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Molecular investigation of ready-to-eat salads for *Giardia duodenalis* and *Cryptosporidium* spp. in Portugal

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ABSTRACT

Foodborne outbreaks are often associated with the consumption of salads. However, published studies on the detection of foodborne pathogens in ready-to-eat salads are scarce. The aim of this study was to detect *Giardia duodenalis* and *Cryptosporidium* DNA in ready-to-eat salads, by applying techniques of molecular biology to study the frequency of contamination in salads. A total of 100 packages of ready-to-eat salads containing assorted leafy green vegetables were randomly purchased from hypermarkets located in central regions of Portugal (Coimbra and Viseu). Nested-PCR and qPCR methods were used to detect *G. duodenalis* and *Cryptosporidium* DNA. Species and assemblages of the parasites were identified by sequence analysis and PCR. Eighteen of the 100 samples (18%) were positive for *G. duodenalis* and twelve were sequenced and identified as assemblage A. *Cryptosporidium* spp. were not detected in any salads. Overall, pre-harvest and post-harvest preventive measures may be need for *G. duodenalis* control throughout the food production industry, from the field to consumers.

1. Introduction

Foodborne zoonotic pathogens have been increasing significantly, thus becoming an emerging problem throughout the world (Ryan et al., 2019). Many of the zoonotic pathogens are responsible for serious public health problems and economic losses (Oliveira et al., 2010; Vestrheim et al., 2016). Many outbreaks of foodborne illness associated with protozoan parasites have been reported (Kirk et al., 2014; Robertson, 2018; Ryan et al., 2018; Ryan et al., 2019). Parasitic protozoa, such as *G. duodenalis* and *Cryptosporidium* spp., have been recognized as having considerable potential to cause zoonotic, waterborne and foodborne diseases (Dawson, 2005; Efstratiou et al., 2017). These two intestinal parasites are transmitted by the fecal-oral route: directly between infected humans or animals and persons; or indirectly via contaminated water or food. The cysts of *Giardia* spp. and oocysts of *Cryptosporidium* spp., are resistant to environmental conditions and water treatments remaining viable for a long time in the environment having high potential to contaminate water, soil and subsequently some foods at risk (Feng and Xiao, 2011).

Generally speaking, salads are potential sources of pathogenic microorganisms, either by their growth medium (soil) or by their processing (cutting, washing, drying and sorting) (Dixon et al., 2013; Caradonna et al., 2017; Ferreira et al., 2018). Over the last two decades, there have been many outbreaks of gastroenteritis caused by pathogenic microorganisms present in salads (Mikhail et al.,

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2018; Kintz et al., 2019; Self et al., 2019). The proportion of these outbreaks tends to increase, given the growing popularity of these salads with consumers.

Vegetable consumption is strongly associated with the adoption of healthy lifestyles by the population (Slavin and Lloyd, 2012). This factor makes people increasingly seek to incorporate vegetables in a balanced and healthy diet. The demand for untouched or minimally processed vegetables has increased because of their practical, accessible and easy-to-use nature, meaning that they have become a very popular product among consumers (Taban and Halkman, 2011; Söderqvist et al., 2016). In many countries these ready-to-eat salads are produced on an industrial scale and sold widely.

A number of studies have shown that *Cryptosporidium* spp. and *G. duodenalis* are present worldwide in vegetables and fruits (Li et al., 2020). On the African continent, cysts of *G. duodenalis* and oocysts of *Cryptosporidium* spp. have been recorded previously in fresh vegetables from Sudan and Ethiopia (Mohamed et al., 2016; Alemu et al., 2019). Similar results were observed in India, where fresh vegetables were contaminated by both parasites (Utaaker et al., 2017). In China, Li et al. (2019) identified three kinds of human pathogenic agents (*Enterocytozoon bieneusi*, *Cyclospora cayetanensis* and *C. parvum*) on the surfaces of the vegetables and fruits. In Italy, Caradonna et al. (2017) using microscope and/or molecular techniques detected *Dientamoeba fragilis*, *Blastocystis hominis*, *G. duodenalis*, *Cryptosporidium* spp., *Toxoplasma gondii* and *C. cayetanensis* in ready-to-eat salads. However, the detection of these protozoa has been performed mostly on unpackaged vegetables (Badri et al., 2022).

