 1991) (**Figure 10 a-d**). Sporulated oocyst has two ovoid structure called sporocysts (4 to 6.3 µm) and each sporocyst has two sporozoites (1.2 to 9 µm) (Ortega *et al.*, 1993, 1994). By light microscopy (high magnification), meronts Type I measure 0.5 X 3–4 mm while meronts Type II measure 0.7 0.8 X 12–15mm. Merozoites from both types of meronts have typical structures of coccidia, including rhoptries, micronemes, and nuclei (Ortega *et al.*, 1997b; Connor *et al.*, 1999). Macrogametocytes have characteristic wall-forming bodies Types I and II, and polysaccharide granules (Ortega *et al.*, 1997b).

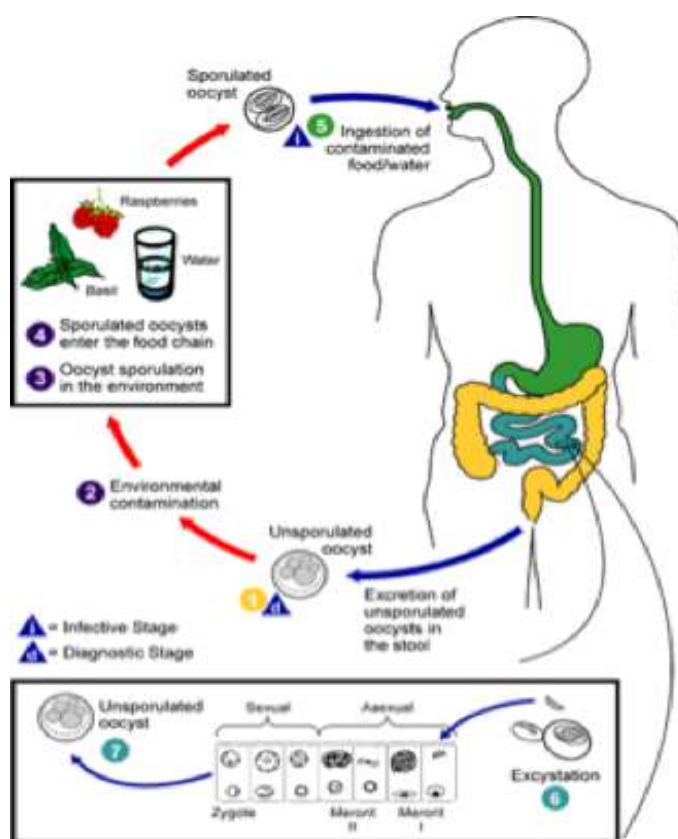
Figure 10 (a-d). *Cyclospora* oocysts. The images show unsporulated (A) and sporulated (B) oocysts and oocysts detected by autofluorescence (C) or modified acid-fast stain (D). (source: Ortega and Sanchez, 2010)



2.3.4.2 Biology and Life Cycle

C. cayetanensis is an obligate intracellular parasite with a life cycle not fully characterized. It appears to be monoxenous, requiring a single human host to complete the entire life cycle, since both asexual and sexual stages have been observed in the same host (Sun *et al.*, 1996; Ortega *et al.*, 1997b; Connor *et al.*, 1999). The life cycle is complex and follows the patterns described for other enteric coccidia, which involve a merogonic cycle with two generations of meronts, a gametogonic cycle with macrogametes and microgametes and zygotes, and a sporogony (Ortega *et al.*, 1997b; Connor *et al.*, 1999) (**Figure 11**).

Figure 11. Life cycle of *Cyclospora cayetanensis* (source: CDC, 2015)



Infection start when a susceptible person ingests food or water contaminated with sporulated oocysts (Ortega and Sanchez, 2010). In the gastrointestinal tract, the oocysts excyst release sporozoites, which infect the epithelium cells lining the small intestine, in particular the jejunum (Sun *et al.*, 1996; Ortega *et al.*, 1997b). The sporozoites and subsequent stages are located within the cytoplasm in a supranuclear position and are surrounded by parasitophorous vacuoles (Sun *et al.*, 1996). Once inside the epithelial

cells (enterocytes), the sporozoites transform into trophozoites, which undergo asexual proliferation by merogony to form meronts, which contain merozoites. Two types of meronts develop; type I meronts contain 8–12 merozoites which penetrate in host cells and form type II meronts which contain four merozoites. Once released, the type II meronts enter in other intestinal cells and starts the sexual phase by producing microgametocytes (male) or macrogametocytes (female). The microgametocyte fertilizes the macrogametocyte and produces a zygote. The zygotes differentiate into unsporulated oocysts, which are released into the lumen of the intestinal and after in the faeces. Unsporulated oocysts (and therefore non-infectious) are released in the environment. Oocysts require a few days to weeks, depending on climatic factors, to mature in the environment into the infective sporulated oocysts (Ortega *et al.*, 1998).

2.3.4.3 Epidemiology

Humans - Cyclosporiasis is endemic in several tropical and subtropical developing countries 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). *Cyclospora* oocysts have been detected in China (0.70%) and *C. cayetanensis* was identified with a prevalence of 0.30% (Zhou *et al.*, 2011). In Turkey, *Cyclospora* oocysts have been observed in 129 (5.7%) out of 2281 individuals (Karaman *et al.*, 2015). In India, *C. cayetanensis* has been reported in immunocompetent and immunocompromised individuals with an overall prevalence of 2.4% (Yadav *et al.*, 2015),

In industrialized countries, cyclosporiasis have been observed mainly in tourists or expatriates visiting countries where the disease is endemic (0.6%) (reviewed by Ortega and Sanchez, 2010). The first case of *C. cayetanensis* infection in international travelers was performed in Germany in 1997 (Jelinek *et al.* 1997); out of 795 patients, infections with *C. cayetanensis* have detected in five subjects (1.1%).

In Italy, a few cases of cyclosporidiosis have been registered; *Cyclospora* has been detected in two patients returned from Nepal and South America (Scaglia *et al.*, 1994; Drenaggi *et al.*, 1998), in two AIDS patients (Maggi *et al.*, 1995). Recently in Central Italy, *C. cayetanensis* has been detected in eight patients, five were Italians, and three of these returned from endemic areas (Masucci *et al.*, 2008, 2011). The highest rate has been reported in adults than 45-65 years of age (Masucci *et al.*, 2011). In southern Italy,

Apulia, 11(27.5%) out of 40 samples were positive to *C. cayetanensis* (Giangaspero *et al.*, 2015b).

Animals -A few reports have described the presence of *Cyclospora* oocysts in the feces of chickens (Mexico) (Garcia-Lopez *et al.*, 1996) and dogs (Brazil) (Yai *et al.*, 1997). Attempts to infect different animals (rodent, chickens, ducks, rabbits, hamsters, ferrets, pigs, dogs and monkeys) with *C. cayetanensis* have been unsuccessful, suggesting host specificity (Eberhard *et al.*, 1999). Chu *et al.* (2004) identified *C. cayetanensis* in domestic animals by PCR, but whether these findings represent true or spurious infections remains to be clarified. Other studies have been described the presence of *Cyclospora*-like oocysts (4.5%) in zoo animals (non-human primates, carnivores, and artiodactyla) in Spain (Perez *et al.*, 2008). Recently, *C. cayetanensis* has been found in non-human primates in Italy, but oocysts of *Cyclospora* for morphological identification/description were not detect (Marangi *et al.*, 2015a).

Surveillance studies reported the presence of *Cyclospora* spp. in different kinds of water such as surface waters, wastewaters and drinking waters worldwide with a prevalence ranging from 0.24% to 63.6%, in particular *C. cayetanensis* was detected in waste and tap water, with a prevalence rate up to 60% (reviewed by Plutzer and Karanis, 2016).

Water - *C. cayetanensis* has been detected or inferred to occur in wastewater in countries endemic for cyclosporiasis, including Peru (Sturbaum *et al.*, 1998), Nepal (Sherchand *et al.*, 1999), Tunisia (Ben Ayed *et al.*, 2012) and USA (Kitajima *et al.*, 2014). *Cyclospora* oocysts have also been detected in water used for the irrigation or processing of vegetables in Cambodia (Vuong *et al.*, 2007) and Vietnam (Tram *et al.*, 2008).

In Europe, *Cyclospora* has been recorded in various water sources only in two countries. In Spain, *Cyclospora* has been found in drinking, river water and wastewater at a prevalence of 6%, 13% and 2%, respectively (Galvan *et al.*, 2013). In Italy, *Cyclospora* DNA has been detected in 30% of the 10 tap water samples from the toilets on Italian trains (Giangaspero *et al.*, 2015a), and in treated water and well water used for the irrigation of the vegetables at a prevalence of 30%, 21.3% and 6.2%, respectively. In this study, *Cyclospora* DNA was also detected in 6 (11.8%) out of 51 soil samples irrigated with treated wastewater (Giangaspero *et al.*, 2015b).

Fresh produce - Surveillance studies reported the presence of *C. cayetanensis* in fresh fruits and vegetables in Asia (i.e. Nepal, Cambodia and Vietnam) and South America (i.e. Costa Rica) with a prevalence ranging from 1.7% to 11.6% (reviewed by Dixon, 2015). In Europe, *Cyclospora* has been reported only in Italy by Giangaspero *et al.* (2015b). In this report *Cyclospora* DNA has been detected on 6 (12.2%) out of 49 vegetable samples as fennel, tomato, cucumber and celery, with the highest prevalence (18.7%) on fennel. Recently, *C. cayetanensis* have been also found in 6 (0.51%) out of 1171 pre-packaged leafy greens (Lalonde and Gajadhar, 2016) and in 9 (1.7%) out of 544 ready-to-eat packaged leafy greens in Canada (Dixon *et al.*, 2013).

Direct person-to-person transmitted is unlikely because the oocysts spread from individuals are not infectious and require extended periods of time outside the host to sporulate (Ortega and Sanchez, 2010), thus, a transmission vehicle must be involved.

Oocysts can survive in water at 4°C for 2 months and at 37°C for 7 days (Smith *et al.*, 1997; Ortega *et al.*, 1998). The oocysts are resistant to many disinfectants including chlorine at levels used in water treatment (Soave *et al.*, 1998). Sathyanarayanan and Ortega (2004) have demonstrated that unsporulated *C. cayetanensis* oocysts are resistant to pesticide commonly used. A study has demonstrated that sporulation can be prevented after 2 days at – 20°C or - 70°C and 70°C for 15 minutes (Sathyanarayanan and Ortega, 2006). Another factor to consider in the transmission of cyclosporiasis is the low infectious dose, although it has not yet been estimated (Sterling and Ortega, 1999).

Epidemiological studies have shown that consumption of untreated water and lack of adequate sanitation are associated with increased risk of *Cyclospora* infections (Ortega and Sanchez, 2010). Up to the year 2010, least 524 water-associated outbreaks of protozoan disease have been reported worldwide and *C. cayetanensis* has been the etiological agent in nine reported waterborne outbreaks (1.7%) (Karanis *et al.*, 2007; Baldursson and Karinis, 2011). The presence of *C. cayetanensis* in aquatic ecosystems suggests the importance of waterborne oocysts in the spread of infection.

Outbreaks of cyclosporiasis have been also associated with contaminated fruits and vegetables in Asia (i.e. Nepal and Indonesia) and Southern America (i.e. Mexico, Columbia, Guatemala and Perú) (Chacin-Bonilla, 2010).

In the United States, foodborne outbreaks of cyclosporiasis since the mid-1990s have been linked to various types of imported fresh produce, including raspberries, basil,

snow peas, and mesclun lettuce. Imported berries from Guatemala were the implicated vehicles whereas mesclun lettuce and basil that have been not imported from endemic countries have been implicated in outbreaks in the United States (CDC, 2015).

Outbreaks of *C. cayetanensis* infections have been registered in Europe. In Germany, a cyclosporiasis outbreak that has involved 34 (85%) out of 40 people has been associated with the consumption of salads, imported from France and Southern Italy (Doller *et al.*, 2002). In Sweden, an outbreak of *C. cayetanensis* infection involving 12 laboratory-confirmed and 6 probable cases was detected during May and June 2009. Imported sugar snap peas from Guatemala was the suspected vehicle (Insulander *et al.*, 2010). In the United Kingdom, *C. cayetanensis* was identified in 79 returned travelers from the Riviera Maya region of Mexico between June and September 2015. The infections have been attributed with consumption of fresh products as fruits (strawberries or raspberries), vegetables and fresh herbs (coriander), and consumption of bottled water, ice and drinks (Nichols *et al.*, 2015).

In Italy, *Cyclospora* foodborne outbreaks associated with fruits and/or vegetables have not been registered.

2.3.4.4 Pathogenesis

The mechanisms of pathogenesis of *Cyclospora* infections by which it interacts with human host target cells and potential virulence factors are poorly understood; however, histological studies have allowed to take on more information about the histopathology of this infection (Connor *et al.*, 1993, 1999; Sun *et al.*, 1996; Ortega *et al.*, 1997b).

Moderate to severe erythema of the distal duodenum, acute and chronic inflammations of the *lamina propria* are observed in patients with *Cyclospora* infections (Connor *et al.*, 1999). Other alterations have been described as alterations of the epithelial tissue as focal vacuolization at the tips of the villi of the surface epithelium, loss of the brush border, alteration of cells from a columnar to cuboid shape, crypt hyperplasia, epithelial disarray, and partial villous atrophy (Connor *et al.*, 1999).

Sun *et al.* (1996) described the presence of multiple parasitic vacuoles containing asexual-stage *Cyclospora* organisms in intestinal biopsies of an HIV-positive patient who had just returned from the Dominican Republic. This patient had a low CD4⁺ cell count (224 cells/ μ l) and presented with chronic inflammation of the duodenum and stomach.

Organisms were present in the supranuclear location of the mucosal villi and absent in the crypts.

Ortega *et al.* (1997b) described an altered mucosal architecture with shortening and widening of the intestinal villi due to diffuse edema and infiltration by a mixed inflammatory cell infiltrate from patients with gastrointestinal disorders. The presence of sexual-stage coccidian organisms has been reported in 17 patients with *Cyclospora* infection.

Biopsies of these patients showed also diffuse edema and infiltration of the villous mucosa by mixed inflammatory cells. Plasma cells and lymphocytes were notoriously present, and eosinophils were numerous in 4 out of 17 cases. In some *C. cayetanensis* infected patients were documented also impairment of D-xylose absorption (Shlim *et al.*, 1991).

2.3.4.5 Clinical signs

Clinical signs of *C. cayetanensis* occur as acute, self-limiting illness lasting for few days, or chronic diarrhea. Diarrhea is usually watery and lacks blood or inflammatory cells, and often occurs in cyclical patterns alternating with constipation (Soave *et al.*, 1998). The other symptoms include anorexia, nausea, flatulence, fatigue, abdominal cramping, bloating, low-grade fever, and weight loss (Ortega *et al.*, 1997b; Connor *et al.*, 1999). The incubation period for *Cyclospora* infection ranges from 2 to 14 days.

Symptoms associated with cyclosporiasis are more severe in HIV/AIDS patients as severe weight losses and a more prolonged illness compared to non-AIDS patients (Sifuentes-Osornio *et al.*, 1995). Biliary diseases have also been reported after *Cyclospora* infections (Sifuentes-Osornio *et al.*, 1995; de Go'rgolas *et al.*, 2001). Studies reported also Guillair-Barrè syndrome and Reiter syndrome after diarrheal illness by *Cyclospora* (Richardson *et al.*, 1998; Connor *et al.*, 2001).

2.3.4.6 Diagnosis in clinical samples

The diagnosis of *Cyclospora* oocysts is based on microscopic observations of fecal samples or in biopsy specimens, or through molecular techniques, or viability studies. Samples can be stored in 2.5% potassium dichromate for sporulation or molecular detection and in 10% formalin for direct microscopy, concentration procedures, and staining or frozen for molecular testing and long-term archiving (Ortega and Sanchez, 2010). Multiple stool samples from the same person at 2 to 3 day intervals may enhance the chance of detecting infection that have intermittent shedding and/or small numbers of oocysts (Eberhard *et al.*, 1997). The most used technique for the concentration of samples is ethyl acetate-formalin sedimentation (Ortega and Sanchez, 2010). *Cyclospora* oocysts can be concentrated prior to the microscopic observation or as a primary step in the purification of the parasite. Other concentration methods are sugar flotation technique (Negm, 1998; Kimura *et al.*, 2004), formalin-ether sedimentation (Negm, 1998; Kimura *et al.*, 2004) and Renocal-sucrose gradient sedimentation (Riner *et al.*, 2007) and discontinuous Percoll gradient (Medina-de la Garza *et al.*, 1997). *Cyclospora* can be identified by phase-contrast microscopy or bright-field microscopy during examination of specimens for the ova and parasites (Ortega and Sanchez, 2010). *Cyclospora* oocysts can be stained using permanent stains. They are acid-fast, and laboratory diagnosis can identify oocysts of *Cyclospora* in stool samples using simple inexpensive staining techniques like Ziehl-Neelsen. *Cyclospora* oocysts stain variably with MZN acid-fast stain and may appear unstained, pink or dark red (Clarke and McIntyre, 1996). Care should be taken during the detection because the oocysts of *Cyclospora* could be confused with *Cryptosporidium* (*Cyclospora* 8-10 μm – twice the size of *Cryptosporidium*). Another stain used is the Safranin; it stains oocysts uniformly (98%) when the fecal smear (Visvesvara *et al.*, 1997). *Cyclospora* could be detected in the tissue biopsies by histological examination of tissue stained with hematoxylin and eosin (Sun *et al.*, 1996)

Particular attention is drawn to the role of fluorescent microscopy in providing a rapid, inexpensive, and sensitive technique for diagnosis of *Cyclospora* infections in stool samples. They are autofluorescence and can be detected under an epifluorescence microscope, using a 450-490nm dichroic mirror excitation filter (Long *et al.*, 1991; Ortega *et al.*, 1994).

Serological assays to determine human exposure to *Cyclospora* are not yet available. However, attempts to determine a serological immune response were done using immunofluorescence-antibody (IFA) microscopy (Clarke and McIntyre, 1997).

Different molecular techniques have been developed for the identification of *Cyclospora*. There are several PCR assays currently available for detection of *C. cayetanensis* DNA, such as conventional PCR (Lalonde and Gajadhar, 2008; Orozco-Mosqueda *et al.*, 2014), nested amplification (nested-PCR) (Lopez *et al.*, 2001; Yadav *et al.*, 2015) and restriction fragment length polymorphism (RFLP-PCR) (Shields and Olson. 2003). Genes normally used for research of protozoan are the internal transcribed spacer 2 (ITS-2) and the 18S ribosomal (rRNA) (Ortega and Sanchez, 2010). Other techniques highly sensitive and specific for reliable detection of *C. cayetanensis* oocysts are the quantitative real-time PCR (Varma *et al.*, 2003; Giangaspero *et al.*, 2015b) and multiplex PCR (Taniuchi *et al.*, 2011).

Currently, there are no animal models or *in vitro* cultivation methods to determine the infectious potential of *Cyclospora* (Eberhard *et al.*, 2000; Ortega and Sanchez, 2010).

Two alternatives have been proposed to determine the viability of *C. caytanensis* oocysts. The first method is to determine if unsporulated oocysts sporulate followed by excystation (Smith *et al.*, 1997). The second alternative is electrorotation, which has been reported to correlate well with vital dyes and morphological indicators for *Giardia* oocysts (Dalton *et al.*, 2001, 2004). Two parameters have been considered, namely, direction of rotation and rotational velocity. This technique has been proposed to determine oocyst viability and compares favorably to oocyst sporulation detection. However, these techniques either cannot be considered completely reliable or are very expensive and/or complex.

2.3.4.7 Detection methods in vegetables

Pathogens are often present in foods in low numbers, or they may be trapped in pores of the food and are subsequently difficult to detect. Methods for the detection of *C. cayetanesis* in vegetables samples are based on concentration. Concentration methods provide detergent solutions for washing of the vegetable and for recovery of the cysts. Detergents used to isolate *Cyclospora* oocysts are i) saline buffer solutions (PBS) (Dixon *et al.*, 2013; Giangaspero *et al.*, 2015b), ii) a glycine buffer solution (1 M, pH 5.5)

followed by a flotation procedure using Sheather's sucrose solution (sp.g 1.26) (Lalonde and Gajadhar, 2016) and iii) distilled water (Sherchand *et al.*, 1999).

The concentration of the oocysts may be performed by centrifugation (Dixon *et al.*, 2013) or centrifugation followed by flotation (Lalonde and Gajadhar, 2016).

After recovery from vegetables, the *Cyclospora* oocysts can be identified by UV epifluorescence microscopy of the wet mount (Tram *et al.*, 2008; Dixon *et al.*, 2013), or by light microscopy adding a drop of Lugol iodine (Al-Megrin *et al.*, 2010), or staining the wet mount with acid-fast stain (Sherchand *et al.*, 1999).

Recently, different molecular methods have been described for *Cyclospora* i.e., nested-PCR based on 18S rRNA gene (Dixon *et al.*, 2013), qPCR assays based on 18S rDNA gene (Lalonde and Gajadhar, 2011) and *ITS-2* gene (Giangaspero *et al.*, 2015b).

3. EXPERIMENTAL PART

3.1 Materials and methods

3.1.1 Sampling design

The sampling design was tailored to provide the highest confidence of detection and quantification of the contamination level, even with the low expected prevalence reported for protozoa in edible salads.

Since detection of parasites at low prevalence requires large samples sizes, in order to keep the study within manageable bounds, the sampling design was based testing pools of salad sample in common and homogenous groups (Cowling *et al.*, 1999). The number of pools test for a given pool size, under a specified expected prevalence, desired confidence and precision has been estimated according to Worlund and Taylor (1983) through the formula:

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

where

m = number of pools

p= expected prevalence

k=pool size

e= desired precision

Z= desired level of confidence.

Detection threshold was considered as a prevalence of the 0.6% and the sampling regime was set in order to provide a 95% confidence level with a precision of 0.6%. Since we chose a pool size composed of 9 salads packages, the number of pools required to estimate the prevalence was 72 pools.

In order to provide a representative sample, the pools (each composed of 9 packages) came from six different selected branded RTE producers: three major industrial companies (indicated as A, B, and C) and three minor companies (indicated as E, F, and G). The industrial brands have a national distribution with a production in Southern Italy (mainly in Apulia and Campania regions) whereas the local brands have a local distribution and production (Northern Apulia).

Each month, from March 2015 to February 2016, nine individual mixed salad (all containing curly and escarole lettuce, red radish, rocket salad and carrots) packages (not less than 100g each) were bought for each brand and subsequently analyzed together as

a single pool. Following this sampling protocol, a total of 648 salad packages were analyzed and their distribution is summarized in Table 3. All salad packages were placed in a cooler bag and transferred to the laboratory, where they were kept refrigerated and then processed before their expiry date.

Table 3. Experimental design of the sampling protocol

Origin of salads packages	Brand	N° of package/pool	N° of pools/month	N° of pools/year	Number of packages/year
National distribution	A	9	1	12	108
	B	9	1	12	108
	C	9	1	12	108
Subtotal			3	36	324
Local distribution	D	9	1	12	108
	E	9	1	12	108
	F	9	1	12	108
Subtotal			3	36	324
TOTAL			6	72	648

3.1.2 Samples processing

After collection, all salad packages were immediately placed in a cooler bag with ice packs and transferred to the laboratory of the Research Unit of Human Microbiome, Bambino Gesù Children's Hospital, San Paolo, Vatican City, Rome where they were refrigerated and then processed before their expiry date.

Salad samples were processed as described by Dixon *et al.*, (2013) and by Giangaspero *et al.* (2015b), but the methods were slightly modified. For each of the nine packaged RTE mixed salads from the same brand, 100g of vegetable material was weighed and placed in stomacher bags 180x320mm (100g for each bag in nine stomacher bags) (BagPage, Interscience, Sant Nom, France). In each stomacher bag 200 ml of buffered detergent solution (containing phosphate-buffered saline 10X [PBS], 0.1% Tween-80, 0.1% sodium dodecyl sulphate [SDS] and 0.05% antifoam B emulsion were added. Bags were placed on an orbital shaker for 15 min at 120 rpm. Then, lavage liquids were

collected into four 50-ml centrifuge tubes and the tubes were centrifuged at 2,000 x g for 15 min at 4°C (Beckman Coulter, TJ-25, Italy). The supernatant was aspirated and discarded. The pellets were suspended in 3 ml of buffered detergent solution, and pooled into one centrifuge tube. Each tube was rinsed with small volume of buffered detergent solution, which was then poured into the next tube until all tubes were rinsed. This rinse liquid was then added to the pooled tube and vortexed for a few seconds. The pooled tube was centrifuged at 3,000 x g for 15 min at 4°C, and the supernatant was again aspirated and discarded. The pellet was resuspended into 2 ml of buffered detergent solution and divided between two different 1.5 ml microcentrifuge tubes by micropipette. The tube was rinsed with 500µl of buffer and the rinse liquid was poured into two microcentrifuge tubes. Microcentrifuge tubes were centrifuged at 10,000 x g for 10min at 4°C (Centrifuge 5430 eppendorf, Germany), and the supernatants were aspirated and discarded. Finally, 500µl and 300 µl of PBS 1X were added in two microcentrifuge tubes for microscopy (first aliquot) and molecular (second aliquot) investigation, respectively, and the pellets were resuspended by vortexing. The first aliquot was tested for microscopy investigation within 3 days after processing whereas the second aliquot for molecular investigation was stored at -20 °C pending molecular analyses.

