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Occurrence and diversity of ciliated protozoa in the most commonly consumed leafy greens in Brazil: a qualitative study

Ocorrência e diversidade de protozoários ciliados nos vegetais folhosos mais consumidos no Brasil: um estudo qualitativo

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Taís Rondello BONATTI, Sandra YAMASHIRO, Pedro Ferreira de Castro Santana e FREITAS, Isabel Cristina Vidal SIQUEIRA–CASTRO, Regina Maura Bueno FRANCO*

*Corresponding author: Protozoology Laboratory, Biology Institute, University of Campinas, Campinas, São Paulo, Brazil. Phone number: +55 19 3521 6285. E-mail: reginamaurafranco@gmail.com

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ABSTRACT

Ciliated protozoa are an ubiquitous group of eukaryotes that have been poorly reported on leafy greens. The present study aimed to verify the occurrence and diversity of ciliated protozoa in the leaves and roots of three of the most commonly consumed leafy greens in Brazil – lettuce, rocket and coriander. The vegetable samples were washed by manual agitation (3 minutes) in two different media (mineral water and Page´s Amoeba Saline solution). After washing, the contents were incubated in Petri dishes and aliquots were removed for microscopic identification and *in vivo* observation. A total of 21 ciliated protozoa species were found, most of which were bacterivorous. Leafy greens have commonly been associated with foodborne outbreaks and ciliated protozoa, which although they are not a Public Health concern, can act as "Trojan Horses" harboring bacteria, viruses and other protozoa cysts and oocysts and can suggest a new route towards microbiological quality related to the food chain. This is the first report of ciliated protozoa on leafy greens consumed in Brazil.

Keywords. ciliated protozoa, biodiversity, leafy greens, food contamination, foodborne diseases, food safety.

RESUMO

Os protozoários ciliados constituem um grupo onipresente de eucariotos pouco relatados em vegetais folhosos. O presente estudo teve como objetivo verificar a ocorrência e a diversidade de protozoários ciliados nas folhas e raízes de três dos vegetais folhosos mais consumidos no Brasil - alface, rúcula e coentro. As amostras de vegetais foram lavadas por agitação manual (3 minutos) em dois meios diferentes (água mineral e Solução Salina para Ameba). Após a lavagem, o conteúdo foi incubado em placas de Petri e alíquotas foram retiradas para identificação microscópica e observação *in vivo* dos organismos. Um total de 21 espécies de protozoários ciliados foi encontrado, a maioria das quais era bacterívora. Tais hortaliças têm sido comumente associadas a surtos transmitidos por alimentos e os protozoários ciliados que, embora não sejam um problema de saúde pública, podem atuar como "Cavalos de Tróia" ao abrigar bactérias, vírus além de cistos e oocistos de protozoários o que pode sugerir uma nova rota para a avaliação da qualidade microbiológica relacionada à cadeia alimentar. Este é o primeiro relato de protozoários ciliados em folhas verdes consumidas no Brasil.

Palavras-chave. protozoários ciliados, diversidade biológica, vegetais folhosos, contaminação alimentar, doenças transmitidas por alimentos, segurança alimentar.

INTRODUCTION

Recent years have seen a growing consensus that eating raw vegetables is essential for health maintenance, as such foods provide several important components such as water, iron, vitamins and mineral elements[1-3]. However, various pathogenic agents as bacteria, viruses and parasitic pathogens, have been implicated in foodborne outbreaks due to raw vegetable consumption[4,5]. According to the WHO[6], the most frequent causes of foodborne illness are diarrheal disease agents, which cause 230,000 deaths per year in the whole world. In Brazil, the consumption of raw salads is very common and 6,632 foodborne outbreaks and 109 deaths were registered from 2007 to 2016, mostly caused by *Salmonella*[7].

Ciliated protozoa are an ubiquitous group of eukaryotic heterotrophic organisms with several feeding habits. These organisms play an important ecological role as they serve as food for other organisms and are fundamental in terrestrial and aquatic food webs[8,9]. They have been widely studied in aquatic environments, often in relation to water quality, but there are few studies about the occurrence of this group in vegetables commonly consumed[4,10,11].

