Detection and prevalence of protozoan parasites in ready-to-eat packaged salads on sale in Italy.

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Abstract

To investigate the prevalence of protozoan contamination by *Giardia duodenalis, Cryptosporidium* spp., *Toxoplasma gondii* and *Cyclospora cayetanensis*, in 'ready to eat' (RTE) salads on sale in Italy, 648 packages from industrial and local brands were purchased. Nine individual packages from each brand were collected per month, pooled and subjected to microscopy and molecular analyses. 864 slides were microscopically examined and *Cryptosporidium* spp. and also *Blastocystis hominis* and *Dientamoeba fragilis* were detected. By molecular tools *G. duodenalis* assemblage A, *Cryptosporidium parvum* and *Cryptosporidium ubiquitum*, *T. gondii* Type I and *C. cayetanensis* were identified. *B. hominis* and *D. fragilis* were also molecularly confirmed. The overall prevalence of each protozoan species was 0.6% for *G. duodenalis*, 0.8% for *T. gondii*, 0.9% for *Cryptosporidium* spp., and 1.3% for *C. cayetanensis*, whereas the prevalence of *B. hominis* and *D. fragilis* were 0.5% and 0.2%, respectively. By microscopy and/or molecular tools, 4.2% of the samples were contaminated by at least one protozoan species, and 0.6% of them showed contamination of two protozoan species with a range number of oocysts from 62 to 554 per g of vegetable for *T. gondii*, and 46 to 1.580 for *C. cayetanensis*. This is the Europe's first large-scale study on the presence of protozoans in packaged salads. RTE sanitation processes does not guarantee a product free from protozoans of fecal origin.

Key words: Prevalence; protozoans; ready-to-eat salads; Italy

1. Introduction

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

After harvesting, ready to-eat vegetables undergo minimum conservation treatments to maintain their organoleptic and sensory characteristics, and are sold already cleaned, cut, washed and packed in a protected atmosphere (**Martín-Belloso and Soliva-Fortuny 2011**).

Italy is Europe's second largest market for fresh-cut products after France. In the period 2010-2015, the Italian fresh cut salad market grew by + 9.9%; RTE salads account for about 75% of these sales, and are at present mostly mixed salads (**Confcooperative, 2016; IsmeaMercati, 2016).**

In Italy, approximately 500 companies and 120 processing plants are involved in the production of RTE vegetables. These companies are mostly in Northern Italy, while the farms that provide the raw material are mostly in Southern Italy (**Casati and Baldi, 2011**).

Vegetables may become contaminated in various ways along the food production chain, i.e. during primary production (contaminated water in irrigation, manure application to croplands, access to crops by livestock/wildlife), harvesting in the field, during transport and market processing (**Chaidez *et al.*, 2005; Francis et al., 1999; Johnston, 2005)** or directly by infected food handlers (**Beuchat and Ryu, 1997**).

Since these products are eaten raw, they are covered by the EU and Italian health laws (L.M 13.05.2011, No.77; EC Reg. 852 of 2004; EC Reg. 20703/2005 and 1441/2007; EC Reg. 209, 2013), defining the presence and microbiological limits for *Escherichia coli*, including some verocytotoxigenic *E. coli*, *Listeria monocytogenes*, and *Salmonella* spp. However, in addition to bacteria, several protozoan parasites from human/animal excreta can also contaminate soil and vegetables. *Giardia duodenalis, Cryptosporidium* spp*., Toxoplasma gondii* and *Cyclospora cayetanensis* are the most important emerging parasitic protozoans (**Dubey, 2008; Fletcher et al., 2012**). *G. duodenalis* and *Cryptosporidium* spp. are well-known causative agents of gastrointestinal disease in humans (particularly children) and animals worldwide (**Bouzid et al., 2013; Feng and Xiao, 2011; Putignani and Menichella, 2010**). Infection occurs via the fecal-oral route through ingestion of *G. duodenalis* cysts and *Cryptosporidium* oocysts. Eight major genetic groups of *G. duodenalis* (assemblages) have been identified (A–H) to date, and assemblage A and, with a lesser extent, assemblage B are considered to be of zoonotic interest **(Feng and Xiao, 2011**). As to

Cryptosporidium, of the 31 *Cryptosporidium* species recognized as valid, over 20 species and genotypes have been identified in humans; however, the majority of human cryptosporidiosis is caused either by the zoonotic *Cryptosporidium parvum* or by the more anthroponotic *Cryptosporidium hominis* (**Ryan et al., 2016**). Other species are associated with human infections, including *Cryptosporidium meleagridis, Cryptosporidium ubiquitum, Cryptosporidium cuniculus* (**Ryan et al., 2014**).

