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Prevention methods of foodborne Chagas disease: Disinfection, heat treatment and quality control by RT-PCR

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ABSTRACT

The most important mode of transmission causing outbreaks of Chagas disease in the Amazon region is the oral route due to the ingestion of contaminated food. Herein, prevention methods for foodborne diseases caused by *Trypanosoma cruzi*, namely, sanitization, thermal treatment were investigated and the use of reverse transcription PCR (RT-PCR) amplification for the mRNA-based detection of viable *T. cruzi* in açai, was developed. Three *T. cruzi* strains (*T. cruzi* I, *T. cruzi* III and Y) were used in the present study. The Amazonian strains *T. cruzi* I (425) and *T. cruzi* III (370) showed higher resistance to sodium hypochlorite treatment and heat treatment than the reference strain Y. The blanching of fruits (70 ± 1 °C for 10 s) and pasteurization of juice (82.5 °C for 1 min) efficiently eliminated *T. cruzi* in food matrices. Additionally, a method that uses RT-PCR amplification of mRNA was developed for the detection of viable *T. cruzi* in açai, which could play a role in examining food samples, ensuring consumer health, and reducing this foodborne disease.

1. Introduction

Trypanosoma cruzi, a flagellated protozoan, is the causative agent of Chagas disease and is transmitted by triatomine insects. Oral transmission is currently the most frequent form of the parasite transmission in Brazil, mainly in the Amazon region (Góes-Costa et al., 2017). Outbreaks have been widely documented in several countries from the Americas, including Brazil, Venezuela, Colombia, and French Guiana (Noya et al., 2015). The possible mechanism for oral transmission is via the ingestion of food and derived products containing whole infected triatomines or their faeces (Pereira et al., 2012). The transmission of Chagas disease by infectious secretions from reservoir hosts, such as opossums, has rarely been observed (Robertson et al., 2016). Sugarcane, guava, bacaba and açai contaminated have already been shown to be responsible for outbreaks (Cardoso et al., 2006; Nóbrega et al., 2009).

In Brazil, açai juice is the most frequent vehicle for infection, particularly in the Amazon region (Barbosa et al., 2012; Góes-Costa et al., 2017). In this region, açai is widely consumed, playing an important socio-economic role and providing high nutritive input to river and urban populations (Rogez, 2000). Açai was initially consumed only in the Amazon region. However, due to its high phenolic compound content, it reached international distribution within a few years (Bichara and Rogez, 2011). Industrialized and exported açai are supposed to be pasteurized. Unfortunately, however, the majority of açai sold for consumption by the population of Brazil and other South American countries is still not pasteurized (Oliveira and Schwartz, 2018).

Attractant factors that draw triatomines to açai fruits have been described. The fruits exhibit accelerated microbial growth kinetics caused by environmental conditions, such as harvesting, storage, shipping and processing, that generate spontaneous fermentation and decay after only 48 h (Rogez et al., 2012). The decay process produces and releases gaseous compounds. Because the production of metabolites (e.g., ethanol, lactic acid, and CO_2) and water which associated with an increase in the fruit temperature of 5 °C attracts triatomines that are sensitive to these gradients (Aguiar et al., 2013; Rogez and Aguiar, 2012). Thus, sanitary management throughout the production chain is necessary for food safety (Pereira et al., 2012).

T. cruzi detection in food matrices is important for the identification of potentially infectious food and for the implementation of prevention procedures in the food chain. The presence of *T. cruzi* in food can be detected by traditional methods, such as culture and microscopic observation (Cardoso et al., 2006). However, microscopic observation methods are difficult and minimally effective when only a few microorganisms exist in a sample, and culture is a labour-intensive, time-

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intensive method subject to contamination risk (Pereira et al., 2012; Godoi et al., 2017). To overcome these limitations, molecular techniques, such as DNA-based PCR methods, have been developed for the rapid detection of *T. cruzi* (Ferreira et al., 2016; Godoi et al., 2017; Mattos et al., 2017). However, a disadvantage of conventional DNA-based PCR techniques is that both viable and non-viable cells can be detected (Postollec et al., 2011; Filion, 2012), whereas reverse transcription PCR (RT-PCR) based on mRNA amplification detects only viable microorganisms (Vaitilingom et al., 1998). The evaluation of viability by RT-PCR is a rapid and sensitive technique. However, a possible problem with this method is the limit of detection, which depends on the matrices and targets utilized and can result in overestimation of the number of viable parasites (Rousseau et al., 2018). Thus, an assay for the rapid identification of *T. cruzi* viability in foods that ensures food safety does not yet exist.

