Universidade de Aveiro
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FABIANA SOUSA RELVAS

Monitoring of *Toxoplasma gondii* and *Cyclospora cayetanensis* in the environment

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro e do Doutor José Manuel Correia da Costa, Investigador principal com habilitação no Instituto Nacional de Saúde Doutor Ricardo Jorge, Porto.

This work was developed within the project PT national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through the project UIDB/ 00211/2020

Dedico este trabalho aos meus pais pelo incansável apoio.

o júri

presidente	Doutora Tânia Isabel Sousa Caetano Investigadora Auxiliar do Departamento de Biologia, Universidade de Aveiro
arguente	Doutora Cláudia Silva Marques Bolseira de Pós-Doutoramento, Universidade do Porto
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agradecimentos

Ao fim de um ano de muito esforço e dedicação, quero agradecer a todos os que caminharam comigo até aqui.

Em primeiro lugar, gostaria de expressar os meus sinceros agradecimentos às instituições que permitiram a realização do presente mestrado, Departamento de Biologia da Universidade de Aveiro e ao Laboratório de Parasitologia, do Departamento de Doenças Infeciosas, no Centro de Saúde Pública Doutor Gonçalves Ferreira, do Instituto Nacional de Saúde Doutor Ricardo Jorge,I.P. (INSA).

Ao meu orientador, Doutor José Manuel Correia da Costa, pela possibilidade de desenvolver o trabalho no INSA e por toda a disponibilidade demonstrada,

À Cláudia Marques, pela dedicação, disponibilidade, paciência, exigências impostas, motivação, preocupação constante, confiança e acima de tudo, pelo apoio e conhecimentos transmitidos.

À Susana, à Maria João e à Lurdes por todas as dicas para o sucesso deste trabalho, por me terem recebido tão bem e terem proporcionado um ótimo ambiente de trabalho.

Ao Dr. António Castro, pela ajuda fundamental na interpretação dos resultados da sequenciação e por todas as explicações

À minha orientadora do Departamento de Biologia da Universidade de Aveiro, Professora Doutora Sónia Mendo, por me ter possibilitado a realização desta Dissertação.

À Madalena que partilhou esta aventura comigo, do início ao fim.

À Tatiana, à Bia, à Mónica, à Aida e à Andreia, as minhas eternas companheiras da Covilhã, por todos os dias terem 5 minutos para mim e por estarem sempre lá. Obrigada por me acompanharem desde o início desta etapa e, mesmo longe, me fazerem sentir perto.

E claro, o que seria de todo o meu percurso sem os meus pais?
Obrigada pai, obrigada mãe, pelo esforço e dedicação de todos os dias.
Vocês, são, sem dúvida, os melhores.

palavras-chave

Doenças causadas por água e alimentos contaminados, *Cyclospora cayetanensis*, *Toxoplasma gondii*, Microscopia, PCR, Sequenciação,

resumo

Toxoplasma gondii e *Cyclospora cayetanensis* são protozoários, de distribuição mundial, com capacidade de infetarem o Homem. A transmissão destes protozoários ocorre, geralmente, por via oral-fecal, sendo os alimentos de origem vegetal consumidos crus e a água contaminada, assumidos, consensualmente, como fontes de contágio.

O objetivo deste estudo foi a deteção e caracterização de *Toxoplasma gondii* e *Cyclospora cayetanensis* em vegetais e água, com o intuito de avaliar o possível risco para a segurança alimentar do consumidor.

Foram recolhidas 25 amostras de uma exploração agrícola, mais especificamente, 13 amostras de água e 12 amostras de vegetais. Os potenciais oocistos de *Toxoplasma gondii* e *Cyclospora cayetanensis* foram concentrados através da filtração de água de alta resolução e por separação imunomagnética (Método 1623.1, EPA, 2012), com o intuito de remover oocistos de *Cryptosporidium* spp. e cistos de *Giardia* spp. e recuperar os parasitas de interesse: *Toxoplasma gondii* e *Cyclospora cayetanensis.* A deteção de *Toxoplasma gondii* e de *Cyclospora cayetanensis* foi realizada através de PCR convencional, utilizando primers específicos para uma sequência de 183 pb da região repetitiva de 529 pb de *T. gondii;* e para uma sequência de 116 pb da região ITS2 e uma sequência de 298 pb do gene 18S rRNA de *C. cayetanensis*. Todas as amostras de ADN positivas para PCR foram purificadas e sequenciadas. Além disso, a presença do parasita foi observada por microscopia de fluorescência, aproveitando a autofluorescência dos oocistos sob luz ultravioleta, assim como também foram utilizadas técnicas de coloração. Nenhuma das amostras mostrou estar contaminada com *T. gondii*, no entanto, 28% das amostras apresentaram resultados positivos para *C. cayetanensis* quando realizada a técnica de PCR. Esses fragmentos foram confirmados por sequenciação. Além disso, a microscopia de fluorescência e técnicas de coloração confirmaram a presença de estruturas compatíveis com oocistos de *C. cayetanensis.*

Os resultados obtidos fornecem evidências relevantes de contaminação de vegetais frescos e água com oocistos de *C. cayetanensis*.

keywords Foodborne and Waterborne diseases, *Cyclospora cayetanensis*, *Toxoplasma gondii* , Microscopy, PCR, Sequencing,

Abstract

Toxoplasma gondii and *Cyclospora cayetanensis* are protozoa, of worldwide distribution, capable of infecting humans. Transmission of these protozoa generally occurs via the oral-fecal route, with foods consumed raw and contaminated water , consensually assumed as sources of contamination.

The aim of this study was the detection and characterization of *Toxoplasma gondii* and *Cyclospora cayetanensis* in fresh vegetables and water in order to assess the possible risk to consumer food safety.

Twenty-five samples were collected from an agriculture exploration, more specifically, 13 samples of water and 12 samples of vegetables. The potential oocysts of *Toxoplasma gondii* and *Cyclospora cayetanensis* were concentrated by applying high resolution water filtration and immunomagnetic separation (Method 1623.1, EPA, 2012) in order to remove oocysts of *Cryptosporidium* spp. and cysts of Giardia spp. and recover the parasites of interest: *Toxoplasma gondii* and *Cyclospora cayetanensis*. The detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* was performed by conventional PCR, using specific primers for a 183 bp sequence of the 529 bp repetitive region of *T. gondii*; and for a 116 bp sequence of the ITS2 region and a 298 bp sequence for 18S rRNA gene of *C. cayetanensis*. All PCR-positive DNA samples were purified and sequenced. In addition, the presence of the parasite was observed by fluorescence microscopy, taking advantage of the autofluorescence of the oocysts under ultraviolet light, as well as staining techniques.

None of the samples showed to be contaminated with *T. gondii*, however, 28% of the samples showed positive results for *C. cayetanensis* when the PCR technique was performed. These fragments were confirmed by sequencing. Moreover, microscopic autofluorescence and staining techniques supported the presence of structures compatible with *C. cayetanensis* oocysts.

The results obtained provide relevant evidence of contamination of fresh vegetables and water with *C. cayetanensis* oocysts.

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List of abbreviations

A: absorptivity

AIDS: Acquired Immunodeficiency Syndrome

$Al_2(SO_4)_3$: Aluminium sulfate

ASAE: Economic and Food Security Authority

BLAST: Basic Local Alignment Search Tool

bp: base pairs

C. cayetanensis: *Cyclospora cayetanensis*

CDC: Centers for Disease Control and Prevention

95 % CI: 95% Confidence Interval

Cl: Chlorine

CT: Congenital toxoplasmosis

DNA: deoxyribonucleic acid

EFSA: European Food Safety Authority

EPA: United States Environmental Protection Agency

FA: Fluorescence Assay

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

FW: Forward

HCl: Hydrochloric acid

HIV: Human Immunodeficiency Virus

HRM: High Resolution Melting

H_2O_2: Hydrogen peroxide

Ig: Immunoglobulins

IMS: Immunomagnetic Separation

ITS: Internal transcribed spacer

LAMP: Loop-mediated isothermal amplification

NCBI: National Center for Biotechnology Information

OT: Ocular toxoplasmosis

PBS: phosphate buffered saline

PBST: phosphate buffered saline containing 0.01% Tween 20

PCR: Polymerase Chain Reaction

qPCR: real-time PCR

REV: Reverse

RNA: ribonucleic acid

rRNA: ribosomal RNA

T. gondii: *Toxoplasma gondii*

TAE: Tris, Acetic Acid and EDTA

TE: Toxoplasmic Encephalitis

USA: United States of America

UV: Ultraviolet

WHO: World Health Organization

Introduction

1. Introduction

1.1. Contextualization

Economic and Food Security Authority (ASAE) refers that about 90% of foodborne illnesses are caused by microorganisms, such as bacterias, virus and parasites. These microorganisms can be found in almost all, or even all foods, and their transmission is closely related to poor hygiene practices and errors in the distribution and preparation of food (ASAE, 2021).

In recent years, new parasites have emerged or re-emerged due to climate change, population increase, deforestation, urbanisation, irrigation and many others (EFSA (European Food Safety Authority) et al., 2020). In addition, with advances in technology, new laboratory tests are available, which allows the identification of previously undetectable parasites (ASAE, 2021).

Protozoans are responsible for causing human and animal diseases and have a substantial impact in socioeconomic burden in many developing countries (Bintsis, 2017; Erickson et al., 2006; Gajadhar et al., 2006; Nievas et al., 2020). The main sources of contamination are water and vegetables or fruits eaten raw contaminated by parasites (Castro-Hermida et al., 2008; Marques et al., 2020; Smith et al., 2007).

This can have significant problems since protozoans can cause serious consequences. So, is necessary to create and invest in methodologies capable of detecting parasites, and thus, controlling possible outbreaks, which have social and monetary consequences and, it is imperative to create rules aimed to food security and thus safety of humans and animals.

1.2. Foodborne and waterborne disease caused by parasites

World Health Organization (WHO) defines foodborne diseases as diseases that "are caused by eating food contaminated with bacteria, viruses, parasites or chemical substance such as heavy metals" and every year, nearly one in 10 people around the world fall ill after eating contaminated food, leading to over 420 000 deaths (WHO, 2021). In the United States of America (USA), the number of the foodborne outbreaks linked to the consumption of fresh produce have increased, with an estimated 14.8% in 1998 to 22.8% in 2007 (Wadamori

et al., 2016). Following the same logic, waterborne diseases are caused by ingesting water contaminated with microorganisms or chemical substances, which represents a more serious problem because it can result in a larger outbreak (Robertson, 2016).

Protozoa and helminths are the food and waterborne parasites. Protozoa are eukaryotic unicellular microscopic organisms than can be free-living or parasitic in nature. Some are able to multiply in humans and animals, which contributes to their survival and the development of serious infection from a single organism (CDC, 2020a).

Protozoa such as *Cryptosporidium* spp., *Giardia intestinalis*, *Cyclospora cayetanensis* and *Toxoplasma gondii*, are the most common parasites to be transmitted through the food chain (Dawson, 2005; Fayer et al., 2004; Gajadhar et al., 2006). In addition to transmission through contaminated fresh products and contaminated water, transmission can occur through products derived from animals (Table 1) (CDC, 2020a).

Table 1: Main parasites and major foods associated with foodborne diseases (Adapted from (ASAE, 2021; Robertson et al., 2013)).

Parasite	**Major foods associated with foodborne diseases transmission**
Toxoplasma gondii	Meat from small ruminants, pork, beef, game meat, fresh produce
Cryptosporidium spp.	Fresh produce, fruit juice, milk, water
Giardia duodenalis	Fresh produce, water
Cyclospora cayetanensis	Berries, fresh produce
Diphyllobothrium latum	Salmon and other fish
Entamoeba histolytica	Fresh produce
Ascaris spp.	Fresh produce
Anisakis simplex	Salmon, codfish and other fish
Taenia solium	Pork, fresh produce
Trichinella spiralis	Meat from boar and pork

In order to reduce diseases caused by protozoa, it is crucial to create mechanisms for detection and inactivation of parasites. However, this process is not easy. Protozoans survive for a long period of time, from days to months, in extreme conditions, thanks to the structure that protects them in exogenous stages. This allow parasites not to be affected by the common inactivation methods, used for bacterias and viruses, such as extreme temperatures, irradiation and chemical disinfectants (Erickson et al., 2006; Fayer et al., 2004; Gajadhar et al., 2006). For instance, chlorine (Cl) as sodium hypochlorite is used as a disinfectant by industrial producers of vegetables because is easy to use but, to destroy the protozoans is necessary a large amount of the product, which is not permissible. On the other hand, chlorine dioxide and the treatment with ozone are effective but hazardous to the operators. Other possibility is the disinfection by ultraviolet (UV) light because is inexpensive and fast, however, it is hard guaranteeing complete inactivation of all contaminating parasites, especially for produce with an irregular surface such as raspberries (Annunziata Giangaspero et al., 2019; Ynés R. Ortega et al., 2017).

As already mentioned, protozoa have a structure that protects them from adverse external environment. The *Toxoplasma gondii* oocyst have a bilayer thickwall with 50 nm. The outer layer has 18 nm, and the inner layer has approximately 30 nm thick. The external layer of the oocysts can be removed or permeabilized and the chemical and physical processes seems to be effective, however the inner layer seems to be stronger and harder to rupture (Dumètre et al., 2013; Ynés R. Ortega et al., 2017). Regarding to *Cyclospora* spp., the oocysts have a double layer wall with 113 nm. The outer layer has 63 nm, and the inner layer has 50 nm. Moreover, when sporulated, the sporozoites have an extra protection because the sporocysts have a cell wall, too (Y. R. Ortega et al., 1998). On other hand, pathogenic protozoa can resist the action of disinfectants due to their retention in irregularities on the surface of vegetables, in small lesions in plant tissue and in biofilms (Robertson, 2016).

In addition to disinfectants, other precautions must be considered. For example, it is known that parasites are found in the feces of several animals. Thus, the adequate removal of fecal matter from animals is recommended to prevent the transmission of parasites, from occurring through water or food. Moreover, contaminated feces, and water, can infect other animals that are implicated on parasites dissemination to other places and other animals (Gajadhar et al., 2006).

Although there is a lot of evidence that diseases caused by some parasites have harmful effects on humans, especially immunocompromised individuals, and their impact is increasing, Protozoa are a neglected group of pathogens due to the fact that they do not cause an immediate response, but rather a chronic and longer one. In addition, this type of disease is related to poorer communities but can also occur in developed countries (Bintsis, 2017; Dorny et al., 2009; Macpherson, 2005; Nievas et al., 2020; Robertson et al., 2020; Slifko et al., 2000).

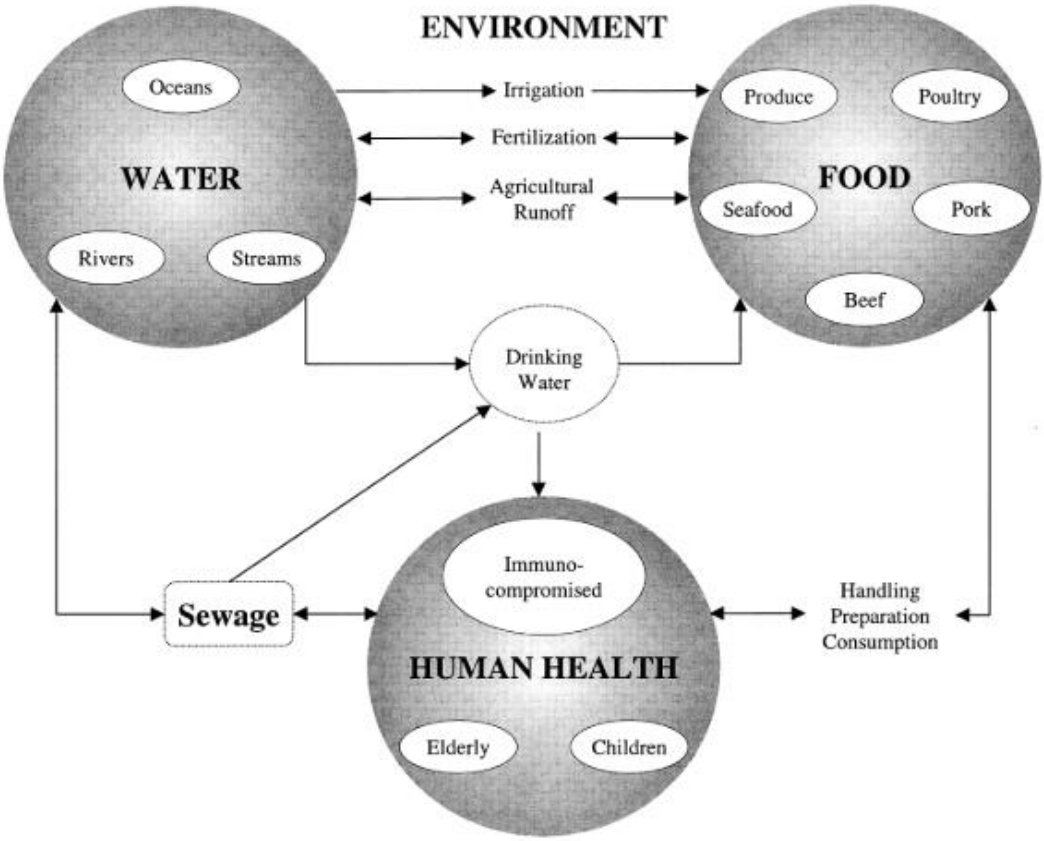

Figure 1: Food and water connection between human health and the environment (Slifko et al., 2000).

When investigating possible control methods, a vital step is to examine the nature of the human pathogenic microorganisms present in produce throughout the production process (Harris et al., 2003). The poor hygiene practices of handlers during the cultivation and processing of these vegetables are also factors that contribute to contamination (Herman et al., 2015; Robertson, 2016). It is important to adopt measures that minimize the risk of infection, such as cleaning and washing food, as well as hands and objects handled for this purpose; separate foods eaten raw to avoid cross-contamination; cook food since the heat kills parasites and store food in the refrigerator (CDC, 2020a; FDA, 2019).

Due to their health and economic effects, several programs to control these parasites have been conducted in different countries. However, in developing countries, these projects are not successful because the implementation of control actions is hampered both by the financial cost of technical measures and by the lack of educational projects that involve the

community (Hoelzer et al., 2018; Ludwig et al., 1999). In addition, to improving socio-economic conditions and general infrastructure, a partnership between industry, government and academia is one of the key aspects for the implementation, development and success of control programs (Hoelzer et al., 2018).

1.3. *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular parasite that belongs to Apicomplexa phylum (Blader et al., 2015; Dubey, 2020) and the organism was first described in 1908 by Nicolle and Manceaux, who were working in Africa on the rodent *Ctenodactylus gundii*, hence the name given to the parasite (Dubey, 2020).

T. gondii is responsible for toxoplasmosis, causing infections in humans and animals of warm blood. The disease is distributed globally and is considered to be a leading cause of death attributed to foodborne illness in the USA (CDC, 2020b; Zamora-Vélez et al., 2020) and may be responsible for an estimated 17% of the total foodborne disease burden in the European Region, affecting more than 1 million people annually through the food chain (WHO, 2017). For instance, in France, a high prevalence of infection has been related to a preference for eating undercooked or raw meat, while a high prevalence in Central America has been related to the frequency of stray cats in a climate favoring survival of oocysts and soil exposure (CDC, 2020b).

1.3.1. Life cycle

The life cycle of *T. gondii* has 2 reproductive phases, the sexual and the asexual one. The sexual life cycle occurs only in cats, more specifically, in the epithelium of the cat's intestine, when *T. gondii* differentiates into gametocytes, male and female, allowing sexual reproduction. Asexual reproduction occurs in different intermediate hosts such as man, pig and other animals (Dubey, 2020; Kochanowsky et al., 2018).