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

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

Giardia, *Cryptosporidium, Toxoplasma* and *Cyclospora* oo/cysts are very robust and unlikely to be inactivated by routine chemical disinfectants or sanitizing water treatments, which explains their diffusion in the environment (**Fletcher et al., 2012; Giangaspero et al., 2009; Jones and Dubey, 2010, 2012;**) and food (**Dixon et al., 2013**). Outbreaks of infections caused by protozoan parasites detected in contaminated fresh produce have been recorded worldwide (**Dixon et al. 2013; Feng and Xiao, 2011; Kozak et al., 2013; Ortega and Sanchez, 2010; Putignani and Menichella, 2010**), including Europe (**Aberg et al., 2015; Doller et al., 2002; McKerr et al., 2015**). However, despite the rules issued by **FAO/WHO (2003),** supporting the need for tracking, monitoring and surveillance of food products, studies on parasite contamination of RTE and pre-packaged/bulked vegetables products are limited to only a few reports from Canada (**Dixon et al., 2013; Lalonde and Gajadhar, 2016**).

The aim of this work was to bridge a gap in knowledge about the safety of RTE salads and potential consumer health risks in Europe, by using both microscopy and molecular tools to investigate the occurrence and prevalence of *G. duodenalis, Cryptosporidium* spp., *T. gondii* and *C. cayetanensis* in packaged RTE mixed salad, sold under industrial and local brands and available in Italian food stores.

2. Materials and methods

2.1 Sampling design

The sampling design was tailored to provide the highest confidence of contamination detection and quantification, even with the low expected prevalence reported for protozoa in edible salads. The detection of parasite at a low prevalence requires large sample sizes, in order to keep the study within manageable limits, the sampling design was based on testing pools of salad samples in common and homogenous groups (**Cowling et al., 1999**). The number of pools to test, for a given pool size, under a specified expected prevalence, desired confidence and precision has been estimated according to **Worlund and Taylor (1983).**

We set a prevalence value of 0.6% as the detection threshold for protozoa (i.e., the lowest prevalence we were able to detect with our sampling regime). The confidence level and precision were set at 95% and 0.6%, respectively.

Since we chose a pool size of 9 salad packages, 72 pools were required to estimate prevalence.

In order to provide a representative sample, the pools (each composed of 9 packages) came from six different selected RTE producers: three major industrial companies (indicated as A, B, and C) with national distribution and three minor companies with local distribution (indicated as E, F, and G). Each month, from March 2015 to February 2016, for each company, nine individual mixed salad (all containing curly and escarole lettuce, red radish, rocket salad and carrots) packages (not less than 100g each) were bought and subsequently analyzed together as a single pool. Following this sampling protocol, a total number of 648 salad packages were analyzed and their distribution is summarized in **Table 1.** All salad packages were placed in a cooler bag and transferred to the laboratory, where they were kept refrigerated and then processed before their expiry date.

2.2 Sample processing

Salad samples were processed as described by **Dixon et al. (2013)** and by **Giangaspero et al. (2015a),** but the methods were slightly modified. For each of the nine packaged RTE mixed salads from the same brand, 100g of vegetable material was weighed and placed in 9 different stomacher bags (BagPage, Interscience, Sant Nom, France). After this, 200 ml of buffered detergent solution

(phosphate-buffered saline 10X [PBS], 0.1% Tween-80, 0.1% sodium dodecyl sulphate [SDS] and 0.05% antifoam B emulsion), was added to each bag. Bags were placed on an orbital shaker for 15 min at 120 rpm. Then, lavage liquids were collected into four 50ml tubes and centrifuged at 2,000 x g for 15 min at 4°C. The supernatant was discarded. The pellets were suspended in 3 ml of buffered detergent solution, and pooled into one tube. Each tube was then rinsed and the rinse liquid was added to the pooled tube. The pooled tube was centrifuged at 3,000 x g for 15 min at 4°C, and the supernatant was again discarded.