In Portugal, the prevalence of *Giardia duodenalis* and *Cryptosporidium* spp. has been poorly documented. Some studies have detected both parasites in humans, domestic animals, livestock and wild ruminants (Sousa et al., 2006; Ferreira et al., 2017). In addition, *Giardia* cysts and *Cryptosporidium* oocysts are widely distributed in Portuguese waters (Almeida et al., 2010a; Almeida et al., 2010b; Júlio et al., 2012), suggesting a potential waterborne transmission.

Considering the impact on public health of giardiasis and cryptosporidiosis, in which the etiological agents of these diseases are mostly transmitted by water and food, and given that there is no research on *G. duodenalis* and *Cryptosporidium* spp. in salads in Portugal, the present work aimed to detect and characterize both protozoa in ready-to-eat salads using molecular methods to evaluate the public health risk associated with their consumption.

2. Material and methods

2.1. Samples

A total of 100 packages of ready-to-eat salads were purchased at three supermarkets located in Coimbra and Viseu cities, Center of Portugal, including industrial brands and the white brand of each supermarket (Table 1). Salads with one type of leaf green vegetable and leaf green vegetables mixtures were processed: leaf lettuce (*Lactuca sativa* var. *Latina),* purple lettuce (*L. sativa* var. *crispa*), rocket salad or arugula (*Eruca sativa*), canon (*Valerianella locusta*), escarole (*Chicorium endivia),* watercress (*Nasturtium officinale*), radicchio (*Cichorium intybus*)*,* and chives (*Allium schoenoprasum*).

Table 1
Composition of ready-to-eat salads tested.

Samples		Composition of salads
Number	Identification code	
21	Aa1, Aa2, **FV1**, FV2, **FV3**, FC1, FC2, **FC3**, FP1, **FP2**, **FP3**, FP4, FP5, FP6, FP7, FP8, FP9, FP10, FP11, FP12, FP13	Purple lettuce
16	AV1, **AV2**, AV3, **AP1**, **AP2**, **AP3**, AP4, AP5, AP6, AP7, AP8, AP9, AP10, AP11, AP12, AP13	Watercress
19	**RV1**, **RV2**, RV3, RC1, RC2, **RC3**, RP1, RP2, **RP3**, RP4, RP5, RP6, RP7, RP8, RP9, RP10, RP11, RP12, RP13	Rocket salad or arugula
3	AC1, AC2, AC3	Watercress baby leaf
2	Va1, Va2	Purple lettuce + Leaf lettuce
4	Fa1, Am1, Fa2, Am2	Escarole + Radicchio
4	VG1, AG1, VG2, AG2	Escarole + Radicchio + Canon
1	FG1	Escarole + Radicchio + Rocket salad or arugula
21	Vm1, Vm2, **IV1**, **IV2**, IV3, IC1, IC2, **IC3**, IP1, IP2, **IP3**, IP4, IP5, IP6, IP7, IP8, IP9, IP10, IP11, IP12, IP13	Leaf lettuce + Purple lettuce + Rocket salad or arugula
2	As1, As2	Sprouts of Leaf lettuce + Sprouts of Purple lettuce + Rocket salad or arugula
2	Fm1, **Fm2**	Purple lettuce baby + Canon + Rocket salad or arugula + Watercress
1	FG2	Escarole +Radicchio + Rocket salad or arugula + Canon
1	Fant.1	Purple lettuce + Canon + Rocket salad or arugula + Watercress
1	Fant.2	Purple lettuce + Canon + Rocket salad or arugula + Chives
2	Vp1, Vp2	Canon + Purple lettuce + Escarole +Chives
Total = 100		

In bold are ready-to-eat green leafy vegetables positive for *Giardia duodenalis* based on nested-PCR amplification of the *ssu rRNA* gene.