Cats are the only definitive hosts, so far known, of *T. gondii* (Zamora-Vélez et al., 2020). The release of *T. gondii* oocysts by cats, whether wild or domestic, contaminates food,

vegetables or fruits, water and other animals, which will act as a vehicle for humans to be infected (Dubey, 2020).

Unsporulated oocysts are released into the feces of infected cats in a very high amount. To become infectious, it is necessary to occur the sporulation process that takes place in the environment between 1 to 5 days after their release (Figure 2).

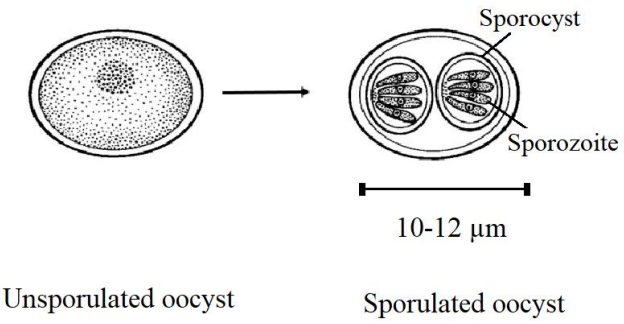

Figure 2: Unsporulated oocysts of *T. gondii* are excreted by infected cats. These oocysts are released in stool and are not infectious. To became infectious, the oocysts require 1 – 5 days to sporulate. The sporulated oocyst measure 10-12 µm in diameter and contains two sporocysts with four sporozoites each (Adapted from (Dubey, 2020; CDC, 2020c)).

When ingested, sporulated oocysts walls are removed by digestive enzymes, in the small intestine, and the sporozoites are released and infected epithelial cells (Attias et al., 2020). These sporozoites develop into tachyzoites, which replicate rapidly and spread throughout the host (Attias et al., 2020; Blader et al., 2015). In the neural and muscular tissues parasites differentiates into cysts that contain bradyzoites, which are more resistant to the immune response, allowing a persistent infection throughout the life of the host (Attias et al., 2020; CDC, 2020b; Dubey et al., 2004; Kochanowsky et al., 2018).

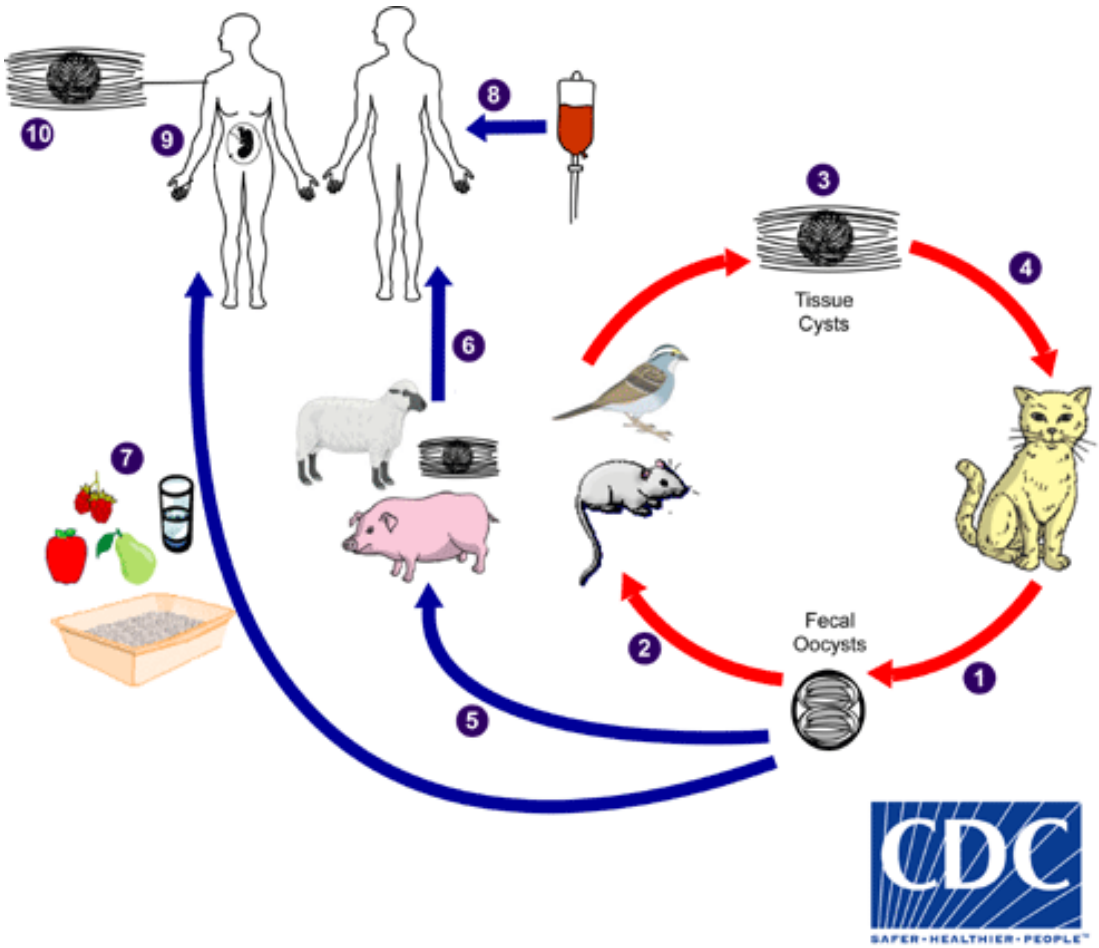

Figure 3: Life cycle of *Toxoplasma gondii*. The only known definitive hosts for *Toxoplasma gondii* are members of family Felidae. Unsporulated oocysts are released in the cat's feces (1) and takes 1–5 days to sporulate in the environment and become infective. Intermediate hosts in nature become infected after ingesting contaminated soil, water or food (2) After ingestion, sporulated oocysts released the sporozoites in the small intestine. These sporozoites develop into tachyzoites, which replicate rapidly and spread throughout the host. In neural and muscle tissue, tachyzoites differentiate into tissue cyst bradyzoites (3). Cats are infected after consuming intermediate hosts harboring tissue cysts (4) or by ingestion of sporulated oocysts. Animals bred for human consumption may also become infected with tissue cysts after ingestion of sporulated oocysts in the environment (5). Humans are infected by several routes, such as, eating undercooked meat of animals harboring tissue cysts (6); consuming food or water contaminated with cat feces or by contaminated environmental samples (7); blood transfusion or organ transplantation (8) and transplacentally from mother to fetus (9). In addition to serological tests, diagnosis can be performed by observing tissue cysts in stained biopsy samples (10) (Adapted from (CDC, 2020)).

1.3.2. Toxoplasmosis in humans

Toxoplasmosis is characterized by diarrhea, vomiting and in people with a compromised immune system can cause serious problems. In immunocompetent persons is generally an asymptomatic infection (CDC, 2020b). However, it is particularly important to monitoring this infection in pregnant women and patients with Acquired Immunodeficiency Syndrome (AIDS).

a. Congenital Toxoplasmosis

Congenital toxoplasmosis (CT) is a serious *T. gondii* infection and the most severe symptoms of the disease are related to it (Christie et al., 2004; FAO/WHO, 2014). CT results from the passage of tachyzoites from the mother to the child, through the placenta, and occurs when the pregnant woman manifests an active infection during pregnancy.

The risk of CT during pregnancy is higher when maternal infection occurs during the last trimester (Jones et al., 2003; McAuley, 2014). In the USA, approximately 1 in 10 000 live births are affected by CT (Hampton, 2015) and in Europe the overall notification rate was 5.8 cases per 100 000 live births (European Centre for Disease Prevention and Control, 2018), so it is crucial to monitor the infection as it can cause serious problems.

CT infection can occur during pregnancy in immunocompetent women who are not immune to this parasite, or by reactivation of an existing infection in immunocompromised pregnant women. In addition, IgG/IgM positive women, can be infected by new and more virulent strains (Maldonado et al., 2017; McAuley, 2014).

Many infants with subclinical infection at birth will subsequently develop signs or symptoms of CT (CDC, 2020b). This symptoms includes chorioretinitis, seizures, mental retardations, and motor or cerebellar dysfunction (Hampton, 2015). In other situations, a miscarriage may occur or a stillborn child (CDC, 2020b).

b. Ocular Toxoplasmosis

Ocular toxoplasmosis (OT) is primarily a retinal disorder (Hovakimyan et al., 2002) and is the most common etiology of posterior uveitis in many countries (Cunningham Jr et al., 2015; Ozgonul et al., 2017).

OT is more common in children and can be the result of congenital infection or infection after birth. Patients are often asymptomatic until the second or third decade of life, when lesions starts to developing in the eye (CDC, 2020b; Hovakimyan et al., 2002). In the worst case scenario it can cause blindness (Mcfarland et al., 2016).

OT is diagnosed based on the appearance of the lesions in the eye, symptoms, course of disease, and often serologic testing (CDC, 2020b).

c. Toxoplasmic Encephalitis

Toxoplasmic encephalitis (TE) causes inflammation of the brain which results from reactivation of *T. gondii* bradyzoites in tissue cysts into tachyzoites, which invade and replicate in new cells, mainly in immunocompromised patients (Milne et al., 2020). Is a rare condition in immunocompetent individuals but can occur by transplacental infection, for example (Mcfarland et al., 2016).

1.3.3. Diagnosis, Treatment and Prevention

Numerous methods have been developed for the detection of *T. gondii* in the environment and animals, but the reliability of many of these techniques is unclear. Diagnostic approaches include serology, parasite isolation and identification by traditional parasitological methods, microscopy bioassays, polymerase chain reaction (PCR) and other molecular assays to detect *T. gondii* DNA (Gajadhar et al., 2006; van Loon, 1989).

The study of immunoglobulins (Ig) is possible since infection by *T. gondii* will trigger a strong humoral immune response of the immune system (CDC, 2020b). The first test

available to detect specific antibodies anti-*T. gondii* was a Sabin-Feldman reaction (1948) (Dubey, 2020). This test is very sensitive with no evidence for false-negative results (Reiter-Owona et al., 1999). When there is a suspicion of acute infection, the patient's serum should be tested for IgG, that determines if a person is or has been infected, and IgM, that estimates the time of infection (CDC, 2020b; Dubey, 2020).

Asymptomatic patients do not require major treatments and individuals with light symptoms such as diarrhea and other gastrointestinal problems can use medication to relieve it. Pyrimethamine is considered the most effective drug against toxoplasmosis and is a standard drug used for therapy (CDC, 2020b). Garin and Eyles (1958) found that spiramycin has antitoxoplasmic activity in mice and this drug has been used in pregnant women, since it is a non-toxic substance and does not cross the placenta, not harming the fetus (Dubey, 2020). Moreover, it is important to monitor pregnant women and immunodrepressed patients to avoid worse scenarios (Maldonado et al., 2017).

Regarding to prevention, cleaning, and washing food, hands and the kitchen tools seems to be effective. As well as separate foods eaten raw to avoid cross-contamination and cook food to kill microorganisms (FDA, 2019).

1.4. *Cyclospora cayetanensis*

Cyclospora spp. are protozoan parasites, belonging to the phylum Apicomplexa, subclass Coccidiasina, order Eucoccidiorida and family Eimeriidae. Cyclosporans were first noted by Eimer (1870) in the intestine of moles and the genus was created by Schneider in 1881 (Y. R. Ortega et al., 1998).

Currently, are recognized various species of *Cyclospora*, and these parasites can infect reptiles, insectivores, and primates (Annunziata Giangaspero et al., 2019). However, *Cyclospora cayetanensis* appears to be the only species that affects humans and is apparently restricted to this host (Almeria et al., 2019; Bintsis, 2017; Mansfield et al., 2004).

C. cayetanensis is responsible for cyclosporiasis and berry fruits and fresh produce are frequently associated with outbreaks of cyclosporiasis (Ynés R. Ortega et al., 2017).

1.4.1. Life cycle

The life cycle of *C. cayetanensis* has not been fully described (Almeria et al., 2019). However, the starting point for infection is the ingestion of contaminated food or water with *C. cayetanensis* oocysts. When ingested, the sporulated oocysts release the sporozoites, in the upper gastrointestinal tract, which, in turn, will infect the cells of the epithelium of the small intestine, with greater incidence in the jejunum. The sporozoites invade the enterocytes where the sporozoites are transformed into trophozoites, which reproduce asexually, through the process of merogony to form meronts, which contain merozoites. Two types of meronts develop: type I, which contains eight to twelve merozoites, which penetrate the host cells and form type II meronts, which contain four merozoites. Once released, type II merozoites enter intestinal cells and begin the sexual phase of the cycle, through the gametogony process, in which some meronts form microgametocytes (male) or macrogametocytes (female). In turn, the microgametocytes fertilize the macrogametocytes, thus giving rise to the zygotes, which differ in unsporulated oocysts. Finally, they are released into the lumen of the intestine, passing through the feces, in a non-infectious phase, and then to the environment, where they sporulate in a 7-15 days (in the sporogony phase) and become

infectious (Figure 4) (Almeria et al., 2019; Annunziata Giangaspero et al., 2019; Hadjilouka et al., 2020; Li et al., 2019; Mansfield et al., 2004; Ynés R. Ortega et al., 2017)

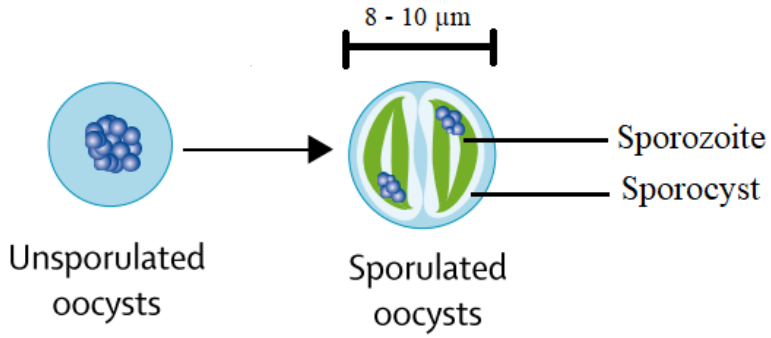

Figure 4: Unsporulated oocysts of *C. cayetanensis* are excreted by an infected human. These oocysts are released in stool and are not infectious. To became infectious, the oocyst requires 7–15 days at 22-30 °C to sporulate. The sporulated oocysts are spheroid, 8–10 µm in diameter, and contains two sporocysts, each with two sporozoites (Adapted from (Annunziata Giangaspero et al., 2019; Ynés R. Ortega et al., 2017)).

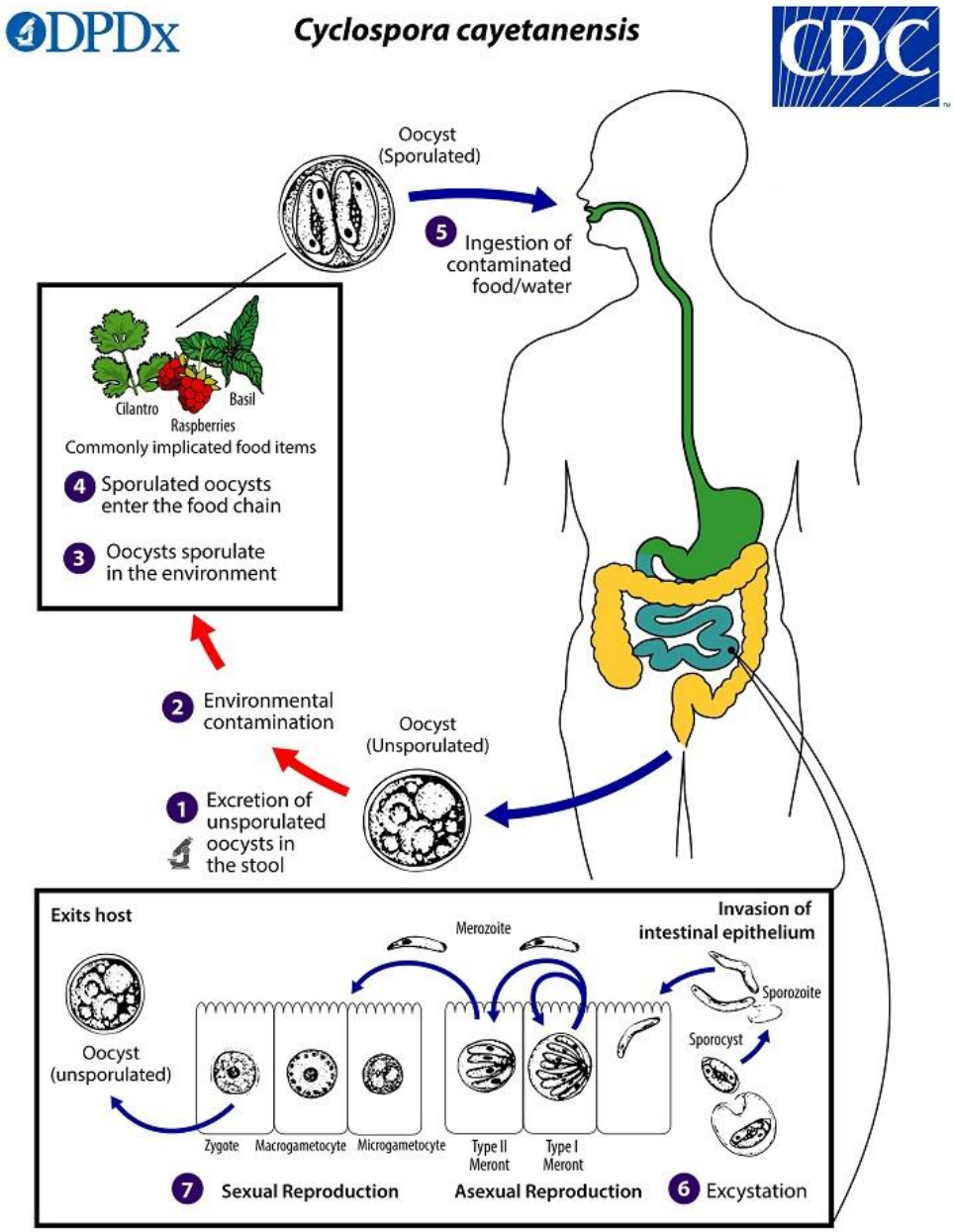

Figure 5: Life cycle of *Cyclospora cayetanensis.* Non-Infective oocysts are released (1) into the environment (2), and after several days/weeks, at temperatures between 22ºC and 32ºC, sporulation occurs, resulting in two sporocysts, each containing two elongated sporozoites (3). In this infectious phase, sporulated oocysts enter the food chain (4), contaminating fresh products and water (5). The oocysts excyst in the gastrointestinal tract, releasing the sporozoites, which invade epithelial cells in the small intestine (6). Inside the cell, they undergo asexual multiplication into type I and type II merontes. Merozoites from type I meronts remain in the asexual cycle, while merozoites from type II undergo sexual cycle into macrogametocytes and microgametocytes. Fertilization occurs, and the zygote develops to an oocyst which is released from the host cell and shed in the stool (7) (CDC, 2018).

1.4.2. Cyclosporiasis in humans

Cyclospora cayetanensis infection has been reported worldwide, in both developed and developing countries, but it is most common in tropical and subtropical areas (CDC, 2018). Cyclosporiasis is an emerging disease of public health concern mainly in the developed countries and it has been identified as the cause of several outbreaks in North America and Europe, and with traveler's diarrhea (Annunziata Giangaspero et al., 2019; Hadjilouka et al., 2020; Karanja et al., 2007).

