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Original Article

The effect of water source and soil supplementation on parasite contamination in organic vegetable gardens

O efeito da fonte de água e suplementação de solo na contaminação parasitária em hortas orgânicas

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Abstract

The objective of this study was to determine factors associated with vegetable contamination with zoonotic protozoan. Samples of water, soil and vegetables were collected from July/2014 to May/2016, totaling 83 samples, 21 properties of Londrina region, Paraná, Brazil. DNA amplification of *Toxoplasma gondii*, *Cryptosporidium* spp. and *Giardia intestinalis* in the samples was conducted using polymerase chain reaction (PCR). The PCR results were positive for *T. gondii* in 12.9% (8/62), *Cryptosporidium* spp. in 11.3% (7/62) and *G. intestinalis* in 25.8% (16/62) of the samples. DNA sequencing identified *C. parvum* in five samples and *G. intestinalis* Assemblage E in three. The statistical associations demonstrated greater probability of positive samples for *T. gondii* and for at least one of the three protozoa when the source of irrigation water was the river; a greater chance of positive samples for *Cryptosporidium* spp. when deer were present on the property; and a smaller chance of positive samples for at least one of the three etiologic agents when soil was supplemented with limestone. The results expose some critical contamination points, providing support for training farmers on good management practices during the production process.

Keywords: Vegetables, water, environmental contamination, *Cryptosporidium* spp., *Toxoplasma gondii*, *Giardia intestinalis.*

Resumo

O trabalho teve como objetivo determinar os fatores associados à contaminação de vegetais por protozoários zoonóticos. Amostras de água, solo e vegetais foram coletadas de julho/2014 a maio/2016, totalizando 83 amostras de 21 propriedades da região de Londrina, Paraná, Brasil. A amplificação de fragmentos de DNA de *T. gondii*, *Cryptosporidium* spp. e *Giardia intestinalis* foi realizada por meio da reação em cadeia da polimerase (PCR). Os resultados da PCR foram positivos para *T. gondii* em 12,9% (8/62), *Cryptosporidium* spp. em 11,3% (7/62) e *G. intestinalis*. em 25,8% (16/62) das amostras. O sequenciamento de DNA identificou *C. parvum* em cinco amostras e *G. intestinalis, Assemblage* E em três amostras. As associações estatísticas evidenciaram maior probabilidade de amostras serem positivas para *T. gondii* ou para pelo menos um dos três protozoários quando a fonte de água de irrigação era o rio; uma maior chance de amostras positivas para *Cryptosporidium* spp. quando havia cervos na propriedade; e uma menor chance das amostras serem positivas para pelo menos um dos três agentes etiológicos quando o solo era suplementado com calcário. Os resultados expõem alguns pontos críticos de contaminação, fornecendo suporte para capacitar os agricultores em boas práticas de gestão durante o processo de produção.

Palavras-chave: Legumes, água, contaminação ambiental, *Cryptosporidium* spp., *Toxoplasma gondii*, *Giardia intestinalis.*

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Introduction

Vegetables, including all vegetables grown in gardens, are foods rich in vitamins, carbohydrates, fiber and minerals; are easy to digest; and offer high satiety, a high antioxidant content and a low caloric content. Due to their nutritional importance, vegetables are essential in human nutrition (SINGH et al., 2001; WORTHINGTON, 2001).

In Brazil, vegetables are produced commonly by the conventional farming system; however, in recent years there was an important growth in organic cultivation. It is mainly related to the search for healthier food, with better quality and flavor, environmental preservation and pesticides' free (ARCHANJO et al., 2001). The main differences between organic and conventional cultivation are the use, in the latter, of agricultural pesticides, chemical fertilizers and genetically modified organisms. Organic agriculture excludes these elements from the production process, using natural implements and predators (BOURN & PRESCOTT, 2002; GARCIA & TEIXEIRA, 2017; GOMIERO et al., 2011; WILKINS & HILLERS, 1994).

Contamination of vegetables can occur during production, transportation, storage and commercialization; however, cultivation conditions involving the quality of irrigation water, fertilizer type, the presence of animals in the property and direct contamination from farm workers are the main risk points (ALMEIDA et al., 2013; AMAHMID et al., 1999; CHAIDEZ et al., 2005; DIXON et al., 2013; DIXON, 2016).

Toxoplasmosis is one of the most common parasitic zoonoses in the world, and it can affect all warm-blooded animals, presenting a wide variability in clinical outcomes (DUBEY, 2010). Cryptosporidiosis and giardiasis are zoonoses and are described as important causes of diarrhea (FAYER, 1997; PEDROSO & AMARANTE, 2006). In an immunocompetent individual, these diseases may assume a self-limiting characteristic with clinical manifestations of low expression, but in an immunocompromised patient they may manifest in a severe and prolonged form (MITSUKA-BREGANÓ et al., 2010; PEDROSO & AMARANTE, 2006; SHIKANI & WEISS, 2014). The description of these protozoa in food and waterborne outbreaks (BERGER et al., 2010; CHEUN et al., 2013; MAC KENZIE et al., 1994; SILVA et al., 2005; VAUDAUX et al., 2010; YEUNG et al., 2013) highlights the importance of leafy vegetables, which are consumed *in natura* and facilitate the transmission of these protozoa and other pathogens.