3.1.3 Microscopy investigation²

3.1.3.1 Staining procedures

After concentration procedures, Lugol's Iodine staining was used for the detection of *Giardia* cysts and *Toxoplasma* oocysts.

Twenty µl of the sample were transferred on six slides and one drop of Lugol's Iodine solution was added. The slides were covered with coverslip and examined by optical microscopy (NIKON Eclipse E600, Tokyo, Japan) at 20X and 40X magnification.

² All microscopy procedures and identification of the parasites were guaranteed by two experienced operators, as internal control, according to Bambino Gesù Hospital analytical ISO procedures (9100-2015).

Modified Ziehl-Neelsen staining (TB Stain Kit ZN, Becton, Dickinson and Company, New Jersey, USA) was used for the detection and identification of *Cryptosporidium* and *Cyclospora* oocysts.

Twenty µl of the sample were transferred on six slides. The samples were dried and then fixed in absolute methanol. Fixed slides were flooded with ZN carbol fuchsin for 30 minutes and then, washed gently with tap water. Acid alcohol 5% was added on slides for 30 seconds for decolorization and then washed gently with tap water. The slides were flooded with methylene blue for 2 minutes, washed gently with tap water and left to dry. After drying, the slides were examined microscopically (NIKON Eclipse E600, Tokyo, Japan) with a drop of oil under high power (100X oil immersion) lens.

The presence of any protozoans, beside those selected for investigation, were also annotated.

3.1.3.2 Immunofluorescence assay

Detection and identification of *Giardia* cysts and *Cryptosporidium* oocysts was also performed using a commercial kit (Merifluor C/G, Meridian Diagnostics, Cincinnati, Ohio, USA) containing a mixture of FITC labeled monoclonal antibodies directed against cell wall antigens of *Giardia* cysts and *Cryptosporidium* oocysts.

Fifty µl of the sample, of the Positive Control and of the Negative Control were added on the wells. The slide was placed at room temperature to allow the complete drying. Then, a drop of Detection Reagent and one drop of Counterstain were placed in each well and the reagents were mixed with an application stick without scratch the treated surface of the slide. Then, the slide was incubated in a dark humidified chamber for 30 minutes at room temperature. The slide was rinsed with Wash Buffer 1X to remove unbound antibodies. One drop of Mounting Medium was added to each well and a coverslip was applied. The slide was examined by fluorescence microscope (NIKON Eclipse E600, Tokyo, Japan) equipped with a filter system for fluorescein isothiocyanate (FITC) with the following parameters: excitation wavelength 490-500nm, barrier filter 510-530nm and at 40X and 100X magnification.

3.1.4 Molecular investigation

The second aliquot, stored at -20 °C, were placed in cooler bag with dry ice and transferred to the Laboratory of Parasitology (Dipartimento di Scienze Agrarie, degli Alimenti e dell' Ambiente, Foggia, Italy) where they were stored at -20 °C, pending molecular analysis.

3.1.4.1 DNA isolation

DNA was extracted from samples using the QiAmp Plant Mini Kit (Qiagen, Inc., Mississauga, Ontario, Canada).

Briefly, 300 µl of final suspension was divided into three aliquots of 100µl each and the aliquots were subjected to 15 cycles of freezing/thawing of 1 minute each in liquid nitrogen and 65°C water bath, respectively.

For each aliquot, 400µl of Buffer AP1 and 4µl RNaseA stock solution (100 mg/ml) were added according to the manufactures' instructions. The mixtures were mixed for 2-3 time during incubation (10 min at 65°C). Then, 130µl of Buffer P3 was added to the lysates and incubated for 5 min on ice. Then, the lysates were centrifuged for 5 min at 20,000 x g. The lysates were added to the QIAshredder spin column sitting in a 2ml collection tube and centrifuged for 2 min at 20,000 x g. The flow-through fractions were transferred in new tubes and 500 µl of Buffer AW1 were added into each tube.

All the aliquots of the same sample were added into one DNeasy mini spin column sitting in a 2ml collection tube and centrifuged for 1 min at $\geq 6,000 \times g$. The flow-through was discarded and the step was repeated with the remaining liquid. Then, 500 µl Buffer AW2 were added to the DNeasy column and centrifuged at 20,000 x g for 1 minute. This step was repeated with another 500 µl Buffer AW2 and centrifuged for 2 min at 20,000 x g. The DNeasy column was transferred into a 1.5 ml microcentrifuge tube and 100 µl of Buffer AE was added directly onto the DNeasy membrane (incubated for 5 min at room temperature). Then, the tube was centrifuged for 1 min at maximum speed. This step was repeated another time. The extracted DNA was quantified by NanoDrop 2000 and stored at -20 °C.

3.1.4.2 Nested-PCR

A nested and semi-nested PCR protocol were used to amplify a fragment of about 500 bp for *G. duodenalis* *TPI* gene and a fragment of about 400 bp for *Cryptosporidium* *COWP* gene.

Each PCR was conducted in a volume of 20 µl containing 5X Ready Red Taq Mix (Sigma-Aldrich, USA), 100µM of each primer and 3.25 µl of distilled water. Two µl of genomic DNA for first PCR, and 1:20 (*Giardia*) and 1:40 (*Cryptosporidium*) diluted PCR product for the second PCR was added to each reaction. Positive and negative controls were included in each PCR run.

For *G. duodenalis* (all assemblages), the *TPI* gene was amplified using primers AL3543 (forward: 5'-AAATTATGCCTGCTCGTCG-3') and AL3546 (reverse: 5'-CAAACCTTTTCCGCAAACC-3') for the first PCR followed by nested amplification with primers AL3544 (forward: 5'CCCTTCATCGGTGGTAACTT3') and AL3545 (reverse: 5'-GTGGCCACCACTCCCGTGCC-3') (Sulaiman *et al.*, 2003). For the primary amplification, the cycling protocol was 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 45 s (denaturation), 50 °C for 45 s (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C for 10 min, and for the second amplification the cycling protocol was 94°C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

For *Cryptosporidium* spp., the *COWP* gene was amplified using primers CRY15D (forward: 5'-GTA GAT AAT GGA AGR GAY TGT G-3') and CRY9D (reverse: 5'-GGA CKG AAA TRC AGG CAT TATCYT G-3') for the first PCR followed by semi-nested amplification with primers CRYINT2D (forward: 5'-TTT GTT GAA GAR GGA AAT AGA TGT G3') and CRY9D (reverse: 5'-GGA CKG AAA TRC AGG CAT TAT CYT G-3') (Traversa *et al.*, 2008). For both amplification, the cycling protocol was 94 °C for 7 min (initial denaturation), followed by 40 cycles of 95 °C for 50 s (denaturation), 50 °C for 40 s (annealing) and 72 °C for 50 s (extension), with a final extension of 72 °C for 10 min.

The PCR products were run on agarose gel and visualized with GelRed Nucleic Acid staining (Biotium, USA).

3.1.4.3 Real-Time PCR and quantitative analysis (qPCR)

A qPCR and melting curve analysis were performed for *T. gondii* and *C. cayetanensis* in a CFX-96 Real Time Instrument (BioRad, Italy). A sequence of *T. gondii* *B1* gene and *C.*

cayetanensis ITS-2 gene were selected as reference target to design the plasmid control. The pEX-A2 vector (Eurofins, MWG/Operon, Ebersberg, Germany) was used to insert a fragment of approximately 129 bp and 116 bp, respectively. The concentration of the pEX-A2 plasmid was measured using a fluorometer, and the corresponding copy number was calculated using the following equation: $\text{pEX-A2 } T. \text{ gondii}/C. \text{ cayetanensis} \text{ (copy numbers)} = 6.02 \times 10^{23} \text{ (copy/mol)} \times \text{pEX-A2 } T. \text{ gondii}/C. \text{ cayetanensis} \text{ amount (0.31/0.21} \times 10^{-5} \text{ g/ml)}/\text{pEX-A2 } T. \text{ gondii}/C. \text{ cayetanensis} \text{ length (129/116 bp} + 2450) \times 660 \text{ (g/mol/bp)}$ (Whelan *et al.*, 2003).

Ten-fold serial dilutions of the pEX-A2 *T. gondii*/*C. cayetanensis* plasmid (from 1.03×10^7 to 1.03×10^{-3} copies/ μl) were used to determine the quantity of the unknown samples based on linear regression calculations of the standard curve. qPCR was performed in a CFX-96 Real Time Instrument (BioRad, Italy). qPCR was carried out in a final volume of 20 μl , utilizing SsoFast™ EvaGreen® Supermix (cat. no. 172–5201; BioRad, Italy), 0.5 μM of each specific primer for *T. gondii* B1 gene (ToxB41f: 5'-TCGAAGCTGAGATGCTCAAAGTC-3' and ToxB169r: 5'-AATCCACGTCTGGGAAGAACTC-3') (Burg *et al.*, 1989) and for *C. cayetanensis* ITS-2 gene (CCITS2-F: 5'-GCAGTCACAGGAGGCATATATCC-3' and CCITS2-R: 5'-ATGAGAGACCTCACAGCCAAAC-3') (Lalonde and Gajadhar, 2008). Genomic DNA (50 to 100 ng) (or 0.5 pg; reference, positive-control) DNA or water (negative control) in 5 μl were added to the reaction. Cycling conditions were: initial denaturation at 98°C for 2 min, followed by 35 cycles at 98°C for 5 s, and 62°C (*T. gondii*) and 59°C (*C. cayetanensis*) for 15 s. Fluorescence data were collected at the end of each cycle as a single acquisition. Melting curve analysis was performed at the end of each PCR run (70°C to 95°C at 0.5°C/5 s). Each sample was analyzed in duplicate, and the amplification cycle threshold (*Ct*) and melting temperature (*Tm*) values were calculated. The diagnostic *Tm* peak was 80°C for *T. gondii* and 83.5°C for *C. cayetanensis*.

The criteria used to define a test-positive sample were: (a) a detectable amplification curve, (b) a *Tm* value of $\pm 0.5^\circ\text{C}$ with reference to the *Tm* value of positive control, and (c) a dF/dT fluorescence value of >2. Raw data were normalized by applying curve-scaling to a line of best fit, so that the highest fluorescence value was 100 and the lowest was zero (standard normalized melt curve). Then, the curves were differentiated, and a composite median curve was constructed using the median fluorescence values for each sample.

Absolute quantification was performed for the positive samples; the DNA amount (copies/ μ l) was calculated by relating the *Ct* mean value of each sample to a standard curve obtained from the respective positive control. Moreover, oocysts numbers were calculated for *T. gondii* and *C. cayetanensis* according to Lass *et al.* (2012) and Varma *et al.* (2003), respectively.

3.1.4.4 End-Point PCR

End-point PCR protocol was used to detect *Blastocystis* and *Dientamoeba*. PCR was conducted in a volume of 20 μ l containing 5X Ready Red Taq Mix (Sigma-Aldrich, USA), 10 μ M of each specific primer, 3.25 μ l of distilled water and 2 μ l of genomic DNA. Positive and negative controls were included in each PCR run. For *Blastocystis*, the *SSrRNA* gene was amplified using primers RD5 (forward: 5'-ATCTGGTTGATCCTGCCAGT-3') and BhRD (reverse: 5' GAGCTTTTAACTGCAACAACG-3') (Scicluna *et al.*, 2006) and for *Dientamoeba* the *SSrRNA* gene was amplified using primers Df-124F (forward: 5' -CAACGGATGTCTTGGCTCTTTA-3') and Df-221R (reverse: 5' -TGCATTCAAAGATCGAACTTATCAC-3') (Verweij *et al.*, 2007). The cycling protocol was 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 45 s (denaturation), 50 °C for 45 s (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C for 10 min. The PCR products were run on agarose gel and visualized with Gel Red Nucleic Acid staining (Biotium, USA).

3.1.4.5 DNA sequencing

Positive samples to one or more protozoans were purified using EXO I and FAST AP enzymes (Fisher Scientific, Netherlands) according to the manufacturer's protocol. Purified PCR products were sequenced in both directions with the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), using the same primers as the respective PCR reactions according to the manufacturer's instructions. An ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) was used to obtain sequences; electropherograms were inspected by eye and consensus sequences were obtained. Subsequently, sequences were aligned using the ClustalW program (BioEdit software v.7.2.5) and each sequence was compared to the nucleotide sequences available in publicly accessible databases using BLASTn software (<https://www.ncbi.nlm.nih.gov/blast/>).

3.1.5 Statistical analyses

Based on the number of positive pools, the prevalence of salads contaminated with each protozoan species was estimated according to Schaarschmidt (2007), and the 95% confidence levels using the exact method of Clopper-Pearson. This approach provides robust estimation of confidence level, without need to retest individual samples from positive pools, so that analyses were kept within manageable limits. Since the salad samples were analyzed through multiple testing, i.e. both microscopic and molecular approaches, we estimated a combined prevalence as testing in parallel; that is a sample was considered positive if it reacted positively to either or both of the diagnostic tests. Moreover, although the risk related to the brands and seasons was not the purpose of this study (the sample size was not tailored to provide such data), for each protozoan species the difference in prevalence between brands and sampling seasons was tested with a group regression model (Vansteelandt *et al.*, 2000), considering the contamination status of the pool (positive/negative) as response variable and pool identity as a/the grouping factor. Statistical analysis was performed using the 'binGroup' package for evaluation of binomial group testing, developed under the software R 3.3.2 (R Development Core Team, 2016) and considering $p < 0.05$ as the threshold for statistical significance.

To assess the performance of the microscopy and molecular tools used to detect the four investigated pathogens (*G. duodenalis*, *Cryptosporidium* spp., *T. gondii* and *C. cayetanensis*), the Cohen's kappa coefficient and proportions of positive and negative agreement were calculated to measure the performance between two tests (Test 1 "microscopy approach" and Test 2 "molecular approach") (Gardner *et al.*, 2000). Since all pool samples were analyzed through both microscopy and molecular approaches to detect the pathogens, in order to have a more robust result, each protozoan species identified by microscopy and/or molecular assay was considered as single testing for a total of 288 tests (72 pools x 4 investigated pathogens). More specifically a pool sample was considered positive for both tests (Test 1+/Test 2 +) for any protozoa (e.g., *Giardia*) if it reacted positively to both of the diagnostic tests, a pool sample was considered negative for both tests (Test 1-/Test 2 -) for any pathogen (e.g., *Giardia*) if it reacted negatively to both of the diagnostic tests, a pool sample was considered positive for a test and negative for another test for any pathogen (e.g., *Giardia*) if it reacted positively or negatively to one of the diagnostic tests (Test 1+/Test 2 - or Test 1-/Test 2 +).

Statistical analysis was performed using the 'EpiTools epidemiological calculators' package for the comparison of two tests, developed under the Ausvet company (Sergeant, ESG, 2017), considering the 95% confidence level and $p < 0.05$ as the threshold for statistical significance.

4. RESULTS

4.1 Microscopy investigation

A total of 864 slides were examined by one or more microscopic techniques. By modified Ziehl-Neelsen staining, *Cryptosporidium* spp. oocysts were observed in 4 pools (**Figure 12a-d**), 2 pools were from Industrial Brands and 2 from Local Brands. By immunofluorescence assay, *Cryptosporidium* oocysts were identified in one of the four pools positive (**Figure 13**).

During the microscopy investigation, additional intestinal protozoan parasites were detected: *Blastocystis hominis* and *Dientamoeba fragilis*. Out of 72 pools, *B. hominis* was observed in 3 pools from Local Brands; the presence of *Blastocystis* was observed in 2 pools by Lugol's Iodine staining (**Figure 14a, b**) and in one pool by modified Ziehl-Neelsen staining (**Figure 15**). *Dientamoeba fragilis* was observed in one pool (Local Brand) through Lugol's Iodine and then, Giemsa staining (**Figure 16a, b**) (**Raw data: Annex 1; Table 4 and 5**).

Figure 12 (a-d). Samples number 31, 48, 54 and 70 stained with modified Ziehl-Neelsen showing *Cryptosporidium* spp. oocysts in red on the blue background and with black granules visible (Magnification 100X).

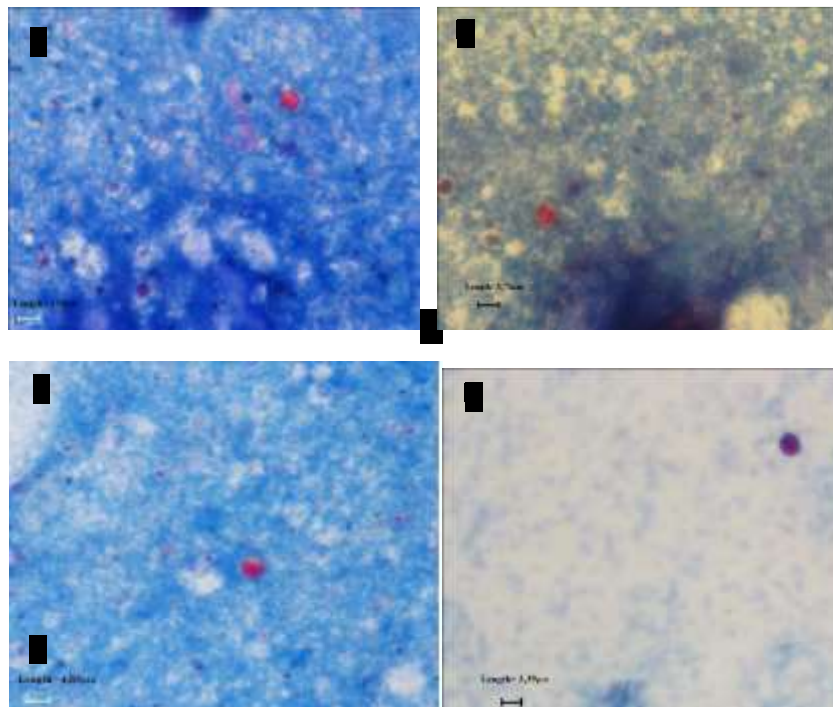


Figure 13. Sample number 31 stained with FITC showing *Cryptosporidium* oocyst detected by immunofluorescence assay (Magnification 40X).

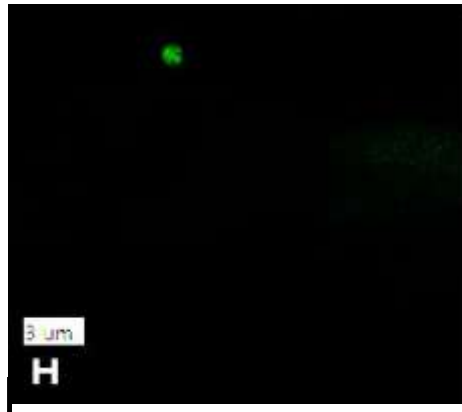


Figure 14 (a, b). Samples number 70 and 71 stained with Lugol's Iodine showing two vacuolar forms of *Blastocystis hominis*. A large central body (like a large vacuole) and in the ring of cytoplasm with black inclusions bodies are visible (Magnification 40X).

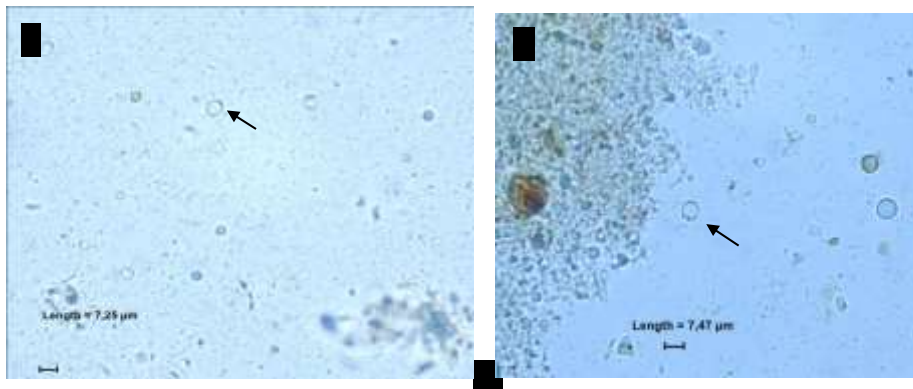


Figure 15. Sample number 19 stained with modified Ziehl-Neelsen showing the vacuolar form of *Blastocystis hominis*. (Magnification 100X).

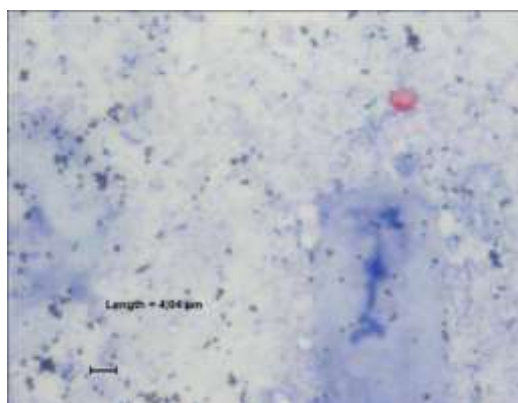
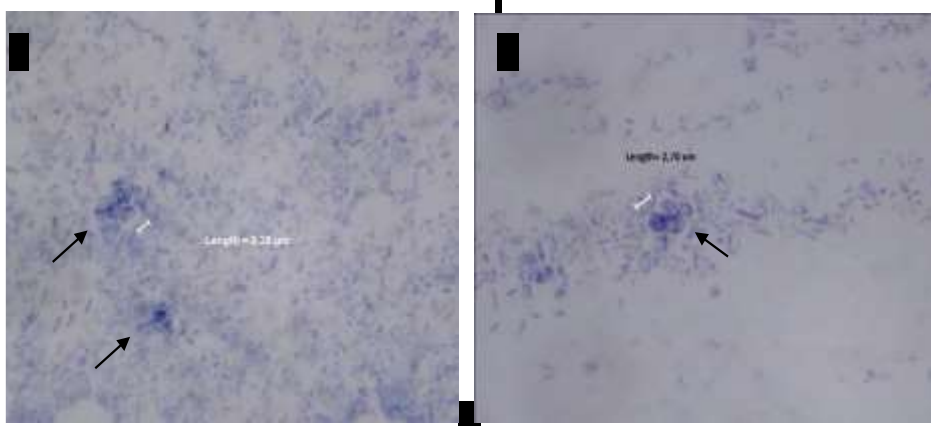


Figure 16 (a, b). Sample number 50 stained with Giemsa showing *Dientamoeba fragilis*. Small binucleate trophozoites (Magnification 100X).



4.2 Molecular investigation

Out of 72 pools, *G. duodenalis* was detected in 4 pools: 3 pools were from Industrial Brands and one from Local Brand (**Figure 17**). *Cryptosporidium* spp. was detected in 5 pools, of which 2 pools were from Industrial Brands and 3 from Local Brands; out of 5 pools, 3 were also positive to microscopy (Figure not provided because of the low image quality). *T. gondii* was detected in 5 pools: 3 pools were from Industrial Brands and 2 from Local Brands (**Figure 18**). *C. cayetanensis* was detected in 8 pools of which 6 pools were from Industrial Brands and 2 from Local Brands (**Figure 19**).

The microscopically positive samples to *B. hominis* and *D. fragilis* – which were outside of the aim of the prevalence study - were molecularly confirmed (Figure not provided because of the low image quality).

Figure 17. Nested PCR (primer pair AL3544 - AL3545) of *Giardia duodenalis* TPI gene (~530 bp): Line 1: size marker; lines 2, 3 and 4: positive controls; lines 5 and 6: negative controls; lines 7 to 17: negative samples; line 18: positive sample (n. 53); lines 19 to 28: negative samples; line 29: size marker.

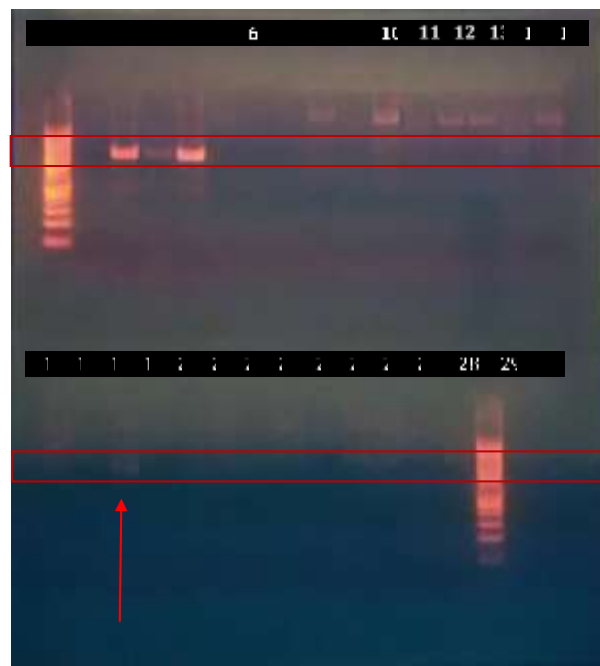


Figure 18. Melting curve peaks and T_m of the positive samples (blue lines), negative control (light green) and positive control (red line) to *Toxoplasma gondii* detected by Real Time PCR.