C. cayetanensis causes prolonged illness and the disease can last more than 6 weeks, both in immunocompromised and immunocompetent patients. In general, children, foreigners, and immunocompromised patients in endemic developing countries will be the most vulnerable to *C. cayetanensis* infection (Almeria et al., 2019). Children (<10-20 years) account for about 70% of infections, and 72-94% of these children are asymptomatic (Helmy, 2012). Cyclosporiasis is more severe in immunocompromised hosts, in particular in Human Immunodeficiency Virus (HIV)-infected patients (Almeria et al., 2019). The symptoms are watery diarrhea, nausea, vomiting, anorexia, abdominal pain, fever and fatigue (Almeria et al., 2019; Li et al., 2019). In addition, *C. cayetanensis* can also cause respiratory infection with the presence of cough, expectoration of purulent sputum and dyspnea (Helmy, 2012).

The clinical cases reported and studied over time, showed that the consumption of fresh products (fruits, herbs and vegetables) is the main source of contamination, followed by water, generally in developing countries, and there is not enough knowledge about zoonotic transmission (Almeria et al., 2019; Annunziata Giangaspero et al., 2019).

1.4.3. Diagnosis, Treatment and Prevention

Diagnosis is usually performed through the analysis of the patient's symptoms and complaints (Annunziata Giangaspero et al., 2019). At a hospital level, *Cyclospora* infection is diagnosed by examining stool specimens, using modified acid-fast staining or modified safranin technique (hot method) staining procedures (CDC, 2018), however some patients with symptoms might not shed enough oocysts in their stool to be readily detectable by laboratory examination (CDC, 2018; Helmy, 2012). The study can also be carried out with the aid of a epifluorescence microscope, since the parasite emit autofluorescence in the UV range (Figure 6) (Almeria et al., 2019; Li et al., 2019; Ynés R. Ortega et al., 2017).

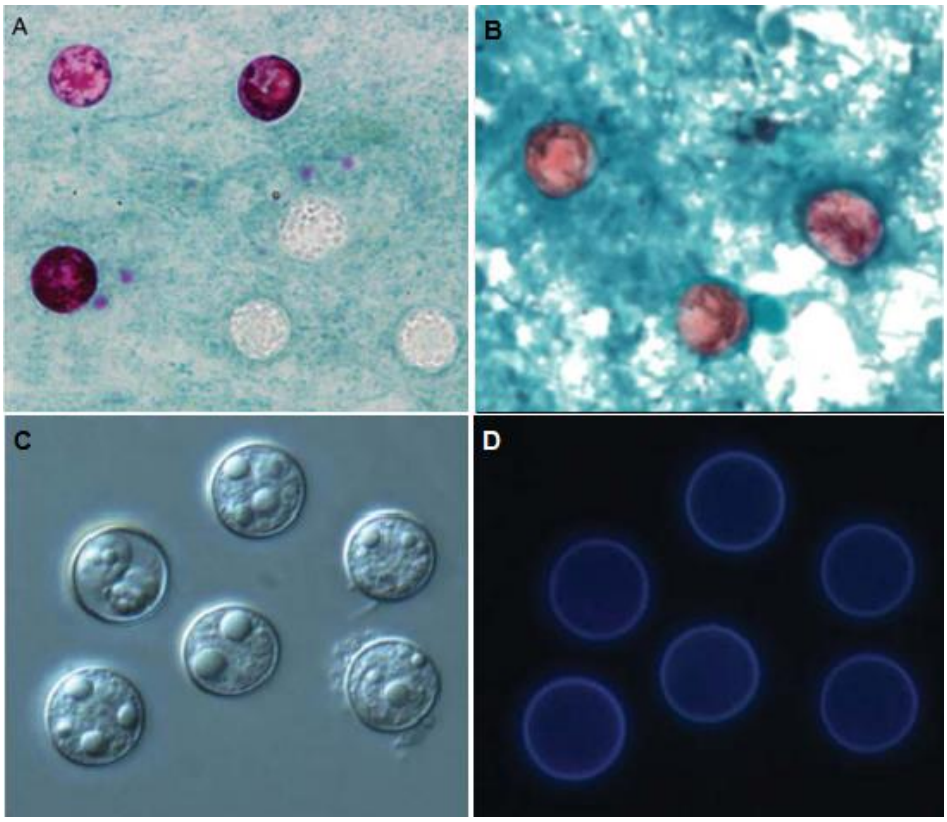

Figure 6: Morphology of *C. cayetanensis* oocysts under microscopy. Oocysts in stool smears stained with modified acid-fast stain (A) and with modified safranin technique (hot method) procedure (B) under light microscopy; oocysts observed by differential interference contrast microscopy of wet mounts (C); Autofluorescent oocysts observed by epifluorescence microscopy with a 330–380 nm UV excitation filter (D) (Adapted from (Li et al., 2019; CDC, 2018)).

Molecular techniques can also be useful, depending on the specificity and sensitivity of the chosen method since they can effectively detect a low parasite load or DNA from a single oocyst, overcoming the limitations of microscope diagnostics (Li et al., 2019). For instance, PCR method can be used to amplify specific fragments of the parasite, such as 18S ribosomal RNA (rRNA) gene and specific sequences based on Internal Transcribed Spacer (ITS) can be used, too (Lalonde et al., 2008; Murphy et al., 2017). ITS sequences are noncoding regions between the 18S, 5.8S, and 28S rRNA genes: ITS1 between the 18S and 5.8S rRNA genes and ITS2 between the 5.8S and 28S rRNA genes (Plutzer et al., 2016).

Patients are usually treated with the administration of trimethoprim / sulfamethoxazole for one week, which has been shown to be effective in relieving the symptoms of cyclosporiasis and in eliminating oocysts in the stool (Ynés R. Ortega et al., 2017).

In order to prevent infection, individuals should be careful about what they eat, cleaning and washing the food products properly and the tools used for that effect. Moreover, it is important to monitor patients with symptoms after traveling to endemic areas in order to prevent outbreaks (CDC, 2018).

1.5. Recovery and detection of *T. gondii* and *C. cayetanensis* in vegetables and water: Data available

The detection of *T. gondii* and *C. cayetanensis* oocysts is a non-consensual and exigent process and until now there is not a validated and universal method. (Slifko et al., 2000). The recovery and concentration of *T. gondii* oocysts in water and vegetables can be performed through the process of flocculation and filtration, using cellulose membranes (A. Lass et al., 2012; Anna Lass et al., 2019; Sánchez et al., 2018). In addition, other authors suggest using a stomaching process with a glycine buffer, to extract oocysts from a solid sample, such as vegetables (Lalle et al., 2017) or even through the formalin / ether method, which has percent recoveries between 76-90% (Lora-Suarez et al., 2016). Recently, Marques et. al, used the Filta-Max® System (IDEXX), to filter large volumes of washing water, which increases the oocyst recovery. Moreover, they also performed the Method 1623.1. This method seems to be an advisable strategy for removing *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts and separating them from potential *T. gondii* (and probably *C.*

cayetanensis) oocysts present in the sample washing water (EPA, 2012; Marques et al., 2020). However, centrifugation seems to be the most common method to recover *T. gondii.*

T. gondii DNA can be detected using real-time PCR (qPCR) coupled to TaqMan probes or SYBR Green (Marques et al., 2020), or conventional PCR to detect the B1 gene or 529 bp repetitive region of *T. gondii* (A. Lass et al., 2012; Anna Lass et al., 2019; Plutzer et al., 2016; Sánchez et al., 2018; Villena et al., 2004). Another method is the Loop-mediated isothermal amplification (LAMP), which is a much more specific method since it uses 6 specific primers, which reduces the possibility of non-specific amplifications (Lalle et al., 2017; Plutzer et al., 2016). Oocysts can be observed by microscopy through the analysis of the protozoan morphology and autofluorescence (CDC, 2020b; Duedu et al., 2014; Lora-Suarez et al., 2016; Slifko et al., 2000). Nevertheless, sequencing the PCR fragments also confirms the specificity of PCR (Marques et al., 2020)

The recovery and concentration of *C. cayetanensis* oocysts can be performed by using cellulose membranes to filter the parasites, or using the Envirochek capsule and Haniffin polypropylene cartridge filter (Quintero-Betancourt et al., 2002; Sánchez et al., 2018). Centrifugation also seems to be effective. For *C. cayetanensis* DNA detection, Murphy el. al, used two types of PCR: nested-PCR and qPCR (Murphy et al., 2017). A. Giangaspero et al., also used the qPCR protocol coupled with high resolution melting (HRM) analysis to test samples of tap water from the toilets on trains (A. Giangaspero et al., 2015). Sequences of the 18S rRNA gene or the ITS2 region of *C. cayetanensis* are normally used in qPCR and nested-PCR (Durigan et al., 2020; Temesgen et al., 2019). The detection of *C. cayetanensis* oocysts can be performed by using microscopy techniques like epifluorescence microscopy and staining procedures (Quintero-Betancourt et al., 2002).

Goals

2. Goals

The aim of this study was the detection and characterization of *Toxoplasma gondii* and *Cyclospora cayetanensis* in fresh vegetables and water in order to assess the possible risk to consumer food safety.

With this objective, a work plan was elaborated with the following steps:

a. Filter and concentrate *T. gondii* and *C. cayetanensis* oocysts present in environmental samples;
b. Extract *T. gondii* and *C. cayetanensis* oocysts DNA;
c. Detect *T. gondii* DNA using the conventional PCR;
d. Detect *C. cayetanensis* DNA using the conventional PCR and nested-PCR;
e. Observe oocysts-like structures by microscopy;
f. Characterization of *T. gondii* and *C. cayetanensis* by DNA sequencing.

Materials and Methods

3. Materials and Methods

3.1. Sampling

Samples were collected in September 2020, from a large-scale agricultural exploration in Portugal. In the present study, a total of 25 samples were analysed, more specifically, 13 samples of water and 12 samples of vegetables. The food products included different types of lettuce (*Lactuca sativa*), arugula (*Eruca vesicaria sativa*) and spinach (*Spinacia oleracea*). The vegetables samples were transported to the parasitology laboratory, in sterile and properly labelled bags and were stored in the cold until analysis.

Regarding the samples of water and sand, these were collected at specific points, considering the flow of water, from the dam to the irrigation of the vegetables, as well as water samples after several treatments (Table 2 and Table 3).

First, water was collected from the Dam (sample 1), in Portugal. The Dam water is conducted to the agriculture exploration into a water channel (sample 2) and finally directed to the reservoirs where water is retained. At this stage, there are two reservoirs: the reservoir used for conventional agriculture system (sample 3) and the reservoir used for organic agriculture system (sample 4). These are the stored waters that are going to be used to irrigate the vegetables through sprinklers (sample 5).

After storage, the water undergoes physical and chemical treatments. The first one is the flocculation. In this process, a chemical coagulant, aluminium sulfate ($Al_2(SO_4)_3$), is added to the water which facilitates the bond between particles, forming aggregates that are easy to separate (Bridle et al., 2014). Then, the water passes through sand and coal filters, in which the small solid particles that may still exist are retained (SIMDOURO, 2017). After the treatments that allow particles to be separated from the water, a treatment of disinfection through Cl is performed and the water is stored in 2 tanks: tank 1, with potable water, and tank 2, where the water undergoes an extra treatment with hydrogen peroxide (H_2O_2). This treated water is used to wash the vegetables, for later distribution for the population. Sampling include collection water after $Al_2(SO_4)_3$ treatment (sample 6), after passing through the sand filter (sample 9 and 10), after Cl treatment (sample 11 and 12) and after H_2O_2 treatment (sample 13). In addition, 36g a 38 g of sand were collected from the sand/coal filter systems (sample 7 and 8) (Table 3).

Table 2: Non-treated water collected. The table shows the volume of water collected in each point.

Sample	Product/ Local	Volume (L)
1	Dam	80
2	Channel	100
3	Reservoir used for conventional agriculture system	100
4	Reservoir used for organic agriculture system	50
5	Sprinklers	100

Table 3: Treated water and sand samples collected. The table shows the volume of water collected in each point. For the sand samples, the sample weight is also represented.

Sample	Product/Local	Volume (L)	Weight (g)
6	Post-treatment $Al_2(SO_4)_3$ flocculation / Pre-sand filter	90	-
7	Sand (1)	10	36
8	Sand (2)	10	38
9	Post-sand filter (1)	100	-
10	Post-sand filter (2)	90	-
11	Post-disinfection Cl/ Potable water (Tank 1)	90	-
12	Tank 1 (Potable Water)	30	-
13	Tank 2 (Disinfection with H_2O_2)	40	-

Fresh vegetables samples were vigorously washed, in large volumes of distilled water. Approximately 20 L of water was used for each 1 kg of sample and the weight of the samples ranged from 500g to 1Kg (Table 4). This process was carried out in the parasitology laboratory.

Table 4: Conventional and organic fresh vegetables samples collected. The table shows the volume of water used to wash the samples and if they been pre-washed or not.

Sample	Product/ Local	Agriculture system	Pre-Wash	Volume (L)	Weight (g)
14	Iceberg lettuce	Conventional	Yes	20	1000
15	Riva salad	Conventional	Yes	20	1000
16	Spinach	Conventional	Yes	20	1000
17	Spinach	Organic	Yes	20	930
18	Wild arugula	Conventional	Yes	20	1000
19	Red lettuce	Organic	No	20	953
20	Green lettuce	Conventional	No	10	500
21	Red lettuce	Conventional	No	10	500
22	Green lettuce	Organic	No	10	500
23	Wild arugula	Conventional	No	20	1000
24	Spinach	Organic	No	20	963
25	Iceberg lettuce	Conventional	No	20	1000

3.2. Recovery of *Toxoplasma gondii* and *Cyclospora cayetanensis*

In order to identify and recovery the protozoa, United States Environmental Protection agency (EPA) Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/ Immunomagnetic Separation (IMS)/ Fluorescence Assay (FA) was used.

This step is important because it allows to separate different parasites, removing *Giardia* spp. and *Cryptosporidium* spp. and concentrating the protozoa of interest: *Toxoplasma gondii* and *Cyclospora cayetanensis*.

3.2.1. Filtration

Water filtration allows the oocysts, cysts and extraneous materials to be retained on the filter (EPA, 2012). The collected water samples, they were directly filtered on site while the filtration of the water used for washing the fresh vegetables was carried out in the laboratory (Figure 7).

Figure 7: Filtration of the water used to wash the vegetable sample was performed on the laboratory (capture by Parasitology laboratory team).

For water filtration, a 1 µm Filta-Max® filter (IDEXX, West-brook, ME, USA) (Figure 8) applied to a peristaltic pump at four bars was used. After the entire sample was passed through the filter, the peristaltic pump was turned off. Then, the filter was removed, identified, and kept it in the cold for later elution.

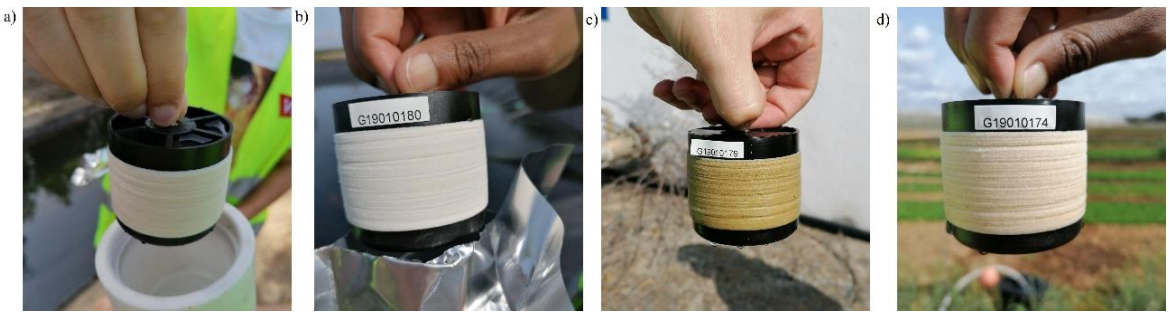

Figure 8: Filters after water filtration of a) water of channel; b) reservoir used for irrigation in conventional agriculture system; c) reservoir used for irrigation in organic agriculture system; d) sprinklers (capture by Parasitology laboratory team).

3.2.2. Elution

Elution is a crucial step and is performed to wash the oocysts from the filter using the Filta-Max® manual wash station (Figure 9) (Berrouch et al., 2020; EPA, 2012).

First, the membrane filter (3- µm pore size; polysulphone) was placed in the base of the sample concentrator and inserting the filter module into the apparatus. Next, 600 ml of phosphate buffered saline (PBS) containing 0.01% Tween 20 (PBST) was added to the reservoir and the filter housing to allow expansion of the foam pads was unscrewed (Castro-Hermida et al., 2009; EPA, 2012).

The foam pads were washed by moving the plunger, smoothy, fully up and down 20 times and, once complete, pushed the plunger handle back to lock into place. The entire volume was filtered under vacuum. The process was repeated with a second volume (600 ml) of PBST, where the foam pads were again washed by pumping the plunger 10 times, and the resulting concentrate was filtered under vacuum.

The filter membrane was transferred to a small sealable plastic bag, and 8–10 ml of PBST was added. The membrane was shaken manually and in the shaking plate to ensure that the oocysts and cysts present come off the membrane and remain in the solution. The solution was resuspended with 50 ml of distilled water and centrifuged at 1500x g for 10 min (durafuge 300R, Thermo Electron Corporation, USA).

Finally, the supernatant was aspirated leaving 3 ml of sediment and supernatant.

Figure 9: Elution was performed in a Filta-Max® manual wash station using a membrane filter (3-µm pore size) (capture by parasitology laboratory team).

3.2.3. Immunomagnetic Separation (IMS)

The IMS allows to separate the protozoa of interest (*T. gondii* and *C. cayetanensis*) by removing the *Giardia* cysts and *Cryptosporidium* oocysts. Dynabeads coated with affinity-purified antibodies against specific surface markers of *Giardia* and *Cryptosporidium* were used (Dynabeads^{TM} GC- Combo, Thermo Fisher Scientific, Waltham, MA, USA).

The sediment obtained in the elution step was transferred to a Leighton tube, containing 1 ml of 10x SL buffer A and 1 ml of 10x SL buffer B, 60 µl Dynabeads anti-*Cryptosporidium* and 60 µl Dynabeads anti-*Giardia*, and were incubated for 1h 30min at room temperature with constant rotation using a Sample Mixer (Dynal®, Norway). Then, the Leighton tubes were placed in a magnetic particle concentrator (Dynal MPC®-1), with the flat side of the tube toward the magnet, and gently rocked for 20-30 times. After this time, the cap was removed with the flat side of the tube on top and all the tube supernatant was kept for later detection of *Toxoplasma gondii* and *Cyclospora cayetanensis*. The tubes were removed from the magnetic particle concentrator, and 1 ml of 1x SL buffer A was added to each. The tubes were gently rocked to resuspend the bead-(oo)cyst complexes; the suspension was transferred to 1.5 ml eppendorfs, with a glass Pasteur pipette, and these tubes were placed in a second magnetic particle concentrator (Dynal MPC®-S) and rocked for 1 min. The supernatants from the tubes were aspirated without disturbing the material attached to the wall of the tube adjacent to the magnet and were added to the previous one for later detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* (Castro-Hermida et al., 2009; EPA, 2012).

3.3. *Toxoplasma gondii* and *Cyclospora Cayetanensis* detection

3.3.1. DNA extraction

The DNA extraction step was carried out with the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany).