Although some reports have shown that ciliated protozoa can prey on pathogenic protozoa cysts and oocysts as well as viruses and bacteria, there is no information about the maintenance of the infectivity of these forms inside feeding organelles, and so they may harbor these agents and act as "Trojan horses"[9,12,13]. Moreover, there is a possibility that these pathogenic forms, as *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts for example, could survive inside ciliated protozoa and become protected from disinfectants such as chlorine, therefore being implicated as reasons for foodborne outbreaks[4,9,11,14].

There are several ways that vegetables can become contaminated by pathogenic organisms, such as through the water used for irrigation and during collection, transport and preparation[10,15]. Furthermore, the role of the microbial communities associated with vegetables and their relationship with human pathogens has been poorly explored[11]. Ciliated protozoa are not considered in microbiological monitoring as they are classified as not dangerous from a Public Health perspective, although studies have shown that they can represent an indirect risk for consumers by harboring infective pathogenic bacteria, such as that by Vaerewijk et al[8].

The aim of the present study was to verify the occurrence and diversity of ciliated protozoa in leaves and roots of three of the most commonly consumed leafy greens in Brazil – lettuce, rocket and coriander.

MATERIAL AND METHODS

Three different types of raw leafy greens were obtained from supermarkets, market-places and central open-air markets in Campinas, São Paulo, Brazil from March to June 2015: lettuce (n=5), rocket (n=6) and coriander (n=8).

Samples were collected under normal purchase conditions, randomly selected, and immediately taken to the Protozoology Laboratory of the Biology Institute of the University of Campinas. Some of the vegetables were pre-washed ("easy-to-eat") before sale.

Approximately 30g of leaves and roots (hydroponic samples) were removed from the heads and put separately in sterile plastic bags for further washings, separately. These washings were performed by manual agitation for three minutes in two different media: mineral water (not sterile) and Page's Amoeba Saline Solution[4], using 50 mL of each media for each sample. There was no control sample processed for this study.

After washing, only the liquid content (50 mL) was immediately transferred to Petri dishes containing raw and crushed rice (approximately 3 grains) which was previously autoclaved to stimulate the growth of indigenous bacteria, flagellates, fungi and amoeba[16], in order to support ciliated protozoa excystment and growth. Two plates were used for each sample: one with 50 mL of mineral water and the other with 50 mL of Page's Amoeba Saline solution. Petri dishes were incubated in room temperature and more mineral water or Page´s Amoeba Saline solution were added one a week to prevent drying out.

Aliquots of each culture were taken for identification and *in vivo* observation. All plates were monitored three times a week, for one month in order to verify ciliated protozoa diversity. Other aliquots were also collected for qualitative examination of the details of the ciliature organization, macro and micronucleus. These features were revealed by the protargol impregnation technique proposed by Dieckman[17].

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The morphological aspects of ciliates *in vivo* and after protargol impregnation were observed and photo-documented by Axiocam MRC - Zeiss model scientific digital color camera attached to a microscope (Axio Imager) by differential interference contrast (DIC) microscopy (400X). Several guides and complementary literature[16,18-24] were consulted for the ciliated protozoa identification.

RESULTS

During this study and despite media used, we identified 16 ciliated protozoa species in lettuce, 12 in rocket and four in coriander (**Table; Figure**).

This is the first report of ciliated protozoa on leafy greens consumed in Brazil.

For lettuce and rocket samples, most of the species identified are bacterivorous ciliates (*Aspidisca cicada*, *Chilodonella* sp., *Colpidium colpoda*, *Colpoda inflata, Cyclidium glaucoma*, *Euplotes affinis, Opercularia* sp., *Paramecium aurelia*- complex, *Paramecium* sp., *Parentocirrus* sp., *Tetrahymena pyriformis*-complex, *Vorticella* sp. and *Uronema nigricans*). For coriander samples, most of the species recorded are bacterivorous and commonly found in terrestrial habitats (*Colpidium colpoda*, *Colpoda inflata* and *Gonostomum affine*).