The pellet was resuspended into 2ml of buffered detergent solution and divided into two different tubes, respectively for microscopy and molecular investigations. Each tube was centrifuged at 10.000 x g for 10min at 4°C, and the supernatants were discarded. Finally, the pellets were resuspended with 500µl of PBS 1X for microscopy, and 300µl for molecular investigation.

The aliquot for microscopy investigation was tested within 3 days after processing, whereas the aliquot for molecular investigation was stored at -20 °C pending molecular analyses.

2.3 Microscopy investigation

From each pooled sample, 20 µl of concentrated solution was transferred on slides for direct observation after adding a Lugol's Iodine solution and for modified Ziehl-Neelsen staining (TB Stain Kit ZN, Becton, Dickinson and Company, New Jersey, USA). Giemsa staining was also used for confirmation when needed. Six slides were prepared for each pooled sample and examined by optical microscopy at 20, 40 and 100X using a NIKON Eclipse E600 (Nikon, Tokyo, Japan) microscope. Moreover, *G. duodenalis* cysts and *Cryptosporidium* oocysts were detected and analysed using a commercial kit (Merifluor C/G, Meridian Diagnostics, Cincinnati, Ohio, USA) and examined by fluorescence microscope (NIKON Eclipse E600 microscope). Filter system for fluorescein isothiocyanate (FITC): excitation wavelength 490-500nm, barrier filter 510-530 nm and magnification power at 40X and 100X. Microscopy procedures and parasite identification were guaranteed by two experienced operators, according to the internal control Bambino Gesù Hospital ISO analytical procedures (9100-2015).

2.4 Molecular investigation

2.4.1 DNA extraction

Genomic DNA was extracted from individual samples using the QiAmp Plant Mini Kit (Qiagen, Inc., Mississauga, Ontario, Canada). Briefly, 300 µl of the final suspension was divided into three aliquots of 100µl, and the aliquots were subjected to 15 one-minute cycles (liquid nitrogen/65°C).Then, 400µl of Buffer AP, 4µl RNaseA stock solution (100 mg/ml) and 130µl of

Buffer P3 were added to each sample and subjected to the QIAshredder spin column, following the manufacturer's instructions. The genomic DNA was quantified by NanoDrop 2000 and stored at −20 °C.

2.4.2 PCR protocols

A nested and semi-nested PCR protocol was used to detect *G. duodenalis* and *Cryptosporidium* spp, respectively. The PCR mixture contained 10.5 µl of Ready Red Taq Mix (Sigma-Aldrich, USA), 10µM of each primer and 3.25 µl of distillated water. Two µl of genomic DNA was added to the reaction for the first PCR, and 1:20 diluted PCR product (*Giardia*) and 1:40 (*Cryptosporidium*) for the second PCR. Positive and negative controls were included in each PCR run. For *G. duodenalis,* the *TPI* gene was amplified using primers AL3543 (forward: 5′-AAATTATGCCTGCTCGTCG-3′) and AL3546 (reverse: 5′-CAAACCTTTTCCGCAAACC-3′) for the first PCR and primers AL3544 (forward: 5′-CCCTTCATCGGTGGTAACTT-3′) and AL3545 (reverse: 5′-GTGGCCACCACTCCCGTGCC-3′) (**Sulaiman et al., 2003**) for the second PCR. For the primary amplification, the cycling protocol was 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 45 s (denaturation), 50 °C for 45 s (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C for 10 min. For the second amplification, the cycling protocol was 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

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

End point PCR protocol was used for *Blastocystis* and *Dientamoeba,* with a PCR mixture containing 10.5 µl of Ready Red Taq Mix (Sigma-Aldrich, USA), 10µM of each specific primer, 3.25 µl of distillated water and 2 µl of genomic DNA. Positive and negative controls were included in each PCR run. For *Blastocystis,* the *SSrRNA* gene was amplified using primers RD5 (forward: 5'-ATCTGGTTGATCCTGCCAGT-3') and BhRD (reverse: 5'-GAGCTTTTTAACTGCAACAACG-3') (**Scicluna et al., 2006**) and for *Dientamoeba* the *SSrRNA* gene was amplified using primers Df-124F (forward: 5' -CAACGGATGTCTTGGCTCTTTA-3') and Df-221R (reverse: 5' -TGCATTCAAAGATCGAACTTATCAC-3') (**Verweij et al., 2007**). The cycling protocol was 94

°C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 45 s (denaturation), 50 °C for 45 s (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C for 10 min.