Two hundred µl of supernatant containing potential *T. gondii* and *C. cayetanensis* were transferred to 2 ml eppendorfs, centrifuged them at 3500 x g for 10 minutes at room

temperature and the supernatant was discarded. Then, 200 µl of Buffer ATL (Qiagen) was added to the pellet. The disruption of the oocyst cell wall was performed 4 freeze (− 20 °C for 10 minutes)/thaw (95 °C for 5 minutes) cycles. After that, it was added 20 µl Proteinase K (Qiagen) and the samples were incubated overnight at 56ºC. The samples were then treated with 1 ml of InhibitEX buffer (Qiagen), that efficiently removes PCR inhibitors commonly present in environmental samples (Marques et al., 2020), vortexed and incubated at 95ºC for 10 minutes. Then, the samples were centrifuged at full speed for 2 minutes and 400 µl of the supernatant was transferred to a 1.5 ml eppendorf where 200 µl of lysis Buffer AL (Qiagen) was added and vortexed for 15 seconds. The eppendorfs were incubated at 70ºC for 10 minutes and centrifuged briefly. After that, 200 µl of ethanol (96-100%) was added, vortexed for 15 seconds and centrifuged briefly. The mixture was pipetted into the QIAmp Mini spin column (in a 2 ml collection tube) and centrifuge at 6000 x g for 1 min and the flow-through and collection tube were discarded. Next, the QIAmp Mini spin column was placed in a new 2 ml collection tube and added 500 µl Washing Buffer AW1 (Qiagen). The spin column was centrifuged at 6000 x g for 1 min and the flow-through and collection tube were again discarded. Then, the QIAmp Mini spin column was placed in a new 2 ml collection tube and 500 µl Washing Buffer AW2 (Qiagen) was added and centrifuged at full speed for 3 min. The supernatant was discarded and the QIAmp Mini spin column was placed in the same 2 ml collection tube and centrifuged at full speed for 1 min. Finally, the QIAmp Mini spin column was placed in a new 1,5 ml eppendorf and 200 µl of Elution Buffer AE (Qiagen) was added. Next, the eppendorfs were centrifuged at 6000 x g for 3 min to elute the DNA.

To avoid degradation of the genetic material, the eppendorfs were kept in the freezer (-20º C) until use (QIAGEN, 2016).

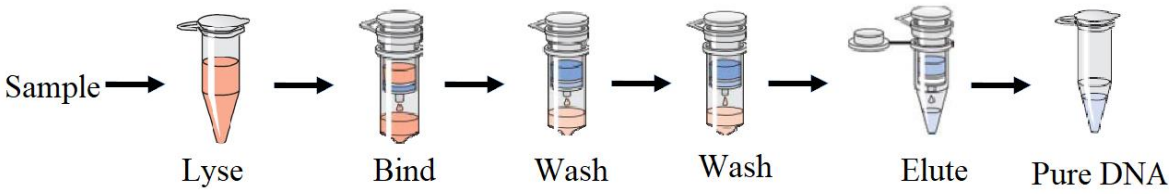

Figure 10: Schematic representation of the DNA extraction. The lyse was performed by doing 4 freeze/thaw cycles and by using Proteinase K. The binding of the genetic material to the QIAamp silica membrane was possible thanks to a brief centrifugation. DNA bound to the membrane was washed in 2 centrifugations, using 2 different wash buffers: Buffer AW1 and Buffer AW2. Finally, purified DNA was eluted from the QIAamp Mini spin column in a concentrated form of Buffer AE (Adapted from (QIAGEN, 2016)).

3.3.2. *Toxoplasma gondii* detection

3.3.2.1. Conventional PCR

The extracted DNA was analysed by conventional PCR method using specific primers (FW: 5′-AGC CAC AGA AGG GAC AGA AG-3′ and REV: 5′-TCC AGG AAA AGC AGC CAA G-3′) targeting a 183 bp sequence of the 529 bp repetitive region of *T. gondii* (Marques et al., 2020). This fragment is repeated 200 to 300 times, which increases the specificity of the reaction (Homan et al., 2000). Two different enzymes were tested to minimize possible enzymes inhibition.

3.3.2.1.1. With Xpert Fast HotStart MasterMix (2x) with dye (GRiSP Research Solutions, Portugal)

Xpert Fast HotStart MasterMix (2x) with dye (GRiSP Research Solutions, Portugal) is a robust enzyme, suitable for the amplification of difficult targets (GRiSP Research Solutions, 2017).

The amplification reaction mixture consisted of 12.5 µl Xpert Fast Hot Start Master Mix (2x) with dye (GRiSP Research Solutions, Portugal), 500 nM of each primer (Eurofins Genomics, Germany) and 5 µl of sample DNA in a 25 µl reaction volume. Cycling conditions

for amplification were 95ºC for 3 min, 40 cycles of denaturation at 95ºC for 15 s, annealing at 62ºC for 15 s, and extension at 72ºC for 15 s, and a final extension at 72ºC for 5 min.

In all PCR experiments, a positive control (genomic DNA isolated from *T. gondii* ME49 oocysts kindly providing by J. P. Dubey, USDA, ARS, Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center) and a negative control (water template) were used.

After the PCR reaction, an agarose gel electrophoresis was performed on 2% agarose gel (GRS Agarose LE, GRiSP Research Solutions, Portugal), in TAE 1X (Tris, Acetic Acid and EDTA) (GRiSP Research Solutions**,** Portugal). This mixture is heated in the microwave, until the agarose is completely dissolved and cooled down to 55-60º C. Then, 5 µL of Xpert Green DNA strain (GRiSP Research Solutions, Portugal) was added to allow viewing of PCR products because when excited by ultraviolet light, it will emit fluorescence. This final mixture was gelled at room temperature.

Each well of the agarose gel was loaded with 10 µl of the PCR product and the molecular weight marker, GRS Ladder 100bp (GRiSP Research Solutions, Portugal) was placed in one of the wells of the gel. Then the electrophoresis system was connected to a power supply at 120V, approximately 45 minutes.

Finally, the visualization of the PCR products on the agarose gel was performed on a transilluminator with UV light (Gel Doc ™ XR + System with Image Lab ™ Software, BIO-RAD).

3.3.2.1.2. With DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA)

DreamTaq Hot Start DNA Polymerase has been engineered to provide increased sensitivity and specificity (ThermoFisher Scientific, 2016).

The amplification reaction mixture consisted in 12.5 µl DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA), 500 nM of the same primers used in the previous reaction and 5 µl of sample DNA in a 25 µl reaction volume. PCR amplification was performed with an initial polymerase activation step (3 min at 95 °C), followed by 35 cycles of denaturation (30 s at 95 °C), annealing (30 s at 62 °C) and extension (30 s at 72

°C), followed by a final extension step of 10 min at 72 °C; Amplifications were performed in a T100 ™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) and PCR products were observed using the same protocol as the previous reaction.

3.3.2.2. PCR from previously amplified product

The concentrations of *T. gondii* can be very low, so a PCR may not be sufficient for its detection in agarose gel. To overcome this, a PCR from the previously amplified product can be performed. The amplification reaction mixture consisted of 12.5 µl DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA), 500 nM of each primer (Eurofins Genomics, Ebersberg, Germany) and 1 µl of previously amplified product, in a 25 µl reaction volume. PCR amplification was performed following the same conditions as conventional PCR with DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA), as well as agarose gel and visualization of results.

3.3.2.3. Gel Band Purification

The positive amplification fragments resulting from the second PCR reaction were purified using the GRS PCR & Gel Band Purification kit (GRiSP Research Solutions, Portugal). Briefly, a agarose gel electrophoresis on 2% low melting gel (Agarose LM sieve, CONDA, Madrid) in TAE 1X, was performed, and the positive DNA bands were cut from the gel in a SXT-F20.M Transilluminator (Uvitec, France) with the aid of a scalpel and transferred to a 1.5 ml eppendorf. 500 µL of Gel Solubilization Solution was added and the eppendorfs were vortexed. Then, the samples were incubated at 60ºC for 10 minutes. A mini spin column was placed in a 2 ml collection tube and the entire content resulting from de previous step, was transferred to the collection tube, and was centrifuged at 16 000x g for 1 minute. Then, the collection tube was discarded and a new one was placed in the column. 600 µL of Wash Buffer 2 was added and the tubes were incubated at room temperature for 1 minute and centrifuged at 16 000x g for 1 minute. The liquid was discarded and placed in the same collection tube for a final centrifugation for 3 minutes at 16 000x g. Finally, the spin column was transferred to a 1.5 ml eppendorf and 40 µL of Elution Buffer was added

directly in the centre, carefully, without touching the membrane and incubated for 5 minutes at room temperature. Then, the eppendorfs were centrifuged for 2 minutes at full speed to elute purified DNA, the spin column was discarded, and the DNA purified was stored in the freezer until use.

3.3.2.4. Spectrophotometric measurements

It is important to know the DNA concentrations of each sample to be sequenced, since a minimum concentration is required. The desired DNA concentration for fragments with 150 to 300 bp is 2 ng/µl (Eurofins Genomics, 2021). Moreover, is crucial to know the quality and purity of DNA.

NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) is the trademark of spectrophotometer that can measure a wide range of absorbance with just 1-2 µl with high accuracy and reproducibility.

Two µl of the purified DNA was pipetted into the measurement pedestal of a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and DNA concentration (ng/µl) and DNA purity (ratio A260/280) was measured. As reference, 2 µl of the Elution Buffer was used.

3.3.2.5. Restriction enzyme digestion

In order to confirm the previous results, purified positive PCR fragments were subject to restriction enzyme digestion with the endonuclease EcoRV (New England Biolabs, Ipswich, MA, USA). EcoRV recognize GAT^ATC sites in the 183 bp DNA fragment of *T. gondii*. Two DNA fragments of 74 bp and 109 bp will be obtained after enzyme digestion (Figure 11).

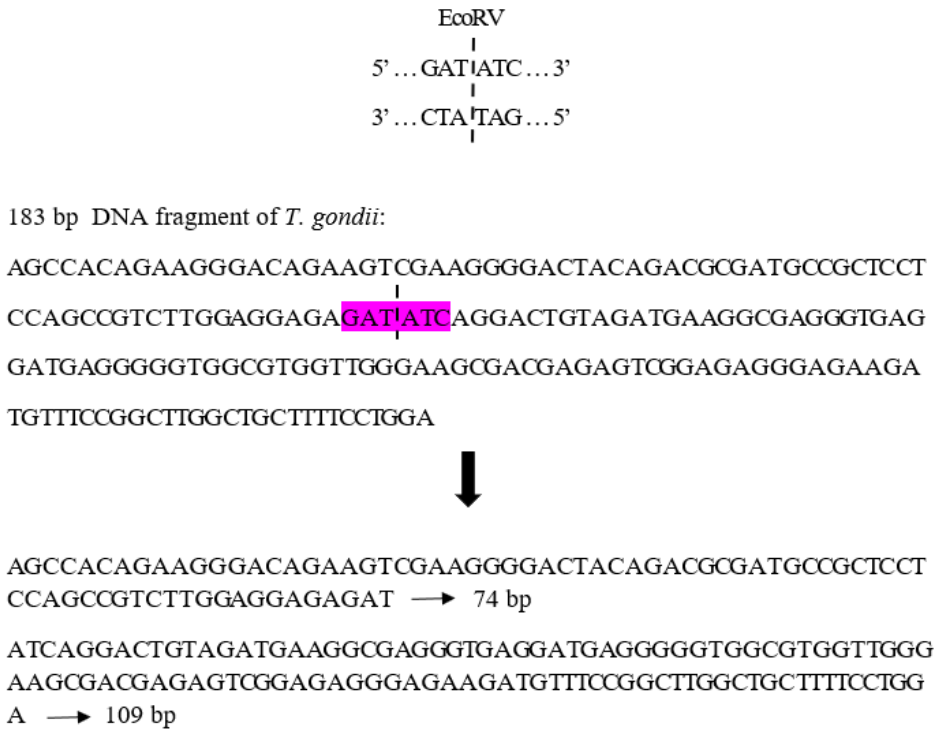

Figure 11: Schematic representation of the binding and cutting DNA regions of the endonuclease EcoRV (pink). EcoRV recognize GAT^ATC sites in the 183 bp DNA fragment of *T. gondii*. Two DNA fragments of 74 bp and 109 bp will be obtained after enzyme digestion (The cutting zone was obtained with the help of the website: http://www.restrictionmapper.org/).

The samples were incubated at 37ºC for 1h and the digested fragments were electrophoresed on a 2.5 % high resolution agarose gel, MetaPhor® Agarose, (Lonza, USA, 2007). As the samples were colourless, there was a need to add GRS DNA Loading Buffer Blue (6X) (GRiSP Research Solutions, Portugal). Each well of the agarose gel was loaded with 10 µl of the digested product and a molecular weight marker, GRS Low Range Ladder (GRiSP Research Solutions, Portugal) was placed in the gel ends. Then the electrophoresis was connected to a power supply at 100V for, approximately 45 minutes. The digested fragments were analysed using a Gel Doc ™ XR+ (Bio-Rad). Components and the volumes used to perform this assay, are described in Table 5.

Table 5: Volumes used for the restriction enzyme digestion assay with the endonuclease EcoRV. The mixture with the enzyme (Volume (1)), if the presence of DNA of *T. gondii* is confirmed, two DNA fragments of 74 bp and 109 bp will be obtained; and the mixture without the enzymes (Volume (2)), serve as a negative control.

Component	Volume (1)	Volume (2)
Restriction enzyme, EcoRV (New England Biolabs, Ipswich, MA, USA)	2 µl	-
Buffer, 10x NEBuffer 3.1 (New England Biolabs, Ipswich, MA, USA)	2 µl	2 µl
Water	6 µl	8 µl
Purified positive PCR fragments	10 µl	10 µl

3.3.2.6. Sequencing

National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) finds regions of similarity between biological sequences . In an eppendorf, 7.5 µl of the purified positive samples (5-20 ng/ µl) and 2.5 µl of the FW primer (10 pmol/µl) were added. Samples were sequenced using Sanger sequencing services from GATC Biotech (Eurofins Genomics, Germany).

After downloading the results, sequence comparison was made with already published sequences using the NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.2.7. Inhibition PCR

All negative samples were retested for the presence of PCR inhibitors by adding 1 µl of *T. gondii*-positive control to the 5 µl of DNA template.

PCR amplification was performed following the same conditions as conventional PCR with DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA), as well as agarose gel and visualization of results, described in 3.3.2.1.2..

3.3.3. *Cyclospora cayetanensis* detection

3.3.3.1. PCR – Primers optimization

Primers target the ITS2 region and the 18S rRNA gene were tested. Of the tested primers, 2 pairs were used considering the bibliography, while 2 were designed (Primer3Plus and BLAST®) by the Parasitology laboratory (Table 6).

Table 6: Primers target the ITS2 region and the 18S rRNA gene. Of the tested primers, 2 pairs were used considering the bibliography, while 2 were designed by the Parasitology laboratory team.

Region or gene to be amplified	References	Expected band (bp)
18S rRNA	(Murphy et al., 2017)	298
	In house	140
ITS2	(Lalonde et al., 2008)	116
	In house	166

As shown in figure 12, the primers to be used for the amplification of the 18S rRNA gene are the primers (a), used in a conventional nested-PCR, and for the ITS2 region are the primers used in the (d) amplification.

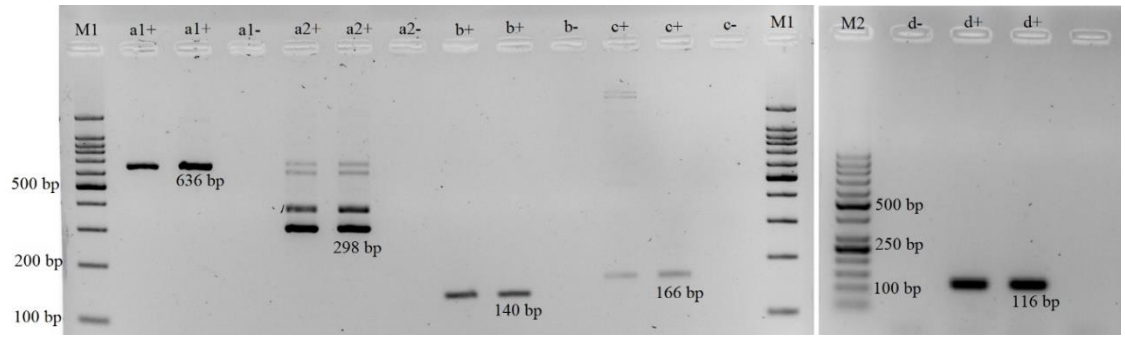

Figure 12: Electrophoretic separation on 1.5 % agarose gel using the different primers. a1: 636 bp sequence of the *C. cayetanensis* 18S rRNA gene; a2: 298 bp sequence of the C. *cayetanensis* 18S rRNA gene by (Murphy et al., 2017); b: 140 bp sequence of the *C. cayetanensis* 18S rRNA gene (in house); c: 166 bp sequence of the *C. cayetanensis* ITS2 region (in house); d: 116 bp sequence of the *C. cayetanensis* ITS2 region by (Lalonde et al., 2008); M1: molecular weight marker of 100 bp (GRS Ladder 100bp, GRiSP Research Solutions, Portugal) ; M1: molecular weight marker of 50 bp (GRS Ladder 50bp, GRiSP Research Solutions); +: positive control; -: negative control.

3.3.3.2. Nested-PCR with primers that amplify the 18S rRNA gene

Nested-PCR is a modification of PCR that was created to improve specificity and sensitivity and requires the use of two primer sets and two successive PCR reactions. The first set of primers are designed to anneal to sequences upstream from the second set of primers and are utilized in an initial PCR reaction. Products resulting from the first PCR reaction are used as a template for the second set of primers, and a second amplification is performed. However, nested-PCR requires extra time and cost, and the risk of contamination increases (Wanger et al., 2017).

The conventional nested-PCR method specifically targeting the *C. cayetanensis* 18S rRNA gene was performed with some modifications following the protocol described in Murphy (Murphy et al., 2017).

3.3.3.2.1. Nested–PCR first amplification

In first amplification, the target are *Cyclospora* spp. and *Eimeria* spp.

Specific primers **(**FW: 5′-TAC CCA ATG AAA ACA GTT T -3′ and REV: 5′-CAG GAG AAG CCA AGG TAG G-3′) targeting a 636 bp sequence of the 18S rRNA gene of *C. cayetanensis* were used (Murphy et al., 2017). The amplification reaction mixture consisted of 12.5 µl DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA), 500 nM of each primer (Eurofins Genomics, Germany) and 5 µl of sample DNA in a 25 µl reaction volume.

Cycling conditions for the primary reaction were 95ºC for 3 min for initial polymerase activation, 35 cycles of denaturation at 95ºC for 30 s, annealing at 54ºC for 30 s, and extension at 72ºC for 90 s, and a final extension at 72ºC for 10 min.

In all PCR experiments, a positive control (genomic DNA extracted from *C. cayetanensis* oocysts kindly providing by Lucy Robertson, Norwegian University of Life Sciences, Oslo, Norway) and a negative control (water template) were used, and amplifications were performed in a T100 ™ Thermal Cycler (Bio-Rad, Hercules, CA, USA).

After the PCR reaction, an electrophoresis, using 1.5 % agarose low melting gel (Agarose LM sieve, CONDA, Madrid) in TAE 1X, was performed and PCR products were observed

in a transilluminator with UV light (Gel Doc ™ XR + System with Image Lab ™ Software, BIO-RAD).

3.3.3.2.2. Nested-PCR second amplification

In order to amplify only *Cyclospora cayetanensis*, a new PCR was performed, with specific primers **(**FW: 5′- GTA GCC TTC CGC GCT TCG-3′ and REV:5′- CGT CTT CAA ACC CCC TAC TGT CG-3′) for this target within the 18S rRNA gene. The amplified product in the previous reaction will serve as a template for this.

The amplification reaction mixture consisted of 12.5 µl DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific), 500 nM of each primer (Eurofins Genomics, Ebersberg, Germany) and 1 µl of previously amplified product, in a 25 µl reaction volume.