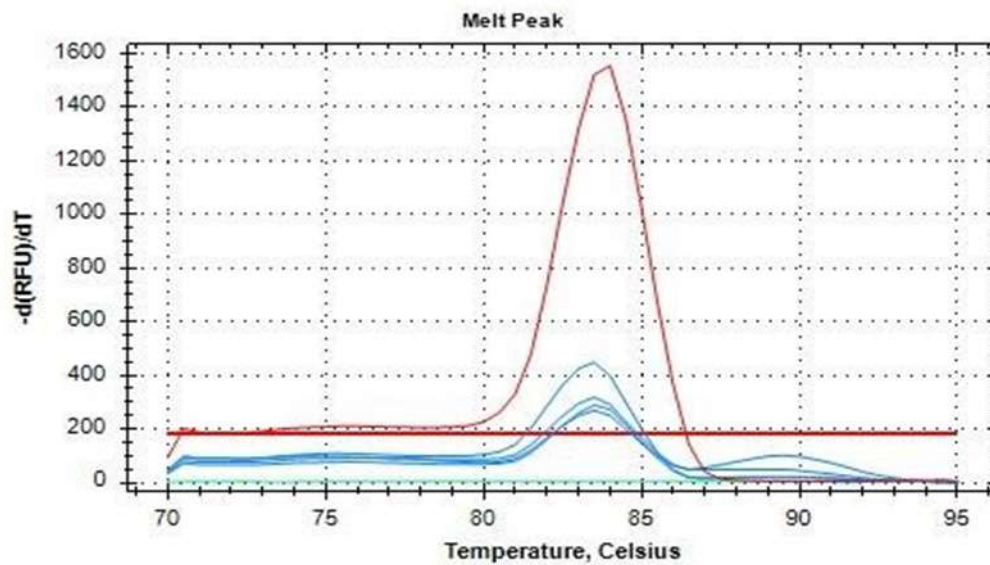
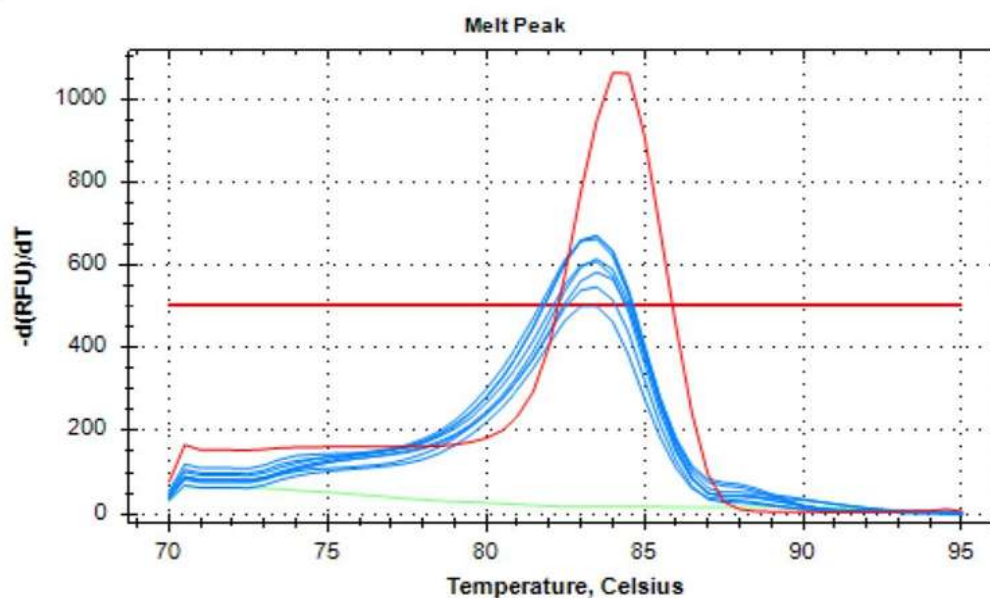


Figure 19. Melting curve peaks and T_m of the positive samples (blue lines), negative control (light green) and positive control (red line) to *Cyclospora cayentanensis* detected by Real Time PCR.



4.2.1 DNA quantification

The number of *T. gondii* and *C. cayetanensis* oocysts in qPCR test-positive samples were predicted to range from 62 to 554 and 46 to 1580 per g of vegetable product, respectively (**Table 6**).

Table 6. Number of oocysts/sample of *Toxoplasma gondii* Type I and *Cyclospora cayetanensis* in thirteen batches of ready-to-eat mixed salads collected from industrial and local Brand Companies from Italy and examined by qPCR.

Number	Sample ID	Protozoan parasites	Oocyst number/g of product
1	6	<i>T. gondii</i>	320
2	8	<i>C. cayetanensis</i>	1.240
3	11	<i>C. cayetanensis</i>	1.580
4	12	<i>C. cayetanensis</i>	833
5	12	<i>T. gondii</i>	62
6	26	<i>T. gondii</i>	65
7	28	<i>T. gondii</i>	221
8	36	<i>T. gondii</i>	554
9	40	<i>C. cayetanensis</i>	1190
10	41	<i>C. cayetanensis</i>	509
11	42	<i>C. cayetanensis</i>	667
12	57	<i>C. cayetanensis</i>	684
13	60	<i>C. cayetanensis</i>	46

4.2.2 Sequencing

Sequencing confirmed a homology of the 98% for *G. duodenalis* Assemblage A, for *C. cayetanensis* and for *B. hominis*. A homology of 99% was found for *C. ubiquitum*, for *T. gondii* Type I and for *D. fragilis*; finally, a homology of 100% for *C. parvum*.

The sequences were deposited in GenBank under accession number **KY554829** - **KY554832** (*G. duodenalis*), **KY554819** - **KY554823** (*C. parvum* - *C. ubiquitum*), **KY554824** - **KY554828** (*T. gondii*), **KY554833** - **KY554840** (*C. cayetanensis*), **KY554841** - **KY554843** (*B. hominis*) and **KY554844** (*D. fragilis*).

4.3 Prevalence data

By both microscopy and molecular tools, 4.2% (95% C.I. 2.6-6.2%) of the samples were contaminated by at least one protozoa species, and 0.6% (95% C.I. 0.2-1.6%) of them presented contamination by *G. duodenalis* and *Cryptosporidium* spp. (Industrial Brand), by *C. cayetanensis* and *T. gondii* (Industrial Brand), by *G. duodenalis* and *C. cayetanensis* (Industrial Brands) and by *C. parvum* and *B. hominis* (Local Brand) (**Annex 1**).

The most detected protozoa species was *C. cayetanensis* (1.3%, 95% C.I. 0.6-2.5%), followed by *Cryptosporidium* spp. (0.9%, 95% C.I. 0.4-2.1), *T. gondii* (0.8%, 95% C.I. 0.3-1.8%), *G. duodenalis* (0.6%, 95% C.I. 0.2-1.6%) (**Table 4**).

However, also *B. hominis* (0.5%, 95% C.I. 0.1-1.4%) and *D. fragilis* (0.2%, 95% C.I. 0.0-0.9%) were detected by microscopy and molecularly confirmed (**Table 5**).

Table 4. Prevalence values of the investigated protozoan parasites in ready-to-eat mixed salads collected from industrial and local brand Companies at retail on Italian market and examined by microscopy and molecular assays.

Pathogens	Diagnostic test results (N° positive pools)		Prevalence % (95% CI*)		Combined values	
	Microscopy	Molecular assay	Microscopy	Molecular assay	N° Positive pools	Prevalence % (95% CI*)
<i>Giardia duodenalis</i> Assemblage A	0	4	0 (0-0.57)	0.63 (0.17-1.61)	4	0.63 (0.17-1.61)
<i>Cryptosporidium parvum</i> / <i>Cryptosporidium ubiquitum</i>	4	5	0.63 (0.17-1.61)	0.80 (0.26-1.85)	6	0.96 (0.35-2.08)
<i>Toxoplasma gondii</i> Type I	0	5	0 (0-0.57)	0.80 (0.26-1.85)	5	0.80 (0.26-1.85)
<i>Cyclospora cayetanensis</i>	0	8	0 (0-0.567)	1.30 (0.559-2.547)	8	1.30 (0.559-2.547)

* 95% Confidence Interval

Table 5. Additional protozoan parasites detected in ready-to-eat mixed salads collected from industrial and local brand Companies at retail on Italian market and examined by microscopy and molecular assays.

Pathogens	Diagnostic test results (N° positive pools)		Percentage % (95% CI*)	
	Microscopy	Molecular assay	Microscopy	Molecular assay
<i>Blastocystis hominis</i>	3	3	0.47 (0.10-1.37)	0.47 (0.10-1.37)
<i>Dientamoeba fragilis</i>	1	1	0.15 (0-0.86)	0.15 (0-0.86)

* 95% Confidence Interval

The comparison test between microscopy and molecular assay showed that the degree of agreement between these two techniques was low ($k=0.2123$). Since the positive agreement was 0.24 and molecular analyses showed an observed prevalence of 0.88% compared to that of 0.63% obtained by microscopy, we can infer the higher detection capacity of the former tool. On the other hand, negative agreement of 0.96 indicate comparable levels of specificity of these approaches.

Prevalence between brands

Prevalence between brands varied between the minimum value of zero for all and the highest values of 3.1% (95% C.I. 0.6-9.0%) for *Cryptosporidium* spp., 2.0% (95% C.I. 0.2-7.1%) for *G. duodenalis*, *T. gondii*, *C. cayetanensis* and *B. hominis*, and 1.0% (95% C.I. 0.0-5.3%) for *D. fragilis* (**Figure 20**). The variability observed in prevalence between salad Brand producers and distribution of protozoan parasites showed a lower prevalence (3.1%, 95% C. I. 0.6-8.9%) in the Local Brands E and F compared to Local Brand D (5.8%, 95% C.I. 1.8-13.3%) and to National Brands A, B and C (4.4%, 95% C.I. 1.1-11.0%) (**Table 6**).

Figure 20. Protozoan prevalence ($\pm 95\%$ confidence intervals) in ‘ready to eat’ mixed salads according to the six producer companies (National and Local brands)

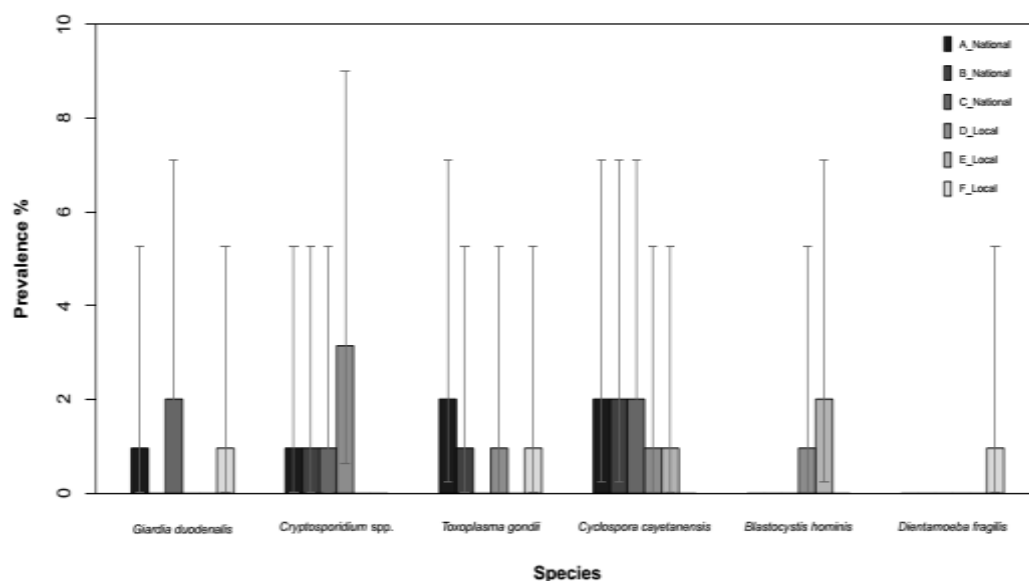


Table 6. Protozoa prevalence (and their 95 % confidence intervals in brackets) in the 6 examined brands

Pathogens	Brands					
	A	B	C	D	E	F
<i>Giardia duodenalis</i> Assemblage A	0.96% (0.02-5.25)	0 (0-3.35)	2.00% (0.23-7.09)	0 (0-3.35)	0 (0-3.35)	0.96% (0.02-5.25)
<i>Cryptosporidium parvum</i> / <i>Cryptosporidium ubiquitum</i>	0.96% (0.02-5.25)	0.96% (0.02-5.25)	0.96% (0.02-5.25)	3.15% (0.62-8.99)	0 (0-3.35)	0 (0-3.35)
<i>Toxoplasma gondii</i> Type I	2.00% (0.23-7.09)	0.96% (0.02-5.25)	0 (0-3.35)	0.96% (0.02-5.25)	0 (0-3.35)	0.96% (0.02-5.25)
<i>Cyclospora cayetanensis</i>	2.00% (0.23-7.09)	2.00% (0.23-7.09)	2.00% (0.23-7.09)	0.96% (0.02-5.25)	0.96% (0.02-5.25)	0 (0-3.35)
<i>Blastocystis hominis</i>	0 (0-3.35)	0 (0-3.35)	0 (0-3.35)	0.96% (0.02-5.25)	2.00% (0.23-7.09)	0 (0-3.35)
<i>Dientamoeba fragilis</i>	0 (0-3.35)	0 (0-3.35)	0 (0-3.35)	0 (0-3.35)	0 (0-3.35)	0.96% (0.02-5.25)
Total	4.40% (1.11-11.04)	4.40% (1.11-11.04)	4.40% (1.11-11.04)	5.81% (1.81-13.3)	3.15% (0.62-8.99)	3.15% (0.62-8.99)

Seasonal prevalence

The distribution of the protozoans investigated in RTE salads from the National Brands (A, B and C) and Local Brands (D, E and F) during a one year, according to season, showed the highest prevalence (11.5%, 95% C.I. 1.1-41.2%) in the National brands A and C during spring and autumn, and Local brands E and F during autumn and winter. The wider distribution of parasites in the two types of brands, was observed in autumn (7.4%, 95% C.I. 3.3-13.9%) (**Table 7**).

The seasonal variation showed the highest prevalence (2.0%, 95% C.I. 0.4-5.8%) in summer for *T. gondii* and in autumn for *G duodenalis* and *Cryptosporidium* spp.

B. hominis and *D. fragilis* showed prevalence values of 1.3% (95% C.I. 0.2-4.6%) in winter and 0.6% (95% C.I. 0.0-3.5%) in autumn, respectively.

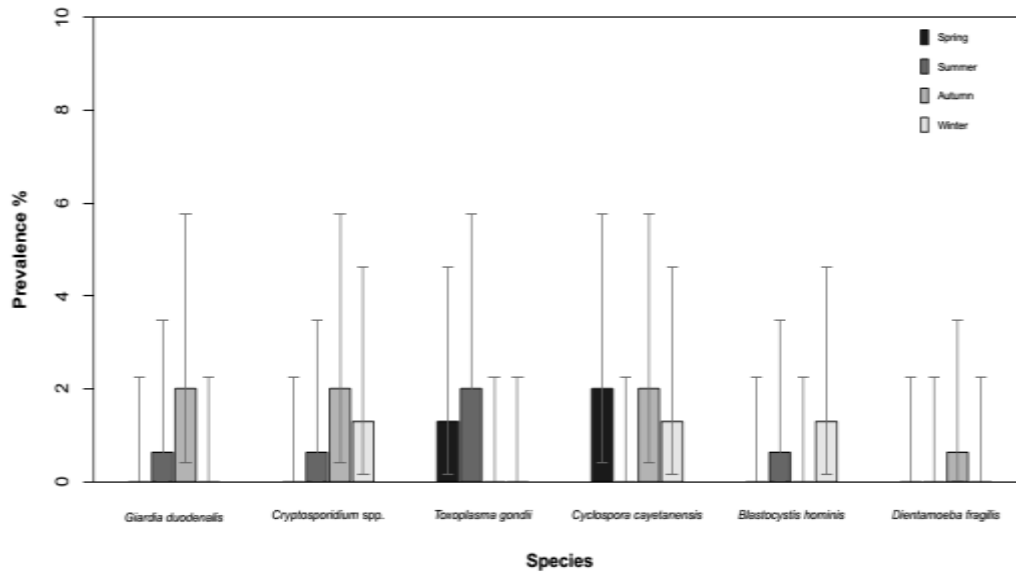
C. cayetanensis showed highest values of 2.0% (95% C.I. 0.4-5.8%) in spring and autumn (**Figure 21**).

Table 7. Distribution of protozoan parasites to microscopy and/or molecular assays in ready to eat mixed salads from three industrial Brands (A, B and C) and three Local Brands (D, E and F) during a one year, according to season (no. of positive samples/no. of examined samples, prevalence, and 95% confidence interval) (p<0.05)

Companies	Season			
	Spring	Summer	Autumn	Winter
A	11.49% (1.09-41.20)	4.40% (0.093-23.08)	0 (0.-12.77)	4.40% (0.09-23.08)
B	0 (0.-12.77)	4.40% (0.093-23.08)	11.49% (1.09-41.2)	4.40% (0.09-23.08)
C	4.40% (0.09-23.08)	0 (0.-12.77)	4.40% (0.09-23.08)	0 (0.-12.77)
D	4.40% (0.09-23.08)	4.40% (0.09-23.08)	4.40% (0.09-23.08)	11.49% (1.094-41.2)
E	0 (0.-12.77)	4.40% (0.09-23.08)	4.40% (0.09-23.08)	4.40% (0.09-23.08)
F	0 (0.-12.77)	4.40% (0.09-23.08)	11.49% (1.09-41.2)	0 (0.-12.77)
TOTAL	2.75% (0.733-6.936)	3.55% (1.127-8.152)	7.41% (3.293-13.89)	3.55% (1.127-8.152)

* 95% Confidence Interval

Figure 21. Seasonal protozoan prevalence ($\pm 95\%$, confidence intervals) in 'ready to eat' mixed salads



However, prevalence variabilities between the two kinds of brands considered in this study (industrial with national distribution and local with regional distribution) and seasons were not statistically significant for each investigated protozoan ($p > 0.05$).

5. DISCUSSION

This is the first large-scale study on detection and prevalence of protozoan parasites in ready-to-eat salads in Europe. RTE salads sold in Italy are contaminated by one or more protozoan pathogens. By microscopy and/or molecular tools, *G. duodenalis* Assemblage A, *C. parvum* and *C. ubiquitum*, *T. gondii* Type I, *C. cayetanensis* were detected with a prevalence ranging from 0.6% to 1.3%, with high oocysts burden (calculated only for *T. gondii* and *C. cayetanensis*) of up to 554 and 1580 per g of vegetable product, respectively (**Table 4**). Moreover, *B. hominis* and *D. fragilis* were also detected (0.5 and 0.2%, respectively) (**Table 5**).

Protozoan parasites have been documented in fresh produce worldwide. In unpackaged marketed fresh vegetables, *Giardia* and/or *Cryptosporidium* (with a prevalence up to 31.6% and 24%, respectively) and/or *Toxoplasma* (up to 6.6%) and/or *Cyclospora* (up to 21.3%), have been recorded mostly from remote areas of developing Asian, African and South American Countries (reviewed by Dixon, 2015; Marchioro *et al.*, 2016; Mohamed *et al.*, 2016; Tiyo *et al.*, 2016).

However, similar percentage of contamination or even higher have been found in several species of fresh marketed unpackaged produce also in European Countries, such as in Norway, where both protozoan parasites were found with a percentage up to 2.1% for *Giardia* and 4% for *Cryptosporidium*) (Robertson and Gjerde, 2001), Spain (up to 52,6% for *Giardia* and 63,1% for *Cryptosporidium*) (Amoros *et al.*, 2010), Poland (3.6% of *Cryptosporidium*) (Rzezutka *et al.*, 2010) and up to 19.5% for *Toxoplasma* (Lass *et al.*, 2012). As to Italy, after a very preliminary research in '60s (Mastrandrea and Micarelli, 1968) in which the presence of *Giardia* cysts were registered in marketed lettuce, later, with a wider approach, *Cryptosporidium* oocysts (9.1%) (Lonigro *et al.*, 2006) and, for the first time in Europe, *Cyclospora* DNA (up to 12.2%) was detected (Giangaspero *et al.*, 2015b) in harvested vegetables (i.e., lettuce, fennel, tomato, cucumber and celery) irrigated with contaminated water.

As to RTE salads, a very first record comes just from Italy, by Di Benedetto *et al.* (2007), who by microscopy detected *Giardia* in one out of 40 samples of RTE mixed salads (leafy vegetables and carrots). Wider studies investigating (by both microscopy and molecular

tools) parasite contamination in these products are indeed very recent and recorded only in Canada (Dixon *et al.*, 2013; Lalonde and Gajadhar, 2016).

In the present study, the contamination by *G. duodenalis* (0.6%) and *Cryptosporidium* spp. (0.9%) is lower than the percentage in packaged or pre-packaged/bulk leafy green recorded in both Canadian studies (Dixon *et al.*, 2013 and Lalonde and Gajadhar, 2016), and, more specifically, for *C. parvum*, and *G. duodenalis* Assemblage A (Dixon *et al.*, 2013). As to *Cyclospora*, the prevalence registered in this study (1.3%) appears similar to the one registered by Dixon *et al.* (2013) and higher to the one registered by Lalonde and Gajadhar (2016). Concerning *Toxoplasma*, the prevalence reported in this study (0.8%) appears higher to the one registered by Lalonde and Gajadhar (2016).

Foodborne outbreaks associated with consumption of fresh produce contaminated with protozoan parasites have been amply documented worldwide (see for details Chapter 2.3). As to Europe, the most recent outbreaks due to *C. parvum* were across England and Scotland (McKerr *et al.*, 2015) and in Finland (Aberg *et al.*, 2015), involving 182 and 250 persons who ate fresh-cut salads leaves and frisee salads, respectively. A cyclosporiasis outbreak was registered in Germany, involving 34 people who in a restaurant ate contaminated butterhead lettuce and mixed lettuce and other vegetables (Doller *et al.*, 2002), and one in Sweden involving 18 persons who ate imported sugar snap peas from Guatemala (Insulander *et al.*, 2010).

The presence of protozoan oo/cysts in the investigated ready-to-eat samples is indicative of extensive contamination by feces of human and/or animal origins also in our country. In fact, the zoonotic assemblage/species *G. duodenalis* Assemblage A and/or *C. parvum* have been documented in humans (Putignani and Menichella, 2010; Masucci *et al.*, 2011), domestic and wild animals (Giangaspero *et al.*, 2007; Paoletti *et al.*, 2011; Papini *et al.*, 2012; De Liberato *et al.*, 2015), and also in wastewater and shellfish (Giangaspero *et al.*, 2009; 2014). *Cyclospora* oocysts (Masucci *et al.*, 2011) or *Cyclospora* DNA have been recorded not only in humans (Giangaspero *et al.*, 2015b), but also in non-human primates (Marangi *et al.*, 2015a), as well as in irrigation water (Giangaspero *et al.*, 2015b), and even in tap water (Giangaspero *et al.*, 2015a). As to *T. gondii*, it has been shown that cats shed oocysts widely across Italy (Mancianti *et al.*, 2015); these then reach the sea and contaminate the shellfish (Putignani *et al.*, 2011).

The detection of two additional species of protozoans i.e. *B. hominis* and *D. fragilis* is the first record on fresh produce in Europe and in the world, respectively.

The presence of these protozoans - which are responsible for several gastrointestinal symptoms in humans and both recognized as responsible or co-responsible for Irritable Bowel Syndrome (Yakoob *et al.*, 2010; Garcia, 2016) - cannot be considered as completely unexpected. As to *B. hominis*, our percentage (0.5%) is sensitively lower to the sole data available in the world, i.e. in raw vegetable purchased in a daily market in Saudi Arabia (Al-Binali *et al.*, 2006; Al-Megrin, 2010).

B. hominis and *D. fragilis* have been frequently detected in humans worldwide (reviewed by Wawrzyniak *et al.*, 2013; Garcia, 2016), including Italy (Lacasella *et al.*, 2013; Manganelli *et al.*, 2012), and, in this country, *D. fragilis* (Crotti *et al.*, 2007; Cacciò *et al.*, 2012,) and *B. hominis* (Zanzani *et al.*, 2016), have been also detected in animals, supporting their zoonotic role.