2.2. Parasites recovery and DNA extraction

After the purchase, the ready-to-eat salads were immediately processed as previously described (Dixon et al., 2013) with some adaptations. Briefly, a portion of each salad (25 g; 1/6 of the total sample) was placed in a plastic stomacher bag (VIDROLAB 2 S.A., Portugal) and 200 mL of sterile phosphate buffered saline (PBS) with 0.01% Tween 80 was added. To recover any (oo)cysts present, this mixture was stirred in the vertical incubator (Model G25, New Brunswick Scientific Co.) at room temperature for 15 min at 120 rpm. The resulting wash buffer was filtered through double layered gauze, transferred to 50 mL sterile conical tubes and then centrifuged (10,000 ×*g* for 10 min at 4 °C). These procedures were repeated two times and then the sediments from the four tubes were consolidated into a single tube (1.5 mL) and stored at 4 °C.

DNA extraction was performed using the QIAamp DNA Stool mini Kit (Qiagen, Germany) according to the manufacturer's instructions but using 0.2 g of glass beads (0.5 mm, BioSpec Products, Inc.) and 170 µL of a polyvinylpyrrolidone solution (10%) in the first step with ASL buffer (Faria et al., 2016).

2.3. Nested-PCR and qPCR

The extracted DNA was analyzed by nested-PCR using primers to the small-subunit ribosomal RNA (*ssu rRNA*) gene of *G. duodenalis* and *Cryptosporidium* spp., as previously described (Appelbee et al., 2003; Xiao et al., 1999) (Table 2). All reactions contained 12.5 µL of NZYTaq 2× Green Master Mix (Nzytech, Portugal), 1 µL of each primer (10 pmol/µL), 1 µL of extracted DNA and 9.5 µL of sterile water, for a final volume of 25 µL. PCR was carried out on the MJ Mini™ Thermal Cycler (BioRad). For the *ssu rRNA* amplification of *G. duodenalis,* after initial denaturation of 96 °C for 4 min, a set of 35 cycles was run, each consisting of 45 s at 96 °C, 30s of annealing (55 °C for the primary reaction, 59 °C for the second), and 45 s at 72 °C, followed by a final extension step of 4 min at 72 °C. For the *ssu rRNA* gene amplification of *Cryptosporidium*, the primary reaction was carried out as follows: 1 cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s and 72 °C for 1 min. A final extension of 72 °C for 7 min and a 4 °C hold was used. The nested PCR was performed with the following amplification conditions: 1 cycle of 94 °C for 3 min, followed by 40 cycles of 95 °C for 30s, 58 °C for 90s and 72 °C for 1 min, and a final extension of 72 °C for 7 min. In all PCR reactions, positive (*G. duodenalis* and *Cryptosporidium* DNA), negative (water) and positive control with added matrix DNA (DNA of each salad sample plus *G. duodenalis* DNA of assemblage A (WBC6), Assemblage B (Ad-28, INI 10 and INI 27); and *Cryptosporidium* DNA) were included. The PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide and visualized using a gel documentation system (Uvitec, UK).

Extracted DNA was also analyzed by a real-time quantitative assay (qPCR) targeting the *18S rDNA* gene of *Cryptosporidium* (Lalonde and Gajadhar, 2011). Amplification reactions contained 12.5 µL of Sso Fast™ EvaGreen SuperMix (BioRad, USA), 1 µL of each primer (10 pmol/µL), 1 µL of extracted DNA and 9.5 µL of sterile water, for a final volume of 25 µL. All reactions were performed in triplicate; positive (*Cryptosporidium* DNA), negative (water) and positive control with added matrix DNA (DNA of salad samples plus *Cryptosporidium* DNA) were also included in each PCR. qPCR assays were performed on CFX 96™ Real Time PCR Detection System (BioRad). All reactions started with a denaturation step at 98 °C for 2 min, followed by 40 cycles of denaturation (98 °C for 30s) and annealing (65 °C for 30s). The melting curve program was performed at the end of each reaction and consisted of 95 °C for 5 s, 65 °C for 1 min, and heating to 95 °C with continuous acquisition (5 acquisitions per degree Celsius).