Cycling conditions for the secondary reaction were 95ºC for 3 min for initial polymerase activation, 30 cycles of denaturation at 94ºC for 30 s, annealing at 64ºC for 30 s, and extension at 72ºC for 90 s, and a final extension at 72ºC for 5 min. and Amplifications were performed in a T100 ™ Thermal Cycler (Bio-Rad, Hercules, CA, USA).

After the PCR reaction, electrophoresis was performed following the same protocol as the previous reaction.

3.3.3.3. Conventional PCR with primers that amplify the ITS2 region

The extracted DNA was analysed by conventional PCR method using specific primers (FW: 5′-GCA GTC ACA GGA GGC ATA TAT CC -3′ and REV:5′-ATG AGA GAC CTC ACA GCC AAA C-3′) targeting a 116 bp sequence of the ITS2 region of *C. cayetanensis*. (Lalonde et al., 2008). As for *T. gondii*, two different enzymes were tested to minimize possible enzyme inhibition.

3.3.3.3.1. With Xpert Fast HotStart Mastermix (2x) with dye (GRiSP Research Solutions, Portugal)

The amplification reaction mixture consisted in 12.5 µl Xpert Fast HotStart MasterMix (2x) (GRiSP Research Solutions, Portugal), 500 nM of each primer (Eurofins Genomics, Germany) and 5 µl of sample DNA, in a 25 µl reaction volume.

Cycling conditions for the reaction were 95ºC for 3 min, 40 cycles of denaturation at 95ºC for 30 s, annealing at 59ºC for 30 s, and extension at 72ºC for 30 s, and a final extension at 72ºC for 5 min.

In all PCR experiments, a positive control (DNA extracted of *C. cayetanensis* oocysts kindly providing by Lucy Robertson, Norwegian University of Life Sciences, Oslo, Norway) and a negative control (water template) were used, and amplifications were performed in a T100 ™ Thermal Cycler (Bio-Rad, Hercules, CA, USA).

After the PCR reaction, was performed an electrophoresis, using 1.5 % agarose low melting gel (Agarose LM sieve, CONDA, Madrid) in TAE 1X and PCR products were observed in a transilluminator with UV light (Gel Doc ™ XR + System with Image Lab ™ Software, BIO-RAD).

3.3.3.3.2. With DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA)

The amplification reaction mixture consisted of 12.5 µl DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA), 500 nM of each primer (Eurofins Genomics, Germany) and 5 µl of sample DNA in a 25 µl reaction volume.

The PCR protocol consisted of 3 min at 95°C followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min and a final extension for 5 min at 72°C.

Amplifications were performed in a T100 ™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) and PCR products were observed using the same protocol as the previous reaction.

3.3.3.4. Gel Band Purification, Spectrophotometric measurements, Sequencing and Inhibition PCR

The positive amplification products resulting from the PCR reaction were purified using the GRS PCR & Gel Band Purification kit (GRiSP Research Solutions, Portugal) following the same protocol described in section 3.3.2.3..

To ensure that there is enough DNA and the purity of DNA, the samples were analysed on the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) following the same protocol described in section 3.3.2.4..

In an eppendorf, 7.5 µl of the purified positive samples (5-35 ng/ µl) and 2.5 µl of the FW primer (10 pmol/ µl) were added. Samples were sequenced using Sanger sequencing services from GATC Biotech (Eurofins Genomics, Germany) and sequence comparison was made with already published sequences using the NCBI (BLAST).

Additionally, all the negative samples, of both reactions, were retested for the presence of PCR inhibitors by adding 1 µl of *C. cayetanensis*-positive control to the 5 µl of DNA template. PCR amplification was performed following the same conditions as conventional PCR, as well as agarose gel and visualization of results described in 3.3.3.3.2..

3.3.3.5. Modified Safranin Technique (Hot Method) Staining Procedure

The modified safranin technique (hot method) staining procedure was used to stain *C. cayetanensis* oocysts for microscopy analysis (CDC, 2018).

Fifty microliters of the sediment suspension, of the confirmed PCR positive samples, were air-dried onto a microscope slide. Fixation was performed for 5 min with acid alcohol (3% v/v HCl/Methanol), and the slides were allowed to dry completely. Then, the slides were placed in boiling 1% w/v safranin O (Merk KGaA, Germany) for 2 minutes, rinsed with distilled water and was added the 3% w/v malachite green (Sigma-Aldrich Laborchemikalien, GmbH, Seelze, Germany) for 30 seconds. Finally, the slides were rinsed with distilled water and completely air-dried before being mounted with DPX mounting media (BDH Chemicals Ltd, Poole, England).

Figure 13: Solutions used in the preparation of the slides. From left to right, acid alcohol (3% HCl/ Methanol), 1% safranin and 3% malachite green (capture by author).

Oocysts were visualized using a EVOS M5000 microscope (Thermo Fisher Scientific, USA), under bright field microscopy. Images were captured using the microscope system itself. In addition, was also used a conventional optical microscope to take imagens at 1000 x magnification.

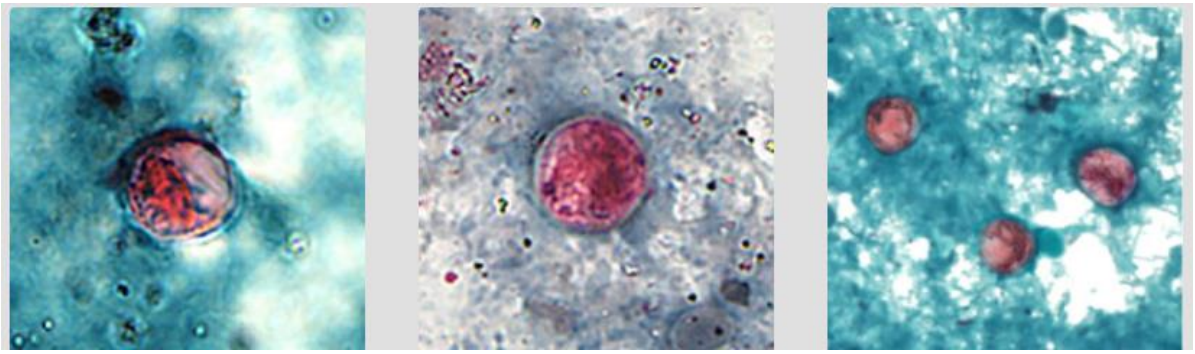

Figure 14: Oocysts of *C. cayetanensis* stained with safranin. (CDC, 2019, https://www.cdc.gov/dpdx/cyclosporiasis/index.html#tabs-2-3).

3.3.3.6. Epifluorescence microscopy

Fifty microliters of the sediment suspension of the positive samples were put onto microscope slide directly, and each one was covered with a coverslip.

As a positive control, a slide was prepared with *T. gondii* ME49 oocysts (kindly provided by J. T. Dubey). *T. gondii* oocysts are used as a control since they have a similar morphology and size to *C. cayetanensis* oocysts (Temesgen et al., 2019). Both emit fluorescence under UV light and when unsporulated they are spherical, with around 10 μm.

Oocysts were visualized using a EVOS M5000 microscope (Thermo Fisher Scientific, USA), under bright field and UV filter block (excitation 335 nm; emission 450 nm) based on the autofluorescent nature of the oocyst wall (presence of high levels of tyrosine) (Garcia et al., 2018). Images were captured using the microscope system itself.

3.4. Statistical analysis

Graphpad Prism version 7.02 (San Diego, CA, USA) was the software used for statistical analysis and the 95% confidence intervals (95% CI) for the population proportions were calculated using the modified Wald method (https://www.graphpad.com/quickcalcs/ConfInterval1.cfm) (Agresti et al., 1998).

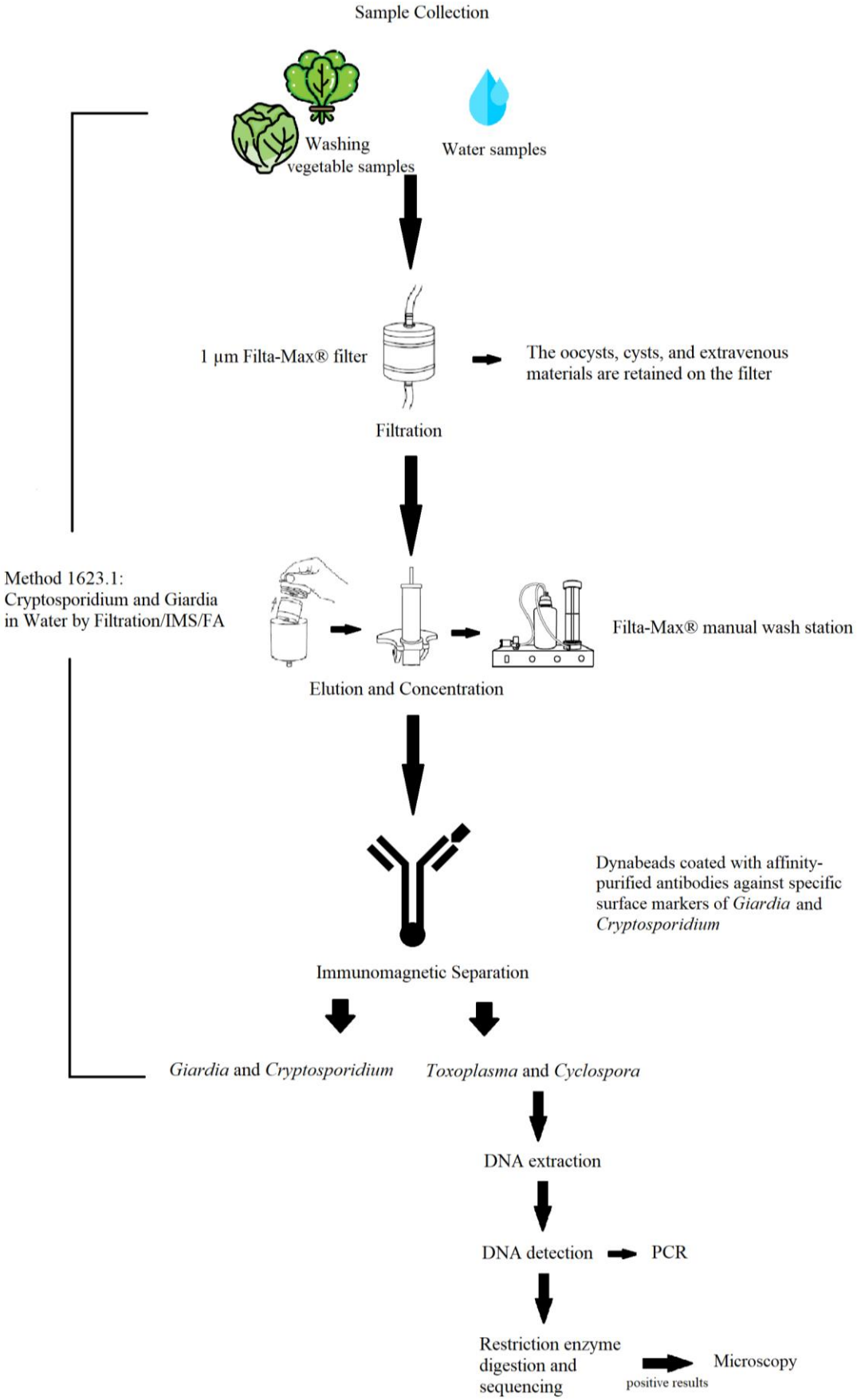

Figure 15: Schematic representation of the methodology.

Results

4. Results

4.1. Sampling

A total of 25 samples were collected from a large-scale agricultural exploration in Portugal. 20% of the samples were non-treated water samples, 32% were treated water samples and sand collected from the sand/coal filter systems and 48% were vegetables samples (Figure 16). 430 L of non-treated water, 440 L of treated water, 74 g of sands collected from the sand/coal filter systems and 10346 g of vegetables samples were analyzed. The vegetables samples included different varieties of lettuce, spinach and arugula. 5 vegetables samples, corresponding to 4930 g were pre-washed and 7 corresponding to 5416 g were not pre-washed. Also, of the 12 vegetables samples, 4 corresponding to 3346 g were samples of organic agriculture system and 8, corresponding to 7000 g, were samples of conventional agriculture system.

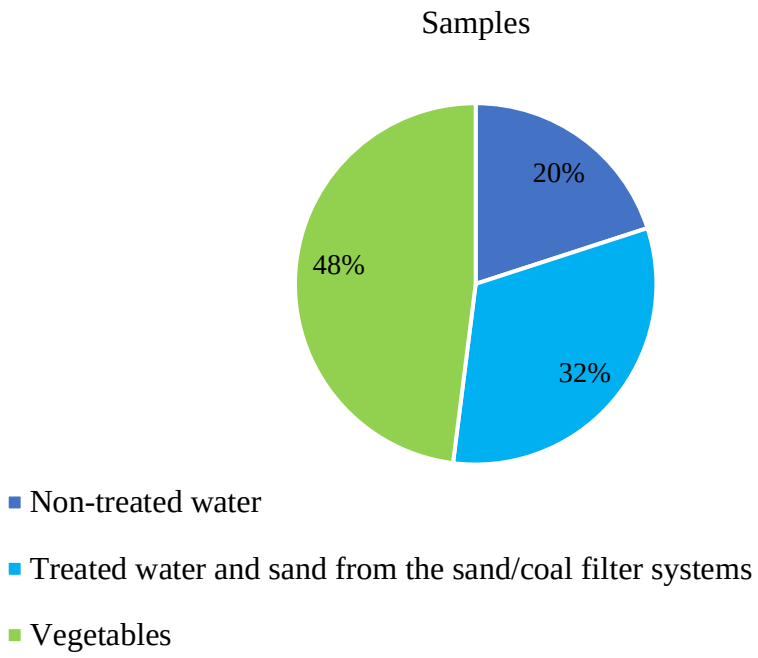

Figure 16: A total of 25 samples were collected from an agricultural exploration in Portugal. 20% of the samples were non-treated water samples, 32% were treated water samples and sand collected from the sand/coal filter systems and 48% were vegetables samples.

4.2.*Toxoplasma gondii* and *Cyclospora Cayetanensis* detection

4.2.1. *Toxoplasma gondii* detection

4.2.1.1. Conventional PCR

T. gondii detection was performed by conventional PCR specifically targeting a 183 bp corresponding to the specific DNA fragment of the 529 bp repetitive region of *T. gondii* (GenBank: AF146527.1). None of the samples amplified the desired product, using both of enzymes: DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA) (Figure 17) and Xpert Fast Hot Start Master Mix (2x) with dye (GRiSP Research Solutions, Portugal).

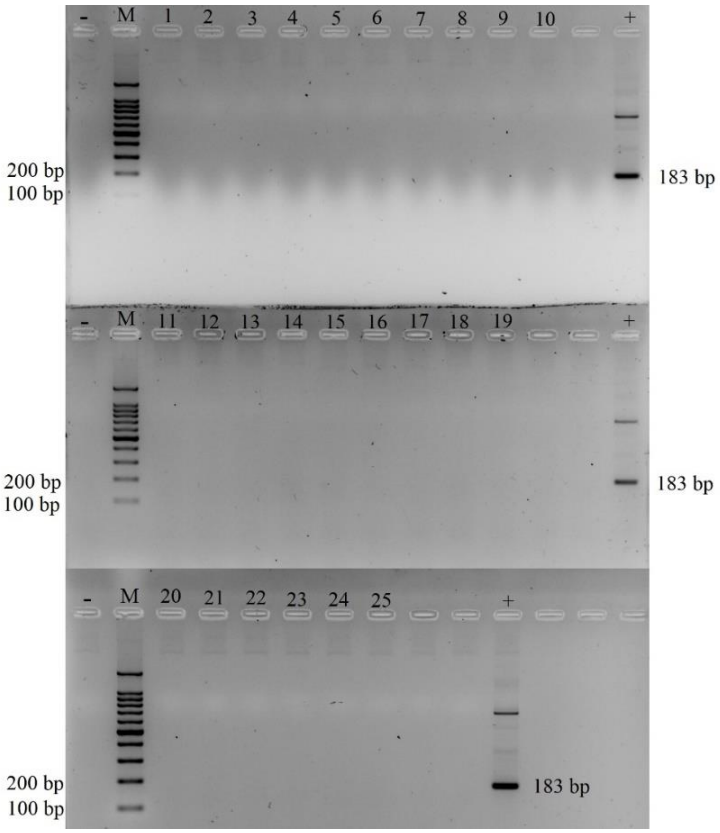

Figure 17: *Toxoplasma gondii* conventional PCR results. Electrophoretic separation on 2% agarose gel of the conventional PCR amplified products, from all samples (1-25), using the DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA).

M: molecular weight marker of 100 bp (GRS Ladder 100bp, GRiSP Research Solutions, Portugal); +: positive control; -: negative control.

4.2.1.2. Conventional PCR from previously amplified product

PCR from previously amplified product allowed the detection of a very small amounts of DNA that might not be visible in the agarose gel in the first PCR reaction.

As shown in figure 18, samples 1 (water from the Dam), 10 (water from post-sand filter (2)) and 18 (pre-washed wild arugula) amplified a band between 100 and 200 bp. Although, the bands seemed to be slightly below 183 bp, meaning that they could be non-specific amplifications, they were purified, quantified and send to sequencing.

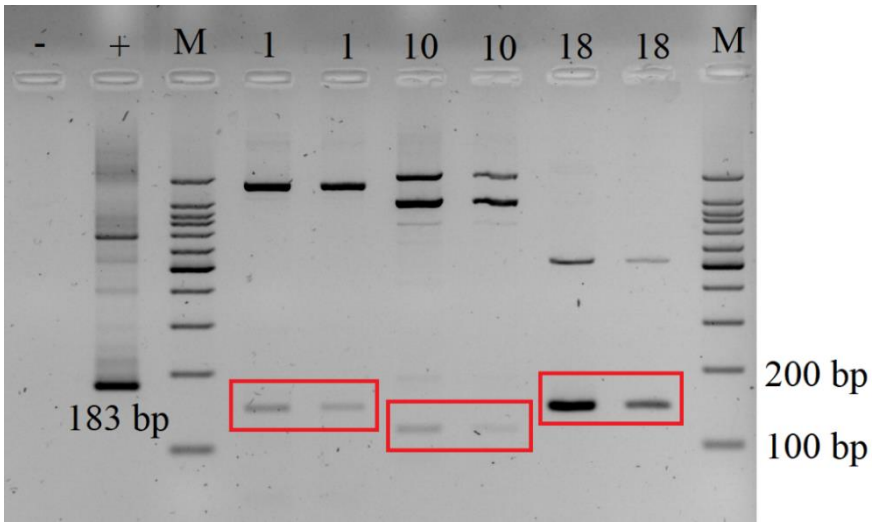

Figure 18: Conventional PCR of previously amplified product of *T. gondii*. Electrophoretic separation on 2% agarose gel of the conventional PCR amplified products, from the samples 1, 10 and 18. Potential positive samples are marked in red.
M: molecular weight marker of 100 bp (GRS Ladder 100bp, GRiSP Research Solutions, Portugal); +: positive control; -: negative control.

4.2.1.3. Spectrophotometric measurements

The purified DNA from the PCR product of the 3 positive samples and the positive control were quantified using the NanoDrop ™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentration (ng/µl) varied between 5 to 13 ng/µl and DNA purity (A260/A280) between 1.60 to 2.10 (Table 7).

Table 7: Spectrophotometric measurements using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) of the potential positive DNA samples and the positive control of *T. gondii*.

Sample	A260/A280	[DNA] ng/µl
1	1.60	5.5
10	2.06	7.9
18	1.71	12.3
Positive Control	1.86	12.4

All nucleic acids exhibit a maximum molar absorptivity at 260 nm, which allows quantitative or non-specific quantitative studies. An example has to do with determining the contamination of a DNA solution with proteins. Proteins have maximum absorptivities at 230 nm and 280 nm. The A260 / A280 ratio of a pure DNA solution is around 1.8. Any lower value than this indicates a possible contamination of the DNA solution with proteins and a higher value indicates a contamination with DNA or RNA (Lucena-Aguilar et al., 2016).