The results of the present research confirm that protozoan parasites circulate widely in Italy. Contamination of RTE salads is just the tip of the iceberg, indicating that the food chain can be a very sensitive “hub”.

Besides *G. duodenalis* and *C. parvum* - whose responsibility is often underestimated due to the relatively long period of incubation time - particular attention should be given to *Cyclospora* and *Toxoplasma*. Although the U.S. Public Health Service classifies *C. cayetanensis* as a foodborne pathogenic microorganism associated with the consumption of fresh fruits and vegetables, due to the number of outbreaks registered oversea, its pathogenic role is underestimated in Europe, including Italy. As said above, although autochthonous cases of this disease have been recorded in Italy (Scaglia *et al.*, 1994; Maggi *et al.*, 1995; Masucci *et al.*, 2008, 2011), *Cyclospora* is not routinely investigated in gastrointestinal disorders and its DNA detection in infected people was found to be higher than previously believed (Giangaspero *et al.*, 2015b). Considering that the infectious dose for *Cyclospora* is unknown but is presumed to be low (Sterling and Ortega, 1999), its prevalence (1.3%) and oocyst burden (up to 1.580 per gram of vegetable product) detected in this study in RTE salads, may explain the potential for transmission to consumers. The report of an outbreak in Germany involving 34 people, associated with contaminated butterhead lettuce (imported from France) and mixed

lettuce and other vegetables (imported from Southern Italy, including the area in which the present study was carried out) (Doller *et al.*, 2002), further highlights the risk.

Consumption of raw fruits and vegetables constitutes a risk of cyclosporiasis in Europe and the same should be underlined for toxoplasmosis. In fact, the prevalence of *T. gondii* found here in RTE salads (0.8%) and the known low infectious dose (Dubey *et al.*, 1996; Fayer *et al.*, 2004), suggests that the dynamics of toxoplasmosis for humans could be different from previous assumptions. *T. gondii* has recently been listed as the second most harmful foodborne pathogen (Scallan *et al.*, 2015), and is responsible for the highest disease burden of all foodborne pathogens (Wells *et al.*, 2015). The source of infection for *Toxoplasma* in humans has always been attributed mainly to the consumption of pork and goat meat. The results obtained in this study highly support the hypothesis that there is a greater risk of human infections associated with a vegetarian diet (Kapperud *et al.*, 1996; Hall *et al.*, 1999), and that the oocyst burden registered in this study (up to 554 oocysts per gram of vegetable product) can contribute greatly to transmission of this protist. The identification of Type I confirms that this lineage (which is the most pathogenic) is present in Europe, including Italy, where it circulates in cats (Mancianti *et al.*, 2015) and pigs (Bacci *et al.*, 2015).

In this study, the identification of *C. ubiquitum* in RTE salads is also interesting. Severe cryptosporidiosis due to this species have been registered in the UK (Elwin *et al.*, 2012), and in Spain, including in an immunocompetent child (Cieloszyk *et al.*, 2012). *C. ubiquitum* is considered an emerging zoonotic species (Li *et al.*, 2014; Zahedi *et al.*, 2016), with sheep and wild rodents suspected to be the key source of *C. ubiquitum* transmission to humans (Li *et al.*, 2014). The detection of this species of *Cryptosporidium* requires further studies on its diffusion in Europe and the related risks.

Although a seasonal variation in prevalence for each parasite emerged in the raw data, the lack of statistical differences in seasonality between the protozoans could be related to the long resistance of the oo/cysts of these parasites in the environment, and to the areas where the vegetables were grown. They are cultivated in Southern Italy (mainly in Apulia and Campania), and intensively irrigated throughout the year due to the low rainfall and high temperatures in these areas.

This aspect, coupled to agricultural practices (such as the use of manure fertilizer) and “on-plain-air” crop management might explain the continuous contamination, probably

due to the entry of the wild animals in the fields, which defecate and contaminate the area; thus, the prevalence recorded suggests that there is a risk of contamination throughout the year.

Although the observed variability in prevalence between six examined brands and investigated protozoans has shown a higher prevalence for *G. duodenalis* (2%) in a National Brand (i.e., C), *C. parvum/ubiquitum* (3.1%) in a Local Brand (i.e., D), *T. gondii* (2%) in a National Brand (i.e., A), *C. cayetanensis* (2%) in a National Brand (i.e., A, B and C), *B. hominis* (2%) in a Local Brand (i.e., E) and *D. fragilis* (0.9%) in a Local Brand (i.e., F), no statistically significant differences have been noted.

Such lack of statistical differences between the two kinds of companies considered in this study (industrial vs. local brands) means that despite the economic (differences in price on the shelves) and target differences (supermarkets and local grocery shops) between the two kinds of brands, their management, technology and processing plant protocols used for the production of ready to eat salads - although we do not know the actual management and organization of each company - could overlap. At the light of obtained results, we can assume that the marketed products by different companies could be similar in terms of quality and safety.

Isolation and detection of the protozoans in fresh produce is very challenging (Dixon *et al.*, 2013; Giangaspero *et al.*, 2015b; Lass *et al.*, 2012), particularly for detection of multiple species of protozoan oo/cysts.

In this study, microscopy coupled to molecular tools have been used in order to improve the chance to detect pathogens. As to microscopy, more than one techniques were used according to the capability of each technique to detect a given pathogen. In fact, the identification of more protozoans requires different temporary and/or permanent staining techniques (see Chapter 2.3) and Lugol for *Giardia*, Ziehl-Neelsen staining for *Cryptosporidium* are considered the gold standard for those pathogens whereas immunofluorescence assays, which allow simultaneous detection of both pathogens, were used to furtherly improving the chance of their detection at microscopy. As to *C. cayetanensis* and *T. gondii*, their detection methods are both not standardized or challenging (see Chapter 2.3); thus, the acid-fast staining and light microscopy assays, used for the previous pathogens, were considered at least advantageous for investigating their presence. Compared to microscopy, which is time-consuming and

requires skilled operators, molecular techniques have the advantages to be more sensitive, specific and rapid and allow the simultaneous identification of protozoa DNA together with genetic characterization of the isolates.

Several molecular protocols have been described for the isolation and detection of protozoan oo/cysts from fresh produce (described in Chapter 2.3); 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 most efficient molecular protocols according to our experience and the combination of both microscopy and molecular assays (higher performance capacity to detect pathogens of the molecular tools has been confirmed also in this study) allowed us to reduce the risk of errors and underestimation, providing a wide overview of the presence of protozoan pathogens.

A limitation of this study is the impossibility of distinguishing between viable and dead parasites, in order to assess the public health risk. However, due to the size of sampling, the lack of univocal techniques for evaluating the viability of all investigated protozoans, the procedures not yet fully validated for all pathogens, the study of the viability was extremely complex and challenging (Jenkins *et al.*, 1997; Slifko *et al.*, 2000; Ortega and Sanchez, 2010).

Considering that a long-lived resistance of *Giardia*, *Cryptosporidium*, *Toxoplasma* and *Cyclospora* on vegetables both to the temperature (4°C for 8 days) (even longer than RTE salads recommended shelf-life) and to the chlorine concentrations used for RTE disinfection (80-100mg/L) have recently been demonstrated (Sathyanarayanan and Ortega, 2006; Hohweyer *et al.*, 2016), and that the coexistence of both viable and non-viable organisms has been ascertained (Dixon *et al.*, 2013), any finding of protozoan oocysts in produce should be considered an indication of risk to public health.

The presence of protozoa in these products indirectly indicates that the disinfection treatments along the food chain, for all investigated brands (both industrial and local), are not effective in the removal of these protists. As expected, in the production processes, a correct disinfection is able to remove or reduce only the bacterial load (Chapter 2.1/Subchapter 2.1.1). In fact, chlorine as sodium hypochlorite is the most widely used disinfectant because of its limited cost and easy-to-use; however, its effectiveness on protozoa is low because the common concentration used (80-100mg/L)

is too low and thus unable to remove these protozoa. The chlorine dioxide is more effective compared to sodium hypochlorite for protozoan inactivation, but it is 5-10 times more expensive and dangerous for the operators compared to sodium hypochlorite. The ozone has a high disinfecting capacity compared to chlorination, but due to its high instability and dangerousness for the health of operators, it is a little-used solution in the industry (Turatti, 2011). Disinfection systems with ultraviolet (UV) can be considered a good alternative disinfection methods, because i) it is a physical process that does not rely on chemical additives; ii) it does not cause changes in the taste or smell of the disinfected water; iii) requires relatively short contact times; iv) it has a low environmental impact: the system does not imply the formation of toxic or harmful byproducts and v) it has low initial operation costs. UV light is highly effective in the inactivation of *G. duodenalis* cysts and *C. parvum* oocysts (Erickson and Ortega, 2006) at doses typically used in water and wastewater treatment practices (30 to 40 mJ/cm²). However, it requires a mechanical filtration of water in order to maintain the transparent fluid without particles in suspension. Despite the advantages, the UV disinfection system is not widely used for disinfection along the food chain by IV game producers. Beside UV technology, the most recent membrane filtration - a technology coming from the treatment plants of municipal and industrial wastewater, and drinking water treatment processes - could be considered another reliable alternative to the removal of oo/cysts thanks to the pore sizes of filters for microfiltration and ultrafiltration used in water treatment processes which range from 0.002 to 0.5 µm (Betancourt and Rose, 2004; Lonigro *et al.*, 2006; Ottoson *et al.*, 2006) while the size of the parasites range from 4 to 15 µm.

In this study, we were unable to know:

i) the origin of the contamination. Any point between the factory and consumer, i.e., during pre-harvesting process *via* fecal contamination of soil (due to the entrance in the field of domestic and/or wild animals or pests, or the improper use of manure-based fertilizer, contaminated irrigation water); during harvesting (dirty equipment, food handlers with poor personal hygiene); during processing practices along the chain (cutting and shredding with dirty equipment, washing with contaminated water, incorrect hygiene practices by workers) could have been the key for risk;

ii) which kind/s of vegetable/s was/were contaminated. Mixed salads were investigated in this study, thus, the greater the number of different types of vegetables the higher becomes the risk of contamination due to the multiple handling processes along the food chain.

iii) if any batches of products tested were associated with reported illness outbreaks. The lack of information on outbreaks or single cases is mostly due to the long incubation period of protozoan diseases. Although, outbreaks have not been reported for RTE salads in Italy, the abovementioned report of an outbreak of cyclosporiasis in Germany associated with contaminated butterhead lettuce (imported from France) and mixed lettuce and other vegetables (interestingly imported from Southern Italy, including the area in which the present study was carried out) (Doller *et al.*, 2002), highlights the risk.

It should be underlined that the rules for good practice adopted along the RTE productive chain (described in the Chapter 2.1/Subchapter 2.1.1) are currently aimed to avoid contamination by bacteria following the current EU and National regulations (see for details Chapter 2.1/Subchapter 2.1.3). Thus, Food Business operators/Food Industries are obviously only keen to follow those rules.

In this view, efforts have to be done by both Food operators and Ministry of Health, in order to push them towards the problem of the contamination by protozoans along the processing chain and convince them that the problem is fully underestimated.

6. CONCLUSION

In conclusion, the results of this survey demonstrate that the prevalence of protozoan species in RTE salads is a cause for concern about human health in Europe, and in particular in Italy. In Italy, the widespread circulation of pathogens in humans, animals, vegetables and water, calls for studies monitoring the use of properly treated irrigation and processing water, the efficiency of wastewater treatment plants, animal access to crops, the proper use of manure as fertilizer, and all the processes along the RTE food chain, such as the observation of personal hygiene regulations by food handlers, and the use of pathogen-free water for washing produce.

Although the role of the RTE salads in increasing consumer exposure to these pathogens and the impact of these protozoans on human health can only be suspected, these results further enhance the need to integrate the microbiological criteria required by EU Law No. 1441/2007 by including these protists in the list of contaminants. Monitoring the absence (or detection limits) only of bacteria, i.e. *E. coli*, *L. monocytogenes* and *Salmonella* spp. in vegetables, definitely can no longer indicate the absence of fecal contamination and guarantee food safety for both immune and immune-compromised consumers.

Considering that the disinfection treatments currently used during the RTE processing are unable to remove protozoans, an increasing of the concentration of chlorine during washing is not a recommended practice, because it could cause chemical contamination, unpleasant odors and the appearance of harmful compounds in the final product (Parish *et al.*, 2003; Soliva-Fortuny and Matin-Belloso, 2003). Alternatively, the development and/or application of innovative technological procedures, - some of them already applied in the waste- and water treatment plants - for the inactivation and removal of oo/cysts from contaminated fresh produce should be promoted (possibly via new and safe methods i.e., UV, membrane filtration, and by a multi-barrier approach), in order to improve the quality and safety of these foods.

Pending the inclusion of protozoan parasites in EU and Italian legislation to reduce the risks of RTE contamination and minimize their foodborne transmission, our results indicate the need for additional surveillance studies of possible sources of food

contamination. Beside the microscopy analysis, molecular techniques are necessary because of their high specificity and sensitivity, in order to allow a rapid and reliable detection of protozoans of concern for human health.

In this view, the support and fostering of epidemiological and surveillance studies on RTE salads, the development of new technologies for protozoan removal along the food chain and the setting up of microscopy and molecular “golden standard procedures” are considered the prerequisites for making aware the food industry and lawmakers on the need to update the current EU legislation by including protozoan parasites.

In the meantime, studies to understand in depth the role of RTE salads in spreading protozoans for a proper risk assessment are necessary.

The sampling method designed in this research - which allowed us to maximize detection even with very low expected prevalence values - and the results obtained can provide the direction for monitoring and surveillance studies on fresh produce in other areas. This method can form the basis for drawing up food safety guidelines, based also on the HACCP system, in order to reduce the risk of RTE contamination and to minimize foodborne disease transmission.

7. ANNEX 1

N° CAMPIONI	MESE E ANNO COLLEZIONE	BRANDS	N° BUSTE	DATA PROCESSAMENTO	LOTTO	DATA DI SCADENZA	TOTALE PROCESSATO	VOLUMI ALIQUOTE (µl o mg)		MICROSCOPIA su aliquote A				MOLECOLARE su aliquote B		RISULTATI MICROSCOPICI							RISULTATI MOLECOLARI						
								Aliquota A	Aliquota B	LUGOL	IFA	ZIEHL-NEELSEN	GEMSA	DNA (µl)	DNA (mg/µl)	Lugol		IF		Ziehl Neelsen			Giemsa other	Giardia duodenalis TP1 gene (nested-PCR)	Cryptosporidium spp. GMP gene (nested-PCR)	Cyclospora cayentensis ITS-2 gene (qPCR)	Toxoplasma gondii B1 gene (qPCR)	Blastocystis spp. SSrRNA gene (End-Point PCR)	Dientamoeba fragilis SSrRNA gene (End-Point PCR)
																Giardia	other	Giardia	Cryptosporidium	Cryptosporidium	Cyclospora	other							
1	MARZO 2015	D	9 (165gr)	09/03/2015	L.15064	13/03/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	34,0	0	0	0	0	0	0	-	0	0	0	0	-	-	
2		F	9 (250gr)	10/03/2015	L. F 068	12/03/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	18,3	0	0	0	0	0	0	-	0	0	0	0	-	-	
3		E	9 (165gr)	16/03/2015	L. 8089	17/03/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	47,4	0	0	0	0	0	0	-	0	0	0	0	-	-	
4		C	9 (250gr)	17/03/2015	L. 73	20/03/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	43,7	0	0	0	0	0	0	-	0	0	0	0	-	-	
5		B	9 (250gr)	18/03/2015	G. 07958	19/03/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	73,2	0	0	0	0	0	0	-	0	0	0	0	-	-	
6		A	9 (250gr)	19/03/2015	B075011841	23/03/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	10,6	0	0	0	0	0	0	-	0	0	0	Type 1	-	-	
7	APRILE 2015	D	9 (165gr)	13/04/2015	L. 15089	14/04/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	104,3	0	0	0	0	0	0	-	0	0	0	0	-	-	
8		F	9 (250gr)	14/04/2015	L. F094	14/04/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	11,6	0	0	0	0	0	0	-	0	0	0	1	-	-	
9		E	9 (165gr)	15/04/2015	L. 0099	17/04/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	26,5	0	0	0	0	0	0	-	0	0	0	0	-	-	
10		C	9 (250gr)	20/04/2015	L. 72	22/04/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	37,8	0	0	0	0	0	0	-	0	0	0	0	-	-	
11		B	9 (250gr)	21/04/2015	L. G0898	23/04/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	6,0	0	0	0	0	0	0	-	0	0	0	1	-	-	
12		A	9 (250gr)	22/04/2015	B066031834	22/04/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	18,9	0	0	0	0	0	0	-	0	0	0	1	Type 1	-	
13	MAGGIO 2015	B	9 (250gr)	11/05/2015	L. G1258	10/05/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	61,9	0	0	0	0	0	0	-	0	0	0	0	-	-	
14		F	9 (250gr)	12/05/2015	L. F122	12/05/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	38,2	0	0	0	0	0	0	-	0	0	0	0	-	-	
15		C	9 (250gr)	14/05/2015	L. 72	16/05/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	134,4	0	0	0	0	0	0	-	0	0	0	0	-	-	
16		E	9 (165gr)	18/05/2015	L. B 132	22/05/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	44,2	0	0	0	0	0	0	-	0	0	0	0	-	-	
17		D	9 (165gr)	19/05/2015	L. 13315	22/05/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	61,2	0	0	0	0	0	0	-	0	0	0	0	-	-	
18		A	9 (250gr)	20/05/2015	B0747231645	24/05/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	67,8	0	0	0	0	0	0	-	0	0	0	0	0	-	-
19	GIUGNO 2015	D	9 (165gr)	04/06/2015	L. 15145	04/06/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	31,8	0	0	0	0	0	0	-	0	0	0	0	B. hominis	-	
20		F	9 (250gr)	08/06/2015	L. F 115	10/06/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	35,9	0	0	0	0	0	0	-	0	0	0	0	-	-	
21		E	9 (165gr)	09/06/2015	L. B 146	10/06/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	27,2	0	0	0	0	0	0	-	0	0	0	0	-	-	
22		C	9 (250gr)	22/06/2015	L. 69	23/06/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	55,7	0	0	0	0	0	0	-	0	0	0	0	-	-	
23		B	9 (250gr)	23/06/2015	G. 06258	23/06/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	38,6	0	0	0	0	0	0	-	0	0	0	0	-	-	
24		A	9 (250gr)	26/06/2015	B0467821920	25/06/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	71,3	0	0	0	0	0	0	-	0	0	0	0	0	-	-
25	LUGLIO 2015	A	9 (165gr)	20/07/2015	B1980122052	24/07/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	22,9	0	0	0	0	0	0	-	0	0	0	0	-	-	
26		C	9 (125gr)	21/07/2015	B202	25/07/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	26,3	0	0	0	0	0	0	-	0	0	0	Type 1	-	-	
27		B	9 (200gr)	25/07/2015	L. 20368	28/07/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	23,1	0	0	0	0	0	0	-	0	0	0	0	-	-	
28		F	9 (150gr)	27/07/2015	C. 201	28/07/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	52,3	0	0	0	0	0	0	-	0	0	0	Type 1	-	-	
29		E	9 (200gr)	01/08/2015	L. 20916	03/08/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	46	0	0	0	0	0	0	-	0	0	0	0	-	-	
30		D	9 (250gr)	01/08/2015	L. 34316209	05/08/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	29,1	0	0	0	0	0	0	-	0	0	0	0	0	-	-
31	AGOSTO 2015	A	9 (125gr)	05/08/2015	B. 2110071442	07/08/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	8 vetrini COLORATI	-	200 µl	55,2	0	0	0	1	1	0	0	-	Assemblage A	0	0	0	-	-
32		C	9 (165gr)	08/08/2015	B216	08/08/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	8 vetrini COLORATI	-	200 µl	54,4	0	0	0	0	0	0	-	0	0	0	0	-	-	
33		B	9 (350gr)	23/08/2015	L. 23168	25/08/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	8 vetrini COLORATI	-	200 µl	35,2	0	0	0	0	0	0	-	0	0	0	0	-	-	
34		F	9 (165gr)	24/08/2015	C. 230	25/08/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	34,4	0	0	0	0	0	0	-	0	0	0	0	-	-	
35		D	9 (250gr)	29/08/2015	L. 32116237	02/09/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	58,4	0	0	0	0	0	0	-	0	0	0	0	-	-	
36		E	9 (165gr)	30/08/2015	L. 23616	01/09/2015	900gr	500µl	300µl	6 vetrini	(2 pacchetti) 50 µl	6 vetrini COLORATI	-	200 µl	176,5	0	0	0	0	0									

8. REFERENCES

- Abe, N., Tanoue, T., Noguchi, E., Ohta, G., H. Sakai, H., 2010. Molecular characterization of *Giardia duodenalis* isolates from domestic ferrets. Parasitol. Res. 106, 733–736.
- Åberg, R., Sjöman, M., Hemminki, K., Pirnes, A., Räsänen, S., Kalanti, A., Pohjanvirta, T., Caccio, S.M., Pihlajasaari, A., Toikkanen, S., Huusko, S., Rimhanen-Finne, R., 2015. *Cryptosporidium parvum* caused a large outbreak linked to frisée salad in Finland, 2012. Zoonoses Public Health. 62, 618-624.
- Adam, R.D., 2001. Biology of *Giardia lamblia*. Clin. Microbiol. Rev. 14, 447-75.
- Adam, E.A., Yoder, J.S., Gould, L.H., Hlavsa, M.C., Gargano, J.W., 2016. Giardiasis outbreaks in the United States, 1971-2011. Epidemiol. Infect. 144, 2790-2801.
- Adams, R.B., Guerrant, R.L., Zu, S., Fang, G., Roche, J.K., 1994. *Cryptosporidium parvum* infection of intestinal epithelium: morphologic and functional studies in an in vitro model. J. Infect. Dis. 169, 170-177.
- Agholi, M., Hatam, G.R., Motazedian, M.H., 2013. HIV/AIDS-associated opportunistic protozoal diarrhea. AIDS Res Hum Retroviruses. 29, 35-41.
- Akyar, I., 2011. Seroprevalence and Coinfections of *Toxoplasma gondii* in Childbearing Age Women in Turkey. Iranian J. Publ. Health. 40, 63-67.
- Al, F.D., Kuştimur, S., Ozekinci, T., Balaban, N., Ilhan, M.N., 2006. The use of enzyme linked immunosorbent assay (ELISA) and direct fluorescent antibody (DFA) methods for diagnosis of *Giardia intestinalis*. Turkiye Parazitolo. Derg. 30, 275-278.
- Al-Binali, A.M., Bello, C.S., El.Shewy, K., Abdulla, S.E., 2006. The prevalence of parasites in commonly used leafy vegetables in South Western Saudi Arabi, Saudi. Med. J. 27, 613-616.
- Alhabbal, A.T., 2015. The prevalence of parasitic contamination on common cold vegetables in Alqalamoun Region. Int J Pharm Sci Rev Res. 30, 94–97.
- Al-Megrin, W.A., 2010. Intestinal parasites infection among immunocompromised patients in Riyadh, Saudi Arabia. Pak. J. Biol. Sci. 13, 390-394.
- Almeida, A.A., Delgado, M.L., Soares, S.C., Castro, A.O., Moreira, M.J., Mendonca, C.M., Canada, N.B., Da Costa, C.M., 2006. Genotype analysis of *Giardia* isolated from asymptomatic children in northern Portugal. J. Eukaryot. Microbiol. 53 Suppl. 1, S177–S178.