The nested-PCR positive results of *G. duodenalis* were confirmed by qPCR using the triosephosphate isomerase (*tpi*) gene (Almeida et al., 2010c). Amplification reactions contained 12.5 µL of Sso Fast™ EvaGreen SuperMix (BioRad, USA), 1 µL of each primer (10 pmol/µL), 1 µL of extracted DNA and 9.5 µL of sterile water, for a final volume of 25 µL. All reactions were performed in triplicate; positive and negative controls were also included in each PCR. A minor modification was done in the thermal profile: reactions started with a denaturation step at 98 °C for 2 min, followed by 40 cycles of denaturation (98 °C for 5 s) and annealing (59 °C for 5 s). The

Table 2
List and sequences of PCR primers.

Locus	Primer	Sequence (5′-3′)	Reference
Giardia			
ssu rRNA[*]	Gia2019	AAGTGTGGTGCAGACGGACTC	Appelbee et al., 2003
	Gia2150c	CTGCTGCCGTCCTTGGATGT	
	RH11	CATCCGGTCGATCCTGCC	
	RH4	AGTCGAACCCTGATTCTCCGCCAGG	
tpi[**]	tpiA_F	TCGTCATTGCCCCTTCCGCC	Almeida et al., 2010c
	tpiA_R	CGCTGCTATCCTCAACTG	
Cryptosporidium			
ssu rRNA[*]	Crypto18SF1	TTCTAGAGCTAATACATGCG	Xiao et al., 1999
	Crypto18SR1	CCCTAATCCTTCGAAACAGGA	
	Crypto18SF2	GGAAGGGTTGTATTTATTAGATAAAG	
	Crypto18SR2	AAGGAGTAAGGAACAACCTCCA	
18S rDNA[**]	Crypto F	AGTGACAAGAAATAACAATACAGG	Lalonde and Gajadhar, 2011
	Crypto R	CCTGCTTTAAGCACTCTAATTTTC	

[*] Primers used in the nested-PCR.
[**] Primers used in the qPCR.

melting curve program was performed at the end of each reaction and consisted of 95 °C for 5 s, 60 °C for 1 min, and heating to 95 °C with continuous acquisition (5 acquisitions per degree Celsius).

2.4. Sequencing analysis

Positive nested-PCR and qPCR products were purified using QIAquick PCR Purification kit (Qiagen), quantified (ND1000, NanoDrop) and sequenced with secondary PCR primers in both directions using the respective forward and reverse primers with an Applied Biosystems 3730 xL DNA Analyser (Applied Biosystems). Chromatograms and nucleotide sequences were analyzed, edited using BioEdit Sequence Alignment Editor Programme (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and were aligned with reference sequences retrieved from the GenBank using Clustal W. In order to identify *Giardia* and *Cryptosporidium* sequences, BLAST software (http://www.ncbi.nlm.nih.gov/blast/) was used for comparison of the nucleotide sequences obtained in the present work with sequences available at GenBank.

2.5. Statistical analysis

The data entry was carried out using Excel software and analyzed using Statistical Package for the Social Sciences (SPSS) version 16. Pearson's chi-squared and Fisher's Exact Test were used for categorical data. The level of statistical significance was set as $p < 0.05$.

3. Results

The fragment of *ssu rRNA* gene (292 bp) of *G. duodenalis* was amplified by nested PCR in the positive control samples and in eighteen samples (18%) of 100 tested ready-to-eat salads (Table 3). No significant differences in positive rates were observed among the varieties of leafy green vegetables. Five (23.8%) of purple lettuce, four (21.05%) of arugula,four (25%) watercress and four (19.4%) Iberian salad (leaf lettuce + purple lettuce + arugula) were nested-PCR positive. One (50%) of leafy green vegetable mix (purple lettuce + canon + arugula + watercress) was positive.