4.2.1.4. Restriction enzyme digestion

Restriction enzyme digestion with endonuclease EcoRV will confirm the presence of a *T. gondii* fragment, presenting two fragments of 74 bp and 109 bp, as shown in image 19 for the positive control. However, in the remaining samples no bands in the range of 74 and 109 bp were observed (Figure 19).

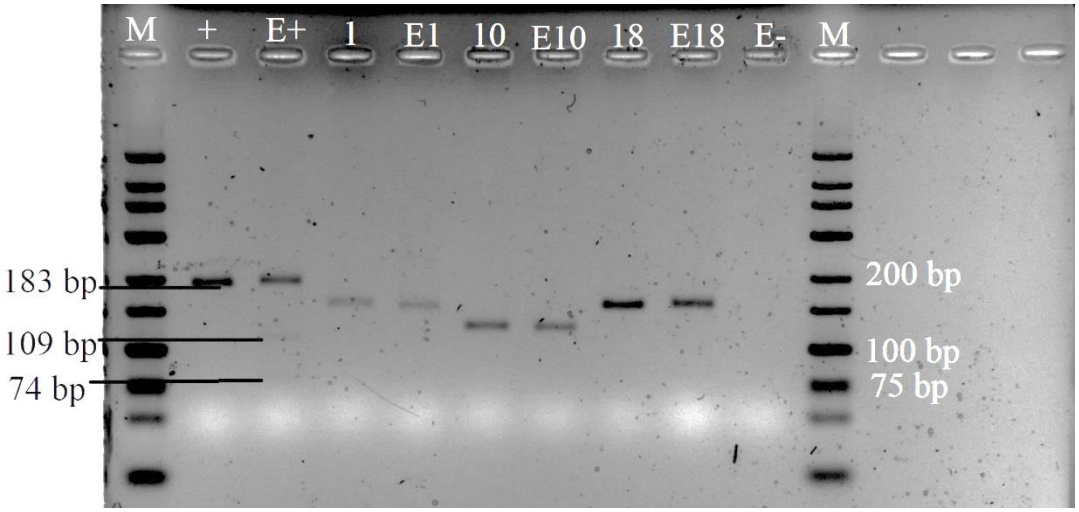

Figure 19: Restriction enzyme digestion with the endonuclease EcoRV. Electrophoretic separation on a 2.5 % high resolution agarose gel, MetaPhor® Agarose, (Lonza, USA) of *T. gondii* subject to restriction enzyme digestion with the endonuclease EcoRV (New England Biolabs, Ipswich, MA, USA).
M: molecular weight marker of Low Range (GRS Low Range Ladder, GRiSP Research Solutions, Portugal); +: positive control; -: negative control. E: samples with the enzyme.

4.2.1.5. Sequencing

Samples 1 (water from the Dam), 10 (water from post-sand filter) and 18 (pre-washed wild arugula) were sequenced using Sanger sequencing services from GATC Biotech (Eurofins Genomics, Germany).

It is also important to have the positive control sequenced. This serves as a control to check if the reaction went well and if the primers are amplifying the intended target. The positive control had more than 95% nucleotide similarity with the 529 bp *T. gondii* repetitive region (GenBank: AF146527.1) (Figure 20). Sample 10 (water from post-sand filter) showed to be 97.26% similar to *Stenotrophomonas maltophilia* (Figure 21) and 1 and 18 showed no similarity to any sequence available in the database.

Toxoplasma gondii repeat region

Sequence ID: AF146527.1 **Length:** 529 **Number of Matches:** 1

Range 1: 223 to 293 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
112 bits(123)	5e-21	68/71(96%)	1/71(1%)	Plus/Plus

```
Query  4    CGCTC-TCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGAAGGCGACGGTGAGG  62
            ||||| ||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  223  CGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGAAGGCGAGGGTGAGG  282

Query  63   ATGAGGCGGTG  73
            |||||| ||||
Sbjct  283  ATGAGGGGGTG  293
```

Figure 20: Multiple alignment of fragments of the *T. gondii* positive control. The BLAST program was used to compare the nucleotide sequences available on GenBank.

Stenotrophomonas maltophilia strain PEG-42 chromosome, complete genome

Sequence ID: CP040435.1 **Length:** 4854802 **Number of Matches:** 1

Range 1: 97961 to 98032 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
116 bits(128)	1e-22	71/73(97%)	2/73(2%)	Plus/Plus

```
Query  1      ATTGGC-ACTTCGCGCGCGCTGCCGGGCATGTTGGCTTCGTCGCGACAGATAGGCACAGG  59
              |||||| |||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  97961  ATTGGCCACTTCGCGCGCGCTGCCGGGCATGTTGGCTTCGTCGCGAC-GATAGGCACAGG  980

Query  60     TGACCTTGGCTGC  72
              |||||||||||||
Sbjct  98020  TGACCTTGGCTGC  98032
```

Figure 21: Multiple alignment of fragments of the sample 10 (water from post-sand filter). The BLAST program was used to compare the nucleotide sequences available on GenBank.

4.2.1.6. Inhibition PCR

Although InhibitEX buffer (Qiagen) is used in the DNA extraction process, which has the function of eliminating possible PCR inhibitors, they may not be completely removed. As environmental samples are used, they are rich in contaminants and inhibitors that can affect PCR enzymes performance. As we can see in figure 22, in all the samples were observed a band of 183 bp, indicated that there were not PCR inhibitors.

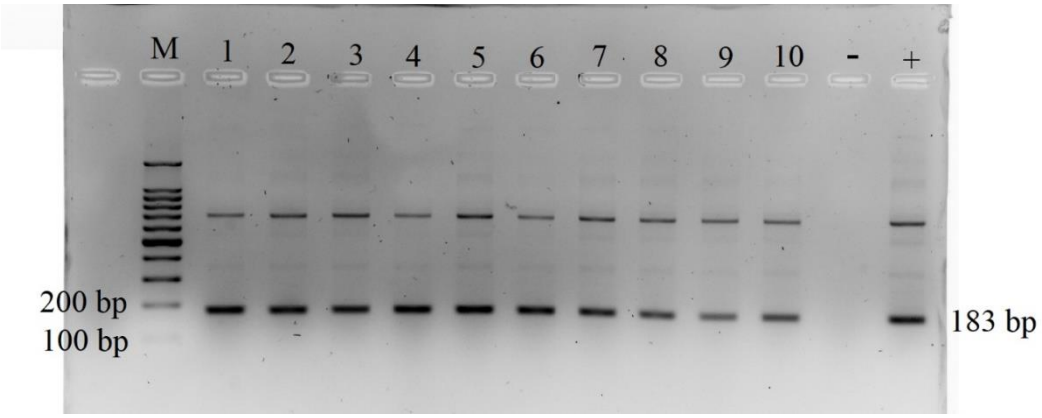

Figure 22: Representative a gel resulting from the inhibition PCR of *T. gondii*. Samples were contaminated with *T. gondii* DNA, and all presented the expected band of 183 bp, which indicates that there is no PCR inhibition.

M: molecular weight marker of 100 bp (GRS Ladder 100bp, GRiSP Research Solutions, Portugal); +: positive control; -: negative control.

4.2.2. *Cyclospora cayetanensis* detection

4.2.2.1. Nested-PCR with primers that amplify the 18S rRNA gene

In the first reaction, none of the experimental samples presented the expected 636 bp for *Cyclospora* spp. or/and *Eimeria* spp. (Figure 23).

The negative result can result from the low sensitivity and the difficulty of amplifying the desired product. Thus, the second reaction serves to increase the sensitivity and amplify, if the sample is positive.

Furthermore, this being the second reaction, it is more specific and only amplifies *C. cayetanensis* fragment. As shown in figure 24, all samples were negative.

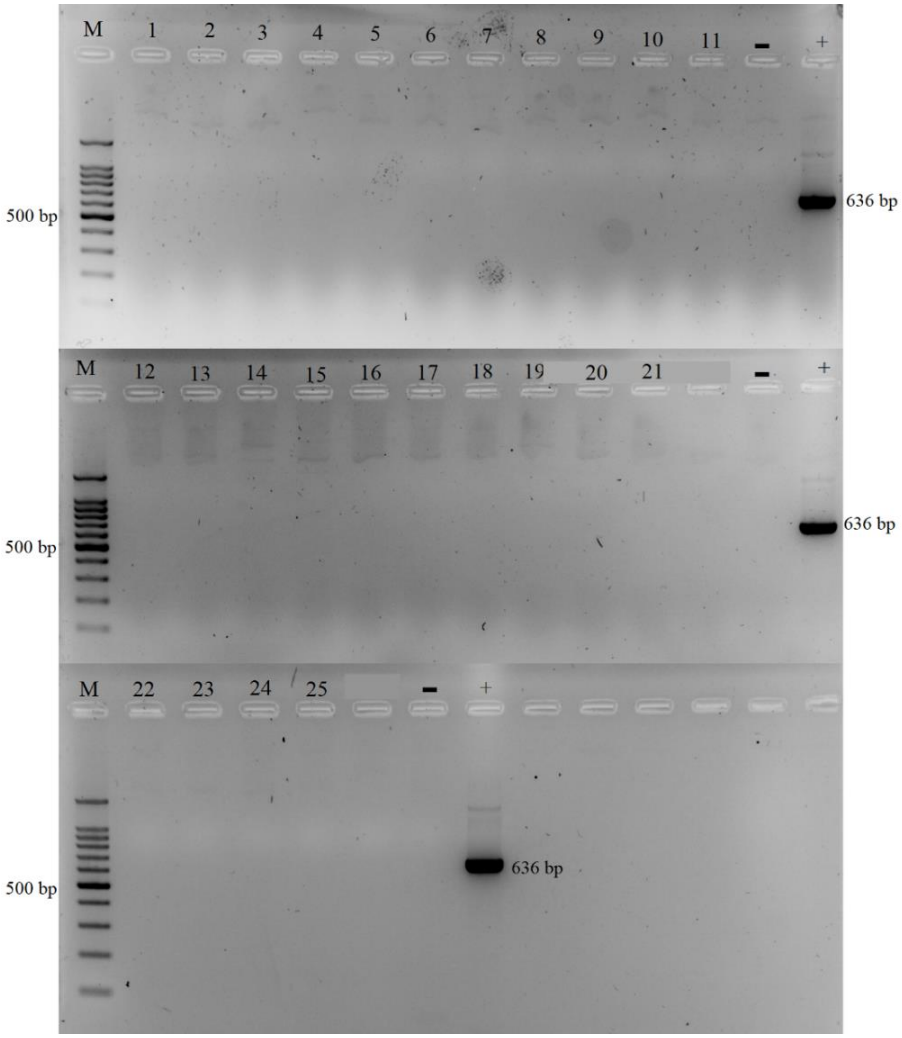

Figure 23: *Cyclospora cayetanensis* nested–PCR first amplification results. Electrophoretic separation on 1.5% agarose gel of nested-PCR amplified products, from all samples (1-25).
M: molecular weight marker of 100 bp (GRS Ladder 100bp, GRiSP Research Solutions, Portugal); +: positive control; -: negative control.

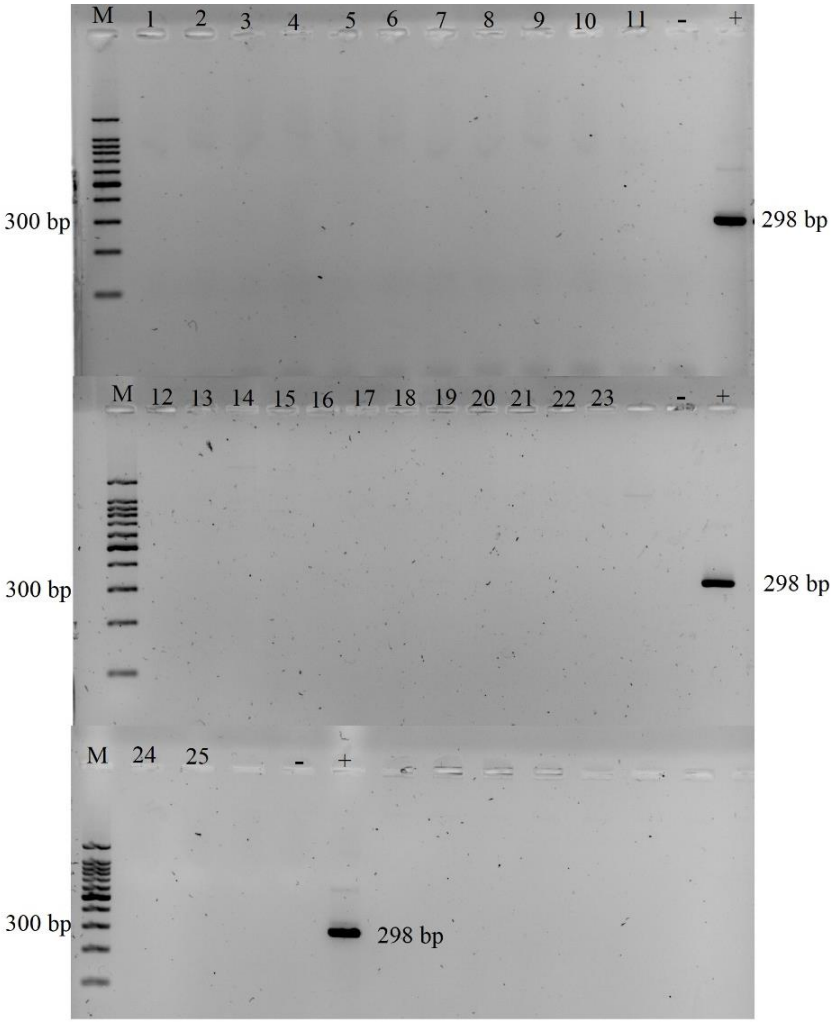

Figure 24: *Cyclospora cayetanensis* nested-PCR second amplification results. Electrophoretic separation on 1.5% agarose gel of nested-PCR amplified products, from all samples (1-25).
M: Molecular weight marker; of 100 bp (GRS Ladder 100bp, GRiSP Research Solutions, Portugal); +: positive control; -: negative control.

4.2.2.2. Conventional PCR with primers that amplify the ITS2 region

C. cayetanensis detection was performed by conventional PCR using the Xpert Fast Hot Start Master Mix (2x) with dye (GRiSP Research Solutions, Portugal) and DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA). A 116 bp gel band corresponding to the specific DNA fragment of the ITS2 region was found in 28% (95% CI: 14.06 – 47.80 %) of all samples, more specifically, 23% (95% CI: 7.50 – 50.94 %) of water

and sand samples and 33% (95% CI: 13.55 – 61.20 %) of vegetables samples when DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA) was used (Figure 25).

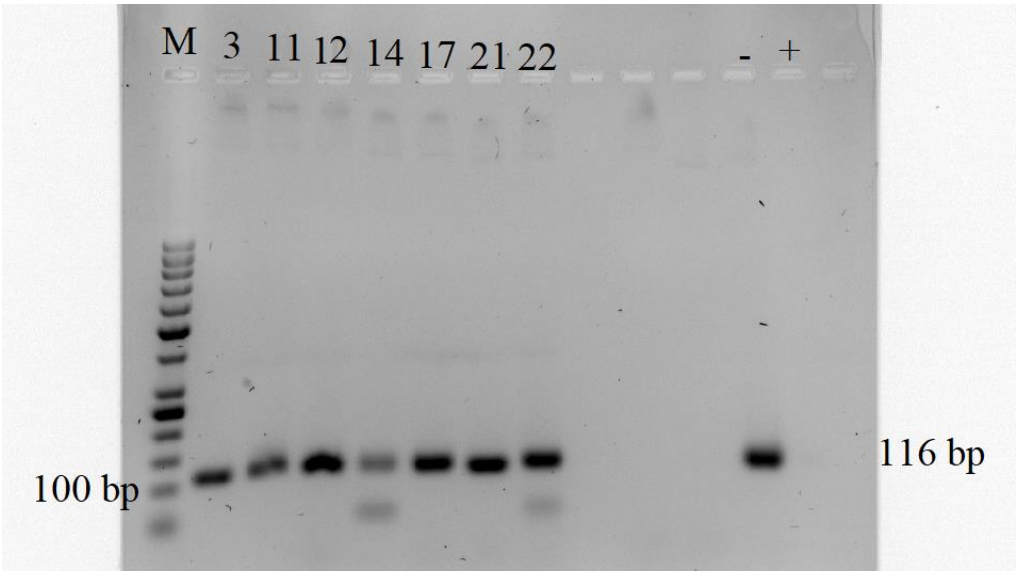

Figure 25: *Cyclospora cayetanensis* conventional PCR results. Electrophoretic separation on 1.5% agarose gel of the conventional PCR amplified products, from all positive samples, using the DreamTaq™ Hot Start Green PCR Master Mix 2x (Thermo Fisher Scientific, USA).
M: molecular weight marker of 50 bp (GRS Ladder 50bp, GRiSP Research Solutions, Portugal); +: positive control; -: negative control.

Thus, from this point on, DreamTaq™ Hot Start Green PCR Master Mix 2× (Thermo Fisher Scientific, USA) was used for the remaining reactions related to *C. cayetanensis*.

4.2.2.3. DNA quantification

The purified DNA from the PCR product of the 7 positive samples and the positive control were quantified using the NanoDrop ™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentration (ng/µl) varied between 5 to 32 ng/µl and DNA purity (A260/A280) between 1.90 to 4.60 (Table 8).

Table 8: Spectrophotometric measurements using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) of the potential positive DNA samples and the positive control of *C. cayetanensis*.

Sample	A260/A280	[DNA] ng/µl
3	2.39	11.6
11	1.90	23.3
12	1.80	17.3
14	4.58	5.3
17	2.43	10.1
21	2.47	11.2
22	1.98	2.9
Positive Control	2.18	31.1

4.2.2.4. Sequencing

Samples 3 (water from reservoir of conventional agriculture system) , 11 (water from the Tank 1, after Cl treatment), 12 (water from Tank 1), 14 (pre-washed iceberg lettuce), 17 (pre-washed organic spinach), 21 (red lettuce) and 22 (green lettuce) were sequenced using Sanger sequencing services from GATC Biotech (Eurofins Genomics, Germany).

It is also important to have the positive control sequenced. This serves as a control to check if the reaction went well and if the primers are amplifying the intended target.