All the PCR products were run on agarose gel and visualized with Gel Red Nucleic Acid staining (Biotium, USA).

A quantitative PCR (*q*PCR) and melting curve analysis were performed for *T. gondii* and *C. cayetanensis* in a CFX-96 Real Time Instrument (BioRad, Italy). A sequence of *T. gondii* B1 gene and of *C. cayetanensis* ITS-2 gene were selected to design the plasmid control. The pEX-A2 vector (Eurofins, MWG/Operon, Ebersberg, Germany) was used to insert a fragment of approximately 129 bp and 116 bp, respectively. The concentration of the pEX-A2 plasmid was measured using a fluorometer, and the corresponding copy number was calculated using the following equation: pEX-A2 *T. gondii*/*C. cayetanensis* (copy numbers) = 6.02 X 10^{23} (copy/mol) X pEX-A2 *T. gondii*/*C. cayetanensis* amount (0.31/0.21 X 10^{-5} g/ml)/pEX-A2 *T. gondii*/*C. cayetanensis* length (129/116 bp + 2450) X 660 (g/mol/bp) (**Whelan et al., 2003**).

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

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

2.4.3 Sequencing

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

2.5 Statistical Analyses

Based on the number of positive pools, the prevalence of salads contaminated with each protozoan species was estimated according to **Schaarschmidt (2007)**, and the 95% confidence levels were estimated using the exact method of Clopper-Pearson. This approach provides robust estimation of confidence levels, without need to retest individual samples from positive pools, so that analyses were kept within manageable limits. Since the salad samples were analyzed via multiple testing, i.e. both microscopic and molecular approaches, we estimated a combined prevalence as testing in parallel; that is a sample was considered positive if it reacted positively to either or both of the diagnostic tests. Moreover, for each protozoan species the difference in prevalence between brands and sampling seasons was tested *via* a group regression model (**Vansteelandt et al., 2000**), considering the contamination status of the pool (positive/negative) as response variable and pool identity as a grouping factor. Statistical analysis was performed using the 'binGroup' package for evaluation of binomial group testing, developed under the software R 3.3.2 (**R Development Core Team, 2016**) and considering $p<0.05$ as the threshold for statistical significance.

3. Results

A total of 864 slides were microscopically examined and by one or more microscopy techniques, among the investigated pathogens, *Cryptosporidium* spp. was detected. However, *B. hominis*, and *D. fragilis* were also microscopically detected. Molecular tools identified *G. duodenalis* assemblage A, *Cryptosporidium parvum* and *Cryptosporidium ubiquitum*, *T. gondii* Type I and *C. cayetanensis*. The microscopically positive samples to *B. hominis* and *D. fragilis* – which were outside of the aim of the prevalence study - were molecularly confirmed**. ok**

The results are summarized in **Table 2.**

Use of both microscopy and molecular tools showed that 4.2% (95% C.I. 2.6-6.2%) of the samples were contaminated by at least one protozoan species, and 0.6% (95% C.I. 0.2-1.6%) of samples presented contamination by two protozoa species. The most prevalent protozoa species was *C. cayetanensis* (1.3%, 95% C.I. 0.6-2.5%), followed by *Cryptosporidium* spp. (0.9%, 95% C.I. 0.4-2.1), *T. gondii* (0.8%, 95% C.I. 0.3-1.8%) and *G. duodenalis* (0.6%, 95% C.I. 0.2-1.6%), but *B. hominis* (0.5%, 95% C.I. 0.1-1.4%) and *D. fragilis* (0.2%, 95% C.I. 0.0-0.9%) were also detected (**Table 2**).