Considering the health benefits of consuming vegetables, their greater inclusion in the diet of people worldwide and their risk as a source of zoonotic protozoa such as *T. gondii*, *Cryptosporidium* spp. and *G. intestinalis*, this work aimed to evaluate the contamination and hygienic-sanitary conditions of their production in organic gardens.

Materials and Methods

The study was carried out from July 2014 to May 2016 on 21 horticultural properties of the municipalities of Apucarana, Marilândia do Sul, Ortigueira, Rolândia and Londrina (District of Guaravera), in the state of Paraná (Figure 1). As an inclusion criterion, small commercial vegetable-producing organic properties were assisted by the Organic Certification Project of the State University of Londrina. On eleven properties, water and vegetable samples were collected, and soil samples were included on the other properties.

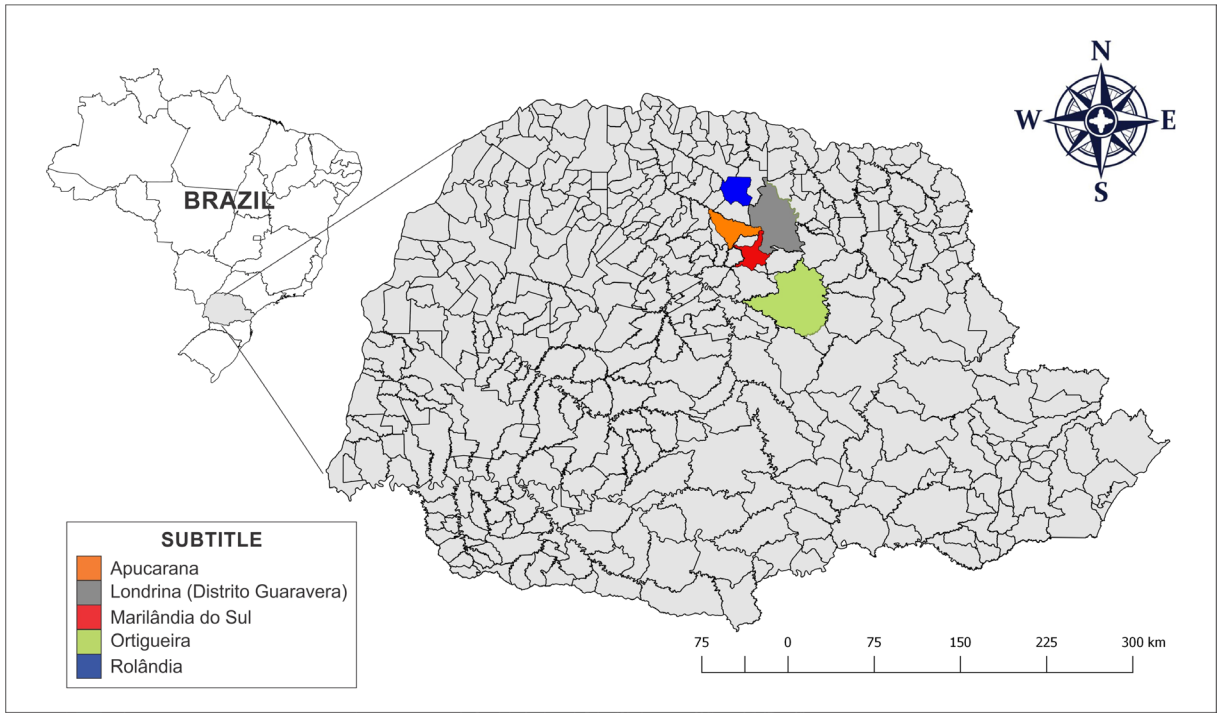

Figure 1. Map of Paraná highlighting the municipalities where samples of vegetables, water and soil were collected and submitted for parasitological and microbiological research from 2014 to 2016, Paraná, Brazil.

Obtaining and analyzing samples of vegetables

Forty-two clumps of leafy vegetables were randomly collected, two per property (from property 1 to 21), packed in plastic bags and kept in refrigeration. For *T. gondii*, *Cryptosporidium* spp. and *G. intestinalis* detection, 50 g of the leaves were washed in 300 mL of 1% Tween 80 extraction solution in a plastic bag under manual shaking for 10 minutes and then filtered into 500 mL glass beakers through two layers of gauze. The wash was divided into conical tubes and subjected twice to centrifugation at 2100 x g for 10 minutes. The pellet was aliquoted into microtubes and stored at -20 °C until DNA extraction.