All the samples showed positive results for *C. cayetanensis* with more than 95% nucleotide similarity with sequences available on GenBank for the 116 bp *C. cayetanensis* ITS2 region (Figure 26 and Figure 27).

a)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Cyclospora cay...	134	134	96%	5e-28	100.00%	1510	AF301390.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Cyclospora cay...	134	134	96%	5e-28	100.00%	1515	AF301387.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, co...	Cyclospora cay...	130	130	96%	2e-26	98.65%	917	AF301385.1
Cyclospora cayetanensis strain NP20 clone 17 internal transcribed spacer 1, partial sequence; 5.8S rib...	Cyclospora cay...	122	122	96%	3e-24	96.05%	1025	AF303963.1
Cyclospora cayetanensis strain NP20 clone 1 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Cyclospora cay...	122	122	96%	3e-24	96.05%	1028	AF303961.1
Cyclospora cayetanensis strain NP21 clone 65 internal transcribed spacer 1, partial sequence; 5.8S rib...	Cyclospora cay...	122	122	96%	3e-24	96.05%	1035	AF303958.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Cyclospora cay...	122	122	96%	3e-24	96.05%	1530	AF301391.1

b)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Cyclospora cay...	127	127	91%	8e-26	97.33%	1528	AF301386.1
Cyclospora cayetanensis strain NP20 clone 17 internal transcribed spacer 1, partial sequence; 5.8S rib...	Cyclospora cay...	125	125	90%	3e-25	97.30%	1025	AF303963.1
Cyclospora cayetanensis strain NP20 clone 1 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Cyclospora cay...	125	125	90%	3e-25	97.30%	1028	AF303961.1
Cyclospora cayetanensis strain NP21 clone 65 internal transcribed spacer 1, partial sequence; 5.8S rib...	Cyclospora cay...	125	125	90%	3e-25	97.30%	1035	AF303958.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Cyclospora cay...	125	125	90%	3e-25	97.30%	1530	AF301391.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, co...	Cyclospora cay...	125	125	90%	3e-25	97.30%	916	AF301384.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, co...	Cyclospora cay...	125	125	90%	3e-25	97.30%	923	AF301382.1

c)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cyclospora cayetanensis strain NP20 clone 17 internal transcribed spacer 1, partial sequence; 5.8S rib...	Cyclospora cay...	124	124	97%	9e-25	96.05%	1025	AF303963.1
Cyclospora cayetanensis strain NP20 clone 1 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Cyclospora cay...	124	124	97%	9e-25	96.05%	1028	AF303961.1
Cyclospora cayetanensis strain NP21 clone 65 internal transcribed spacer 1, partial sequence; 5.8S rib...	Cyclospora cay...	124	124	97%	9e-25	96.05%	1035	AF303958.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Cyclospora cay...	124	124	97%	9e-25	96.05%	1530	AF301391.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, co...	Cyclospora cay...	124	124	97%	9e-25	96.05%	916	AF301384.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, co...	Cyclospora cay...	124	124	97%	9e-25	96.05%	923	AF301382.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Cyclospora cay...	122	122	97%	3e-24	96.05%	1510	AF301390.1

Figure 26: Some sequences producing significant alignments of water samples. a) Sample 3; b) Sample 11; c) Sample 12.

a)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cyclospora cayetanensis isolate cc2 internal transcribed spacer 2, partial sequence	Cyclospora cayet...	64.4	64.4	85%	1e-07	100.00%	64	MZ088044.1
Cyclospora cayetanensis strain NP20 clone 17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN...	Cyclospora cayet...	62.6	62.6	82%	5e-07	100.00%	1025	AF303963.1
Cyclospora cayetanensis strain NP20 clone 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Cyclospora cayet...	62.6	62.6	82%	5e-07	100.00%	1028	AF303961.1
Cyclospora cayetanensis strain NP21 clone 68 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN...	Cyclospora cayet...	62.6	62.6	82%	5e-07	100.00%	1027	AF303960.1
Cyclospora cayetanensis strain NP21 clone 66 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN...	Cyclospora cayet...	62.6	62.6	82%	5e-07	100.00%	1032	AF303959.1
Cyclospora cayetanensis strain NP21 clone 65 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN...	Cyclospora cayet...	62.6	62.6	82%	5e-07	100.00%	1035	AF303958.1
Cyclospora cayetanensis strain NP21 clone 46 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN...	Cyclospora cayet...	62.6	62.6	82%	5e-07	100.00%	1031	AF303957.1

b)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spac...	Cyclospora cay...	136	136	96%	2e-28	97.50%	1528	AF301386.1
Cyclospora cayetanensis strain NP20 clone 23 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Cyclospora cay...	133	133	96%	2e-27	96.25%	1038	AF303964.1
Cyclospora cayetanensis strain NP20 clone 17 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Cyclospora cay...	132	132	96%	7e-27	96.25%	1025	AF303963.1
Cyclospora cayetanensis strain NP20 clone 1 internal transcribed spacer 1, partial sequence; 5.8S ribos...	Cyclospora cay...	132	132	96%	7e-27	96.25%	1028	AF303961.1
Cyclospora cayetanensis strain NP21 clone 65 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Cyclospora cay...	132	132	96%	7e-27	96.25%	1035	AF303958.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spac...	Cyclospora cay...	132	132	96%	7e-27	96.25%	1530	AF301391.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, com...	Cyclospora cay...	132	132	96%	7e-27	96.25%	916	AF301384.1

c)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S r...	Cyclospora cayet...	124	124	88%	1e-24	96.05%	1510	AF301390.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S r...	Cyclospora cayet...	124	124	88%	1e-24	96.05%	1515	AF301387.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequ...	Cyclospora cayet...	120	120	88%	1e-23	94.74%	917	AF301385.1
Cyclospora cayetanensis strain NP20 clone 17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Cyclospora cayet...	112	112	88%	7e-21	92.31%	1025	AF303963.1
Cyclospora cayetanensis strain NP20 clone 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Cyclospora cayet...	112	112	88%	7e-21	92.31%	1028	AF303961.1
Cyclospora cayetanensis strain NP21 clone 65 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Cyclospora cayet...	112	112	88%	7e-21	92.31%	1035	AF303958.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S r...	Cyclospora cayet...	112	112	88%	7e-21	92.31%	1530	AF301391.1

d)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cyclospora cayetanensis isolate cc2 internal transcribed spacer 2, partial sequence	Cyclospora cayet...	84.2	84.2	100%	3e-13	96.08%	64	MZ088044.1
Cyclospora cayetanensis strain NP20 clone 17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Cyclospora cayet...	78.8	78.8	94%	1e-11	95.83%	1025	AF303963.1
Cyclospora cayetanensis strain NP20 clone 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Cyclospora cayet...	78.8	78.8	94%	1e-11	95.83%	1028	AF303961.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S r...	Cyclospora cayet...	78.8	78.8	94%	1e-11	95.83%	1510	AF301390.1
Cyclospora cayetanensis small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S r...	Cyclospora cayet...	78.8	78.8	94%	1e-11	95.83%	1515	AF301387.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequ...	Cyclospora cayet...	78.8	78.8	94%	1e-11	95.83%	916	AF301384.1
Cyclospora cayetanensis 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequ...	Cyclospora cayet...	78.8	78.8	94%	1e-11	95.83%	923	AF301382.1

Figure 27: Some sequences producing significant alignments of vegetable samples, a) Sample 14; b) Sample 17; c) Sample 21; d) Sample 22.

4.2.2.5.Inhibition PCR

As already done for the detection of *T. gondii*, an inhibition PCR was also performed to rule out false negatives**.** When adding 1 µl of the positive control of *C. cayetanensis* to all samples, it was found that they all amplified (Figure 28).

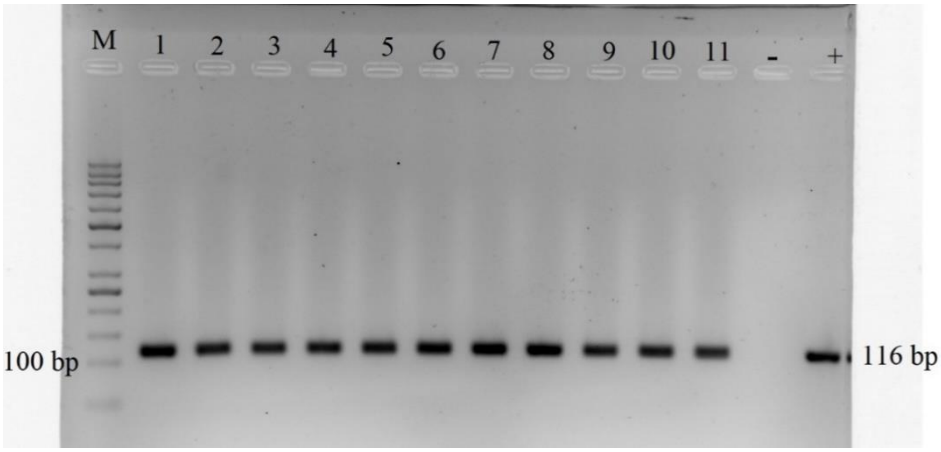

Figure 28: Representative gel resulting from the inhibition PCR of *C. cayetanensis*. Samples were contaminated with *C. cayetanensis* DNA, and all presented the expected band of 116 bp, which indicates that there is no PCR inhibition.

M: molecular weight marker of 50 bp (GRS Ladder 50 bp, GRiSP Research Solutions, Portugal); +: positive control; -: negative control.

4.2.2.6. Modified Safranin Technique (Hot Method) Staining Procedure

Slides from PCR-positive samples were examined using the modified safranin technique (hot method) staining procedure, and structures, compatible with *C. cayetanensis* oocysts in morphology and size (between 8–10 µm in diameter) were observed for all PCR-positive samples (Figure 29 and 30).

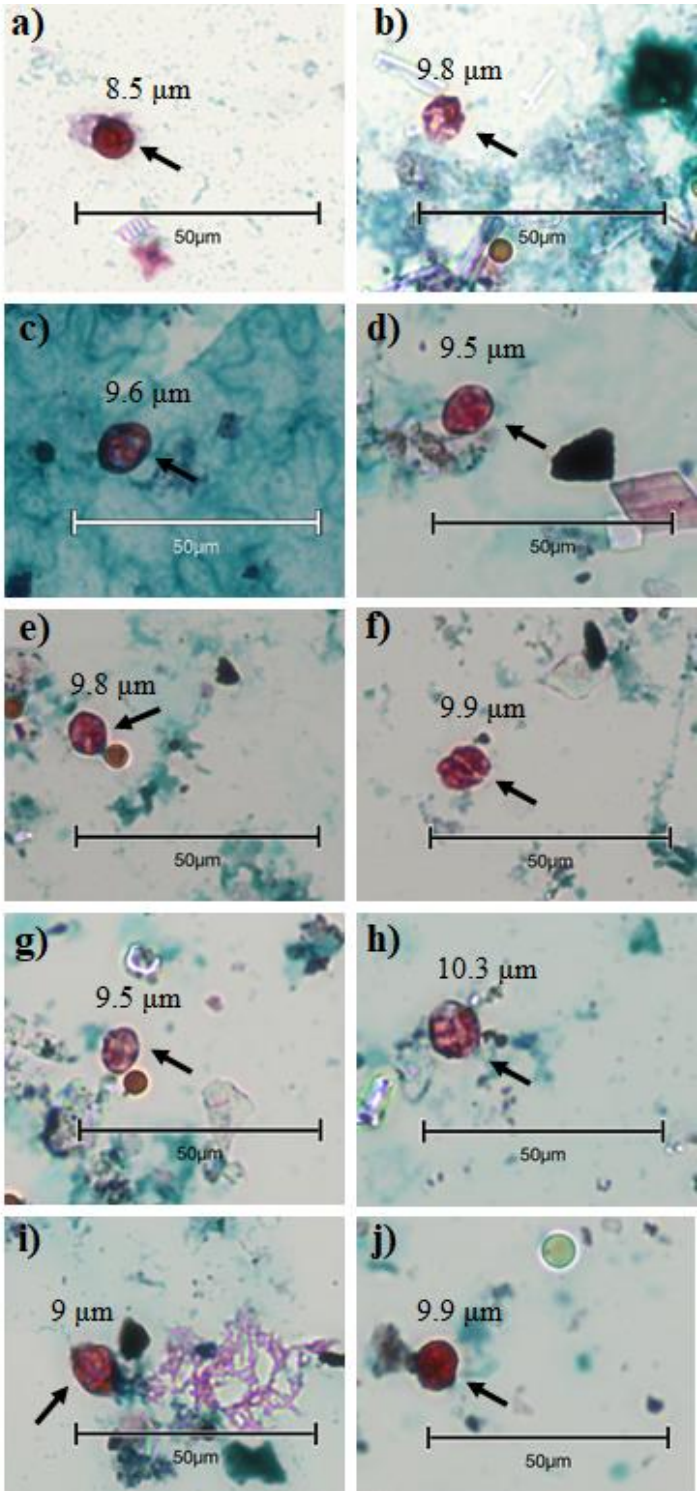

Figure 29: Structures compatible with oocyst of *C. cayetanensis* stained with safranin (black arrow). a) and b) Sample 3; c) Sample 12; d) Sample 14; e) and f) Sample 17; g), h), i) and j) Sample 21. Images taken at 400x magnification (capture by author).

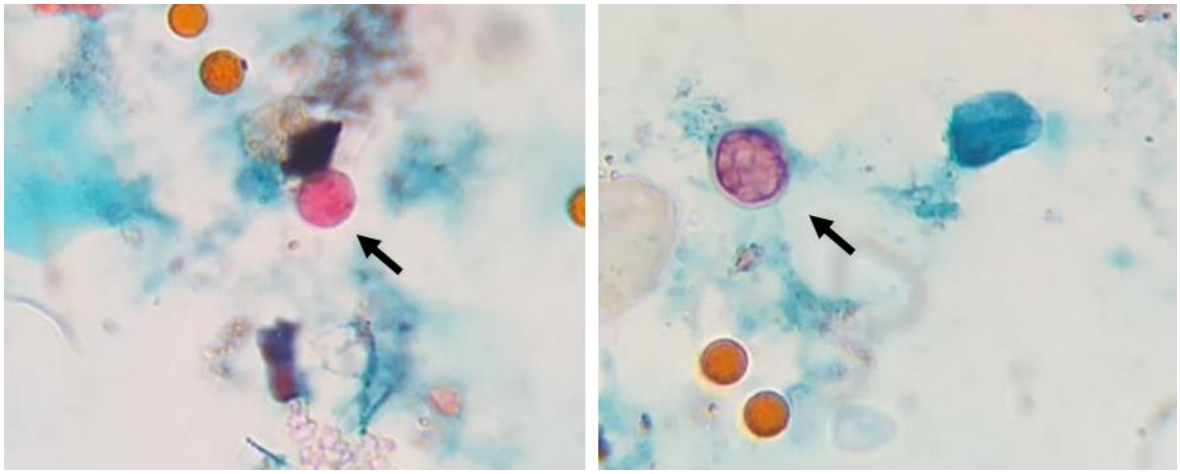

Figure 30: Structures compatible with oocysts of *C. cayetanensis* stained with safranin of the sample 11 (black arrow). Imagens taken at 1000x magnification under a conventional optical microscope (capture by author).

4.2.2.7. Epifluorescence microscopy

Slides from PCR-positive samples were also examined under bright field microscopy and UV and autofluorescent structures, compatible with *C. cayetanensis* oocysts in morphology and size (between 8–10 µm in diameter) were also observed. The majority of the samples presented unsporulated oocysts (Figure 32-35).

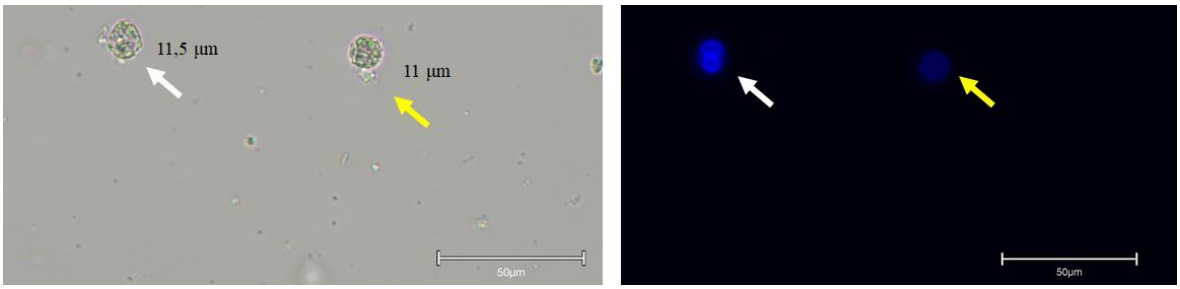

Figure 31: Since there is no *C. cayetanensis* oocysts available, as a positive control, a slide was prepared with *T. gondii* ME49 oocysts (kindly provided by J. T. Dubey) and oocysts were viewed under bright field microscopy (left) and UV microscopy (right). Sporulated (white arrow) and unsporulated oocyst (yellow arrow). Image taken at 400x magnification (capture by author)

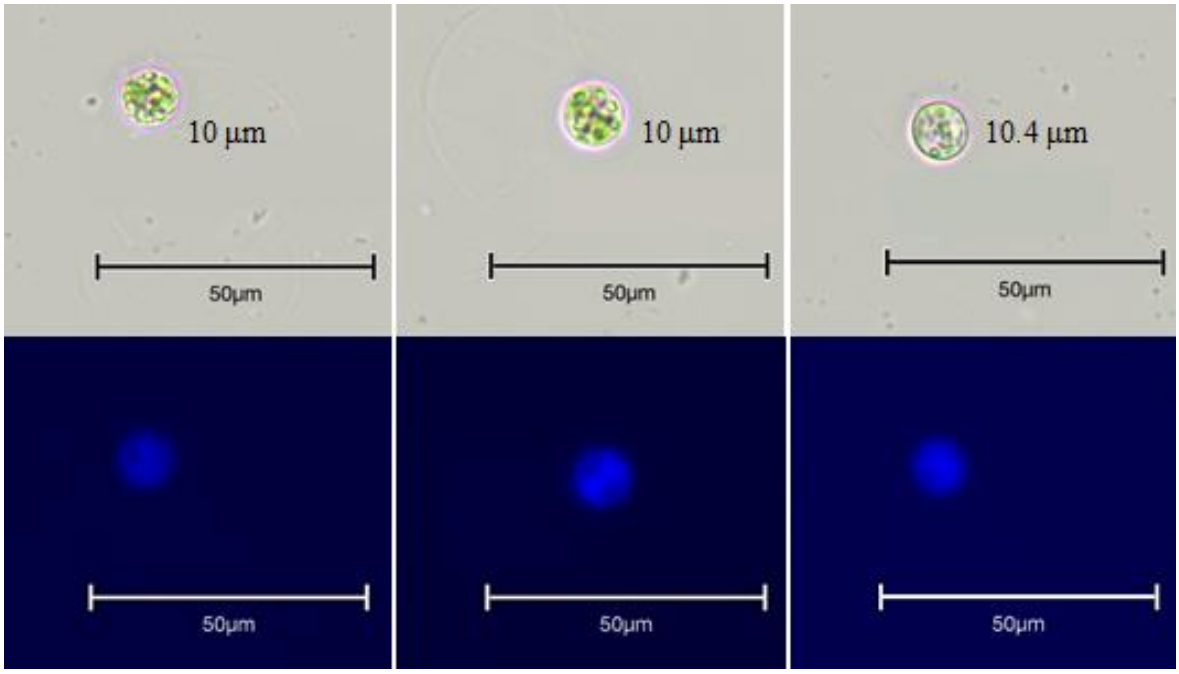

Figure 32*:* Structures compatible with oocysts of *C. cayetanensis* seen under bright field microscopy (top) and UV microscopy (bottom) of the sample 3. Image taken at 400x magnification (capture by author).