- Althaus, D., Hofer, E., Corti, S., Julmi, A., Stephan, R., 2012. Bacteriological survey of ready-to-eat lettuce, fresh-cut fruit, and sprouts collected from the Swiss market. *J. Food Prot.* 75, 1338-1341.
- Alves, M., Xiao, L., Antunes, F., Matos, O., 2006. Distribution of *Cryptosporidium* subtypes in humans and domestic and wild ruminants in Portugal. *Parasitol Res.* 99, 287-292.
- Amoros, I., Alonso, J.L., Cuest, G., 2010. *Cryptosporidium* oocysts and *Giardia* cysts on salads products irrigated with contaminated water. *J. Food Prot.* 73, 1138-1140.
- Angarano, G., Maggi, P., Di Bari, M.A., Larocca, A.M., Congedo, P., Di Bari, C., Brandonisio, O., Chiodo, F., 1997. Giardiasis in HIV: a possible role in patients with severe immune deficiency. *Eur. J. Epidemiol.* 13, 485-487
- Angelini, R., Gullino, M.L., Ponti, I., 2011. *Insalate*. ART Servizi Editoriali S.p.A, Bologna.
- Argenzio, R.A., Laicos, J.A., Levy, M.L., Meuten, D.J., Lecce, J.G., Powell, D.W., 1990. Villous atrophy, crypt hyperplasia, cellular infiltration and impaired glucose-Na absorption in enteric cryptosporidiosis of pigs. *Gastroenterology* 98, 1129-140.
- Ashford, R.W. 1979. Occurrence of an undescribed coccidian in man in Papua New Guinea. *Ann. Trop. Med. Parasitol.* 73, 497-500.
- Bacci, C., Vismarra, A., Mangia, C., Bonardi, S., Bruini, I., Genchi, M., Kramer, L., Brindani, F., 2015. Detection of *Toxoplasma gondii* in free-range, organic pigs in Italy using serological and molecular methods. *Int. J. Food Microbiol.* 202, 54-56.
- Bajer, A., 2008. *Cryptosporidium* and *Giardia* spp. infections in humans, animals and the environment in Poland. *Parasitol. Res.* 104, 1-17.
- Baldi, L., 2007. Speciale IV gamma - Evoluzione e prospettive del settore. *L'informatore Agrario*, n. 16.
- Baldursson, S., Karanis, P., 2011. Water transmission of protozoan parasites: review of worldwide outbreaks-an update 2004-2010. *Water Res.* 15, 6603-6614.
- Barratt, J.L., Harkness, J., Marriott, D., Ellis, J.T., Stark, D., 2010. Importance of nonenteric protozoan infections in immunocompromised people. *Clin. Microbiol. Rev.* 23, 795-836.
- Batchelor, D.J., Tzannes, S., Graham, P.A., Wastling, J.M., Pinchbeck, G.L., German, A.J., 2008. Detection of endoparasites with zoonotic potential in dogs with gastrointestinal disease in the UK. *Transbound. Emerg. Dis.* 55:99-104.

- Belosevic, M., Faubert, G.M., Maclean, J.D., 1989. Disaccharidase activity in the small intestine of gerbils (*Meriones unguiculatus*) during primary and challenge infections with *Giardia lamblia*. Gut. 30, 1213-1219
- Ben Ayed, L., Yang, W., Widmer, G., Cama, V., Ortega, Y., Xiao, L., 2012. Survey and genetic characterization of wastewater in Tunisia for *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Cyclospora cayetanensis* and *Eimeria* spp. J. Water Health. 10, 431 –444.
- Berrilli, F., Prisco, C., Friedrich, K.G., Di Cerbo, P., Di Cave, D., De Liberato, C., 2011 *Giardia duodenalis* assemblages and *Entamoeba* species infecting non-human primates in an Italian zoological garden: zoonotic potential and management traits. Parasit. Vectors. 4, 199.
- Betancourt, W.Q., Rose, J.B., 2004. Drinking water treatment processes for removal of *Cryptosporidium* and *Giardia*. Vet. Parasitol. 126, 219-234.
- Beuchat, L.R., Ryu, J.H., 1997. Produce Handling and Processing Practices. Emerg. Infect. Dis. 3, 459-565.
- Bossi, P., Bricaire, F., 2004. Severe acute disseminated toxoplasmosis. Lancet. 364, 579–579.
- Bouzid, M., Hunter, P.R., Chalmers, R.M., and Tyler, K.M., 2013. *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev. 26, 115-34.
- Brandonisio, O., 2006. Waterborne transmission of *Giardia* and *Cryptosporidium*. Parassitologia. 48, 91-4.
- Brandonisio, O., Maggi, P., Panaro, M.A., Lisi, S., Andriola, A., Acquafredda, A., Angarano, G., 1999. Intestinal protozoa in HIV-infected patients in Apulia, South Italy. Epidemiol Infect 123:457–462.
- Briancesco, R., Bonadonna, L., 2005. An Italian study on *Cryptosporidium* and *Giardia* in wastewater, fresh water and treated water. Environ. Monit. Assess. 104, 445–457.
- Brandonisio, O., Fumarola, L., Spinelli, R., Donadio, F., Montemurro, P., Portincasa, F., 2004. *Giardia* e *Cryptosporidium* spp.: rassegna critica e monitoraggio in acque superficiali e reflue. Ig. Mod. 122, 137–160
- Broglia, A., Kapel, C., 2011. Changing dietary habits in a changing world: emerging drivers for the transmission of foodborne parasitic zoonoses. Vet. Parasitol. 182, 2-13.

- Buchel, L.A., Gorenflot, A., Chochillon, C., Savel, J., Gobert, J.G., 1987. In vitro excystation of *Giardia* from humans: a scanning electron microscopy study. *J. Parasitol.* 73, 487-93.
- Bukhari, Z., Smith, H.V., 1995. Effect of three concentration techniques on viability of *Cryptosporidium parvum* oocysts recovered from bovine feces. *J. Clin. Microbiol.* 33, 2592-2595.
- Buret, A.G., 2007. Mechanisms of epithelial dysfunction in giardiasis. *Gut.* 56, 316-317.
- Buret, A.G., Hardin, J.A., Olson, M.E., Gall, D.G., 1992. Pathophysiology of small intestinal malabsorption in gerbils infected with *Giardia lamblia*. *Gastroenterology.* 103, 506-513.
- Buret, A.G., Mitchell K., Muench, D.G., Scott K.G.E., 2002. *Giardia lamblia* disrupts tight junctional ZO-1 and increases permeability in non-transformed human small intestinal epithelial monolayers: Effects of epidermal growth factor. *Parasitology,* 125, 11-19.
- Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J. Clin. Microbiol.* 27, 1787-1792
- Cacciò S.M., Putignani L., 2014. Epidemiology of Human Cryptosporidiosis. In: Simone M. Cacciò, S.M, Widmer, G. (eds.), *Cryptosporidium: parasite and disease.* Springer, Wien Heidelberg New York Dordrecht London, pp. 43-79.
- Cacciò, S.M., Beck, R., Lalle, M., Marinculic, A., Pozio, E., 2008. Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. *Int. J. Parasitol.* 38, 1523-1531.
- Cacciò, S.M., Sannella, A.R., Manuali, E., Tosini, F., Sensi, M., Crotti, D., Pozio, E., 2012. Pigs as natural hosts of *Dientamoeba fragilis* genotypes found in humans. *Emerg. Infect. Dis.* 18, 838841.
- Calvo, M., Carazo, M., Arias, M.L., Chaves, C., Monge, R., Chinchilla, M., 2004. Prevalence of *Cyclospora spp.*, *Cryptosporidium spp.*, microsporidia and fecal coliform determination in fresh fruit and vegetables consumed in Costa Rica. *Arch. Latinoam. Nutr.* 54, 428-432.
- Cama, V.A., Mathison, B.A., 2015. Infections by Intestinal Coccidia and *Giardia duodenalis*. *Clin. Lab. Med.* 35, 423-444.

- Campbell, A.T., Robertson, L.J., Smith, H.V., 1992. Viability of *Cryptosporidium parvum* oocysts: correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. *App Environ Microbiol*, 58, 3488-3493.
- Campos, J., Mourão, J., Pestana, N., Peixe, L., Novais, C., Antunes, P., 2013. Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. *Int. J. Food Microbiol.* 166, 464-470.
- Carruthers, V., Boothroyd, J.C., 2007. Pulling together: an integrated model of *Toxoplasma* cell invasion. *Curr. Opin. Microbiol.* 10, 83-89.
- Casati, D., Baldi, C., 2011. Mondo e mercato: Insalate e quarta gamma nel mondo, in: Angelini, R., Gullino, M.L., Ponti, I. (Eds), *Le Insalate*. ART Servizi Editiriali S.p.A, Bologna, pp. 507-531. <http://www.colturaecultura.it/capitolo/insalate-e-quarta-gamma-nel-mercato>. (accessed 27.04.2016)
- Casemore, D.P., 1991. ACP Broadsheet 128: June 1991. Laboratory methods for diagnosing cryptosporidiosis. *J. Clin. Pathol.* 44, 445-451.
- Castro-Hermida, J.A., Delafosse, A., Pors, I., Ares-Mazas, E., Chartier, C., 2005. *Giardia duodenalis* and *Cryptosporidium parvum* infections in adult goats and their implications for neonatal kids. *Vet. Rec.* 157, 623- 627.
- Castro-Hermida, J.A., Almeida, A., Gonzalez-Warleta, M., Correia da Costa, J.M Mezo, M., 2006. Prevalence and preliminary genetic analysis of *Giardia* isolated from adult sheep in Galicia (northwest Spain). *J. Eukaryot. Microbiol.* 53, S172-S173.
- Castro-Hermida, J.A., Almeida, A., Gonzalez-Warleta, M., Correia da Costa, J.M., Rumbo-Lorenzo, C., Mezo, M., 2007. Occurrence of *Cryptosporidium parvum* and *Giardia duodenalis* in healthy adult domestic ruminants. *Parasitol. Res.* 101, 1443-1448.
- Castro-Hermida J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Correia, Da Costa, J.M., Mezo, M., 2008. Presence of *Cryptosporidium* spp. and *Giardia duodenalis* through drinking water. *Sci. Total. Environ.* 405, 45-53.
- Cavaiuolo, M., Cocetta, G., Bulgari, R., Spinardi, A., Ferrante, A., 2015. Identification of innovative potential quality markers in rocket and melon fresh-cut produce. *Food Chem.* 188, 225-233.
- CDC (Centers for Disease Control and Prevention), 2015. U.S. Foodborne Outbreaks of Cyclosporiasis—2000–2014.

<https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html>
l (accessed 30.10.2016)

- Chacín-Bonilla, L., 2010. Epidemiology of *Cyclospora cayetanensis*: A review focusing in endemic areas. *Acta Trop.* 115, 181-193.
- Chaidez, C., Soto, M., Gortares, P., Mena, K., 2005. Occurrence of *Cryptosporidium* and *Giardia* in irrigation water and its impact on the fresh produce industry. *Int J Environ Health Res.* 15, 339-345.
- Chalmers, R.M., Davies, A.P., 2010. Minireview: Clinical cryptosporidiosis. *Experimental Parasitology.* 124, 138-146.
- Chalmers, R.M., Elwin, K., Hadfield, S.J., Robinson, G., 2011. Sporadic human cryptosporidiosis caused by *Cryptosporidium cuniculus*, United Kingdom, 2007-2008. *Emerg Infect Dis.* 17, 536-538.
- Chalmers, R.M., Katzer, F., 2013. Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. *Trends Parasitol.* 29, 237-251.
- Chin, A.C., Teoh, D.A., Scott, K.G., Meddings, J.B., Macnaughton, W.K., Buret, A.G., 2002. Strain dependent induction of enterocyte apoptosis by *Giardia lamblia* disrupts epithelial barrier function in a caspase-3-dependent manner. *Infect Immun.* 70, 3673-3680.
- Chu, D.M., Sherchand J.B., Cross J.H. and Orlandi P.A., 2004. Detection of *Cyclospora cayetanensis* in animal fecal isolates from Nepal using an FTA filter-base polymerase chain reaction method. *Am J Trop Hyg.* 71, 373-379.
- Cieloszyk, J., Goñi, P., García, A., Remacha, M.A., Sánchez, E., Clavel, A., 2012. Two cases of zoonotic cryptosporidiosis in Spain by the unusual species *Cryptosporidium ubiquitum* and *Cryptosporidium felis*. *Enferm Infecc Microbiol. Clin.* 30,549-551.
- Claerebout, E., Casaert, S., Dalemans, A.C., De Wilde, N., Levecke, B., Vercruysse, J., Geurden, T., 2009. *Giardia* and other intestinal parasites in different dog populations in Northern Belgium. *Vet. Parasitol.* 161, 41-46.
- Clarke, C.S., McInyre, M., 1996. Modified detergent Ziehl Nelsen technique for the staining of *Cyclospora Cayetanensis*. *J. Clin. Pathol.* 49, 511-512.
- Clavel, A., Arnal, A., Sánchez, E., Varea, M., Quílez, J., Ramírez, I., Castillo, F.J., 1996. Comparison of two centrifugation procedures in the formalin-ethyl acetate stool concentration technique for the detection of *Cryptosporidium* oocysts. *Int J Parasitol.* 26, 671- 672.

- Colelli, G., Elia, A., 2009. Physiological and technological aspects of fresh-cut horticultural products. *Italus Hortus* 16, 55-78.
- Confcooperative, 2016. N. 10 Le insalate di quarta gamma, mercati e tendenze - Ottobre 2016.
<http://www.internazionalizzazione.confcooperative.it/LENEWS/ArtMID/500/ArticleID/468/Export-Mercati-Le-insalate-di-quarta-gamma-mercati-etendenze> (accessed 15.01.2017)
- Connor, B.A., Reidy, J., Soave, R., 1999. Cyclosporiasis: clinical and histopathologic correlates. *Clin. Infect. Dis.* 28, 1216–1222.
- Connor, B.A., Johnson, E.J., Soave, R., 2001. Reiter syndrome following protracted symptoms of Cyclospora infection. *Emerg. Infect. Dis.* 7, 453-454.
- Conrad PA, Miller, M.A., Kreuder, C., James, E.R., Mazet, J., Dabritz, H., Jessup, D.A., Gulland, F., Grigg, M.E., 2005. Transmission of *Toxoplasma*: clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment. *Int. J. Parasitol.* 35, 1155–1168
- Cook, N., Nichols, R.A.B., Wilkinson, N., Paton, C.A, Barker, K., Smith, H.V, 2007. Development of a method for detection of *Giardia duodenalis* cysts on lettuce and for simultaneous analysis of salad products for the presence of *Giardia* cysts and *Cryptosporidium* oocysts. *Appl. Environ. Microbiol.* 73, 7388–7391.
- Coppola, M.G., 2010. Il laboratorio di protozoologia “La diagnosi di laboratorio delle malattie da protozoa”. In: Gringoli G. (ed) *Mappe Parassitarie*. 12 – 128.
- Cowling, D.W., Gardner, I.A., Johnson, W.O., 1999. Comparison of methods for estimation of individual-level prevalence based on pooled samples. *Prev. Vet. Med.* 39, 211-25.
- Crotti, D., Sensi, M., Crotti, S., Grelloni, V., Manuali, E., 2007. *Dientamoeba fragilis* in swine population: a preliminary investigation. *Vet. Parasitol.* 145, 349-351.
- Cultrera, R., Seraceni, S., Contini, C., 2002. Efficacy of a novel reverse transcriptase-polymerase chain reaction (RT-PCR) for detecting *Toxoplasma gondii* bradyzoite gene expression in human clinical specimens. *Mol Cell Probes.* 16, 31-9.
- Current, W.L., Reese, N.C., 1986. A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. *Journal Protozool.* 33, 98–108.

- da Silva, P.C., Shiraishi, C.S., Silva, A.V., Goncalves, G.F., Sant'Ana, D., de, M., Araujo, E.J., 2010. *Toxoplasma gondii*: a morphometric analysis of the wall and epithelial cells of pigs intestine. *Exp. Parasitol.* 125, 380–383.
- Dado, D., Montoya, A., Blanco, M.A., Miró, G., Saugar, J.M., Bailo, B., Fuentes, I., 2012. Prevalence and genotypes of *Giardia duodenalis* from dogs in Spain: possible zoonotic transmission and public health importance. *Parasitol Res.* 111. 2419-2422.
- Dagci, H., Ustun, S., Taner, M.S., Ersoz, G., Karacasu, F., Budak, S., 2002. Protozoan infections and intestinal permeability. *Acta Trop.* 81, 1-5.
- Dalton, C., Goater, A.D., Pethig, R., Smith, H.V., 2001. Viability of *Giardia intestinalis* cysts and viability and sporulation state of *Cyclospora cayetanensis* oocysts determined by electroration. *Appl. Environ. Microbiol.* 67,586–590.
- Dalton, C., Goater, A.D., Burt, J.P., Smith, H.V., 2004. Analysis of parasites by electroration. *J. Appl. Microbiol.* 96, 24–32.
- Davies, A.P., Campbell, B., Evans, M.R., Bone, A., Roche, A., Chalmers, R.M., 2009. Asymptomatic carriage of protozoan parasites in children in day care centers in the United Kingdom. *Pediatr. Infect. Dis. J.* 28 ,838– 840.
- Dawson D., 2005. Foodborne protozoan parasites. *Int J Food Microbiol*, 103, 207-227.
- de Go'rgolas, M., Fortes, J., Fernandez Guerrero, M.L., 2001. *Cyclospora cayetanensis* cholecystitis in a patient with AIDS. *Ann. Intern. Med.* 134, 166.
- De Liberato, C., Berrilli, F., Marangi, M., Santoro, M., Trogu, T., Putignani, L., Lanfranchi, P., Ferretti, F., D'Amelio, S. and Giangaspero, A., 2015. *Giardia duodenalis* in Alpine (*Rupicapra rupicapra rupicapra*) and Apennine (*Rupicapra pyrenaica ornata*) chamois. *Parasit Vectors.* 8, 650.
- Desai, N.T., Sarkar, R., Kang, G., 2012. Cryptosporidiosis: An under-recognized public health problem. *Trop Parasitol.* 2, 91-98.
- Di Benedetto, M.A., Di Piazza, F., Maida, C.M., Firenze, A., Oliveri, R., 2005. Occurrence of *Giardia* and *Cryptosporidium* in wastewater, surface water and ground water samples in Palermo (Sicily). *Ann. Ig.* 17,367–375.
- Di Benedetto, M.A., Cannova, L., Di Piazza, F., 2007. Hygienic sanitary quality of ready to eat salad vegetables on saled in the city of Palermo (Sicily). *Ig. Sanità Pubbl.* 63, 659-670.

- Dixon, B., 2015. Transmission Dynamics of Foodborne Parasites on Fresh Produce. In: Gajadhar, A.A. (ed.), Foodborne Parasites in the Food Supply Web: Occurrence and Control. Woodhead Publishing, Cambridge, United Kingdom. 317 – 353.
- Dixon, B., Fayer, R.R., Santin, M., Hill, D.E., Dubey, J.P., 2011. Protozoan parasites: *Cryptosporidium*, *Giardia*, *Cyclospora* and *Toxoplasma*. In Hoorfar, J. (ed.), Rapid detection, characterization and enumeration of foodborne pathogens. ASM Press, Washington, DC. 349-370.
- Dixon, B., Parrington, L., Cook, A., Pollari, F., Farber, J., 2013. Detection of *Cyclospora*, *Cryptosporidium*, and *Giardia* in ready-to-eat packaged leafy greens in Ontario, Canada. J Food Prot. 76, 307-313.
- Döller, P.C., Dietrich, K., Filipp, N., Brockmann, S., Dreweck, C., Vonthein, R., Wagner-Wiening, C., Wiedenmann, A., 2002. Cyclosporiasis outbreak in Germany associated with the consumption of salad. Emerg Infect Dis. 8, 992-994.
- Drenaggi, D., Cirioni, O., Giacometti, A., Fiorentini, A., Scalise, G., 1998. Cyclosporiasis in a traveler returning from South America. J. Travel. Med. 5, 153-155.
- Drumo, R., Widmer, G., Morrison, L.J., Tait, A., Grelloni, V., D'Avino, N., Pozio, E., and Cacciò, S.M., 2012. Evidence of host-associated populations of *Cryptosporidium parvum* in Italy. Appl. Environ. Microbiol. 78, 3523-3529.
- Dubey, J.P., 1988. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. Am. J. Vet. Res. 49, 910-913.
- Dubey, J.P., 1993. *Toxoplasma*, *Neospora*, *Sarcocystis*, and other tissue cyst-forming coccidian of humans and animals.. In: Kreier, J.P., (ed.) Parasitic protozoa, vol. 6. Academic Press, Inc., New York, pp. 1-158.
- Dubey, J.P., 1996. Infectivity and pathogenicity of *Toxoplasma gondii* oocysts for cats. J. Parasitol, 82, 957-960.
- Dubey, J.P., Lunney, J.K., Shen, S.K., Kwok, O.C.H., Ashford, D.A., Thulliez, P., 1996. Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. J. Parasitol. 82, 438 – 443.
- Dubey, J.P., 1998a. Advances in the life cycle of *Toxoplasma gondii*. Int. J. Parasitol. 28, 1019-1024.
- Dubey, J.P., 1998b. *Toxoplasma gondii* oocyst survival under defined temperatures. J. Parasitol. 84, 862-865.

- Dubey, J.P., 2008. The history of *Toxoplasma gondii* – the first 100 years. J. Eukaryot. Microbiol. 55, 467-475.
- Dubey, J.P., Frenkel, J.K., 1972. Cyst-induced toxoplasmosis in cats. J. Protozool. 19, 155–177.
- Dubey, J.P., Frenkel, J.K., 1976. Feline toxoplasmosis from acutely infected mice and the development of *Toxoplasma* cysts. J. Protozool. 23, 537–546.
- Dubey, J.P., Carpenter, J.L., 1993. Histologically confirmed clinical toxoplasmosis in cats, 100 cases (1952–1990). J. Am. Vet. Med. Assoc. 203, 1556–1566.
- Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin. Microbiol. Rev. 11, 267-299.
- Dubremetz, J.F., 2007. Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction. Cell. Microbiol. 9, 841–848.
- Duedu, K., Yarnie, E., Tetteh-Quarcoop, P., Attah, S., Donkor, E., Ayeh-Kumi, P., 2014. A comparative survey of the prevalence of human parasites found in fresh vegetables sold in supermarkets and open-aired markets in Accra. Ghana. BMC Res. Notes. 7, 836.
- Dumètre, A., Le Bras, C., Baffet, M., Meneceur, P., Dubey, J.P., Derouin, F., Duguet, J.P., Joyeux, M., Moulin, L., 2008. Effects of ozone and ultraviolet radiation treatments on the infectivity of *Toxoplasma gondii* oocysts. Vet. Parasitol. 153, 209–213.
- DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B., and Jakubowski, W., 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. New Engl. J. Med. 332, 855–859.
- Dworkin, L.L., Gibler, T.M., Van Gelder, R.N., 2002 Real-time quantitative polymerase chain reaction diagnosis of infectious posterior uveitis. Arch. Ophthalmol. 120, 1534–1539.
- Eberhard, M.L., Pieniazek, N.J., Arrowood, M.J., 1997. Laboratory diagnosis of Cyclospora infections. Arch. Pathol. Lab. Med. 121, 792–797.
- Eberhard, M.L., da Silva, A.J., Lilley, B.G., Pieniazek, N.J., 1999. Morphologic and molecular characterization of new Cyclospora species from Ethiopian monkeys: *C. cercopithecii* sp.n., *C. colobi* sp.n., and *C. papionis* sp.n. Emerg. Infect. Dis. 5, 651–658.
- Eberhard, M.L., Ortega, Y.R., Hanes, D.E., Nace, E.K., Do, R.Q., Robl, M.G., Won, K.Y., Gavidia, C., Sass, N.L., Mansfield, K., Gozalo, A., Griffiths, J., Gilman, R., Sterling, C.R.,

- Arrowood, M.J., 2000. Attempts to establish experimental *Cyclospora cayetanensis* infection in laboratory animals. J Parasitol. 86, 577-582.
- Ebrahimzadeh, A., Jamshidi, A., Mohammadi, S., 2013. The parasitic contamination of raw vegetables consumed in Zahedan, Iran. Health Scope 1, 205-209.
 - ECDS (European center disease prevention control), 2013. Surveillance report. Annual epidemiological report. Reporting on 2011 surveillance data and 2012 epidemic intelligence data 2013. <http://ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf> (accessed 17.01.2017)
 - Eckmann, L., Laurent, F., Langford, T.D., et al., 2000. Nitric oxide production by human intestinal epithelial cells and competition for arginine as potential determinants of host defense against the lumen-dwelling pathogen *Giardia lamblia*. J. Immunol. 164, 1478-1487.
 - Eckmann, L., Gillin, F.D., 2001. Microbes and microbial toxins, Paradigms for microbial-mucosal interactions. Pathophysiological aspects of enteric infections with the lumen-dwelling protozoan pathogen *Giardia lamblia*. Am. J. Physiol. Gastrointest Liver Physiol. 280, G1-6.
 - Ehsan, A., Geurden, T., Casaert, S., Paulussen, J., De Coster, L., Schoemaker, T., Chalmers, R., Grit, G., Vercruysse, J., Claerebout, E., 2015. Occurrence and potential health risk of *Cryptosporidium* and *Giardia* in different water catchments in Belgium. Environ. Monit. Assess. 187, 6.
 - Elwin, K., Hadfield, S.J., Robinson, G., Chalmers, R.M., 2012. The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000-2008. Epidemiol. Infect. 140, 673-683.
 - Epe, C., Coati, N., Schnieder, T., 2004. Results of parasitological examinations of faecal samples from horses, ruminants, pigs, dogs, cats, hedgehogs and rabbits between 1998 and 2002. Dtsch. Tierarztl. Wochenschr. 111, 243-247.
 - Eraky M.A., Rashed, S.M., Nasr Mel-S, El-Hamshary, A.M., Salah El-Ghannam, A., 2014. Parasitic contamination of commonly consumed fresh leafy vegetables in benha, Egypt. J Parasitol Res. 2014, 613960.
 - Erickson, M.C., Ortega, Y.R., 2006. Inactivation of protozoan parasites in food, water, and environmental systems. J. Food Prot. 69, 2786-2808.