Obtaining and analyzing soil samples

For *T. gondii*, *Cryptosporidium* spp. and *G. intestinalis* detection, a total of 10 samples of approximately 10 g of soil, collected from the surface, one per property (from property 12 to 21) and stored in 50 mL conical tubes with 30 mL of 1M glycine, were homogenized with the aid of a stirrer for 30 minutes. They were then kept at rest for five minutes for sedimentation. After the pellet was discarded, the supernatant was centrifuged at 1500 x g for 15 minutes. The final concentrate was aliquoted into microtubes and stored at -20 °C until DNA extraction.

Obtaining and analyzing water samples

For the microbiological analysis by means of the chromogenic substrate technique (APHA, 2005), 21 samples of water, one per property (from property 1 to 21), were collected from the irrigation tap of the vegetable gardens, as recommended by the protocol in Brazil (BRASIL, 2013).

For *T. gondii*, *Cryptosporidium* spp. and *G. intestinalis* detection, a total of 10 samples (from property 12 to 21) of 10 mL were collected in clean plastic bottles from irrigation tap. The water was filtered through a cellulose ester membrane with a 47 mm-diameter and 1.2 µm porosity (Millipore®, Billerica, Massachusetts, USA) in a filter holder system using a vacuum pump (4 L/min). After filtration, the material was eluted in 0.1% Tween 80 with the aid of flexible plastic loops (Thermo Fisher Scientific, Massachusetts, USA) (BRANCO et al., 2012). The obtained material was concentrated by centrifugation twice at 1050 x g for 15 min at 4°C. The obtained pellet was stored in microtubes at -20 °C until DNA extraction.

Molecular analyses

The samples were previously submitted to freeze-thaw (5 cycles of freezing at -80 °C and thawing at 56 °C), then DNA extraction was performed using a commercial kit (NucleoSpin Tissue®, Macherey-Nagel, Düren, Germany) in accordance with the manufacturer's instructions and DNA was collected in a final volume of 100 µL. DNA extracted were stored at -20°C until polymerase chain reaction (PCR) processing.

PCR assays for *T. gondii* were performed as previously described by Homan et al. (2000), to amplify a fragment of 529 bp. *Cryptosporidium* spp. were detected using a nested-PCR reaction with primers described by Xiao et al. (1999), which target a fragment of 18S rRNA gene between 826 and 840 bp. For *G. intestinalis* DNA detection, the samples were subjected to nested-PCR to amplify an approximately 300-bp fragment of the 18S rRNA gene from *G. intestinalis* (COKLIN et al., 2007), in addition to 530 bp of the TPI (triose-phosphate isomerase) gene with primers described by Sulaiman et al. (2003) and 432 bp of the GDH (glutamate dehydrogenase) gene with primers described by Read et al. (2004) in a semi-nested PCR.

PCR products were visualized by 1.5% agarose gel electrophoresis stained with SYBR Safe DNA (Invitrogen®, California, USA). In addition, when positive for *Cryptosporidium* spp. and for *G. intestinalis*, the PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and the DNA was quantified by a Picodrop (Thermo Fisher Scientific, Wilmington, USA). DNA sequencing was performed with the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA) with the corresponding forward or reverse primers on a 3500 genetic analyzer (Applied Biosystems, Carlsbad, USA), according to the instructions of the manufacturer. The sequences obtained were examined with PHRED software (EWING & GREEN, 1998) and inspected with CHROMAS software for quality analysis of chromatograms. Consensus sequences were determined by the CAP3 software (http://asparagin.cenargen.embrapa.br/cgi-bin/phph/cap3.pl), and their identities were compared to the sequences deposited in GenBank using BLAST software (CAMACHO et al., 2009). The nucleotide sequence data reported in this article were deposited in GenBank under the following accession numbers: *Cryptosporidium parvum* (MF353924 to MF353928) and *Giardia intestinalis* (MF425817 to MF425819). The phylogenetic relationships between *G. intestinalis* sequences of the present study and GenBank standard sequences were characterized by the alignment of 390 nucleotides of the GDH coding gene using the maximum likelihood method with 250 bootstraps in the MEGA 4.0 program. The tree was rooted with *Giardia ardeae* (AF069060) and the GenBank sequences used in this tree are standards of assemblages A1 (AY178735), A2 (AY178737), B (AY826193), D (U60986), E (AY178741) and F (AF069057).