Table. Ciliated protozoa taxa found on leafy green samples (leaves and roots)

Lettuce	Rocket	Coriander
Aspidisca cicada	*Acineria incurvata*	*Colpidium colpoda*
Chilodonella sp.	*Chilodonella sp.*	*Colpoda inflata*
Colpidium colpoda	*Colpidium colpoda*	*Gonostomum affine*
Colpoda inflata	*Colpoda inflata*	*Tetrahymena pyriformis* - complex
Cyclidium glaucoma	*Cyclidium glaucoma*	
Euplotes affinis	*Euplotes aediculatus*	
Gonostomum affine	*Opercularia sp.*	
Heliophrya sp.	*Paramecium aurelia* - complex	
Himenostomatida	*Paramecium sp.*	
Hipotrichida	*Tetrahymena pyriformis* - complex	
Parentocirrus sp.	*Uronema nigricans*	
Prostomatida	*Vorticella sp.*	
Spathidium spatula		
Sterkiella cavicola		
Tetrahymena pyriformis - complex		
Vorticella sp.		
Total: 16	**Total: 12**	**Total: 4**

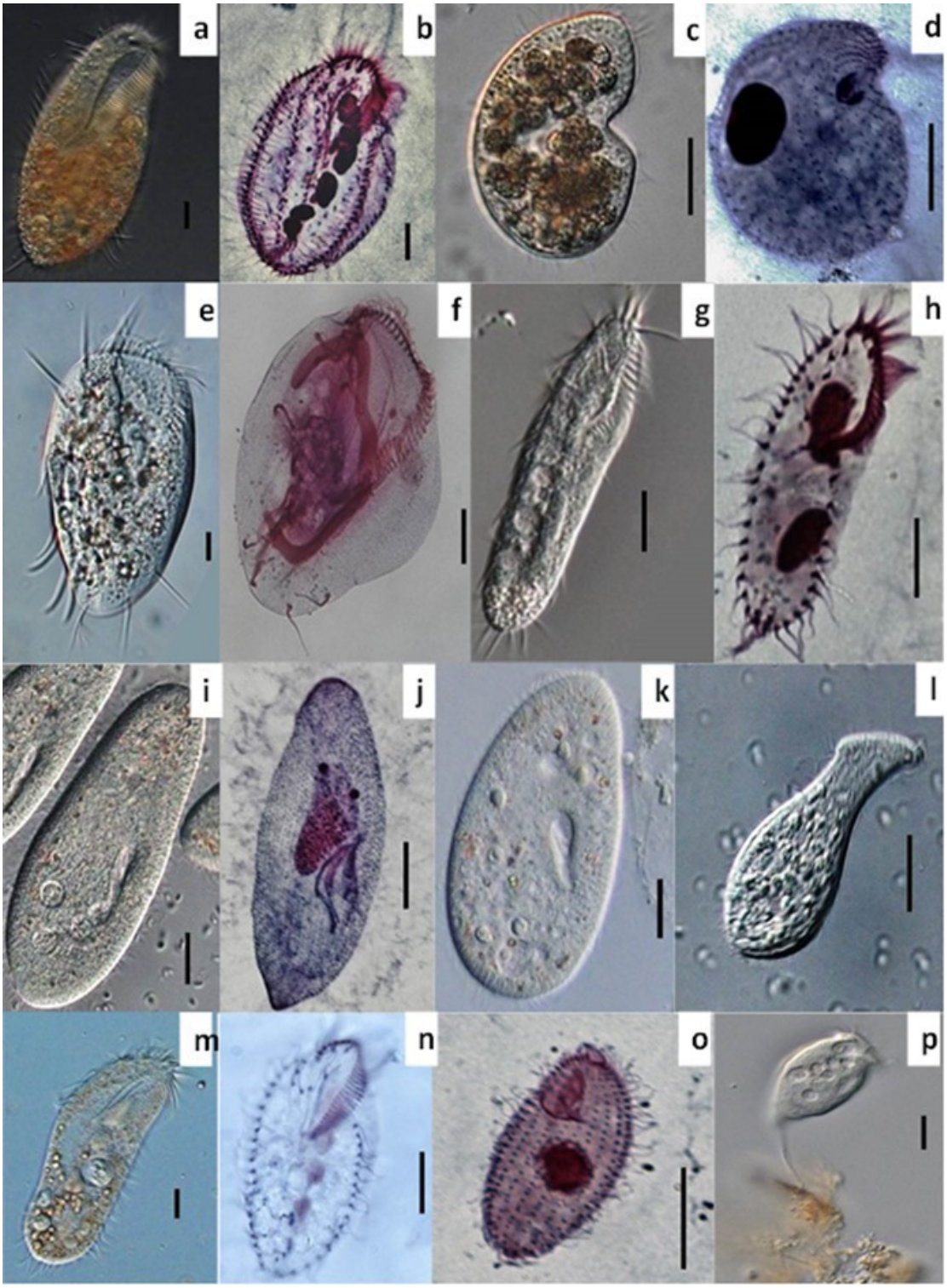

Figure. Photomicrographs of ciliated protozoa found on leafy green samples from Campinas, São Paulo, Brazil, observed in differential interference contrast (DIC) microscopy. Organisms *in vivo* (a; c; e; g; I; k; l; m; p) and following protargol impregnation (b; d; f; h; j; n; o); a-b: *Parentocirrus* sp.; c-d: *Colpoda inflata*; e-f: *Euplotes aediculatus*; g-h: *Gonostomum affine*; i-j: *Paramecium aurelia*; k: *Paramecium sp*; l: *Spathidium spatula*.; m-n: *Sterkiella cavicola*; o: *Tetrahymena pyriformis*; p: *Vorticella* sp. Scale Bars = 10 µm

DISCUSSION

The habit of eating raw vegetables is critical for foodborne disease epidemiology[15,25]. According to the WHO[6], beyond affecting individuals, foodborne diseases harm economic development, creating particular challenges for the tourism, agricultural and food (export) industries.

There are no criteria for ciliates in raw vegetables in contrast to pathogenic bacteria, worms and parasitic protozoa where several national and international microbiological limits have been published[8]. The presence of pathogens in vegetables is problematic as they can survive and even grow inside ciliated protozoa cells[10,13,26] such as *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Arcobacter butzleri* and *Yersinia enterocolitica*[27]. In addition, these bacteria are resistant to protozoan grazing[27]. Nadhanan and Thomas[28] reported that bacteria encapsulated by the fecal pellets of ciliated protozoa may be resistant to disinfectants commonly used in food manufacturing. In the present study, the most commonly registered ciliated protozoa were bacterivorous, which may indirectly evidence the presence of pathogens in these vegetables.