- Erlandsen, S.L., Bemrick, W.J., Pawley, J., 1989. High-resolution electron microscopic evidence for the filamentous structure of the cyst wall in *Giardia muris* and *Giardia duodenalis*. J. Parasitol. 75, 787–797.
- Erlandsen, S.L., Bemrick, W.J., Schupp, D.E., Shields, J.M., Jarroll, E.L., Sauch, J.F., Pawley, J.B., 1990. High-resolution immunogold localization of Giardia cyst wall antigens using field emission SEM with secondary and backscatter electron imaging. J. Histochem. Cytochem. 38, 625–632.
- Ethelberg, S., Lisby, M., Vestergaard, L.S., Enemark, H.L., Olsen, K.E., Stensvold, C.R., Nielsen, H.V., Porsbo, L.J., Plesner, A.M., Mølbak, K., 2009. A foodborne outbreak of *Cryptosporidium hominis* infection. Epidemiol. Infect. 137,348–356.
- Ezatpour B., Chegeni A.S., Abdollahpour F., Aazami M., Alirezaei M., 2013. Prevalence of parasitic contamination of raw vegetables in Khorramabad, Iran. Food Control 34, 92-95
- FAO/WHO (Food and Agriculture Organization of the United Nations and World Health Organization), 2003. Assuring Food Safety and Quality: Guidelines for Strengthening National Food Control Systems. <http://www.fao.org/docrep/006/y8705e/y8705e00.htm> (accessed 12.10.2016).
- FAO (Food and Agriculture Organization), 2016. FAO-WHO Fruit and Vegetable for Health Initiative (PROFAV/PROFEL), 2016. <http://www.fao.org/agriculture/crops/thematic-sitemap/theme/hort-indust-crops/fao-who-fruit-and-vegetable-for-health-initiative-profavprofel/en/> (accessed 09.09.2016).
- Farrington, M., Winters, S., Walker, C., Miller, R., Rubenstein, D., 1994. Cryptosporidium antigen detection in human feces by reverse passive haemagglutination assay. J. Clin. Microbiol. 32, 2744- 2764.
- Farthing, M.J.G., 2000. Clinical aspects of human cryptosporidiosis. In: Petry, F. (Ed.), Cryptosporidiosis and Microsporidiosis. Contributions in Microbiology, vol. 6. Karger, Basel, 50–74.
- Fayer, R., Dubey, J.P., Lindsay, D.S., 2004. Zoonotic protozoa: from land to sea. Trends Parasitol. 20, 531 –536.
- Fayer, R., Morgan, R., Upton, S.J., 2000. Epidemiology of *Cryptosporidium*: transmission, detection and identification. Int. J. Parasitol. 30, 1305-1322.

- Fayer, R., Speer, C.A., Dubey, J.P., 1997. General biology of *Cryptosporidium* and *cryptosporidiosis*. In: Fayer R (Ed.), *Cryptosporidium* and *cryptosporidiosis*. CRC Press, Boca Raton, 1-41.
- Fayer, R., Ungar, B.L.P., 1986. *Cryptosporidium* spp. and *cryptosporidiosis*. Microbiol. Rev. 50, 458–483.
- Feng, Y., Xiao, L., 2011. Zoonotic Potential and Molecular Epidemiology of *Giardia* Species and Giardiasis. Clin Microbiol Rev. 24, 110-140.
- Ferguson, D.J.P., 2004. Use of molecular and ultrastructural markers to evaluate stage conversion of *Toxoplasma gondii* in both the intermediate and definitive host. Int. J. Parasitol. 34, 347–360.
- Ferguson, D.J.P., Dunachie, W.M., Siim, J.C., 1974. Ultrastructural study of early stages of asexual multiplication and microgametogony of *Toxoplasma gondii* in the small intestine of the cat. Acta Pathol. Microbiol. Scand. Sect. 82, 167–181.
- Ferguson, D.J.P., Birch-Andersen, A., Siim, J.C., Hutchison, W.M., 1979a. Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii*. I. Development of the zygote and formation of sporoblasts. Acta Pathol. Microbiol. Scand. Sect. B 87, 171–181.
- Ferguson, D.J.P., Birch-Andersen, A., Siim, J.C., Hutchison, W.M., 1979b. Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii*. II. Formation of the sporocysts and structure of the sporocysts wall. Acta Pathol. Microbiol. Scand. B 87, 183–190. 58.
- Ferguson, D.J.P., Birch-Andersen, A., Siim, J.C., Hutchison, W.M., 1979c. Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii* III. Formation of sporozoites within the sporocysts. Acta Pathol. Microbiol. Scand. Sect. B 87, 253–260. 59.
- Ferreira, C.S., Amato Neto, V., Alarcan, R.S.R., Gakiya, E., 2001. Identification of *Cryptosporidium* spp. oocysts in fecal smears stained with Heidenhain's iron hematoxylin. Rev. Inst. Med. Trop. Sao Paulo 43, 341–342.
- Fiocchi, C., 1998. Inflammatory bowel disease etiology and pathogenesis. Gastroenterology. 115, 182–205
- Fletcher, S.M., Stark, D., Harkness, J., Ellis, J., 2012. Enteric Protozoa in the Developed World: a Public Health Perspective. Clin Microbiol Rev. 25, 420-449.

- Francis, G.A., Thomans, C., O'Beirne, D., 1999. The microbiological safety of minimally processed vegetables. *Int J Food Sci Technol.* 34, 1-22.
- Freitas-Martinez, A.D., Colmenares, D., Perez, M., et al, 2009 *Cryptosporidium* spp. infections and other intestinal parasites in food handlers from Zulia state, Venezuela. *Invest. Clin.* 50, 13-21.
- Frenkel, J.K., Dubey, J.P., 1973. Effects of freezing on the viability of *Toxoplasma* oocysts. *J. Parasitol.* 59, 587-588.
- Frenkel, J.K., Dubey, J.P., Miller, N.L., 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. *Science.* 167, 893-896.
- Galván, A.L., Magnet, A., Izquierdo, F., Fenoy, S., Rueda, C., Fernández Vadillo, C., Henriques-Gil, N., del Aguila, C., 2013. Molecular characterization of humanpathogenic microsporidia and *Cyclospora cayetanensis* isolated from various water sources in Spain: a year-long longitudinal study. *Appl. Environ. Microbiol.* 79, 449 -459.
- Garcia, L.S., Shimizu, R.Y., 1997. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. *J. Clin. Microbiol.* 35, 1526-1529.
- Garcia, L.S., Shimizu, R.Y., 2000. Detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens using the ColorPAC combination rapid solid-phase qualitative immunochromatographic assay. *J. Clin. Microbiol.* 38, 1259-1275.
- Garcia, L.S., 2016. *Dientamoeba fragilis*, one of the Neglected Intestinal Protozoa. *J. Clin. Microbiol.* 54, 2243-2250.
- García-López, H.L., Rodríguez-Tovar, L.E., Medina-De la Garza, C.E., 1996. Identification of *Cyclospora* in poultry. *Emerg. Infect. Dis.* 2, 356-357.
- Gardner I.A., Stryhn H., Lind P., Collins M.T., 2000. Conditional dependence between tests affects the diagnosis and surveillance of animal diseases. *Prev. Vet. Med.* 45, 107-22.
- Gardner, T.B., Hill, D.R., 2001. Treatment of giardiasis. *Clin Microbiol Rev.* 14, 114-128.
- Geurden, T., Claerebout, E., Vercruysse, J., Berkvens, D., 2004. Estimation of diagnostic test characteristics and prevalence of *Giardia duodenalis* in dairy calves in Belgium using a Bayesian approach. *Int J Parasitol.* 34, 1121-1127.

- Geurden, T., Geldhof, P., Levecke, B., Martens, C., Berkvens, D., Casaert, S., Vercruysse, J., Claerebout, E., 2008a. Mixed *Giardia duodenalis* assemblage A and E infections in calves. *Int. J. Parasitol.* 38, 259–264.
- Geurden, T., Thomas, P., Casaert, S., Vercruysse J., Claerebout, E., 2008b. Prevalence and molecular characterisation of *Cryptosporidium* and *Giardia* in lambs and goat kids in Belgium. *Vet. Parasitol.* 155, 142–145.
- Geurden, T., Levecke, B., Caccio, S.M., Visser, A., De Groote, G., Casaert, S., Vercruysse, J., Claerebout, E., 2009. Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhoea in human patients in Belgium. *Parasitology* 136, 1161–1168.
- Giacometti, A., Cirioni, O., Fortuna, M., Drenaggi, D., Vecchia, S., D’Errico, M.M., Scalise, G., 2000. Giardiasis: a parasitic disease of continued topicality. Study of prevalence among a selected adult population. *Infez. Med.* 8, 82–86.
- Giangaspero, A., Berrilli, F., Brandonisio, O., 2007. *Giardia* and *Cryptosporidium* and public health: the epidemiological scenario from the Italian perspective. *Parasitol Res* 101, 1169–1182.
- Giangaspero, A., Cirillo, R., Lacasella, V., Lonigro, A., Marangi, M., Cavallo, P., Berrilli, F., Di Cave, D., Brandonisio, O., 2009. *Giardia* and *Cryptosporidium* in inflowing water and harvested hellfish in a lagoon in Southern Italy. *Parasitol. Int.* 58,12-17.
- Giangaspero, A., Papini, R., Marangi, M., Koehler, A.V., Gasser, R.B., 2014. *Cryptosporidium parvum* genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets. *Int J Food Microbiol.* 171, 62-67.
- Giangaspero, A., Marangi, M., Arace, E., 2015a. *Cyclospora cayetanensis* travels in tap water on Italian trains. *J. Water Health.* 13, 210-216.
- Giangaspero, A., Marangi, M., Koehler, A.V., Papini, R., Normanno, G., Lacasella, V., Lonigro, A., Gasser, R.B., 2015b. Molecular detection of *Cyclospora* in water, soil, vegetables and humans in southern Italy signals a need for improved monitoring by health authorities. *Int. J. Food Microbiol.* 211, 95-100.
- Gil H, Cano L, de Lucio A, Bailo B, de Mingo MH, Cardona GA, Fernández-Basterra JA, Aramburu-Aguirre J, López-Molina N, Carmena D., 2017. Detection and molecular diversity of *Giardia duodenalis* and *Cryptosporidium* spp. in sheltered dogs and cats in Northern Spain. *Infect Genet Evol.* pii: S1567-1348(17)30055-2.

- Gillhuber, J., Pallant, L., Ash, A., Thompson, R.C., Pfister, K., Scheuerle, M.C., Molecular identification of zoonotic and livestock-specific *Giardia*-species in faecal samples of calves in Southern Germany. *Parasit Vectors*. 6, 346.
- Guidetti, C., Ricci, L., Vecchia, L., 2010. Prevalence of intestinal parasitosis in Reggio Emilia (Italy) during 2009. *Infez. Med*. 18, 154-161.
- Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005. *Giardia* in shellfish-farming areas: detection in mussels, river water and waste waters. *Vet. Parasitol*. 133, 13-18.
- Gomez-Munoz, M.T., Navarro, C., Garijo-Toledo, M.M., Dea-Ayuela, M.A, Fernandez-Barredo, S., M. T. Perez-Gracia, M.T., Dominguez-Marquez, M.V., Borrás, R., 2009. Occurrence and genotypes of *Giardia* isolated from lambs in Spain. *Parasitol. Int*. 58, 297–299.
- Gomez-Samblas, M., Vilchez, S., Racero, J.C., Fuentes, M.V., Osuna, A., 2016. *Toxoplasma gondii* detection and viability assays in ham legs and shoulders from experimentally infected pigs. *Food Microbiol*. 58, 112-120.
- Gordon, S.M., Gal, A.A., Hertzler, G.L., Bryan, J.A., Perlino, C., Kanter, K.R., 1993. Diagnosis of pulmonary toxoplasmosis by bronchoalveolar lavage in cardiac transplant recipients. *Diagn. Cytopathol*. 9,650–654.
- Gow, A.G., Gow, D.J., Hall, E.J., Langton, D., Clarke, C., Papasouliotis, K., 2009. Prevalence of potentially pathogenic enteric organisms in clinically healthy kittens in the UK. *J. Feline Med. Surg*. 11, 655–662.
- Graczyk, T.K., Cranfield, M.R., Fayer, R., 1996. Evaluation of commercial enzyme immunoassay (EIA) and immunofluorescent antibody (IFA) test kits for detection of *Cryptosporidium* oocysts of species other than *Cryptosporidium parvum*. *Am. J. Trop. Med. Hyg*. 54, 274–279.
- Griffiths, J.K., Moore, R., Dooley, S., Keusch, G.T., Tzipori, S., 1994. *Cryptosporidium parvum* infection of Caco-2 cell monolayers induces an apical monolayer defect, selectively increases transmonolayer permeability, and causes epithelial cell death. *Infection and Immunity* 62, 4506–4514.
- Grigoriev, G.A., Walmsley, S., Law, L., Chee, S.L., Yang, J., Keystone, J., Krajden, M., 1994. Evaluation of the Merifluor immunofluorescent assay for the detection of *Cryptosporidium* and *Giardia* in sodium acetate formalin-fixed stools.. *Diagn. Microbiol. Infect Dis*. 19, 89-91.

- Guo, M., Dubey, J.P., Hill, D., Buchanan, R.L., Gamble, H.R., Jones, J.L., Pradhan, A.K., 2015. Prevalence and risk factors for *Toxoplasma gondii* infection in meat animals and meat products destined for human consumption. J. Food Prot. 78, 457-476.
- Gurler, Z., Pamuk, S., Yildirim, Y., Ertas, N., 2015. The microbiological quality of ready-to-eat salads in Turkey: a focus on *Salmonella* spp. and *Listeria monocytogenes*. Int. J. Food Microbiol. 196, 79-83.
- Guzman-Herrador, B., Carlander, A., Ethelberg, S., Freiesleben de Blasio, B., Kuusi, M., Lund, V., Löfdahl, M., MacDonald, E., Nichols, G., Schönning, C., Sudre, B., Trönnberg, L., Vold, L., Semenza, J.C., Nygård, K., 2015. Waterborne outbreaks in the Nordic countries, 1998 to 2012. Eur Surveill. 20, 21160.
- Hadfield, S.J., Robinson, G., Elwin, K., Chalmers, R.M., 2011. Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of real-time PCR. J. Clin. Microbiol. 49, 918-924.
- Hall, S.M., Pandit, A., Golwilkar, A., Williams, T.S., 1999. How do Jain get *Toxoplasma* infection? Lancet. 354, 486-487.
- Hall, B.R., Oliver, G.E., Wilkinson, M., 2009. A presentation of longstanding toxoplasmosis chorioretinitis. Optometry. 80, 23-28.
- Hamnes, I.S., Gjerde, B.K., Robertson, L.J., 2006. Prevalence of *Giardia* and *Cryptosporidium* in dairy calves in three areas of Norway. Vet Parasitol. 140, 204-216.
- Hamnes, I.S., Gjerde, B.K., Robertson, L.J., 2007a. A longitudinal study on the occurrence of *Cryptosporidium* and *Giardia* in dogs during their first year of life. Acta Vet. Scand. 49, 22.
- Hamnes, I.S., Gjerde, B.K., Forberg, T., Robertson, L.J., 2007b. Occurrence of *Giardia* and *Cryptosporidium* in Norwegian red foxes (*Vulpes vulpes*). Vet. Parasitol. 143, 347-353.
- Hampton, M.M., 2015. Congenital Toxoplasmosis: A Review. Neonatal Netw. 34, 274-278.
- Harris, J.R., Petry, F., 1999. *Cryptosporidium parvum*, structural components of the oocysts wall. J Parasitol 85, 839-849.
- Heyworth, M.F., 2016. *Giardia duodenalis* genetic assemblages and hosts. Parasite 23, 13.

- Hohweyer, J., Cazeaux, C., Travaill  , E., Languet, E., Dum  tre, A., Aubert, D., Terryn, C., Dubey, J.P., Azas, N., Houssin, M., Lo  c, F., Villena, I., La Carbona, S., 2016. Simultaneous detection of the protozoan parasites *Toxoplasma*, *Cryptosporidium* and *Giardia* in food matrices and their persistence on basil leaves. Food Microbiol. 57, 36-44.
- Horman, A., Korpela, H., Sutinen, J., Wedel, H., Hanninen, M.L., 2004. Meta-analysis in assessment of the prevalence and annual incidence of *Giardia* spp. and *Cryptosporidium* spp. infections in humans in the Nordic countries. Int. J. Parasitol. 34, 1337-1346.
- Howe D.K., Sibley L.D. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis. 172, 1561-1566.
- Hunter, P., Nichols, G., 2002. Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. Clin. Microbiol. Rev. 15, 145-154.
- IFPA (International Fresh-cut Produce Association), 2016. About IFPA – Fresh cut facts. <http://www.creativew.com/sites/ifpa/fcf.html> (accessed 09.09.2016)
- Insulander, M., de Jong, B., Svenungsson, B., 2008. A foodborne outbreak of cryptosporidiosis among guests and staff at a hotel restaurant in Stockholm County, Sweden. Euro Surveill 13, 19071.
- Insulander, M., Svenungsson, B., Lebbad, M., Karlsson, L., de Jong, B., 2010. A foodborne outbreak of *Cyclospora* infection in Stockholm, Sweden. Foodborne Pathog. Dis. 7, 1585-1587.
- IsmeaMercati, 2016. IV gamma: nel 2015 crescono gli acquisti di ortaggi, 2016. <http://www.ismeamercati.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/6439> (accessed 10.11.2016)
- Jacobs, L., Remington, J.S., Melton, M.L., 1960. The resistance of the encysted form of *Toxoplasma gondii*. J. Parasitol. 46, 11-21.
- Jafari, R., Maghsood, A.H., Safari, M., Latifi, M., Fallah, M., 2015. Comparison of fecal antigen detection using enzyme linked immunosorbent assay with the auramine phenol staining method for diagnosis of human cryptosporidiosis. Jundishapur J Microbiol. 8, 16470.
- Jeddi, M.Z, Yunesian, M., Gorji, M.E., Noori, N., Pourmand, M.R., Khaniki, G.R., 2014. Microbial evaluation of fresh, minimally-processed vegetables and bagged sprouts from chain supermarkets. J Health Popul Nutr. 32, 391-399.

- Jelinek, T., Lotze, M., Eichenlaub, S., Löscher, T., Nothdurft, H.D., 1997. Prevalence of infection with *Cryptosporidium parvum* and *Cyclospora cayetanensis* among international travellers. Gut 41, 801–804.
- Jenkins, M.B., Anguish, L.J., Bowman, D.D., Walker, M.J., Ghiorse, W.C., 1997. Assessment of a dye permeability assay for determination of inactivation rates of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 63, 3844-3850.
- Jenkins, M.C, Trout, J., Abrahamsen, M.S., Lancto, C.A., Higgins, J., Fayer, R., 2000. Estimating viability of *Cryptosporidium parvum* oocysts using reverse transcriptase-polymerase chain reaction (RT-PCR) directed at mRNA encoding amyloglucosidase. J Microbiol. Methods, 43, 97–106.
- Johnston, S.P., Ballard, M.M., Beach, M.J., Causer, L., Wilkins, P.P., 2003 Evaluation of three commercial assays for detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. J. Clin. Microbiol. 41, 623–626.
- Johnston, L.M., Jaykus, L.A., Moll, D., Martinez, M.C., Anciso, J., Mora, B., Moe, C., 2005. A field study of the microbiological quality of fresh produce. J. Food Prot. 68, 1840-1847.
- Jokipii, L., Jokipii, A.M.M., 1986. Timing of symptoms and oocyst excretion in human cryptosporidiosis. New Engl. J. Med. 315, 1643–1647.
- Jones, J.L, Dubey, J.P., 2010. Waterborne toxoplasmosis—recent developments. Exp. Parasitol. 124, 10–25.
- Jones, J.L., Dubey, J.P., 2012. Foodborne Toxoplasmosis. Clin Infect Dis. 55, 845-851.
- Jones, J.L., Lopez, A., Wilson, M., Schulkin, J., Gibbs, R., 2001. Congenital toxoplasmosis: a review. Obstet Gynecol Surv. 56, 296-305.
- Jones, J.L., Lopez, A., Wilson, M., 2003. Congenital toxoplasmosis. Am. Fam. Physician. 67, 2131– 2138.
- Jung, Y., Jang, H., Matthews, K.R., 2014. Effect of the food production chain from farm practices to vegetable processing on outbreak incidence. Microb. Biotechnol. 7, 517-27.
- Kapperud, G., Jenum, P.A., Stray-Pedersen, B., Melby, K.K., Eskild, A., Eng, J., 1996. Risk factors for *Toxoplasma gondii* infection in pregnancy. Results of a prospective case-control study in Norway. Am. J. Epidemiol. 144, 405-412.

- Karaman, U., Daldal, N., Ozer, A., Enginyurt, O., Erturk, O., 2015. Epidemiology of *Cyclospora* Species in Humans in Malatya Province in Turkey. Jundishapur J. Microbiol. 8, 18661.
- Karanis P, Kourenti C, Smith H. 2007. Waterborne transmission of protozoanparasites: a worldwide review of outbreaks and lessons learnt. J. Water Health. 5, 1–38.
- Kasper, D.C., Sadeghi, K., Prusa, A.R., Reischer, G.H., Kratochwill, K., Förster-Waldl, E., Gerstl, N., Hayde, M., Pollak, A., Herkner, K.R., 2009. Quantitative real-time polymerase chain reaction for the accurate detection of *Toxoplasma gondii* in amniotic fluid. Diagn Micr. Infect. Dis. 63, 10–15.
- Khan, A., Su, C., German, M., Storch, G.A., Clifford, D.B., Sibley, L.D., 2005. Genotyping of *Toxoplasma gondii* strains from immunocompromised patients reveals high prevalence of type I strains. J Clin Microbiol. 43, 5881-5887.
- Kimura, K., Kumar Rai, S., Takemasa, K., Ishibashi, Y., Kawabata, M., Belosevic, M., Uga S, 2004. Comparison of three microscopic techniques for diagnosis of *Cyclospora cayetanensis*. FEMS Microbiol. Lett. 238, 263-266.
- Kitajima, M., Haramoto, E., Iker, B.C., Gerba, C.P., 2014. Occurrence of *Cryptosporidium*, *Giardia*, and *Cyclospora* in in fluent and effluent water at wastewater treatment plants in Arizona. Sci. Total Environ. 15, 129 –136.
- Kompalic-Cristo, A., Frotta, C., Suarez-Mutis, M., Fernandes, O., Britto, C., 2007. Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of *Toxoplasma gondii* in human peripheral blood. Parasitol. Res. 101, 619–625.
- Kong, J.T., Grigg, M.E., Uyetake, L., Parmley, S., Boothroyd, J.C., 2003. Serotyping of *Toxoplasma gondii* infections in humans using synthetic peptides. J. Infect. Dis. 187, 1484–1495.
- Kovačević, M., Burazin, J., Pavlović, H., Kopjar, M., Piližota, V., 2013. Prevalence and level of *Listeria monocytogenes* and other *Listeria* sp. in ready-to-eat minimally processed and refrigerated vegetables. World J Microbiol Biotechnol. 29, 707-712.
- Kozak, G.K., MacDonald, D., Landry, L., Farber, J.M., 2013. Foodborne outbreaks in Canada linked to produce: 2001 through 2009. J Food Prot. 76, 173-183.
- Lacasella, V., Brandonisio, O., Capolongo, C., Marangi, M., Giangaspero. A., 2013. The importance of being *Dientamoeba fragilis*. Infez. Med. 21, 1-9.

- Lainson, R., 2005. A genus *Cyclospora* (Apincomplexa; Eimeriidae), with a description of *Cyclospora schneideri* n.sp. in the snake *Anilius scytale* (Aniliidae) from Amazonian Brazil—a review. *Mern Inst Oswaldo Cruz*. 100, 103-110.
- Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Caccio, S.M., 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int. J. Parasitol.* 35,207–213.
- Lalonde, L.F., Gajadhar, A.A., 2008. Highly sensitive and specific PCR assay for reliable detection of *Cyclospora cayetanensis* oocysts. *Appl. Environ. Microbiol.* 74 , 4354–4358.
- Lalonde, L.F., Gajadhar, A.A., 2016. Detection of *Cyclospora cayetanensis*, *Cryptosporidium* spp., and *Toxoplasma gondii* on imported leafy green vegetables in Canadian survey. *Food and Waterborne Parasitology* 2, 8-14.
- Lasek-Nesselquist, E. Bogomolni, A.L., Gast, R.J., Welch, D.M., Ellis, J.C., Sogin, M.L., Moore, M.J., 2008. Molecular characterization of *Giardia intestinalis* haplotypes in marine animals: variation and zoonotic potential. *Dis. Aquat. Organ.* 81, 39–51.
- Lass, A., Pietkiewicz, H., Szostakowska, B., Myjak, P., 2012. The first detection of *Toxoplasma gondii* DNA in environmental fruits and vegetables samples. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 1101–1108.
- Laupland, K.B., Church, D.L., 2005. Population-based laboratory surveillance for *Giardia* sp. and *Cryptosporidium* sp. infections in a large Canadian health region. *BMC Infect Dis.* 5, 72.
- Lebbad, M., J. G. Mattsson, B. Christensson, B. Ljungstrom, A. Backhans, J. O. Andersson, and S. G. Svard. 2010. From mouse to moose: multilocus genotyping of *Giardia* isolates from various animal species. *Vet. Parasitol.* 168, 231–239.
- Legnani, P.P., Leoni, E., 2004. Effect of processing and storage conditions on the microbiological quality of minimally processed vegetables. *Int J Food Sci Tech.* 39, 1061-1068.
- Lemos, V., Graczyk, T.K., Alves, M., Lobo, M.L., Sousa, M.C., Antunes, F., Matos, O., 2005. Identification and determination of the viability of *Giardia lamblia* cysts and *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts in human fecal and water supply samples by fluorescent in situ hybridization (FISH) and monoclonal antibodies. *Parasitol. Res.* 98, 48-53.