Statistical Analysis

A semi-structured questionnaire was applied to all horticulturists participating in the study, who were questioned about the type of planting and fertilization, soil supplementation, presence of animals on the property, irrigation system, toilet and sewage characteristics. The program EpiInfo 3.5.4 (DEAN et al., 1990) was used to tabulate the variables together with the microbiological and molecular results found. Statistical analysis was performed using the EpiInfo programs 3.5.4 and R 3.3.2 (R CORE TEAM, 2013) by means of the Chi-square test or Fisher's exact test, where appropriate. The analysis of these associations with a control of the confounding variables was performed applying simple and multiple logistic regression analysis (HOSMER et al., 2013). Only the variables whose p-values were less than 0.10 in the

screening analysis and had some biological meaning were included in the logistic regression models. The association measurement was obtained by the Odds Ratio (OR) calculation with a 95% confidence interval (CI). The multiple correspondence analysis was performed with program R 3.3.3 (R CORE TEAM, 2013) through the FactoMineR (HAIR et al., 2009) package, an exploratory technique that does not rely on statistical tests but allows the visualization of the most important relationships of a large set of variables among each other (HOFFMAN & FRANKE, 1986). In addition, it can help in visualizing the multivariate relationship between the categories of the different variables, and their geometric proximity in the graph, suggesting the possibility of associations between them.

Results

In total, 83 samples were collected, being 42 of vegetables, 31 of water (21 for microbiological analysis and 10 for parasitological analysis) and 10 of soil; from 21 properties of the municipalities of Apucarana (6/21), Marilândia do Sul (7/21), Ortigueira (6/21), Rolândia (1/21) and Londrina (District of Guaravera) (1/21). Among the leafy vegetables, 17 samples were of crisp lettuce (*Lactuca sativa*), seven of arugula (*Eruca sativa*), nine of chicory (*Cichorium intybus* and *Cichorium endivia*), five of chives (*Allium fistulosum*), two of purple lettuce, one spinach (*Spinacia oleracea*) and one chard (*Beta vulgaris* subsp. *Vulgaris*). On the first eleven properties, water was not submitted for molecular analysis, and no soil samples were collected.

Eighteen properties were organic cultivation certified, and three (14.3%) were in the certification phase. The organic fertilizer source was variable among the properties: 23.8% (5/21) used chicken manure, 14.3% (3/21) used mixed manure, 14.3% (3/21) used chicken litter, 14.3% (3/21) used commercial compounds and 33.3% (7/21) used bovine manure. Eleven (52.3%) of the properties underwent mineral supplementation on a regular basis, six with limestone (54.5%), three with a potassium supplement (27.2%) and one with mineral present in the fertilizer.

Wild birds and domestic animals, such as dogs, cats, horses, and cattle, were present on all the properties studied, and in five (23.8%) of them, the animals had access to the gardens. Wild animals, such as cervids, hare, armadillos, capybara, fox and cougar were present on 13 of the studied properties (61.9%). All the properties were washing their vegetables: 52.4% (11/21) of the properties in treated tap water and 47.6% (10/21) in rinsing tanks. Irrigation in 57.1% (13/21) was automated. The source of irrigation water on 57.1% (12/21) of the properties was spring, 14.3% (3/21) of the properties used untreated river water, and 28.6% (6/21) of the properties used artesian wells.

Of the water samples, 95.2% (20/21) presented total coliforms, with a variation of 1 to > 2419.6 CTNMP/100 mL; for CTT and *Escherichia coli,* 76.2% (16/21) of the samples were positive, ranging from 1 to 218.7 CTTNMP/100 mL.

Regarding the PCR results, 12.9% (8/62) of the samples were positive for *T. gondii*, 11.3% (7/62) for *Cryptosporidium* spp., and 25.8% (16/62) for *G. intestinalis* (two in TPI, seven in GDH, five in 18S rRNA and two in TPI and GDH) (Table 1). It was possible to identify the parasite species by DNA sequencing in

Table 1. Vegetable, water and soil samples positive for the protozoa *Toxoplasma gondii*, *Cryptosporidium* spp. and *Giardia intestinalis* from organic crop properties, from 2014 to 2016 in Paraná, Brazil. Results are based on molecular analyses.

Property	Municipality	*Toxoplasma gondii*			*Cryptosporidium* spp.			*G. intestinalis*		
		Vegetable	Soil	Water	Vegetable	Soil	Water	Vegetable	Soil	Water
1	Marilândia do Sul	-	NT	NT	-	NT	NT	+ Let	NT	NT
2	Marilândia do Sul	+ Let	NT	NT	-	NT	NT	+ Aru	NT	NT
3	Marilândia do Sul	-	NT	NT	-	NT	NT	-	NT	NT
4	Marilândia do Sul	+ Let	NT	NT	+ Aru*	NT	NT	+Let**+Aru**	NT	NT
5	Guaravera	+ Let Aru	NT	NT	-	NT	NT	+ Let**	NT	NT
6	Ortigueira	-	NT	NT	-	NT	NT	-	NT	NT
7	Ortigueira	-	NT	NT	-	NT	NT	-	NT	NT
8	Ortigueira	-	NT	NT	-	NT	NT	-	NT	NT
9	Ortigueira	-	NT	NT	-	NT	NT	-	NT	NT
10	Ortigueira	-	NT	NT	-	NT	NT	-	NT	NT
11	Ortigueira	-	NT	NT	-	NT	NT	+ Chic	NT	NT
12	Rolândia	-	+	-	-	-	-	+ Chic	-	-
13	Apucarana	-	-	+	-	-	-	+ Chic	+	-
14	Apucarana	-	-	-	-	-	+*	-	-	+
15	Apucarana	-	-	-	-	-	-	+ Let	-	-
16	Apucarana	-	+	+	-	+*	+*	+ Chiv	+	+
17	Apucarana	-	-	-	-	-	+*	-	-	+
18	Marilândia do Sul	-	-	-	-	-	-	+ Cha	-	-
19	Marilândia do Sul	-	-	-	-	-	-	-	-	-
20	Marilândia do Sul	-	-	-	+ Let	-	-	-	-	-
21	Apucarana	-	-	-	-	+	-	-	-	-