Therefore, the aim of this work was to develop a rapid method for the detection of viable *T. cruzi* based on the amplification of mRNA by RT-PCR. We also aimed to investigate prevention and control methods for orally transmitted Chagas disease.

2. Materials and methods

2.1. Food matrix

Açai (*Euterpe oleracea*) fruit and juice were purchased from a local market (Belém, Brazil). To reduce the initial microbial load, the açai juice was subjected to heat treatment at 90 °C for 1 min before being frozen. The fruits and juice were frozen at −20 °C and thawed prior to experimental inoculation with *T. cruzi* strains.

2.2. Trypanosoma cruzi strains and culture conditions

The reference strain of *T. cruzi* (Y, *T. cruzi* I) isolated from an acute human case and characterized by Silva and Nussenzweig (1953) and two strains isolated from *Rhodnius pictipes* from the Amazon region (425, *T. cruzi* I and 370, *T. cruzi* III) were used for the experimental inoculation of fruits and açai juice. The strains were kindly supplied by the Collection of *Trypanosoma* from Wild and Domestic Mammals and Vectors (COLTRYP)-FIOCRUZ.

Parasites were reactivated in Novy, McNeal and Nicolle (NNN) medium and then cultured in liver infusion triptose (LIT) (supplemented with 10% foetal bovine serum and 100 U of penicillin) and subcultured every seven days during the exponential growth phase. The cultures were maintained at 26 °C, and used after differentiation forms according to Kessler et al. (2017).

2.3. Experimental inoculation

The inoculum for experimental inoculation was obtained from LIT liquid medium by sub-culturing every week during two-week period. The total concentrations of the *T. cruzi* epimastigote, recently differentiated epimastigote (rdEpi) and trypomastigote forms from the Y, 370 and 425 strains were estimated before experimental inoculation. The parasite count was performed in a Neubauer chamber with an optical microscope at 400× and expressed as parasites/mL. Aliquots of all chemical and thermal treatments used for *T. cruzi* and açai fruit and juice inoculation were subjected to a survival test, viability analysis in culture, PCR and RT-PCR. All assays were performed in triplicate.

2.3.1. Açai fruits

The evaluation of *T. cruzi* survival and viability in açai fruits (50 ± 1.0 g) was performed in inoculated samples *i*) maintained at 25 °C for 1, 5, 10, 24 and 32 h after inoculation and *ii*) submitted to blanching treatment at 70 °C for 10 s. One millilitre of parasites inoculum suspension was distributed on the surface of the fruits. Next, the parasites were recovered from the surface of the fruits by adding 10 mL of physiological saline solution (0.9%) and homogenized with the aid of a spatula. Then, the total volume was centrifuged at 1500 *g* for 10 min. The pellet was maintained at −80 °C until DNA and mRNA extraction. All experiments were carried out in triplicate.

2.3.2. Açai juice

Açai juice was inoculated with 1×10^6 parasites/mL and homogenized with the aid of a spatula. Cell survival and viability in juice were monitored in samples *i*) without treatment at room temperature (25 °C) immediately after inoculation and 1, 5, 10, 24 and 32 h after inoculation and *ii*) with heat treatment after pasteurization (82.5 °C for 1 min). The pH of the açai juice was measured prior to inoculation using a pH meter. For the açai juice experimentally inoculated, the parasites were recovered using a triple centrifugation technique previously described by Godoi et al. (2017). The first centrifugation was at 1500 *g* for 10 min at 4 °C. Then, the recovered supernatant was centrifuged a second time in the same conditions 1500 *g* for 10 min at 4 °C. Finally, the recovered supernatant was centrifuged at 14.000 *g* for 15 min at 4 °C. All procedures were performed aseptically and in triplicate.

2.3.3. Chemical and thermal treatment

Experiments involving pure cultures were performed in a physiological saline solution. For this experiment, glass tubes containing 9 mL of physiological saline solution were inoculated with 1 mL of the *T. cruzi* inoculum at a final concentration of 1×10^6 parasites/mL. For the homogenization, the tubes were closed and gently homogenized by inversion. The samples were treated with different concentrations of sodium hypochlorite (50, 100, 125, 150 or 200 ppm for 1, 5, 10 or 20 min) or subjected to thermal treatment at 45 ± 1 °C for 10 min, 45 ± 1 °C for 60 min or 70 ± 1 °C for 10 s. For viability tests in culture, aliquots were collected after 5 min of sodium hypochlorite treatment. The parasite survival and viability tests were also verified in non-distilled water. The negative and positive controls consisted of a physiological saline solution that was not inoculated and an aliquot of inoculum without hypochlorite, respectively. All experiments were carried out in triplicate for each temperature and period.