Also, even when preyed upon these pathogen or protozoan resistant forms such as cysts and oocysts may not be inactivated by the digestive process or by common vegetable disinfectants such as chlorine, which is unable to inactivate protozoa parasitic resistance forms[8]. Siqueira-Castro et al[9] found for the first time that *Giardia* cysts were preyed upon by ciliated protozoa, and confirmed predation on *Cryptosporidium* oocysts, also described in other works[13,29-31].

There are few studies that show the presence of ciliated protozoa in vegetables and no register has been done in Brazil so far, although there are many other reports about pathogenic protozoa in these vegetables. Chavatte et al[10] found 30 ciliated protozoa morphospecies, with *Tetrahymena* sp. dominant among other ciliates on vegetable sprouts. In the present study species were found with *Tetrahymena pyriformis*–complex and *Parentocirrus* sp. the most commonly identified in the samples. Some studies that found morphologically similar hypotrich ciliates have used morphogenetic data to discriminate between species[21,32].

Tetrahymena sp. is commonly used as a model in studies of its interaction with bacteria, such as *Campylobacter jejunii* and *Salmonella enterica*[8,10]. This ciliated protozoan is bacterivorous and, via the cytostome, ingests food particles, forming a food vacuole that is later digested[33]. In response to adverse conditions, the protozoan undergoes encystment, accompanied by the formation of resting non-feeding particles and cysts[33]. It has also been demonstrated *in vitro* that *Tetrahymena pyriformis* cysts were able to harbor viable and virulent *Listeria monocytogenes* cells[33]. McNealy et al[34] verified that *L. monocytogenes* cells were not destroyed by protozoa, as they escaped within the fecal pellets eliminated by the ciliates, such as *Tetrahymena* spp.

Gourabathini et al[11] found intact *Salmonella enterica* and *Escherichia coli* in the expelled vesicles of ciliated protozoa and reported that these prokaryotic organisms can multiply inside these organelles, or even in the cytoplasm. Moreover, these authors suggest that ciliated protozoa can trap these enteric pathogen organisms in their cysts.

Parentocirrus sp. has been reported in an ephemeral garden birdbath[35], activated sludge samples[36-38] and in mosses growing in the shore zone of a mesosaprobic river in Slovakia[39].