NT: Not tested; + positive sample; - negative sample; Let: Lettuce; Aru: Arugula; Cha: Chard; Chic: Chicory; Chiv: Chive. **C. parvum* or ***G. intestinalis* Assemblage E confirmed by the DNA results from Sanger sequencing.

seven of the positive samples. *C. parvum* was the species present in three water samples and one soil sample sample showing 100% of similarity with other sequences from GenBank. *G. intestinalis* Assemblage E was present in two vegetable samples showing 99% of similarity with other sequences from GenBank. In order to confirm the *G. intestinalis* Assemblage, the phylogenetic analysis with the partial sequences of the GDH gene of *G. intestinalis* obtained was performed, a clustering of samples with standard sequences of Assemblage E (Figure 2), commonly associated with giardiasis in cattle, was observed. In other PCR-positive samples, there was no success in sequencing due to the low amount of DNA.

Factors associated with the presence of DNA of zoonotic protozoa such as *T. gondii*, *Cryptosporidium* spp. and *G. intestinalis* in vegetables, soil and water are listed in Tables 2, 3 and 4.

The graph generated by the multiple correspondence analysis (Figure 3) confirmed the main findings of this study related to the risk factors for the presence of zoonotic protozoa in vegetables, water and soil of properties with organic production systems. It is shown in dimension one of the graph (x-axis) that the categories of negative variables for *T. gondii*, *Cryptosporidium* spp. and *G. intestinalis* and at least one of the three zoonotic protozoa in question are on the left along with the categories of limestone supplementation, spring water source and absence of cervids. On the other hand, on the right side of the graph we have the categories of positive variables for *T. gondii, G. intestinalis*, *Cryptosporidium* spp. and at least one of the three zoonotic protozoa together with the categories of potassium and fertilizer supplementation, river water source and the presence of cervids. The interpretation of

Table 2. Characteristics of the organic vegetable producing properties of northern Paraná, Brazil, significantly associated with the occurrence of *Toxoplasma gondii* and *Giardia intestinalis*.

Toxoplasma gondii	**Positive/Total (%)**	**Univariate Logistic Regression**	
		p-Value	**O.R. Gross (CI 95%)**
Water Source		< 0.05	
Spring	1/34 (2.9)		1.00
Well	4/22 (18.2)		7.11 (0.74 - 68.57)
River	3/6 (50.0)		32.00 (2.49 - 411.45)
G. intestinalis	**Positive/Total (%)**	**p-Value**	**O.R. Gross (CI 95%)**
Cryptosporidium spp. positive		< 0.05	
No	11/55 (20.0)		1.00
Yes	5/7 (71.4)		10.00 (1.71 - 58.59)

Table 3. Characteristics of the organic vegetable producing properties of northern Paraná, Brazil, significantly associated with the occurrence of *Cryptosporidium* spp.

	Positive/Total (%)	**Univariate Logistic Regression**		**Multiple Logistic Regression**	
		p-Value	**O.R. Gross (CI 95%)**	**p-Value**	**O.R. Adjusted (CI 95%)**
Presence of cervids		< 0.05		< 0.05	
No	3/52 (5.7)		1.00		1.00
Yes	4/10 (40.0)		10.89 (1.95 - 60.83)		7.87 (1.22 - 50.64)
***Giardia intestinalis* positive**		< 0.05		< 0.05	
No	2/46 (4.3)		1.00		1.00
Yes	5/16 (31.2)		10.00 (1.71 - 58.59)		7.51 (1.20 - 51.44)

Table 4. Characteristics of the organic vegetable producing properties of northern Paraná, Brazil, significantly associated with the occurrence of at least one of the protozoa (*Toxoplasma gondii*, *Cryptosporidium* spp. or *G. intestinalis*).