Prevalence between salad producers varied between the minimum value of zero for all and the highest values of 3.1% (95% C.I. 0.6-9.0%) for *T. gondii* (**Figure 1**). Seasonal variation showed the highest prevalence (2.0%, 95% C.I. 0.4-5.8%) in summer for *T. gondii*, in autumn for *G. duodenalis* and in spring and autumn for *C. cayetanensis* (**Figure 2**).

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

The number of *T. gondii* and *C. cayetanensis* oocysts in *q*PCR test-positive samples were estimated to range from 62 to 554 and 46 to 1.580 per g of vegetable product, respectively (**Table 3**).

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

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

4. Discussion

The present survey is the first European study on detection and prevalence of protozoan parasites in ready-to-eat salads. It found that RTE salads sold in Italy are contaminated by one or more protozoan pathogens. Microscopy and/or molecular tools detected *G. duodenalis* Assemblage A*, C. parvum* and *C. ubiquitum*, *T. gondii* Type I, *C. cayetanensis* with a prevalence ranging from 0.6% to 1.3%, with a high oocyst burden calculated only for *T. gondii* and *C. cayetanensis* (up to 554 and 1.580 per g of vegetable product, respectively) (**Table 2**). Although outside the aims of the project, *B. hominis* and *D. fragilis* were also detected (0.5 and 0.2%, respectively).

The present study found that contamination by *G. duodenalis* (0.6%) and *Cryptosporidium* spp. (0.9%) is lower than the percentage recorded for packaged or pre-packaged/bulk leafy greens purchased at retail outlets in Canada by **Dixon et al. (2013)** and **Lalonde and Gajadhar (2016)**, and this is also the case for *C. parvum*, and for *G. duodenalis* Assemblage A (**Dixon et al., 2013**). As to *Cyclospora*, the prevalence of 1.3% found here appears close to that recorded by **Dixon et al. (2013)** in Canada, where *T. gondii* was also detected but with a lower prevalence (**Lalonde and Gajadhar, 2016)** than in the present study (0.8%).

In Europe, *G. duodenalis* and/or *Cryptosporidium* oo/cysts have been documented in fresh produce in Norway (**Robertson and Gjerde, 2001; Robertson et al., 2002**), Turkey (**Erdogrul and Sener, 2005**), Spain (**Amoros et al., 2010**) and Poland (**Rzezutka et al., 2010**), and also in Italy since 1968 (**Mastandrea and Micarelli, 1968)** and later **(Di Benedetto et al., 2007)**. *T. gondii* has been recorded on leafy vegetables in Poland (**Lass et al., 2012**), and *C. cayetanensis* on fennels, cucumbers and tomatoes harvested in Italy **(Giangaspero et al., 2015a)**, but this is the first record of *B. hominis* on fresh produce in Europe, and the world's first record of *D. fragilis*.

Confirmed outbreaks of foodborne illness linked to fresh produce contaminated with protozoan parasites (directly or through contaminated water) have also been documented in Europe. The most recent outbreaks of *C. parvum* occurred across England and Scotland (**McKerr et al., 2015**), and in Finland (**Aberg et al., 2015**), whereas cyclosporiasis outbreaks were registered in Germany **(Doller et al., 2002)**, and in Sweden **(Insulander et al., 2010)**.

The presence of protozoan oo/cysts in the ready-to-eat samples we investigated is indicative of contamination by feces of human and/or animal origin. In Italy, *G. duodenalis* Assemblage A and/or *C. parvum* are widespread among humans (**Masucci et al., 2011; Putignani and Menichella, 2010)** and animals (both domestic and wild animals) (**De Liberato et al., 2015; Giangaspero et al., 2007; Paoletti et al., 2011; Papini et al., 2012**), and also in wastewater and shellfish (**Giangaspero et al., 2009, 2014)**. *Cyclospora* oocysts (**Masucci et al., 2011)** or *Cyclospora* DNA have been recorded not only in humans (**Giangaspero et al., 2015a)**, but also in non-human primates (**Marangi et al., 2015**), as well as in environmental samples, including vegetables, in the water used to irrigate them (**Giangaspero et al., 2015a**), and even in tap water (**Giangaspero et al., 2015b**). As to *T. gondii*, it has been shown that cats shed oocysts widely in Italy (**Mancianti et al., 2010, 2015**) and that the oocysts then reach the sea and contaminate shellfish (**Putignani et al., 2011).** The presence of two additional species of protozoans i.e. *B. hominis* and *D. fragilis* - responsible for several gastrointestinal symptoms in humans, and both recognized as responsible or co-responsible for Irritable Bowel Syndrome (**Garcia, 2016; Yakoob et al., 2010**) - cannot be considered as completely unexpected, since these pathogens are frequently detected in humans worldwide