- Levecke, B., Geldhof, P., Claerebout, E., Dorny, P., Vercammen, F., Cacciò, S.M., Vercruysse, J., Geurden, T., 2009. Molecular characterisation of *Giardia duodenalis* in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. *Int. J. Parasitol.* 39, 1595-1601.
- Li, N., Xiao, L., Alderisio, K., Elwin, K., Cebelinski, E., Chalmers, R., Santin, M., Fayer, R., Kvac, M., Ryan, U., Sak, B., Stanko, M., Guo, Y., Wang, L., Zhang, L., Cai, J., Roellig, D., Feng, Y., 2014. Subtyping *Cryptosporidium ubiquitum*, a zoonotic pathogen emerging in humans. *Emerg. Infect. Dis.* 20, 217-224.
- Liu, Q., Wang, Z.D., Huang, S.Y., Zhu, X.Q., 2015. Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*. *Parasit. Vectors.* 8,292.
- Llorente, M.T., Clavel, A., Goñi M.P., Varea, M., Seral, C., Becerril, R., Suarez, L., Gómez-Lus, R., 2007. Genetic characterization of *Cryptosporidium* species from humans in Spain. *Parasitol Int.* 56, 201-205.
- Lobo, M.L., Xiao, L., Antunes, F., Matos, O., 2009. Occurrence of *Cryptosporidium* and *Giardia* genotypes and subtypes in raw and treated water in Portugal. *Lett. Appl. Microbiol.* 48, 732-7.
- Long, E.G., White, E.H., Carmichael, W.W., Quinlisk, P.M., Raja, R., Swisher, B.L., Daugharty, H., Cohen, M.T., 1991. Morphologic and staining characteristics of a *Cyanobacterium*-like organism associated with diarrhea. *J. Infect. Dis.* 164, 199-202.
- Lonigro, A., Pollice, A., Spinelli, R., Berrilli, F., Di Cave, D., D'Orazi, C., Cavallo, P., Brandonisio, O., 2006. *Giardia* cysts and *Cryptosporidium* oocysts in membrane-filtered municipal wastewater used for irrigation. *Appl. Environ. Microbiol.* 72, 7916-7918.
- Lopez, A.S., Dodson, D.R., Arrowood, M.J., Orlandi, Jr. P.A., da Silva, A.J., Bier, J.W., Hanauer, S.D., Kuster, R.L., Oltman, S., Baldwin, M.S., Won, K.Y., Nace, E.M., Eberhard, M.L., Herwaldt, B.L., 2001. Outbreak of cyclosporiasis associated with basil in Missouri in 1999. *Clin. Infect. Dis.* 32, 1010-1017.
- Losio, M.N., Pavoni, E., Bilei, S., Bertasi, B., Bove, D., Capuano, F., Farneti, S., Blasi, G., Comin, D., Cardamone, C., Decastelli, L., Delibato, E., De Santis, P., Di Pasquale, S., Gattuso, A., Goffredo, E., Fadda, A., Pisanu, M., De Medici, D., 2015. Microbiological survey of raw and ready-to-eat leafy green vegetables marketed in Italy. *Int. J. Food Microbiol.* 210, 88-91.

- Luft, B.J., Conley, F., Remington, J.S., Laverdiere, M., Wagner, K.F., Levine, J.F., Craven, P.C., Strandberg, D.A., File, T.M., Rice, N., Meunier-Carpentier, F., 1983. Outbreak of central-nervous-system toxoplasmosis in Western Europe and North America. *Lancet* 1, 781–784.
- Maçin, S., Kaya, F., Çağdaş, D., Hizarcioglu-Gulsen, H., Saltik-Temizel, I.N., Tezcan İ, Demir, H., Ergüven, S., Akyön, Y., 2016. Detection of parasites in children with chronic diarrhea. *Pediatr. Int.* 58, 531-533.
- Magi, B., Canocchi, V., Tordini, G., Cellesi, C., Barberi, A., 2006. *Cryptosporidium* infection: diagnostic techniques. *Parasitol. Res.* 98, 150–152
- Maggi, P., Brandonisio, O., Larocca, A.M., Rollo, M., Panaro, M.A., Marangi, A., Marzo, R., Angarano, G., Pastore, G., 1995. *Cyclospora* in AIDS patients: not always an agent of diarrhoeic syndrome. *New Microbiol.* 18, 73–76.
- Mahon, M., Doyle, S., 2017. Waterborne outbreak of cryptosporidiosis in the South East of Ireland: weighing up the evidence. *Ir J Med Sci.* In print
- Manabe, Y.C., Clark, D.P., Moore, R.D., Lumadue, J.A., Dahlman, H.R., Belitsos, P.C., Chaisson, R.E., Sears, C.L., 1998. Cryptosporidiosis in patients with AIDS: correlates of disease and survival. *Clin. Infect. Dis.* 27, 536– 542.
- Mancianti, F., Nardoni, S., Ariti, G., Parlanti, D., Giuliani, G., Papini, R.A., 2010. Cross-sectional survey of *Toxoplasma gondii* infection in colony cats from urban Florence (Italy). *J. Feline Med Surg.* 12, 351-354.
- Mancianti, F., Nardoni, S., Mugnaini, L., Zambernardi, L., Guerrini, A., Gazzola, V., Papini, R.A., 2015. A retrospective molecular study of select intestinal protozoa in healthy pet cats from Italy. *J Feline Med Surg.* 17, 163-167.
- Manganelli, L., Berrilli, F., Di Cave, D., Ercoli, L., Capelli, G., Otranto, D., Giangaspero, A., 2012. Intestinal parasite infections in immigrant children in the city of Rome, related risk factors and possible impact on nutritional status. *Parasit. Vectors.* 5, 265.
- Manthey, M.W., Ross, A.B., Soergel, K.H., 1997. Cryptosporidiosis and inflammatory bowel disease. Experience from the Milwaukee outbreak. *Digestive Disease Science* 42, 1580–1586.
- Manzardo, C., B. Trevino, J. Gomez i Prat, J. Cabezos, E. Mongui, I. Claveria, J. Luis Del Val, E. Zabaleta, F. Zarzuela, and R. Navarro. 2008. Communicable diseases in the immigrant population attended to in a tropical medicine unit: epidemiological aspects and public health issues. *Travel Med. Infect. Dis.* 6, 4–11.

- Marangi M., Koehler A.V., Zanzani S.A., Manfredi M.T., Brianti E., Giangaspero A., Gasser R.B., 2015a. Detection of *Cyclospora* in captive chimpanzees and macaques by a quantitative PCR-based mutation scanning approach. *Parasit Vector*. 15, 274.
- Marangi, M., Giangaspero, A., Lacasella, V., Lonigro, A., Gasser, R.B., 2015b. Multiplex PCR for the detection and quantification of zoonotic taxa of *Giardia*, *Cryptosporidium* and *Toxoplasma* in wastewater and mussels. *Mol. Cell. Probes*. 29, 122-125.
- Marchioro, A.A., Tiyo, B.T., Colli, C.M., de Souza, C.Z., Garcia, J.L., Gomes, M.L., Falavigna-Guilherme, A.L., 2016. First Detection of *Toxoplasma gondii* DNA in the Fresh Leafs of Vegetables in South America. *Vector Borne Zoonotic Dis*. 16, 624-646.
- Martinez-Moreno, F.J., Hernandez, S., Lopez-Cobos, E., Becerra, C., Acosta, I., Martinez-Moreno, A., 2007. Estimation of canine intestinal parasites in Cordoba (Spain) and their risk to public health. *Vet. Parasitol*. 143, 7-13.
- Martínez-Vaz, B.M., Fink, R.C., Diez-Gonzalez, F., Sadowsky, M.J., 2014. Enteric pathogen-plant interactions: molecular connections leading to colonization and growth and implications for food safety. *Microbes Environ*. 29, 123-135.
- Mastrandrea, G., Micarelli A., 1968. Ricerca parassitaria nei prodotti vegetali prelevati da alcuni mercati rionali della città di Roma. *Arch. Ital. Sci. Med. Trop. Parassitol*. 49, 55-59.
- Masucci, L., Graffeo, R., Siciliano, R., Franceschelli, A., Bugli, F., Fadda, G., 2008. First Italian case of cyclosporiasis in an immunocompetent woman: local acquired infection. *New Microbiol*. 31, 281-284.
- Masucci, L., Graffeo, R., Bani, S., Bugli, F., Boccia, S., Nicoletti, N., Fiori, B., Fadda, G., Spanu, T., 2011. Intestinal parasites isolated in a large teaching hospital, Italy, 1 May 2006 to 31 December 2008. *Euro Surveill*, 16, 19891.
- Mattison, K., Harlow, J., Morton, J., Cook, A., Pollari, F., Bidawid, S., Faber, J.M., 2010. Enteric viruses in ready-to-eat packaged leafy greens. *Emerg. Infect. Dis*. 16, 1815-1817.
- McDonald, V., McCrossan, M.V., Petry, F., 1995. Localization of parasite antigens in *Cryptosporidium parvum*-infected epithelial cells using monoclonal antibodies. *Parasitol*. 110, 259-268.
- McKerr, C., Adak, G.K., Nichols, G., Gorton, R., Chalmers, R.M., Kafatos, G., Cosford, P., Charlett, A., Reacher, M., Pollock, K.G., Alexander, C.L., Morton, S., 2015. An Outbreak

of *Cryptosporidium parvum* across England & Scotland Associated with Consumption of Fresh Pre-Cut Salad Leaves, May 2012. PLoS One 10, e0125955.

- Medina-de la Garza, C. E., Garcia-Lopez, H.L., Salinas-Carmona, M.C., Gonzalez-Spencer, D.J., 1997. Use of discontinuous Percoll gradients to isolate *Cyclospora* oocysts. Ann. Trop. Med. Parasitol. 91, 319–321.
- Meisel, J.L., Perera, D.R., Meligro, C., Rubin, C.E., 1976. Overwhelming watery diarrhea associated with a *Cryptosporidium* in an immunosuppressed patient. Gastroenterology 70, 1156–1160.
- Melhorn, H., Frenkel, J.K., 1980. Ultrastructural comparison of cysts and zoites of *Toxoplasma gondii*, *Sarcocystis muris*, and *Hammondia hammondi* in skeletal muscle of mice. J. Parasitol, 66, 59–67.
- Mendonça, C., Almeida, A., Castro, A., de Lurdes Delgado, M., Soares, S., da Costa, J.M., Canada, N., 2007. Molecular characterization of *Cryptosporidium* and *Giardia* isolates from cattle from Portugal. Vet. Parasitol. 147, 47-50.
- Mercanoglu Taban, B., Halkman, A.K., 2011. Do leafy green vegetables and their ready-to-eat (RTE) salads carry a risk of foodborne pathogens? Anaerobe. 17, 286-287.
- Miron, D., Kenes, J.D.K., 1991. Calves as a source of an outbreak among young children in an agricultural closed community. Pediatr. Infect. Dis J. 10, 438–441.
- Mohamed, M.A., Siddig, E.E., Elaagip, A.H., Edris, A.M., Nasr, A.A., 2016. Parasitic contamination of fresh vegetables sold at central markets in Khartoum state, Sudan. Ann. Clin. Microbiol. Antimicrob. 11, 15-17.
- Monge, R., Arias, M.L., 1996. Presence of various pathogenic microorganisms in fresh vegetable in Costa Rica. Arch. Latinoam. Nutr. 46, 192–294.
- Montoya, J.G., Remington, J.S., 2008. Management of *Toxoplasma gondii* infection during pregnancy. Clin. Infect. Dis. 47, 554–566.
- Moretti, M.V., Fiorio, M., Antonelli, S., Baldelli, F., Stagni, G., Pauluzzi, S., 1988. Study on the presence of *Cryptosporidium* in Umbria. Boll. Ist. Sieroter. Milan. 67, 226–231
- Najdrowski, M., Joachim, A., Dauschies, A., 2007. An improved in vitro infection model for viability testing of *Cryptosporidium parvum* oocysts. Vet Parasitol, 150, 150-154.

- Nam, S., Lee, G., 2010. A new duplex reverse transcription PCR for simultaneous detection of viable *Cryptosporidium parvum* oocysts and *Giardia duodenalis* cysts. Biomed. Environ. Sci. 23, 146-150.
- Nazemi, S., Raei, M., Amiri, M., Chaman, R., 2012. Parasitic contamination of raw vegetables in Shahroud, Semnan. Zahedan J Res Med Sci. 14, 84-86.
- Negm, A.Y., 1998. Identification of *Cyclospora cayetanensis* in stool using different stains. J Egypt Soc Parasitol, 28, 429-436.
- Nichols, B.A., Chiappino, M.L., O'Connor, G.R., 1983. Secretion from the rhoptries of *Toxoplasma gondii* during host-cell invasion. J. Ultrastruct. Res. 83, 85-98.
- Nichols, B.A., Chiappino, M.L., Pravesio, C.E.N., 1994. Endocytosis at the micropore of *Toxoplasma gondii*. Parasitol. Res. 80, 91-98.
- Nichols, G.L., Freedman, J., Pollock, K.G., Rumble, C., Chalmers, R.M., Chiodini, P., Hawkins, G., Alexander, C.L., Godbole, G., Williams, C., Kirkbride, H.A., Hamel, M., Hawker, J.I., 2015. *Cyclospora* infection linked to travel to Mexico, June to September 2015. Euro Surveill. 20(43).
- Nicola, S., Fontana, E., Torassa, C., Hoeberechts, J., 2006. Fresh-cut Produce: Postharvest Critical Issues. Acta horticulturae. 712, 223-230.
- Nicolle, C., Manceaux, L., 1908. Sur une infection a` corps de Leishman (ou organismes voisins) du gondi. C. R. Hebd. Seances Acad. Sci. 147, 763- 766.
- Nogui, F.L., Mattas, S., Turcato, Jr.G., Lewi, D.S., 2009. Neurotoxoplasmosis diagnosis for HIV-1 patients by real-time PCR of cerebrospinal fluid. Braz. J. Infect. Dis. 13, 18-23.
- Nyarango, R.M., Aloo, P.A., Kabiru, E.W., Nyanchongi, B.O., 2008. The risk of pathogenic intestinal parasite infections in Kisii Municipality, Kenya. BMC Public Health. 8, 237.
- O'Hara, S.P., Chen, X.M., 2011. The cell biology of cryptosporidium infection. Microbes Infect. 13, 721-730.
- Okhuysen, P.C., Chappell, C.L., 2002. *Cryptosporidium* virulence determinants-are we there yet? Int J Parasitol. 32, 517-525.
- Orozco-Mosqueda, G.E., Martínez-Loya, O.A., Ortega, Y.R., 2014. *Cyclospora cayetanensis* in a pediatric hospital in Morelia, México. Am. J. Trop. Med. Hyg. 91, 537-540.

- Ortega, Y.R., Adam, R.D., 1997. *Giardia*: overview and update. Clin. Infect. Dis. 25, 545-549.
- Ortega, Y.R., Sanchez, R., 2010. Update on *Cyclospora cayetanensis*, a Food-Borne and Waterborne Parasite. Clin Microbiol Rev. 23, 218-234.
- Ortega, Y.R., Sterling, C.R., Gilman, R.H., Cama, V.A., Diaz, F., 1993. *Cyclospora* species—a new protozoan pathogen of humans. N Engl J Med 328, 1308–1312.
- Ortega, Y.R., Gilman, R.H., Sterling, C.R., 1994. A new coccidian parasite (Apicomplexa: Eimeriidae) from humans. J. Parasitol. 80,625–629.
- Ortega, Y.R., Roxas, C.R., Gilman, R.H., Miller, N.J., Cabrera, L., Taquiri, C., Sterling, C.R., 1997a. Isolation of *Cryptosporidium parvum* and *Cyclospora cayetanensis* from vegetables collected in markets of an endemic region in Peru. Am. J. Trop. Med. Hyg. 57, 683 –686.
- Ortega, Y.R., Nagle, R., Gilman, R.H., Watanabe, J., Miyagui, J., Quispe, H., Kanagusuku, P., Roxas, C., Sterling, C.R., 1997b. Pathologic and clinical findings in patients with cyclosporiasis and a description of intracellular parasite life-cycle stages. J. Infect. Dis. 176, 1584-1589.
- Ortega, Y.R., Sterling, C.R., Gilman, R.H., 1998. *Cyclospora cayetanensis*. Advances in Parasitology 40, 399–418.
- Ottoson, J., Hansen, A., Bjorlenius, B., Norder, H., Stenstrom, T.A., 2006. Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. Water Res. 40, 1449-1457
- Overgaauw, P.A., van Zutphen, L., Hoek, D., Yaya, F.O., Roelfsema, J., Pinelli, E., van Knapen, F., Kortbeek, L.M., 2009. Zoonotic parasites in fecal samples and fur from dogs and cats in the Netherlands. Vet. Parasitol. 163, 115–122.
- Palmieri, F., Cicalini, S., Froio, N., Rizzi, E.B., Goletti, D., Festa, A., Macrí, G., Petrosillo, N., 2005. Pulmonary cryptosporidiosis in an AIDS patient: successful treatment with paromomycin plusazithromycin. Int. J. STD AIDS. 16, 515-517.
- Paoletti, B., Otranto, D., Weigl, S., Giangaspero, A., Di Cesare, A., Traversa, D., 2011. Prevalence and genetic characterization of *Giardia* and *Cryptosporidium* in cats from Italy. Res. Vet. Sci. 91, 397-399
- Paoletti, B., Traversa, D., Iorio, R., De Berardinis, A., Bartolini, R., Salini, R., Di Cesare, A., 2015 Zoonotic parasites in feces and fur of stray and private dogs from Italy. Parasitol Res. 114, 2135-2141.

- Papazahariadou, M., Founta, A., Papadopoulos, E., Chliounakis, S., Antoniadou-Sotiriadou, K., Theodorides, Y., 2007. Gastrointestinal parasites of shepherd and hunting dogs in the Serres Prefecture, Northern Greece. *Vet. Parasitol.* 148, 170–173.
- Papini R, Cardini G, Paoletti B, Giangaspero A., 2007. Detection of *Giardia* assemblage A in cats in Florence, Italy. *Parasitol. Res.* 100, 653-656.
- Papini, R., Girivetto, M., Marangi, M., Mancianti, F., Giangaspero, A., 2012. Endoparasite infections in pet and zoo birds in Italy. *ScientificWorldJournal*. 2012, 253127.
- Pappas, G., Roussos, N., Falagas, M.E., 2009. Toxoplasmosis snapshots: global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. *Int. J. Parasitol.* 39, 1385-1394.
- Parish, M.E., Beuchat, L.R., Suslow, T.V., Harris, L.J., Garrett, E.H., Farber, J.N., Busta, F.F., 2003. Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive Rev. Food Sci. Food Safety*. 2, 161-173.
- Parghi, E., Dash, L., Shastri, J., 2014. Evaluation of different modifications of acid-fast staining techniques and stool enzyme-linked immunosorbent assay in detecting fecal *Cryptosporidium* in diarrheic HIV seropositive and seronegative patients. *Trop. Parasitol.* 4, 99-104.
- Peeters, J.E., Mazas, E.A., Masschelein, W.J., Villacorta Martinez de Maturana, I., Bebacker, E., 1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. *J. Appl. Environ. Microbiol.* 55, 1519–1522.
- Pereira, K.S., Franco, R.M., Leal, D.A., 2010. Transmission of toxoplasmosis (*Toxoplasma gondii*) by foods. *Adv. Food Nutr. Res.* 60, 1-19.
- Perez, C. G., P. A. Hitos, D. Romero, M. M. Sanchez, A. Pontes, A. Osuna, and M. J. Rosales. 2008. Intestinal parasitism in the animals of the zoological garden “Pena Escrita” (Almunecar, Spain). *Vet. Parasitol.* 156, 302– 309.
- Perkins, M.E., Riojas, Y.A., Wu, T.W., Le Blancq, S.M., 1999. CpABC, a *Cryptosporidium parvum* ATP-binding cassette protein at the host-parasite boundary in intracellular stages. *Proc. Natl. Acad. Sci. USA*. 96, 5734-5739.
- Plutzer, J., Karanis, P., 2016. Neglected waterborne parasitic protozoa and their detection in water. *Water Res.* 101, 318-332.

- Ponka, A., Kotilainen, H., Rimhanen-Finne, R., et al., 2009. A foodborne outbreak due to *Cryptosporidium parvum* in Helsinki, November 2008. *Euro Surveill.* 14, 19269.
- Potoka, D.A., Upperman, J.S., Zhang, X.R., Kaplan, J.R., Corey, S.J., Grishin, A., Zamora, R., Ford, H.R., 2003. Peroxynitrite inhibits enterocyte proliferation and modulates Src kinase activity in vitro. *Am. J. Physiol. Gastrointest. Liver Physiol.* 285, G861-G869.
- Puccio, G., Cajozzo, C., Canduscio, L.A., Cino, L., Romano, A., Schimmenti, M.G., Giuffrè, M., Corsello, G., 2014. Epidemiology of *Toxoplasma* and CMV serology of GBS colonization in pregnant and neonatal outcome in Sicilian population. *Ital. J. Pediatr.* 40, 23.
- Putignani, L., Menichella, D., 2010. Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. *Interdiscip Perspect Infect Dis.* 2010, 753512.
- Putignani, L., Mancinelli, L., Del Chierico, F., Menichella, D., Adlerstein, D., Angelici, M.C., Marangi, M., Berrilli, F., Caffara, M., di Regalbono, D.A., Giangaspero, A., 2011. Investigation of *Toxoplasma gondii* presence in farmed shellfish by nested-PCR and real-time PCR fluorescent amplicon generation assay (FLAG). *Exp. Parasitol.* 127, 409-417.
- R Development Core Team R, 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL <http://www.r-project.org>.
- Ramirez, N.E., Ward, L.A., Sreevatsan S., 2004. A review of the biology and epidemiology of cryptosporidiosis in humans and animals. *Microbes and Infection* 6, 773-785
- Ranjbar-Bahadori, Sh., Mostoophi, A., Shemshadi, B., 2013. Study on *Cryptosporidium* contamination in vegetable farms around Tehran. *Trop. Biomed.* 30, 193-198.
- Ramirez-Martinez, M.L., Olmos-Ortiz, L.M., Barajas-Mendiola, M.A., Giono Cerezo, S., Avila, E.E., Cuellar-Mata, P., 2015. A PCR procedure for the detection of *Giardia intestinalis* cysts and *Escherichia coli* in lettuce. *Lett. Appl. Microbiol.* 60, 517-523.
- Richardson, R.F.Jr., Remler, B.F., Katirji, B., Murad, M.H., 1998. Guillain-Barre syndrome after *Cyclospora* infection. *Muscle Nerve.* 21, 669- 671.
- Rimhanen-Finne, R., Enemark, H.L., Kolehmainen, J., Toropainen, P., Hanninen, M.L., 2007. Evaluation of immunofluorescence microscopy and enzyme-linked

immunosorbent assay in detection of *Cryptosporidium* and *Giardia* infections in asymptomatic dogs. Vet. Parasitol. 145, 345–348.