	Positive/Total (%)	**Univariate Logistic Regression**	
		p-Value	**O.R. Gross (CI 95%)**
Type of supplement		< 0.05	
Not supplemented	15/38 (39.5)		1.00
Limestone	1/16 (6.2)		0.10 (0.01 - 0.85)
Potassium Base	4/6 (66.7)		3.07 (0.50 - 18.89)
Water source		< 0.05	
Spring	7/34 (20.6)		1.00
Well	10/22 (45.4)		3.21 (0.98 - 10.47)
River	5/6 (83.3)		19.28 (1.92 - 192.82)

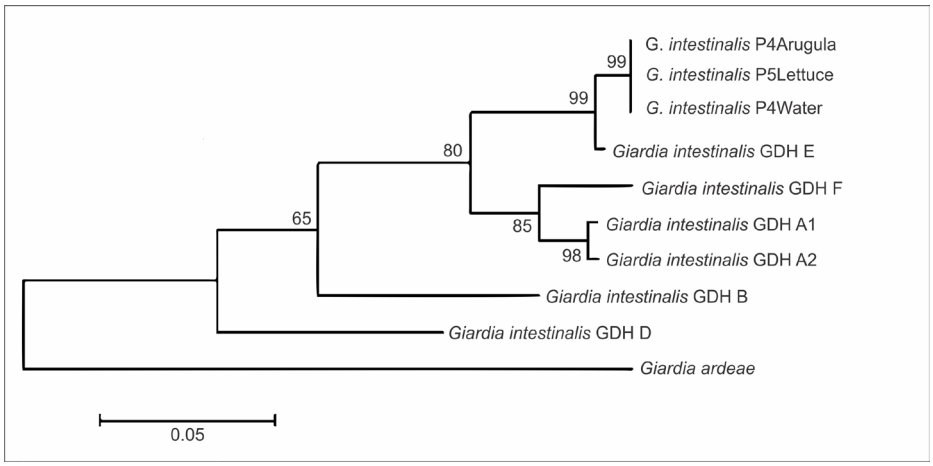

Figure 2. Phylogenetic relationships among *G. intestinalis* isolates characterized by the alignment of 390 nucleotides of the GDH coding gene using the maximum likelihood method with 250 bootstraps in the MEGA 4.0 program. The tree is rooted with *Giardia ardeae* (AF069060), and the GenBank sequences used in this tree are standards of the assemblies A1 (AY178735), A2 (AY178737), B (AY826193), D (U60986), E (AY178741) and F (AF069057).

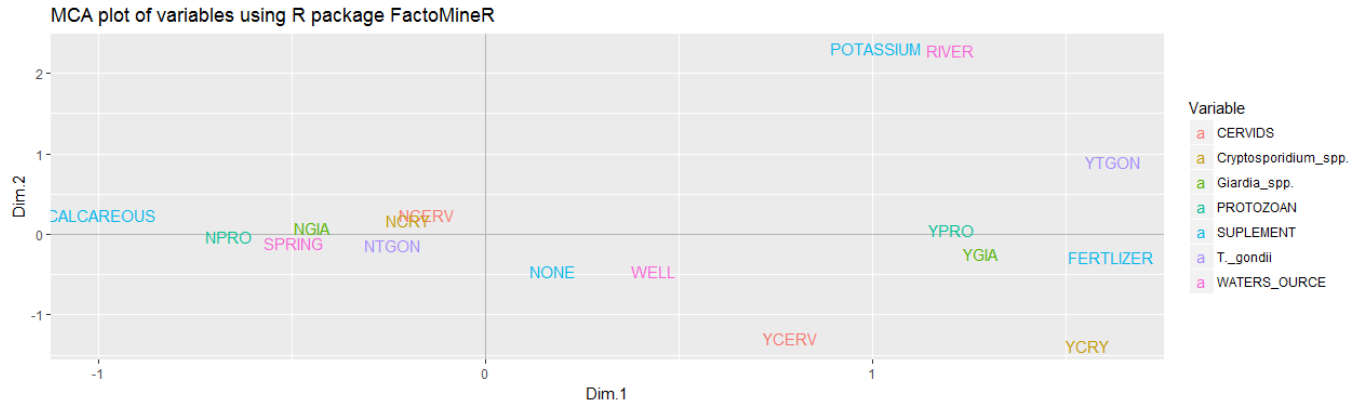

Figure 3. Graph resulting from the multiple correspondence analysis. Presence of cervids (NCERV, YCERV); positive results of *T. gondii* (NTGON, YTGON), *Cryptosporidium* spp. (NCRY, YCRY), *Giardia* spp. (NGIA, YGIA), and one of the three protozoa (NPRO, YPRO); water source (SPRING, WELL, RIVER) and supplement used in growing (LIMESTONE, NONE, POTASSIUM and FERTILIZER).

dimension two (y-axis) of the graph was not possible due to the lack of biological meaning.

Discussion

Resolution 357/05 of CONAMA (National Council for the Environment) (BRASIL, 2005) recommends that for the irrigation of vegetables and fruits with development on the ground, the Thermotolerant coliforms (CTT) count should not exceed 200/100 mL; *Escherichia coli* is the main species of the CTT group, whose exclusive habitat is the intestine (BRASIL, 2005). *E. coli* was present in 76.2% (16/21) of the analyzed water samples, and of these, 18.7% (3/16), one of spring (209.8 CTNMP/100mL), one of river (328.2 CTNMP/100mL) and another of artesian well (201.2 CTNMP/100mL), with higher counts than that allowed by the resolution. The use of faecal indicators, particularly, *E. coli*, is a better practical approach to identify contaminated water sources or food (ALLENDE & MONAGHAN, 2015; NATARO & KAPER, 1998). Recent studies associate the presence of parasites such as *G. intestinalis* and *Cryptosporidium* spp. to the presence of CTT (TOLEDO et al., 2017) in the water, indicating the necessity of monitoring parasite presence in vegetable gardens for the production of innocuous vegetables.