(**Garcia, 2016; Wawrzyniak et al., 2013)**. This includes Italy (**Lacasella et al., 2013; Manganelli et al., 2012)**, where both *D. fragilis* (**Cacciò et al., 2012; Crotti et al., 2007**) and *B. hominis* (**Zanzani et al., 2016**) have also been detected in animals, thus hypotizing their zoonotic role.

The results of the present research demonstrate that these protozoan parasites circulate widely in Italy. Contamination of RTE salads is just the tip of the iceberg, indicating that the food chain can be a very sensitive hub. Particular attention should be given to *Cyclospora* and *Toxoplasma*. While the U.S. Public Health Service classifies *C. cayetanensis* as a foodborne pathogenic microorganism associated with the consumption of fresh fruits and vegetables, due to the number of outbreaks registered overseas, its pathogenic role is underestimated in Europe, including Italy. As said above, although autochthonous cases have been recorded in Italy (**Maggi et al., 1995; Masucci et al., 2008, 2011; Scaglia et al., 1994**), *Cyclospora* is not routinely investigated in gastrointestinal disorders and *Cyclospora* DNA detection in infected people was found to be higher than previously believed (**Giangaspero et al., 2015a**). The prevalence (1.3%) and oocyst burden for *C. cayetanensis* (up to 1.580 per gram of vegetable product) detected in this study of RTE salads, may explain its great potential for transmission to consumers. The report of an outbreak in Germany involving 34 people, associated with contaminated butterhead lettuce (imported from France) and mixed lettuce and other vegetables (imported from Southern Italy, including the area in which the present study was carried out) (**Doller et al., 2002**), further highlights the risk. Consumption of fruits and raw vegetables involves a risk of cyclosporiasis in Europe, and it should be stressed that the same is true for toxoplasmosis. In fact, the prevalence of *T. gondii* found in the RTE salads (0.8%) suggests that the dynamics of toxoplasmosis for humans may be different from previous assumptions. *T. gondii* has recently been listed as the second most harmful foodborne pathogen (**Scallan et al., 2015**), and is responsible for the highest disease burden of all foodborne pathogens (**Wells et al., 2015**). Previously, the source of infection for *Toxoplasma* in humans has always been attributed to consumption of pork and goat meat. The results obtained in this study, however, greatly support the hypothesis that a vegetarian diet constitutes a higher risk of human infection (**Hall et al., 1999; Kapperud et al., 1996**). Therefore, the type of fresh produce contamination registered in this study (up to 554 oocysts per gram of vegetable product) can contribute greatly to the transmission of this protist. The identification of Type I confirms that this lineage is present in Europe, including Italy, where it has been detected in cats (**Mancianti et al., 2015**) and pigs (**Bacci et al., 2015).**

Another interesting find in this study is the identification of *C. ubiquitum* in RTE salads. Severe cryptosporidiosis due to this species has been registered in the UK (**Elwin et al., 2012**), and in Spain, where it was also detected in an immunocompetent child (**Cieloszyk et al., 2012**). *C. ubiquitum* is considered an emerging zoonotic species (**Li et al., 2014; Zahedi et al., 2016**), of

which, sheep and wild rodents are suspected to be the key source of transmission to humans (**Li et al., 2014**). The detection of this species of *Cryptosporidium* requires further studies on its diffusion in Europe and the related risks.

