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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 were purchased from industrial and local brands. Nine individual packages from each brand were collected per month, pooled and subjected to microscopy and molecular analyses. Microscopic examination of 864 slides detected *Cryptosporidium* spp. but also *Blastocystis hominis* and *Dientamoeba fragilis*. Molecular tools identified *G. duodenalis* assemblage A, *Cryptosporidium parvum* and *Cryptosporidium ubiquitum*, *T. gondii* Type I and *C. cayetanensis*. *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*, while prevalence for *B. hominis* was 0.5% and for *D. fragilis* 0.2%. Microscopy and/or molecular tools revealed that 4.2% of the samples were contaminated by at least one protozoan species, and 0.6% of samples presented contamination by two protozoan species, with a number of oocysts ranging from 62 to 554 per g of vegetable matter for *T. gondii*, and 46 to 1.580 for *C. cayetanensis*. This is Europe's first large-scale study on the presence of protozoans in packaged salads, and shows that RTE sanitation processes do not guarantee a product free from protozoans of fecal origin.

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 also have economic consequences for individuals, communities, businesses and countries. In industrialized countries, in addition to other drivers (i.e., environment, climate, land use, trade), the risk of food-borne disease transmission is also enhanced by 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,

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2011).

Vegetables may become contaminated in various ways along the food production chain, i.e. during primary production (contaminated irrigation water, manure used on croplands, livestock/wildlife access to crops), harvesting in the field, during transport and market processing (Chaidez et al., 2005; Francis et al., 1999; Johnston et al., 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 foodborne 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, to 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, including soil. 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 Dardé, 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* (Chacín-Bonilla, 2010), but since its zoonotic role is suspected, this 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 (Åberg et al., 2015; Döller et al., 2002; McKerr et al., 2015). However, despite the guidelines 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 parasites 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 detectable with our sampling regime). The confidence level and precision level 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 100 g 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, 100 g 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 50 ml tubes and centrifuged at 2000 × g for 15 min at 4 °C. The supernatant was