Regarding the PCR results, 12.9% (8/62) of the samples had DNA fragments compatible with *T. gondii*, and of these, 50.0% (4/8) were vegetables, 25.0% (2/8) were water and 25.0% (2/8) were soil. Lass et al. (2012) studied *T. gondii* in 216 fruits and vegetables in Poland and observed a frequency of 9.7% positive samples. The eight positive samples came from six properties, of which 50.0% (3/6) had cats, all with access to the cultivation area, suggesting direct contamination of vegetables by definitive hosts on these properties (FRENKEL et al., 1970). In vegetable gardens that did not have cats (3/6), the source of irrigation water from the river may have influenced the contamination due to the flow during rains, when the river receives several fecal pathogens

from domestic and wild animals (WELLS et al., 2015); its use in irrigation should be avoided, unless it is properly treated. Water was related to outbreaks of human toxoplasmosis previously in Panamá (BENENSON et al., 1982), Canada (BOWIE et al., 1997), Atlanta (DUBEY et al., 1981) and Brazil (VAUDAUX et al., 2010). The use of treated water with regular microbiological analysis is essential to ensure its quality and safe use in irrigation.

From the analyzed samples, 11.3% (7/62) were positive for *Cryptosporidium* spp. Of these, 42,9% (3/7) water samples (two of artesian well and one of spring), 28.6% (2/7) soil sample and 28,6% (2/7) vegetable sample were identified as containing *C. parvum*, an important zoonotic species and the main cause of human cryptosporidiosis, which represents a threat to public health because it is associated with diarrhea and death in immunocompromised individuals (SANTÍN, 2013). The main known reservoirs are production and wild animals (RYAN et al., 2014). In this study, the presence of cervids on the properties was associated with the presence of *Cryptosporidium* spp., which could explain environmental contamination by water drained. Wells et al. (2015), when conducting a study in a water supply network in Scotland, detected *C. parvum* and related the high prevalence of the protozoan to cervids inhabiting the region studied. Ranjbar-Bahadori et al., (2013) suggested that plants may provide a route of infection by *Cryptosporidium* spp. for humans when they observed a frequency of 6.6% (33/496) positive samples with risk factors related to the type of vegetable and irrigation water in gardens of Tehran, Iran. Poulton et al. (1991) suggested that the fertilizer used in agriculture was one of the main sources responsible for the high levels of oocysts of *Cryptosporidium* spp. observed in the River Thames in the south of England. In this study, the type of fertilizers used on the positive properties were 50.0% (3/6) chicken litter, 16.7% (1/6) bovine manure and 33.3% (2/6) mixed manure (chicken and bovine), the type of fertilizer had no statistical association with positivity by *Cryptosporidium* spp., however the parasite was previously described in these animals (AL MAWLY et al., 2015; EWALD et al., 2017; HELMY et al., 2017).

G. intestinalis was found in 25.8% (16/62) of the analyzed samples, of which 12 samples were vegetables, two were water of artesian well and two were soil, on nine properties. The presence of cysts of *G. intestinalis* at any stage of the production of vegetables is of extreme concern, as this parasite is resistant in the environment, remaining viable for weeks or months (ALMEIDA et al., 2010). The *G. intestinalis* samples from the present study showed similarity to the Assemblage E (Figure 2), commonly described in cattle (EY et al., 1997; PLUTZER et al., 2010). Fertilization using bovine or mixed manure may facilitate environmental contamination when not properly processed. Taking into account that infected young animals can release up to 10^6 cysts per gram of feces (GEURDEN et al., 2010), the presence of bovine animals on a farm may lead to contamination of soil and water, since environmental contamination may precede the contamination of water and vegetables (ALMEIDA et al., 2010). The three positive samples for non-zoonotic Assemblage E came from vegetables (two lettuces, one arugula) from two properties that did not have cattle, so the contamination could have occurred due to the use of bovine fertilizer on one of the properties or by contaminated irrigation water in both.