Subsequently, 12 of these *G. duodenalis* positive samples (Fm2, FP2, FV3, FC3, IC3, IP3, RV1, RV2, RP3, AV2, AP2, AP3) were successfully sequenced and showed 100% homology to the previously described *Giardia duodenalis* (accession numbers M54878 and AF199446). Based on the nucleotide positions of the *ssu rRNA* gene, the sequenced samples were all identified as *G. duodenalis* assemblage A: Guanine in positions 22, 24 and 44; Cytosine in position 23 and 73; Adenine in position 38 and 93; and Thymine in position 63 (Table 4). These data were confirmed by qPCR targeting the *tpi* gene (Suppl.1). All samples were identified as *G. duodenalis* assemblage A.

The *ssu rRNA* gene (825 bp) of *Cryptosporidium* was amplified by nested-PCR in the control samples, but was not amplified in tested salad samples.

In qPCR, DNA fragments of *18S rDNA* gene of *Cryptosporidium* was amplified on positive control and in two salad samples (Vm1 and Va1). However, after sequencing these qPCR amplicons, the Vm1 sequence showed 95% identity with *Cryptococcus peneaus* (KR336839), and the Va1 sequence showed 99% identity with *Dioszegia antartica* (KF036667).

4. Discussion

Giardia duodenalis and *Cryptosporidium* spp. are recognized as important foodborne pathogens and are associated with severe gastrointestinal illness (Ryan et al., 2018; Ryan et al., 2019). It is well documented that conventional water and sewage treatment processing are not completely effective in removing or destroying protozoa (oo)cysts (Savioli et al., 2006; Betancourt and Rose, 2004). With regard to zoonotic potential, special attention should be given to the relationship between *Giardia* infections in humans and animals (Thompson and Ash, 2016). *G. duodenalis* is divided into eight distinct genetic assemblages (A to H), but only assemblages A and B are responsible for most (> 99%) human infections (reviewed in Ryan and Cacciò, 2013).

The present study represents the first determination of the prevalence and genetic characterization of *G. duodenalis* and *Cryptosporidium* DNA in ready-to-eat salads in Portugal. We observed a relatively high prevalence of *G. duodenalis* in ready-to-eat salads

Table 3
Ready-to-eat salads positive for *Giardia duodenalis* based on nested-PCR amplification of the ssu rRNA gene.

Samples		Nested-PCR	Detection rate No (%)	*p* values
Control (positive)	assemblage A	+		
	assemblage B	+		
Control (internal)	assemblage A	+		
	assemblage B	+		
Control (negative)		−		
Purple lettuce		+	5/21 (23.8)	0,523
Watercress		+	4/16 (25)	0,479
Rocket salad or arugula		+	4/19 (21.05)	0,742
Leaf lettuce + Purple lettuce + Rocket salad or arugula		+	4/21 (19.04)	0,999
Purple lettuce baby + Canon + Rocket salad or arugula + Watercress		+	1/2 (50.0)	0,329

Table 4
Differentiation of *G duodenalis* assemblages (A-G) based on nucleotide positions of the *ssu rRNA* gene.

Assemblages	Nucleotides position										
	22	23	24	38	44	45	63	73	93	168	173
A	G	C	G	A	G	–	T	C	A	G	G
B	A	T	C	A	A	C	G	G	A	G	G
C	A	T	C	A	A	C	A	G	A	T	G
D	A	T	C	A	A	C	A	A	A	T	A
E	G	C	G	A	G	–	T	C	G	G	G
F	G	C	G	C	G	–	T	C	A	G	G
G	A	T	C	A	G	–	A/G	G	A	G	A
Positive leafy green vegetables samples	G	C	G	A	G	–	T	C	A	G	G

Accession numbers of reference sequences:
Assemblage A: M54878, AF199446; Assemblage B: AF199447, AF113898; Assemblage C: AF113899, AY775200; Assemblage D: AF113900, AY775199; Assemblage E: AF113902, AF199448, AY 297957; Assemblage F: AF113901, AF199444, DQ836339; Assemblage G: AF199450, AF113896.

(18%) when compared to studies conducted in Canada (Dixon et al., 2013) and Italy (Caradonna et al., 2017). Indeed, our result is more similar to the values achieved in unwashed vegetables (Alemu et al., 2019; Li et al., 2020). Sequence analysis and qPCR showed that 12 of 18 positive samples were classified as Assemblage A. The presence of double peaks and ambiguous nucleotides in the chromatogram observed in six samples hindered the classification of *G. duodenalis* assemblages.