Although the seasonal risk was not the main purpose of this study, the lack of statistical seasonal differences between the protozoan species is related to oo/cysts ability to survive for long periods of time, and also to production areas. Vegetables are grown in Southern Italy (mainly in Apulia and Campania Regions), where low rainfall and high temperatures make intensive irrigation necessary throughout the year. This, and other common agricultural practices (e.g. use of manure and "on-plain-air" crops) may explain continuous contamination. Thus, the recorded prevalence suggests that there is a risk of contamination throughout the year. In addition, the lack of statistical differences between the two kinds of branded company considered in this study (industrial *vs.* local brands) indicates that management, technology and protocols adopted in the processing plants overlap, despite the economic and target differences between the brands. Isolation and detection of protozoans in fresh produce is very challenging (**Dixon et al., 2013; Giangaspero et al., 2015a; Lass et al., 2012**), particularly when this involves the detection of multiple species of protozoan oo/cysts, as in this study. A combination of both microscopy and molecular assays (we used what our experience indicated as the most efficient) allowed us to provide an overview of the presence of protozoan pathogens. A limitation of this study is the lack of the data on the viability, in order to assess the public health risk. However, due to the size of sampling, the lack of univocal techniques for evaluating the viability of all investigated protozoans, the procedures not yet fully validated for all pathogens, the study of the viability was extremely complex and challenging (**Ortega and Sanchez 2010; Slifko et al., 2000**). Considering that the lengthy resistance of *Toxoplasma, Cryptosporidium* and *Giardia* on vegetables (even beyond the recommended shelf-life of RTE salads) has recently been demonstrated (**Hohweyer et al., 2016**), and that the coexistence of both viable and non-viable organisms has been ascertained (**Dixon et al., 2013**), any finding should be considered an indicator of risk**.**

In this study, we did not know *i)* the source of contamination. Any of the stages between the factory and consumer, i.e., primary production (i.e. via contaminated soil, manure, irrigation water), harvest, food preparation, packaging, washing water, equipment, or food handlers could have been the key point of risk. Nor did we know *ii)* which specific vegetables were contaminated. We investigated mixed salads in this study, therefore, the greater the number of vegetables the higher becomes the risk of contamination due to the multiple handling processes along the food chain. In addition, we did not know *iii)* if any of the batches of produce tested were associated with reported

outbreaks. However, the lack of information regarding outbreaks or single cases is mainly due to the long incubation period.

5. Conclusions

In conclusion, the results of our survey demonstrate that the prevalence of protozoan species in RTE salads is a cause for concern about human health in Europe, in particular in Italy. Since pathogens circulate widely in humans, animals, vegetables and water in Italy, it is necessary to monitor the use of correctly treated irrigation and processing water and to ensure the efficiency of wastewater treatment plants. In addition, monitoring must involve animal access to crops, the use of manure as fertilizer, and all the processes along the RTE food chain, such as the respect of personal hygiene education rules by food handlers, and the use of pathogen-free water for washing produce.

Although the role of the RTE salads in increasing consumer exposure to these pathogens and the impact of these protozoans on human health can only be suspected, these results further enhance the need to integrate the microbiological criteria required by EU Law No. 1441/2007 by adding these protists to the list of contaminants. Monitoring the absence (or detection limits) only of bacteria, (i.e. *E. coli, L. monocytogenes* and *Salmonella* spp.) on vegetables can no longer indicate the absence of fecal contamination nor guarantee food safety; protozoan parasites – whose high resistance to temperatures and disinfectants and low infectious doses have been amply demonstrated (**Dawson et al., 2005**) - constitute a major risk for both immunocompetent and immune-compromised consumers. Policy decisions should promote development of increasingly advanced procedures and technological treatments for the inactivation and removal of oo/cysts from contaminated fresh produce (possibly using a multi-barrier approach) in order to improve the quality and safety of these foods. The sampling methods designed in this research - which allowed us to maximize detection even with very low expected prevalence values - and the results obtained can provide the direction for monitoring fresh produce in other areas, and for surveillance studies on produce. In addition, they can provide the basis for food safety guidelines, based also on the HACCP system, in order to reduce the risk of RTE contamination and to minimize foodborne disease transmission.

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FIGURE LEGENDS

Figure 1 – Protozoan prevalence (± 95% confidence intervals) in ready-to-eat mixed salads according to the six producer companies (National and Local brands).

Figure 2 – Seasonal protozoan prevalence (± 95% confidence intervals) in ready-to-eat mixed salads.