Statistically, there was a significant association between being positive for *Cryptosporidium* spp. and *G. intestinalis*, we suggest that this association may be due to the fact that both pathogens are known to be water-borne. Before the 1980s, diseases of bacterial and viral origin were associated with water, but with the inclusion of chlorination in the treatment process, there was an effective reduction of these microorganisms (FURTADO et al., 1998). In contrast, *Cryptosporidium* spp. and *G. intestinalis* have become the main etiological agents of water transport and the cause of outbreaks (CHEUN et al., 2013; MAC KENZIE et al., 1994). They are of great importance in public health because of their low infective doses, resistance to conventional water treatment and adverse environmental conditions (SLIFKO et al., 2000). Water, once contaminated, acts as an efficient carrier of pathogens, either because of its great dispersion capacity or because of its direct and indirect compulsory consumption (FRANCO, et al., 2012). Wild animals play an important role in the contamination of water by their aquatic activities or by feces flowing to the rivers and springs (HAMNES et al., 2006; WELLS et al., 2015).

Vegetables do not develop in soil with acidic pH, which is the case of Brazilian soil (ERNANI et al., 2002). Acidic soils are neutralized, most of the times, by the application of limestone (ALBUQUERQUE et al., 2003; PRADO & FERNANDES, 2000). The use of this supplement was a factor associated with a lower chance of positive samples for at least one of the three parasitic agents studied, it is known that its use reduces the viability of helminth eggs (CAPIZZI-BANAS et al., 2004). Franco et al. (2012) have described that the use of alkaline reagents in water, such as calcium carbonate, can produce deformations in cysts of *G. intestinalis* and oocysts of *Cryptosporidium* spp. Transferring this description to this study, the direct contact of the limestone with the cysts and oocysts for a prolonged period may have led to their deformation, DNA exposure and degradation, thus diminishing the positive samples on these properties. Further studies are needed to clarify this result.

Among the analyzed gardens, 57.1% (12/21) presented at least one of the protozoa studied, and with respect to the samples, the frequency was 52.5% (22/42) in vegetables, 20.0% (2/10) in soil and 20.0% (2/10) in water. As in Falavigna et al. (2005), there was no significant difference between the types of leafy greens tested. Commonly used decontamination treatments, such as vinegar and sodium hypochlorite (YUCEL SENGUN & KARAPINAR, 2005), are not able to destroy the resistant forms of *T. gondii*, *Cryptosporidium* spp., and *Giardia* spp. (HUNTER & THOMPSON, 2005). The morphological characteristics of leafy vegetables, such as compact structure and presence of multiple leaves facilitate a greater fixation by parasites, making manual mechanical cleaning difficult (FALAVIGNA et al., 2005). According to Guilherme et al. (1999), the clarification of food safety to producers is extremely important because it is a determining factor for the production of safe food.

The main forms of contamination of fruits and vegetables during production are fertilizer (ALMEIDA et al., 2013; ERDOGRUL & SENER., 2005), direct contamination from farm workers, access of wild animals (WELLS et al., 2015) and

domestic animals to gardens, and contaminated irrigation water (BERALDO & FARACHE, 2011; CHAIDEZ et al., 2005), the latter was a factor associated with the presence on the samples of at least one of the agents. River water, in turn, had a greater number of positive samples than expected; the use of this type of water for irrigation is a big problem in Brazil (MAROUELLI & SILVA, 1998), because a significant number of rivers are polluted, probably due to the extensive area of agriculture and livestock around the surface water sources and the contamination by untreated municipal effluents, mainly sewage, constituting another important vehicle of transmission and dissemination of pathogens (AMORÓS et al., 2010; BERALDO & FARACHE, 2011; DIXON et al., 2013; MAROUELLI & SILVA, 1998; SILVA et al., 2005). Toledo et al. (2017) in a study in dairy farms in Paraná, Brazil, observed that the absence of vegetation and protective structures around the springs was associated with the frequency of positive water samples to *Cryptosporidium* spp. and *G. intestinalis*. The vegetation around the springs acts as a physical barrier, thus reducing the drainage of animal waste and other contaminants (TOLEDO et al., 2017). Most of the springs visited in this study were protected, which may have been an important factor for a smaller number of positive samples than expected in this source.

Of the 12 properties with at least one positive sample for one of the three agents, 66.7% (8/12) produced their own fertilizer with bovine manure or chicken litter, and of these, 87.5% (7/8) did not respect the period of composting. The maturity of the compost is related to the complete microbiological decomposition and the transformation of the organic matter into humus, and this process occurs after approximately 90 days of tanning; correct composting can promote the inactivation of some pathogens (DÉPORTES et al., 1998; KIEHL, 1998; VINNERÅS et al., 2003). Inadequate fertilizer production, in addition to causing contamination of vegetables and water, prevents the inactivation of pathogens present in the raw material, making the fertilizer unfit for use in edible vegetables.

Conclusion

The control of parasitological contamination of raw vegetables is a major challenge, since contamination can occur in all the stages that precede arrival to the consumers' table. The results indicate that inadequate hygienic-sanitary conditions in organic gardens, as well as the risk of infection by protozoa are of public health importance. In addition, the results expose some of the points of contamination risk, such as the water source, soil supplementation and presence of animals on properties. Thus, there is a need to raise awareness among producers and consumers about the implementation of good handling practices during the cultivation process and before the time of consumption.

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