The detection of DNA of *G. duodenalis* Assemblage A in the salad samples is potentially alarming for human health since this assemblage is usually associated with human infections worldwide. Moreover, that assemblage was associated with zoonotic transmission (Sprong et al., 2009; Ryan and Cacciò, 2013) and we know that *G. duodenalis* are found in the feces of humans, domestic animals, wild ruminants and livestock in Portugal (Sousa et al., 2006; Ferreira et al., 2017). Additionally, given that the infective dose of this parasite is very low (10 to 100 cysts) (Nguyen et al., 2016), the amount of salad analyzed (~25 g) potentially contains ten times greater than the infectious dose of *Giardia*. Although a relative small number of ready-to-eat salads was analyzed, it should be noted that the lots used in this study were distributed throughout the country in other supermarkets and could potentially be purchased and consumed by a large segment of the population. Nevertheless, the potential risk to human health of these salads should be carefully analyzed. The detection of *G. duodenalis* DNA in the salads indicates exposure to fecal contamination, representing a potential risk to human health (Dixon et al., 2013).

In ready-to-eat salads, product contamination may occur during different stages of processing, between the farm and the consumer, through contaminated soil, manure, irrigation or wash water, via equipment, or from handlers (Amorós et al., 2010; Shields et al., 2012; Dixon et al., 2013). Since *Giardia* cysts may survive in the environment for weeks or even months, contamination of water and food can arise from this source. Moreover, *Giardia* cysts are resistant to conventional routine disinfectants used in drinking water treatment (Betancourt and Rose, 2004). So, the detection of *G. duodenalis* DNA in our samples is likely to be associated with the inability or inefficacy of the wash water systems to remove the cysts and/or with the quality wash water. It is well documented that *G. duodenalis* are present in river waters (Almeida et al., 2010a; Almeida et al., 2010b; Júlio et al., 2012) and water for human consumption in Portugal (Lobo et al., 2009).

Regarding the detection of *Cryptosporidium*, the qPCR assay was performed in the present study based on previous validation for the diagnosis of *Cryptosporidium parvum*, *C. cayetanensis*, *T. gondii* and several species of *Eimeria*, *Sarcocystis*, and *Isospora* in samples of human and animal feces (Lalonde and Gajadhar, 2011). As mentioned above, after the analysis by qPCR, positive results were observed in two samples and in the *Cryptosporidium* positive control. Sequencing was performed and both samples aligned with fungal sequences. The authors who developed and validated this qPCR technique supported their potential application in environment and food samples, but with the need for adequate validation and the use of appropriate controls. Our *Cryptosporidium* positive controls correctly amplified in the qPCR and had positive results in the nested-PCR. Together, the results lead us to conclude that DNA amplification of fungi instead of *Cryptosporidium* is due to a cross-reaction with microorganisms present in the salads and so sequencing the qPCR product is necessary to confirm the presence of *Cryptosporidium*.

It is worth pointing out that non-amplification of *Cryptosporidium* DNA does not mean that the salads are not contaminated with this protozoan. Several factors influence the detection of parasites, such as the oocysts recovery rate, the efficacy of oocyst rupture during DNA extraction and the sensitivity of PCR. The recovery rates of oocysts in salads are never 100%, as this mainly depends on the solution used during processing and the vegetable type (Shields et al., 2012). Moreover, *C. parvum* oocysts were found to infiltrate through the stomatal openings in leafy greens, and to persist at the mesophyll level (Macarisin et al., 2010), impairing the oocysts recovery.

Studies to assess the level of survival of *G. duodenalis* cysts and *Cryptosporidium* oocysts under varying environmental conditions are imperative, so that control measures can be implemented to minimize food contamination.

Despite the assay limitations, the molecular biology methods in this study were successfully used for the detection of protozoan parasites in ready-to-eat salad samples. It is important to establish measures for parasite prevention and control throughout the production, processing, packaging and transportation of prepackaged and ready-to-eat salads.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2023.e00190.

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