- Riner, D.K., Mullin, A.S., Lucas, S.Y., Cross, J.H., Lindquist, H.D., 2007. Enhanced concentration and isolation of *Cyclospora cayetanensis* oocysts from human fecal samples. J. Microbiol. Methods 71, 75–77.
- Ripabelli, G., Leone, A., Sammarco, M.L., Fanelli, I., Grasso, G.M., McLauchlin, J., 2004. Detection of *Cryptosporidium parvum* oocysts in experimentally contaminated lettuce using filtration, immunomagnetic separation, light microscopy, and PCR. Foodborne Pathog. Dis. 1, 216–222.
- Robertson, L.J., Gjerde, B., 2000. Isolation and enumeration of *Giardia* cysts, *Cryptosporidium* oocysts, and *Ascaris* eggs from fruits and vegetables. J Food Prot. 63, 775–778.
- Robertson, L.J., Gjerde, B., 2001. Occurrence of parasites on fruits and vegetables in Norway. J. Food Prot. 64, 1793–1798.
- Robertson, L.J., Campbell, A.T., Smith, H.V., 1992. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Applied and Environmental Microbiology 58, 3494–3500.
- Robertson, L.J., Forberg, T., Hermansen, L., Hamnes, I.S., Gjerde, B., 2007. *Giardia duodenalis* cysts isolated from wild moose and reindeer in Norway: genetic characterization by PCR-rflp and sequence analysis at two genes. J Wildl Dis. 43, 576–585.
- Robertson, L.J., Gjerde, B.K., Hansen, E.F., 2010. The zoonotic potential of *Giardia* and *Cryptosporidium* in Norwegian sheep: a longitudinal investigation of six flocks of lambs. Vet. Parasitol. 171, 140–145.
- Robertson, L.J., Casaert S., Valdez-Nava, Y., Ehsan, M.A., Claerebout, E., 2014. Drying of *Cryptosporidium* oocysts and *Giardia* cysts to slides abrogates use of vital dyes for viability staining. J. Microbiol. Methods. 96, 68–69.
- Robert-Gangneux, F., Dardé, M.L., 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev, 25, 264–296.
- Rochelle, P.A., Di Giovanni, G.D., 2014. *Cryptosporidium* oocysts in drinking water and recreational water. In: Cacciò, S.M., and Widmer, G., 2014. (Eds.) *Cryptosporidium: parasite and disease*. Springer Wien Heidelberg New York Dordrecht London.

- Rojas-Graü, M.A., Garner, E., Martín-Belloso, O., 2011. The Fresh-Cut Fruit and Vegetables Industry Current Situation and Market Trends. In: Taylor and Francis Group (eds). *Advances in Fresh-Cut Fruits and Vegetables Processing*. CRC Press. Taylor and Francis Group. New York, pp 1-11.
- Roos, D.S., Crawford, M.J., Donald, R.G., Kissinger, J.C., Klimczak, L.J., Striepen, B., 1999. Origin, targeting, and function of the apicomplexan plastid. *Curr. Opin. Microbiol.* 2, 426–432.
- Roser, D., Nielsen, H.V., Petersen, E., Saugmann-Jensen, P., Norgaard-Pedersen, B., 2010. Congenital toxoplasmosis - A report on the Danish neonatal screening programme 1999-2007. *Journal of Inher. Metab. Dis.* 33, S241–S247.
- Rostami, A., Ebrahimi, M., Mehravar, S., Fallah Omrani, V., Fallahi, S., Behniafar, H., 2016. Contamination of commonly consumed raw vegetables with soil transmitted helminth eggs in Mazandaran province, northern Iran. *Int J Food Microbiol.* 225, 54-58.
- Roy, S.L., DeLong, S.M., Stenzel, S.A., Shiferaw, B., Roberts, J.M., Khalakdina, A., Marcus, R., Segler, S.D., Shah, D.D., Thomas, S., Vugia, D.J., Zansky, S.M., Dietz, V., Beach, M.J., 2004. Emerging Infections Program FoodNet Working Group, 2004. Risk factors for sporadic cryptosporidiosis among immunocompetent persons in the United States from 1999 to 2001. *J. Clin. Microbiol.* 42, 2944–2951.
- Ruiz, A., Foronda, P., Gonzalez, J.F., Guedes, A., Abreu-Acosta, N., Molina, J.M., Valladares, B., 2008. Occurrence and genotype characterization of *Giardia duodenalis* in goat kids from the Canary Islands, Spain. *Vet. Parasitol.* 154, 137–141.
- Ryan, U., Fayer, R., Xiao, L., 2014. *Cryptosporidium* species in humans and animals: current understanding and research needs. *Parasitology* 141, 1667–1685.
- Ryan, U., Paparini, A., Monis, P., Hijjawi, N., 2016. It's official - *Cryptosporidium* is a gregarine: What are the implications for the water industry? *Water Res.* 105, 305-313.
- Rzezutka, A., Nichols, R.A., Connelly, L., et al, 2010. *Cryptosporidium* oocysts on fresh produce from areas of high livestock production in Poland. *Int. J. Food Microbiol.* 39, 96–101.
- Sacco, C., Bianchi, M., Lorini, C., Burrini, D., Berchielli, S., Lanciotti, E., 2006. Removal of *Cryptosporidium* and *Giardia* in drinking water treatment in a Tuscan area. *Ann. Ig.* 18, 117–126.

- Sacks, J.J., Roberto, R.R., Brooks, N.F., 1982. Toxoplasmosis infection associated with raw goat's milk. JAMA 248, 1728–1732.
- Saffer, L.D., Mercareau-Puijalon, O., Dubrematz, J.F., Schwartzman, J.D., 1992. Localization of a *Toxoplasma gondii* rhoptry protein by immunoelectron microscopy during and after host cell penetration. J. Protozool. 39, 526–530.
- Sagebiel, D., Weitzel, T., Stark, K., Leitmeyer, K., 2009. Giardiasis in kindergartens: prevalence study in Berlin, Germany, 2006. Parasitol. Res. 105, 681–687.
- Saghaug, C.S., Sørnes, S., Peirasmaki, D., Svärd, S., Langeland, N., Hanevik, K., 2015. Human Memory CD4+ T Cell Immune Responses against *Giardia lamblia*. Clin Vaccine Immunol. 23, 11-18.
- Sagoo, S.K., Little, C.L., Ward, L., Gillespie, I.A., Mitchell, R.T., 2003. Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. J Food Prot. 66,403-409.
- Samie, A., Bessong, P.O., Obi, C.L., Sevilleja, J.E., Stroup, S., Houpt, E., Guerrant, R.L., 2006. *Cryptosporidium* species: preliminary descriptions of the prevalence and genotype distribution among school children and hospital patients in the Venda region, Limpopo Province, South Africa. Exp Parasitol. 114, 314-322.
- Sansavini, S., Ranalli, P., 2012. Utilizzazione industriale, IV e V gamma. In: Sansavini, S., Ranalli, P., (ed). Manuale di ortofrutticoltura. Innovazioni tecnologiche e prospettive di mercato, Edagricole- New Business Media, Milano, pp 380-395.
- Sathyanarayanan, L., Ortega, Y., 2006. Effects of temperature and different food matrices on *Cyclospora cayetanensis* oocyst sporulation. J. Parasitol. 92, 218-222.
- Sayers, G.M., Dillon, M.C., Connolly, E., 1996. Cryptosporidiosis in children who visited an open farm. Commun. Dis. Rep. CDR Rev. 6, R140–144.
- Scaglia, M., Strosselli, M., Brutia, R., Gatti, S., Rondanelli, G., 1987. *Giardia* and *Giardiasis*: morphological features of the Protozoon and pathogenetic hypotheses of Human Infection. Giornale di Malattie infettive e Parassitarie. 39, 789-798.
- Scaglia, M., Gatti, S., Bassi, P., Viale, P.L., Novati, S., Ranieri, S., 1994. Intestinal co-infection by *Cyclospora sp.* and *Cryptosporidium parvum*: first report in an AIDS patient. Parasite. 1, 387-390.
- Scallan, E., Hoekstra, R.M., Mahon, B.E., Jones, T.F., Griffin, P.M., 2015. An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. Epidemiol. Infect. 143, 2795-2804.

- Schaarschmidt, F., 2007. Experimental design for one-sided confidence intervals or hypothesis tests in binomial group testing. *Comm. Biometry. Crop. Scie.* 2, 32-40.
- Scicluna, S.M., Tawari, B., Clark, C.G., 2006. DNA barcoding of *Blastocystis* Protist. 157, 77-85.
- Sergeant, ESG, 2017. Epitools epidemiological calculators. Ausvet Pty Ltd. Available at: <http://epitools.ausvet.com.au>
- Shahnazi, M., Jafari-Sabet, M., 2010. Prevalence of parasitic contamination of raw vegetables in villages of Qazvin Province, Iran. *Foodborne Pathog Dis.* 7, 1025-1030.
- Shepherd, R.C., Reed, C.L., Sinha, G.P., 1988. Shedding of oocysts of *Cryptosporidium* in immunocompetent patients. *J Clin Pathol.* 41, 1104-1106.
- Sherchand, J.B., Cross, J.H., Jimba, M., Sherchand, S., Shrestha, M.P., 1999. Study of *Cyclospora cayetanensis* in health care facilities, sewage water and green leafy vegetables in Nepal. *Southeast Asian J. Trop. Med. Public Health* 30, 58 –63.
- Shields, J. M., Olson, B.H., 2003. PCR-restriction fragment length polymorphism method for detection of *Cyclospora cayetanensis* in environmental waters without microscopic confirmation. *Appl. Environ. Microbiol.* 69, 4662–4669.
- Shlim, D.R., Cohen, M.T., Eaton, M., Rajah, R., Long, E.G., Unger, B.L.P., 1991. An alga like organism associated with an outbreak of prolonged diarrhea among foreigners in Nepal. *Am. J. Trop. Med. Hyg.* 45, 383–389.
- Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature (London).* 359, 82–85.
- Sifuentes-Osornio, J., Porras-Cortes, G., Bendall, R.P., Morales-Villarreal, F., Reyes-Teran, G., Ruiz-Palacios, G.M., 1995. *Cyclospora cayetanensis* infection in patients with and without AIDS: biliary disease as another clinical manifestation. *Clin. Infect. Dis.* 21, 1092–1097.
- Šlapeta, J., 2013. *Cryptosporidiosis* and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? *Int J Parasitol.* 43, 957-970.
- Slavin, J.L., Lloyd B., 2012. Health Benefits of Fruits and Vegetables. *Adv. Nutr.* 3, 506–516.
- Slifko, T.R., Huw V., Smith, H.V., Joan, B., Rose, J.B., 2000. Emerging parasite zoonoses associated with water and food. *Int. J. Parasitol.* 30, 1379-1393.
- Smith, H.V., Paton, C.A., Mitambo, M.M., Girdwood, R.W., 1997. Sporulation of *Cyclospora* sp. oocysts. *Appl. Environ. Microbiol.* 63, 1631-1632.

- Soave, R., Herwaldt, B.L., Relman, D.A., 1998. *Cyclospora*. Infect. Dis. Clin. North. Am. America 12, 1–12.
- Solarczyk, P., Majewska, A.C., 2010. A survey of the prevalence and genotypes of *Giardia duodenalis* infecting household and sheltered dogs. Parasitol. Res. 106:1015–1019.
- Soliva-Fortuny, R.C., Martin-Belloso, O., 2003. New advances in extending the shelf-life of fresh-cut fruits: a review. Trends Food Sci. Technol. 14, 341–353.
- Speer, C.A., Clarke, S., Dubey, J.P., 1998. Ultrastructure of the oocysts, sporocysts and sporozoites of the VEG strain of *Toxoplasma gondii*. J. Parasitol. 84, 505–512.
- Stark, D., Al-Qassab, S.E., Barratt, J.L., Stanley, K., Roberts, T., Marriott, D., Harkness, J., Ellis, J.T., 2011. Evaluation of multiplex tandem real-time PCR for detection of *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica*, and *Giardia intestinalis* in clinical stool samples. J. Clin. Microbiol. 49, 257–262.
- Sterling, C.R., Ortega, Y.R., 1999. *Cyclospora*: an enigma worth unraveling. Emerg. Infect. Dis. 5, 48–53.
- Sturbaum, G.D., Ortega, Y.R., Gilman, R.H., Cabrera, L., Klein, D.A., 1998. Detection of *Cyclospora cayetanensis* in wastewater. Appl. Environ. Microbiol. 64, 2284 –2286.
- Sturdee, A.P., Bodley-Tickell, A.T., Archer, A., Chalmers, R.M., Long-term study of *Cryptosporidium* prevalence on a lowland farm in the United Kingdom. Vet. Parasitol. 116, 97–113.
- Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A., Xiao, L., 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg. Infect. Dis. 9, 1444–1452.
- Sun, T., Ilardi, C.F., Asnis, D., Bresciani, A.R., Goldenberg, S., Roberts, B., Teichberg, S., 1996. Light and electron microscopic identification of *Cyclospora* species in the small intestine. Evidence of the presence of asexual life cycle in human host. Am. J. Clin. Pathol. 105, 216–220.
- Sunil, B., Thomas, D., Latha, C., Shameem, H., 2014. Assessment of parasitic contamination of raw vegetables in Mannuthy, Kerala state, India. Vet. World. 7, 253–256.
- Tahvildar-Biderouni, F., Salehi, N., 2014. Detection of *Cryptosporidium* infection by modified ziehl-neelsen and PCR methods in children with diarrheal samples in pediatric hospitals in Tehran. Gastroenterol Hepatol Bed Bench. 7, 125–130.

- Tamburrini, A., Pozio, E., 1999. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). International J. Parasitol. 29, 711–715.
- Taniuchi, M., Verweij, J.J., Sethabutr, O., Bodhidatta, L., Garcia, L., Maro, A., Kumburu, H., Gratz, J., Kibiki, G., Houpt, E.R., 2011. Multiplex polymerase chain reaction method to detect *Cyclospora*, *Cystoisospora*, and *Microsporidia* in stool samples. Diagn. Microbiol. Infect. Dis. 71, 386-90.
- Teixeira, L.E., Kanunfre, K.A., Shimokawa, P.T., Targa, L.S., Rodrigues, J.C., Domingues, W., Yamamoto, L., Okay, T.S., 2013. The performance of four molecular methods for the laboratory diagnosis of congenital toxoplasmosis in amniotic fluid samples. Rev. Soc. Bras. Med. Trol. 46, 584–588.
- Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30, 1217–1258.
- Thompson, R.C., Reynoldson, J.A., Mendis, A.H., 1993. *Giardia* and giardiasis. Adv Parasitol. 32, 71-160.
- Tiyo, R., de Souza, C.Z., Arruda Piovesani, A.F., Tiyo, B.T., Colli, C.M., Marchioro, A.A., Gomes, M.L., Falavigna-Guilherme, A.L., 2016. Predominance of *Giardia duodenalis* Assemblage AII in Fresh Leafy Vegetables from a Market in Southern Brazil. J Food Prot. 79, 1036-1039.
- Tram, N.T., Hoang, L.M., Cam, P.D., Chung, P.T., Fyfe, M.W., Isaac-Renton, J.L., Ong, C.S., 2008. Cyclospora spp. in herbs and water samples collected from markets and farms in Hanoi, Vietnam. Trop. Med. Int. Health. 13, 1415 –1420.
- Traversa, D., Iorio, R., Otranto, D., Modrý, D., Slapeta, J., 2008. *Cryptosporidium* from tortoises: Genetic characterisation, phylogeny and zoonotic implications. Mol. Cell. Probes. 22: 122-128.
- Troeger, H., Epple, H.J., Schneider, T., Wahnschaffe, U., Ullrich, R., Burchard, G.D., Jelinek, T., Zeitz, M., From, M., Schulzke J.D., 2007. Effect of chronic *Giardia lamblia* infection on epithelial transport and barrier function in human duodenum. Gut. 56, 328-335.
- Turatti, A., 2011. Processo di lavorazione delle insalate di quarta gamma, in: Angelini, R., Gullino, M.L., Ponti, I. (eds). Le Insalate. ART Servizi Editiriali S.p.A, Bologna, pp 420-437.

- Ul-Haq, S., Maqbool, A., Javed Khan, U., Yasmin, G., Sultana, R., 2014. Parasitic contamination of vegetables eaten raw in Lahore, Pakistan. J Zool. 46, 1303–1309.
- Upjohn, M., Cobb, C., Monger, J., Geurden, T., Claerebout, E., Fox, M., 2010. Prevalence, molecular typing and risk factor analysis for *Giardia duodenalis* infections in dogs in a central London rescue shelter. Vet. Parasitol. 172, 341–346.
- Uttah, E., Ogban, E., Okonofua C, 2013. *Toxoplasmosis*: A global infection, so widespread, so neglected. International J. S. R. P. Volume 3, Issue 6.
- van der Giessen, J.W., de Vries, A., Roos, M., Wielinga, P., Kortbeek, L.M., Mank, T., 2006. Genotyping of *Giardia* in Dutch patients and animals: a phylogenetic analysis of human and animal isolates. Int. J. Parasitol. 36: 849–858.
- Vansteelandt, S., Goetghebeur, E., Verstraeten, T., 2000. Regression models for disease prevalence with diagnostic tests on pools of serum samples. Biometrics. 56. 1126-1133.
- Varma, M., Hester, J.D., Schaefer, F.W.3rd, Ware, M.W., Lindquist, H.D., 2003. Detection of *Cyclospora cayetanensis* using a quantitative real-time PCR assay. J. Microbiol. Methods. 53, 27-36.
- Varoquaux, P., Mazollier, J., 2002. Overview of the European Fresh-cut Produce Industry. In: Lamikanra, O., (ed). Fresh-cut fruits and vegetables, Science, Technology, and Market. Taylor and Francis Group. New York, chapter 3.
- Vernile, A., Nabi, A.Q., Bonadonna, L., Briancesco, R., Massa, S., 2009. Occurrence of *Giardia* and *Cryptosporidium* in Italian water supplies. Environ. Monit. Assess. 152, 203-207.
- Verweij, J.J., Mulder, B., Poell, B., van Middelkoop, D., Brienens, E.A., van Lieshout, L., 2007. Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples. Mol. Cell. Probes 21, 400-404.
- Vesey, C.J., Peterson, W.L., 1999. Review article: the management of Giardiasis. Aliment Pharmacol Ther. 13, 843-850.
- Villena, I., Ancelle, T., Delmas, C., Garcia, P., Brezin, A.P., Thulliez, P., Wallon, M., King, L., Goulet, V., 2010. Congenital toxoplasmosis in France in 2007: First results from a national surveillance system. Euro Surveill. 15, pii: 19600.
- Visvesvara, G.S., Moura, H., Kovacs-Nace, E., Wallace, S., Eberhard, M.L., 1997. Uniform staining of *Cyclospora* oocysts in fecal smears by a modified safranin technique with microwave heating. J. Clin. Microbiol. 35, 730-733.

- Vuong, T.A., Nguyen, T.T., Klank, L.T., Phung, D.C., Dalsgaard, A., 2007. Faecal and protozoan parasite contamination of water spinach (*Ipomoea aquatica*) cultivated in urban wastewater in Phnom Penh, Cambodia. Trop. Med. Int. Health 12, 73 –81.
- Wainwright, K.E., Lagunas-Solar, M., Miller, M.A., Barr, B.C., Gardner, I.A., Pina, C., Melli, A.C., Packham, A.E., Zeng, N., Truong, T., Conrad, P.A., 2007. Physical inactivation of *Toxoplasma gondii* oocysts in water. Appl. Environ. Microbiol. 73, 5663–5666.
- Waldman, E., Tzipori, S., Forsyth, J.R., 1986. Separation of *Cryptosporidium* species oocysts from feces by using a percoll discontinuous density gradient. J. Clin. Microbiol. 23, 199-200.
- Wawrzyniak, I., Poirier, P., Viscogliosi, E., Dionigia, M., Texier, C., Delbac, F., Alaoui, H.E., 2013. *Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis. Ther. Adv. Infect. 1, 167-178.
- Weber, R., Bryan, R.T., Juranek, D.D., 1992. Improved stool concentration procedure for detection of *Cryptosporidium* oocysts in fecal specimens. J Clin Microbiol. 30, 2869–2873.
- Wells, B., Shaw, H., Innocent, G., Guido, S., Hotchkiss, E., Parigi, M., Opsteegh, M., Green, J., Gillespie, S., Innes, E.A., Katzer, F., 2015. Molecular detection of *Toxoplasma gondii* in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR. Water Res. 87, 175-181.
- Whelan, J.A., Russell, N.B., Whelan, M.A., 2003. A method for the absolute quantification of cDNA using real time PCR. J. Immunol. Methods. 278, 261–269.
- Wicki, M., Svoboda, P., Tanner, M., 2009. Occurrence of *Giardia lamblia* in recreational streams in Basel-Landschaft, Switzerland. Environ. Res. 109, 524-527.
- Wieseahn, G.P., Jarroll, E.L., Lindmark, D.G., Meyer, E.A., Hallick, L.M., 1984. *Giardia lamblia*: autoradiographic analysis of nuclear replication. Exp. Parasitol. 58, 94–100.
- Wolfe, M.S., 1992. Giardiasis. Clin Microbiol Rev 5, 93–100.
- Wong, B., Gold, J.W., Brown, A.E., Lange, M., Fried, R., Grieco, M., Mildvan, D., Giron, J., Tapper, M.L., Lerner, C.W., Armstrong, D., 1984. Central-nervous-system toxoplasmosis in homosexual men and parenteral drug abusers. Ann. Intern. Med. 100, 36–42.
- WHO (World Health Organization), 2009. Risk Assessment of *Cryptosporidium* in Drinking-water.

http://apps.who.int/iris/bitstream/10665/70117/1/WHO_HSE_WSH_09.04_eng.pdf
(accessed 20.12.2015)

- Worlund, D.D., Taylor, G., 1983. Estimation of disease incidence in fish populations. Can. J. Fish Aquat. Sc. 40, 2194-2197.
- Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: an update. Exp. Parasitol. 124, 80-89.
- Xiao, L., Feng, Y., 2008. Zoonotic cryptosporidiosis. FEMS Immunol. Med. Microbiol. 52, 309-323.
- Yadav, P., Khalil, S., Mirdha, B.R., Makharia, G.K., Bhatnagar, S., 2015. Molecular characterization of clinical isolates of *Cyclospora cayentanensis* from patients with diarrhea in India. Indian. J. Med. Microbiol. 33, 351-356.
- Yai, L.E., Bauab, A.R., Hirschfeld, M.P., de Oliveira, M.L., Damaceno, J.T., 1997. The first two cases of *Cyclospora* in dogs, São Paulo, Brazil. Rev Inst Med Trop Sao Paulo. 39, 177-179.
- Yakoob, J., Jafri, W., Beg, M.A., Abbas, Z., Naz, S., Islam, M., Khan, R., 2010. *Blastocystis hominis* and *Dientamoeba fragilis* in patients fulfilling irritable bowel syndrome criteria. Parasitol. Res. 107, 679-684.
- Zahedi, A., Monis, P., Aucote, S., King, B., Paparini, A., Jian, F., Yang, R., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2016. Zoonotic *Cryptosporidium* Species in Animals Inhabiting Sydney Water Catchments. PLoS One. 11, e0168169.
- Zajac, A. M., Johnson, J., King, S.E., 2002. Evaluation of the importance of centrifugation as a component of zinc sulfate fecal flotation examinations. Journal of American Animal Hospital Association, 38, 221-224.
- Zanzani, S.A., Gazzonis, A.L., Epis, S., Manfredi, M.T., 2016. Study of the gastrointestinal parasitic fauna of captive non-human primates (*Macaca fascicularis*). Parasitol. Res. 115, 307312.
- Zapata, F., Perkins, M.E., Riojas, Y.A., Wu, T.W., Le Blancq, S.M., 2002 The *Cryptosporidium parvum* ABC protein family. Mol. Biochem. Parasitol. 120, 157-161.
- Ziegler, G.B., Buchholz, H.M., 1975. Vital fluorochromasia of mikroorganisms using fluorescein-3',6'-diacetate (FDA). II; Communication: The influences of humoral and cellular defence-mechanisms on vitality of *Toxoplasma gondii*. Zentralbl. Bakteriolog. Orig. A. 231, 333-348.

- Zintl, A., Proctor, A.F., Read, C., Dewaal, T., Shanaghy, N., Fanning, S., Mulcahy, G., 2009. The prevalence of *Cryptosporidium* species and subtypes in human faecal samples in Ireland. *Epidemiol. Infect.* 137, 270-277.
- Zhou, Y., Lv, B., Wang, Q., Wang, R., Jian, F., Zhang, L., Ning, C., Fu, K., Wang, Y., Qi, M., Yao, H., Zhao, J., Zhang, X., Sun, Y., Shi, K., Arrowood, M.J., Xiao, L., 2011. Prevalence and molecular characterization of *Cyclospora cayetanensis*, Henan, China. *Emerg. Infect. Dis.* 17, 1887-1890.
- Zilelidou E.A., Tsourou V., Poimenidou S., Loukou A. and Skandamis P.N., 2015. Modelling transfer of *Echerichia coli* O15:H7 and *Listeria monocytogenes* during preparation of fresh-cut salads: Impact of cutting and shredding practices. *Food Microbiol.* 45, 254-265.
- Zimmerman, S.K., Needham, C.A., 1995. Comparison of conventional stool concentration and preserved-smear methods with Merifluor *Cryptosporidium*/*Giardia* Direct Immunofluorescence Assay and ProSpecT *Giardia* EZ Microplate Assay for detection of *Giardia lamblia*. *J. Clin. Microbiol.* 33, 1942-1943.

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