It is important to highlight the finding of *Colpoda* sp. in coriander samples: Nadhanan and Thomas[28] verified *in vitro* experiments that the fecal pellets of this ciliate contained intact *Listeria monocytogenes* cells. Therefore, the protection provided by fecal pellets can explain the difficulty of decontaminating food-preparation facilities and the presence of *Colpoda* in vegetables may be a concern. It is important to address that this ciliated protozoan is bacterivore and commonly found in polisaprobic environments (associated with a high amount of organic matter available). On the other hand, Zou et al[40] indicate that bacterivore ciliated protozoa found in roots are related to the increase on primary production by raising nutrient availability.

Ciliated protozoa are a useful tool for water quality monitoring. The saprobic index is based on the degree of tolerance of the ciliate species to organic pollution, and this index has seven levels ranging from water lacking in or with a very low organic load, to exceptionally polluted water[20,41,42].

On a recent study of the ciliated protozoa present in the Atibaia River, the main water supply for Campinas, it was found that most ciliated protozoa species were classified as polysaprobic and alfamesosaprobic[43], revealing that this river, the waters of which are used for irrigating the vegetable production areas of the region, is strongly polluted. Some of these species found in the present study are classified as polysaprobic and alphamesosaprobic, such as *Acineriain curvata*, *Colpidium, Paramecium caudatum*, *Tetrahymena pyriformis–complex* and *Cyclidium glaucoma*. *T. pyriformis-complex, Acineria* sp., *Paramecium* sp., and *C. glaucoma* were also detected in interstitial liquid samples from a combined wastewater treatment system at the University of Campinas[44] and from an activated sludge system in southeastern Brazil[45]. Therefore, the presence of these ciliates in vegetables may indicate the contamination of water irrigation with untreated sewage.

Studies of the parasitic contamination of fresh vegetables show that lettuce has the highest prevalence. This may be related to the aspect of the leaf surface and the presence of structures such as stomata, which are capable of harboring parasitic forms and ciliated protozoa[25,46]. In the present study, most of the species were found in lettuce leaves.

In Brazil, the consumption of raw or lightly cooked vegetables throughout the year is common. This habit, combined with poor sanitation, can lead to foodborne outbreaks. According to Machado et al[47], the pathogenic microorganism's contamination on vegetables is considered a national problem due to these high frequencies. According to the Brazilian Health Regulatory Agency[48], fruit and vegetables are associated with almost 3% of the foodborne outbreaks in the country and a recent decree from the Health Ministry[7]made the notification of food-related diseases compulsory. In addition, the irrigation water has a variety of sources, and is a microbial risk, especially for leafy greens[49]. Decol et al[49] found a generic *E. coli* prevalence of 84.8% and 38.3% in irrigation water samples and on lettuces in southern Brazil. In these water samples, *E. coli* O157:H7 was detected by polymerase chain reaction.

Despite the importance of the presence of these organisms on leafy greens harboring pathogenic organisms, it is relevant to recognize that this is the first report about their diversity on these vegetables. In Brazil, there are a few studies that had been conducted on ciliated protozoa diversity in different environments such as fresh waters. Some authors, as Cotterill et al[50], reinforce the importance of local and global research on these organism's diversity once they are excluded from environmental management programs and biodiversity conservation. In addition, the taxonomic survey of the ciliates species is very important due to the rapid responses of these organisms to human activities such as the discharge of organic matter in water bodies. Also, the importance of biodiversity knowledge is also related to the great biotechnological potential they present. Several foodborne outbreaks have been reported in the USA and Europe relating to fresh vegetables and fruit and water used for irrigation. These products usually come from developing countries and, due to globalization, food distribution routes are shorter and the products can be used promptly[51]. The access of developing countries to food export markets will depend on their capacity to meet the international regulatory requirements determined by the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) of the World Trade Organization (WTO). Unsafe exports can lead to significant economic losses[6]. The present study is the first report of ciliated protozoa in fresh vegetables in Brazil and was performed over four months in 2015. It would be interesting to assess whether the compositions remain stable or change throughout the year. Also, it is necessary more studies to contribute on the occurrence and diversity of ciliated protozoa as there is a great potential to use these organisms as bioindicators and also for food monitoring purposes.

CONCLUSIONS

Several species of ciliated protozoa with the potential to host pathogenic microorganisms related to foodborne outbreaks have been found in all vegetables samples considered the ones most commonly consumed in Brazil. The presence of these protozoa in fresh food, as well as the survival of pathogens within them, require further investigation and indicate new directions for microbiological quality related to the food chain.

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