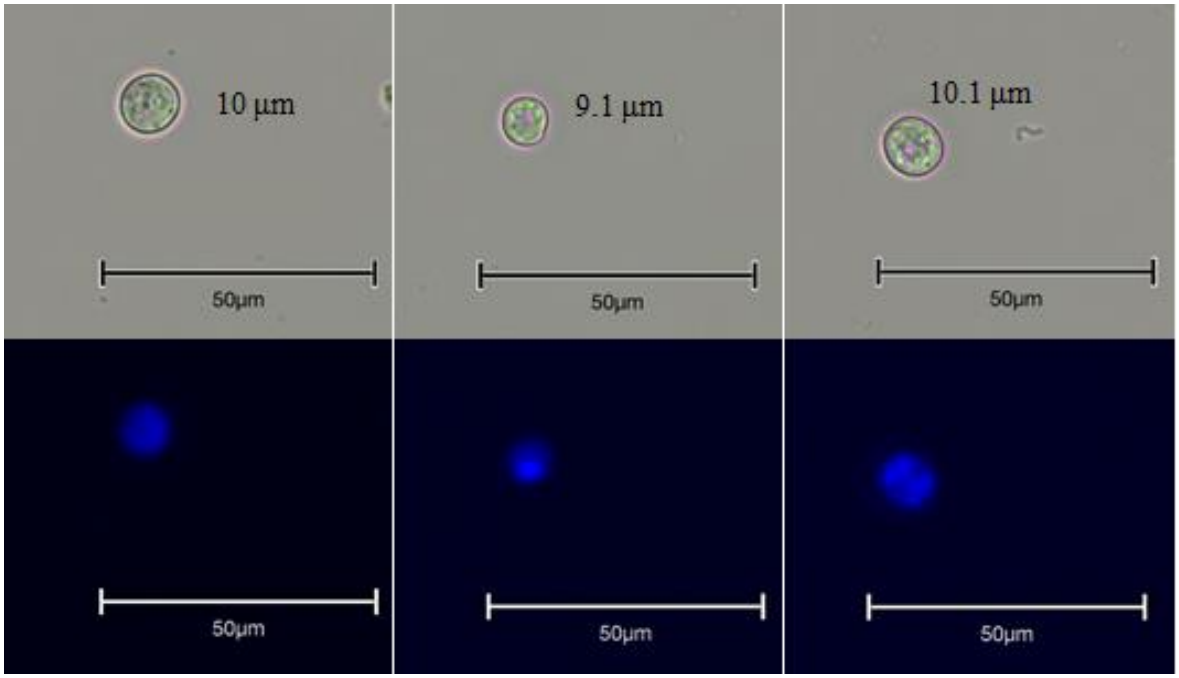

Figure 33: Structures compatible with oocysts of *C. cayetanensis* seen under bright field microscopy (top) and UV microscopy (bottom) of the sample 11. Image taken at 400x magnification (capture by author).

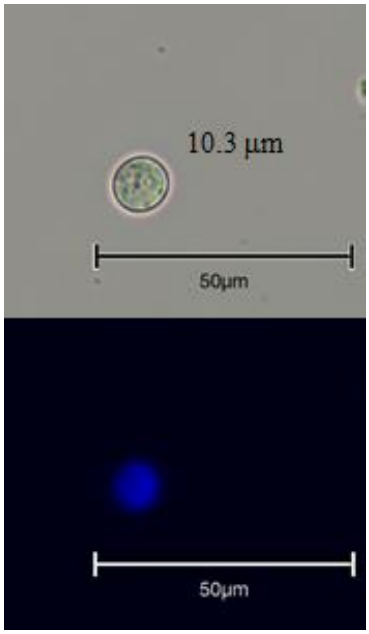

Figure 34: Structures compatible with oocysts of *C. cayetanensis* seen under bright field microscopy (top) and UV microscopy (bottom) of the sample 12. Image taken at 400x magnification (capture by author).

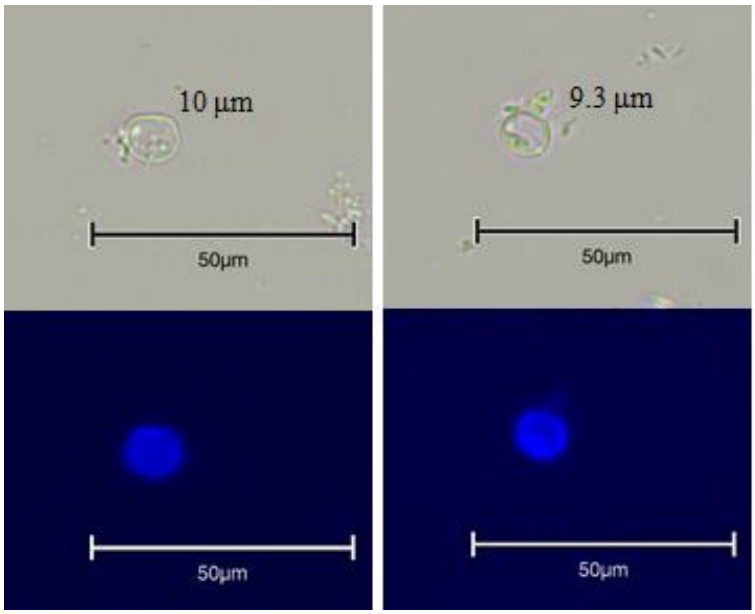

Figure 35: Structures compatible with oocysts of *C. cayetanensis* seen under bright field microscopy (top) and UV microscopy (bottom) of the sample 14. Image taken at 400x magnification (capture by author).

Discussion

5. Discussion

Pathogenic protozoans are responsible for a wide range of human and animal diseases and cause a substantial socioeconomic burden in many developing nations (Bintsis, 2017; Erickson et al., 2006; Gajadhar et al., 2006; Nievas et al., 2020). Water and vegetables can be contaminated by environmental protozoans, such as, oocysts of *T. gondii* and *C. cayetanensis*. The methodology used in this study allows the recovery and detection of these protozoan parasites from water and vegetables samples.

Non-treated and treated water used for irrigation and for washing vegetables were collected in order to see if they are a potential source of contamination. In addition, it is important to evaluate different foods and see if the structure of the vegetables are involved in the parasitic contamination. For instance, leafy greens as lettuce, cabbage and arugula are more susceptible for contamination compared to other vegetables with smooth surfaces (Berrouch et al., 2020). Moreover, in this study were used vegetables samples with 500 – 1000 g in order to work with the whole product and larger volumes of water samples to have a more complete study. Other authors use smaller portions of the vegetables and smaller volumes of water which can lead to errors by default (Table 9 to Table 12).

A standard method currently available for recovery of *Cryptosporidium* spp. and *Giardia* spp. in water and vegetables was used ((Method 1623.1(EPA, 2012)). This approach allowed to separate different parasites, removing *Giardia* spp. and *Cryptosporidium* spp. and concentrating the protozoa of interest: *Toxoplasma gondii* and *Cyclospora cayetanensis.* Then, it was assumed that the most appropriate DNA extraction protocol for *C. cayetanensis* oocysts would be similar to that for *T. gondii* oocysts, taking in to account their morphology and size similarities (Temesgen et al., 2019). Moreover, molecular methodologies were used to detect *T. gondii* and *C. cayetanensis*. For *T. gondii* detection it was used a specific DNA fragment of the protozoa 529 bp repetitive region. This fragment is repeated 200 to 300 times, which increases the specificity of the reaction (Homan et al., 2000). In the first amplification reaction, all samples were negative for the presence of *T. gondii* DNA. Thus, one can put 4 hypotheses: (i) the samples were not contaminated with *T. gondii* DNA; (ii) there were PCR inhibitors to prevent the reaction; (iii) quantity of the DNA in the sample was so low that were not detected in the agarose gel and (iv) the conditions or components

of the PCR reaction were not the most suitable. In order to clarify this issue, a PCR of the amplified product was performed, as well as an inhibition PCR and a new PCR with another enzyme and under other conditions, to rule out false negatives. 3 samples of the 25 analyzed (2 of water and one of vegetable) presented a positive band, between 100 to 200 bp, in the second PCR reaction. So, those samples, with possible positive fragments, and the positive control were sequenced, and DNA was digested with EcoRV endonuclease. Enzymatic digestion with EcoRV endonuclease suggests specificity and excludes cross-amplification of non-target organisms (Marques et al., 2020). The positive control had more than 95 % nucleotide similarity with the 529 bp *T. gondii* repetitive region (GenBank: AF146527.1) and one sample (sample 10: water from post-sand filter) was contaminated with *Stenotrophomonas maltophilia* DNA, that is an environmental bacterium found in aqueous habitats, including animals, foods, and water sources. *S. maltophilia* is not a highly virulent pathogen, but it has emerged as an important nosocomial pathogen (Brooke, 2012). In addition, when adding 1 µl of the positive control of *T. gondii* to samples, all amplified, which indicates that were not inhibitors to interfere in the reaction and, when the PCR was performed with another enzyme, the results were the same, that is, there was no amplification of the fragment of the 529 bp repetitive region of *T. gondii*.

All samples showed negative results for *T. gondii*, which indicates that disinfection methods are suitable for this protozoa. However, when *T. gondii* DNA amplification does not occur, it does not mean that the samples are not contaminated with the parasite's oocysts. There are several factors that influence the detection of the parasites such as the rate of recovery of the parasitic structures when processing the samples, the effectiveness of the rupture of the oocysts during DNA extraction, since the resistant wall of oocyst that difficult the process, and the sensitivity of the amplification technique (A. Lass et al., 2012).

Table 9: Some available data on the detection of *T. gondii* oocysts in water and sand collected from the sand/coal system, under non-experimental conditions.

Reference	Product	Origin	Sample size	Oocyst method recovery	Detection method	PCR target	*T. gondii* prevalence (%)	Sequence confirmation
(Villena et al., 2004)	Water	France	n = 139 (7– 100L)	Concentration using Envirochek capsules; centrifugation.	qPCR	B1 gene	8	-
(Triviño-Valencia et al., 2016)	Water	Colombia	n = 46 (10L)	Formalin-ether concentration method	Conventional nested-PCR	B1 gene	58.6	-
(Ajonina et al., 2017)	Water	Germany	n = 25 (1L)	Centrifugation, filtration using nitrate cellulose membrane	Conventional nested-PCR	B1 gene	0	-
(Sánchez et al., 2018)	Water	Colombia	n = 117	Filtration using cellulose membranes; centrifugation	qPCR	B1 gene	0	-
Present study	Water and sand	Portugal	n = 13 (10-100L)	IDEXX Filta-Max® filtration EPA method 1623.1	Conventional PCR, restriction enzyme, sequencing	529 bp REP	0	No

Table 10: Some available data on the detection of *T. gondii* oocysts in fresh vegetables and fruits, under non-experimental conditions.

Reference	Product	Origin	Sample size	Oocyst method recovery	Detection method	PCR target	*T. gondii* prevalence (%)	Sequence confirmation
(A. Lass et al., 2012)	Carrot, lettuce, radish, strawberries	Poland	n = 216	Washing, centrifugation, flocculation ($CaCO_3$)	qPCR	B1 gene	9.7	-
(Anna Lass et al., 2019)	Lettuce, spinach, pak choi, chinese cabbage, rape, endives, chinese chives	China	n = 279	Washing, $Al_2(SO_4)_3$ flocculation methodology	qPCR	B1 gene	3.6	Yes
(Marques et al., 2020)	RTE/packaged and bulk (organic and nonorganic: lettuce, watercress, coriander, parsley, carrots, arugula, straw-berries, raspberries, blueberries, mixed salads)	Portugal, Spain	n = 35 (64 – 3600g)	IDEXX Filta-Max® filtration EPA method 1623.1	Microscopy, conventional PCR, qPCR, restriction enzyme, sequencing	529 bp REP	40	Yes
Present study	Lettuce, spinach, arugula	Portugal	n = 12 (500 – 1000g)	IDEXX Filta-Max® filtration EPA method 1623.1	Conventional PCR, restriction enzyme, sequencing	529 bp REP	0	No

Molecular approaches were also used to detect *C. cayetanensis* oocysts in environmental samples. Primers target the ITS2 region and 18S rRNA were tested, 2 pairs were used considering the bibliography, while 2 were designed by the Parasitology laboratory team. The pairs considering the bibliography showed more satisfactory results, so these were the primers used. Using the primers that amplify a 298 bp fragment of the 18S rRNA gene (Murphy et al., 2017), all the samples showed negative results. However, using the DreamTaq™ Hot Start Green PCR Master Mix 2x (Thermo Fisher Scientific, USA) and specific primers that amplify in the ITS2 region (Lalonde et al., 2008), a 116 bp fragment occurred in several samples. This indicates that different reagents will have different reactions and conditions that potentiate, or not, the amplification. In addition, when adding 1 µl of the positive control of *C. cayetanensis* to samples, all amplified, which indicates that were not inhibitors to interfere in the reaction.

The 116 bp DNA fragment was amplified in 28% of all samples, more specifically, 23% of water and sand samples and 33% of vegetables samples and our findings indicate a higher prevalence compared to other studies (Tables 11 and 12).

Critical analysis of the data available, evidence major differences related to: (i) sampling weight; (ii) DNA target strategy and (iii) the choice of oocyst recovery method (Tables 11 and 12). The choice of the Filta-Max® System (IDEXX), for high- resolution filtration of large volumes of washing water or raw water, increases oocyst recovery and the detection method. Complementary to this, the execution of Method 1623.1 (EPA, 2012) seems to be an advisable strategy for removing *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts and separating them from potential *C. cayetanensis* oocysts present in the sample washing water.

Slides from PCR-positive samples were examined under bright field microscopy and UV microscopy, and autofluorescent structures, compatible with *C. cayetanensis* unsporulated oocysts in morphology and size (between 8–10 µm in diameter) were observed. However, a major constraint on this research, to address such issues, was the lack of *C. cayetanensis* oocysts to be used as a control in this study, because *C. cayetanensis* appears to be the only species that affects humans and is apparently restricted to this host (Almeria et al., 2019). In addition, the modified safranin technique (hot method) was used to stain *C. cayetanensis* oocysts for microscopy analysis. This technique produces a more uniform staining of these oocysts when compared to modified acid-fast stain procedure (Almeria et al., 2019; CDC, 2018). Microscopy is often labour intensive, particularly if the sample has

lots of debris, such as environmental samples like these, and requires a well-trained microscopist who can distinguish the morphological features of *Cyclospora* from those of impostors and artifacts, which are common in environmental samples (Garcia et al., 2018).

All samples that presented a positive fragment were confirmed by sequencing and this step is important for a correct and assertive diagnosis. Only one of the positive samples (Sample 3, water from reservoir used for conventional agriculture system) did not undergo any treatment, which may explain the presence of *C. cayetanensis*. The remaining positive water samples, (samples 11 and 12 are treated water) underwent a series of processes to eliminate residues and microorganisms. This fact proves that protozoa are organisms that resist the usual treatments and that companies must invest on effective ways to eliminate them (Erickson et al., 2006; Fayer et al., 2004; Gajadhar et al., 2006).

Regarding the vegetables samples, 4 were confirmed by sequencing. The samples were the pre-washed iceberg lettuce , pre-washed organic spinaches , red lettuce and organic green lettuce. As already mentioned, rougher leaves such as lettuce are more likely to have protozoa since they adhere better (Berrouch et al., 2020) and this fact is compatible with the results. Of the 4 samples, 2 were from the organic agriculture system. Indeed there is a higher possibility of parasitic contamination of organic vegetables when compared to conventional agriculture system, once chemical treatments may reduce the presence of *C. cayetanensis* on the raw products, but no significant differences were found between both systems (conventional vs. organic) as well as the pre-washing of the vegetables did not bring significant changes. It is important to note that all the results obtained in the sequencing were compared with the sequence of interest and with other sequences from other environmental protozoa, such as *Eimeria* spp., *Isospora* spp., *Hammondia* spp., *Neospora caninum*, *Toxoplasma gondii*, *Cryptosporidium* spp. and G*iardia duodenalis*. Sequencing results from six samples (3,11,12,14,17 and 22) had similarity values above 90% with *Eimeria* spp. sequences. However, in the majority of them, query cover was relatively low (10-50%).

The results of this study confirmed that fresh vegetables and water available were contaminated with *C. cayetanensis* and they may pose a potential threat for public safety, particularly, people consuming them raw and unwashed (FAO/WHO, 2014). Although, the viability of *C. cayetanensis* isolates was not investigated in this study, and consequently their ability to infect humans and/or animals remains unknown, the detection of *C. cayetanensis*

DNA and the presence of structures compatible with *C. cayetanensis* oocysts in vegetable and water samples is a clear evidence for the presence of the parasite in the food chain and indicates a potential risk for humans. However, the risk to human health when ingesting water or vegetables contaminated with *C. cayetanensis* should be carefully analysed, considering the level of recovery of the oocysts present in the samples, the infectious dose, and the exposure of groups of risk.

Control measures to reduce the possibility of contamination of produce with *C. cayetanensis* and *T. gondii*, include the use of good quality water for irrigation, washing, and processing of the vegetables; the adequate removal of fecal matter from animals (Gajadhar et al., 2006); checking the health of farmworkers and the animals, and encouraging good hygiene (FAO/WHO, 2014). In addition, it is important to adopt measures at home that minimize the risk of infection such as using good quality water for washing and cleaning food, as well as hands and objects handled for the purpose; separate foods eaten raw to avoid cross-contamination; cook food and store the food in refrigerator (FDA, 2019).

Table 11: Some available data on the detection of *C. cayetanensis* oocysts in water and sand collected from the sand/coal system, under non-experimental conditions.

Reference	Product	Origin	Sample size	Oocyst method recovery	Detection method	PCR target	*C. cayetanensis* prevalence (%)	Sequence confirmation
(A. Giangaspero et al., 2015)	Water	Italy	n = 10 (1L)	Nucleospin Soil kit (Macherey-Nagel, The Netherlands)	qPCR, HRM assay	ITS2	30	Yes
(Annunziata Giangaspero et al., 2015)	Water	Italy	n = 113 (94 samples of treated water; 16 samples of well water; 3 samples of drinking water) (100L)	Yarn-wound cartridge filter; centrifugation	qPCR	ITS2	21.3% of treated water 6.2% of well water 0% of drinking water	Yes
(Sánchez et al., 2018)	Water	Colombia	n = 117	Filtration using cellulose membranes; centrifugation	qPCR	18S rRNA	0	-
Present study	Water and sand	Portugal	n = 13 (10-100L)	IDEXX Filta-Max® filtration EPA method 1623.1	Nested-PCR; conventional PCR; Microscopy; Sequencing	18S rRNA; ITS2	23	Yes

Table 12: Some available data on the detection of *C. cayetanensis* oocysts in fresh vegetables and fruits, under non-experimental conditions.

Reference	Product	Origin	Sample size	Oocyst method recovery	Detection method	PCR target	*C. cayetanensis* prevalence (%)	Sequence confirmation
(Duedu et al., 2014)	Carrot, onion, tomato, green pepper, cabbages, lettuce	Ghana	n = 395 (100 - 400 g)	Washing, centrifugation	Microscopy	-	5	-
(Annunziata Giangaspero et al., 2015)	Lettuce, fennel, celery, tomatoes, cucumber, melon	Italy	n = 49 (100g – 1000g)	Washing, centrifugation, Percoll-sucrose flotation	qPCR	ITS2	12.2	Yes
(Sim et al., 2017)	Perilla leaves, winter-grown cabbages, chives, sprouts, blueberries, cherry tomatoes	Korea	n = 404 (20 – 50 g)	Washing, centrifugation,	qPCR	ITS2	1.2	Yes
Present study	Lettuce, spinach, arugula	Portugal	n = 12 (500 – 1000 g)	IDEXX Filta-Max® filtration EPA method 1623.1	Nested-PCR; conventional PCR; microscopy; sequencing	18S rRNA; ITS2	33	Yes

Conclusion

6. Conclusion

Public health agencies, regulatory agencies, the food industry and consumers need to make continuous efforts to prevent contamination of foods on the farm, in processing, in restaurants and at homes.

The molecular biology techniques selected for this study were successfully applied and allowed to achieve the proposed aims for the detection of *T. gondii* and *C. cayetanensis* in samples of water and fresh vegetables. The presence of *T. gondii* DNA was not detected in any sample. However, several samples have been shown to be contaminated with *C. cayetanensis*, indicative of fecal contamination of the products, which may represent a risk to the consumer's health.

It would be interesting to expand the number of agricultural explorations visited, as well as the number of samples analyzed and different biological products, such as berry fruits and small herbs. In addition, repeating the study in different seasons can be useful to understand the behavior of parasite distribution and how it is influenced by heat, humidity and rain. Moreover, considering the possibility of working with contaminated vegetables and fruit as a risk factor of toxoplasmosis and cyclosporiasis, a comparison with the meat industry can be performed.

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7. References

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