2.4. Survival test

T. cruzi survival after the experiments was evaluated by microscopic visualization in triplicate, and only living motile parasites were enumerated. After homogenization, 5 µL of each sample was deposited on a slide. The number of active parasites was enumerated in fifty fields covering the total area of the slide, as described by Brener (1962).

2.5. Viability of Trypanosoma cruzi in culture

The parasite viabilities in the samples after inoculation and various treatments were analysed according to the viability of the parasite form. Viability was defined as the growth capacity of the parasite in vitro by the culture method in NNN and LIT media compared to the positive control. The culture tubes were examined weekly with an optical microscope at 400× for four weeks. Cultures that did not present active parasites were considered negative.

2.6. DNA extraction

DNA was extracted from pure culture strains; from inoculated açai fruit and juice samples; and from chemical and thermal treatment. Before extraction, the parasites were washed twice with Tris-HCl buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and centrifuged at 10,000 *g* for 5 min at 4 °C after each wash. DNA extraction was performed using the adapted phenol/chloroform protocol (Sambrook et al., 1989). The DNA was stored at −80 °C until use.

2.7. RNA extraction and cDNA synthesis

mRNA was extracted from parasites obtained in culture; from inoculated açai fruit and juice samples; and from chemical and thermal treatments. Before extraction, the parasites were washed twice with Tris-HCl buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and centrifuged at 10.000 *g* for 5 min at 4 °C after each wash. The RNeasy Mini Kit (QIAGEN, Hilden, Germany) was used for RNA extraction following the manufacturer's recommendations. The RNA purity and concentration were determined on a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) apparatus at 260/280 nm and 260 nm, respectively. Contaminating genomic DNA was removed from RNA with the TURBO DNA-free™ Kit (Ambion, Austin, TX, USA). The First Strand cDNA Kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis from 10 ng of poly(A) mRNA following the manufacture's recommendations.

2.8. PCR and reverse transcription PCR

The PCR assay was performed using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR amplification of the repetitive nuclear region designated TCZ (TCZ1-5′CGAGCTCTTGCCCA CACGGGTGCT3′/TCZ2-5′CCTCCAAGCAGCGGATAGTTCAGG3′) described by Moser et al. (1989) was performed in a 25 µL reaction mixture containing 10 mM Tris-HCl buffer (pH 8.4), 0.8 mM $MgCl_2$, 0.01% gelatine, 0.2 mM dNTPs, 0.6 µM each primer (TCZ1 and TCZ2) and 1.0 U of Platinum Taq DNA polymerase (Invitrogen). As DNA template was used 2 µL (5 ng/µL) of genomic DNA for the PCR and complementary DNA (cDNA) for the RT-PCR. The amplification consisted of a denaturing step at 95 °C for 5 min; 30 cycles of 95 °C for 1 min, 70 °C for 1 min and 72 °C for 1 min; and a final extension step at 72 °C for 5 min. The amplified products has 188 bp and were separated by electrophoresis in a 1.5% agarose gel and visualized under UV light after ethidium bromide staining. Several controls were routinely run: a positive control with DNA and mRNA from *T. cruzi*, and DNA from inoculated açai fruits and juice; and a negative control from no inoculated açai and without template.

2.9. Statistical analysis

The results were expressed as the mean ± standard deviation when appropriate. The experimental data were analysed using the analysis of variance (ANOVA) module in the program STATISTIC for Windows 7.1 (STAT-SOFT) at significance levels of 95% ($p < 0.05$). The Duncan test was then used to compare sets of data with the same level of significance.

3. Results

3.1. Açai fruits and açai juice

Fig. 1 shows the evaluation of the concentration of active *T. cruzi* in açai fruits maintained at 25 °C over time. The concentration of parasites with apparent motility decreased over time. Surprisingly, the parasites remained alive for up to 32 h after experimental inoculation. In general, most of the parasites were active in the first 5 h, and the parasite concentration decreased by half from 10 h. For the viability test in culture, the presence of the parasite was not observed 10 h after experimental inoculation.

In açai juice inoculated with *T. cruzi* and maintained at room temperature and without treatment, the presence and intense motility of the protozoans were verified immediately after inoculation and for up to 5 h after inoculation, except for the 425 strain (Table 1). For the 370 strain at 10 h and for the 425 strain at 5 h and 10 h after inoculation, only non-active parasites were observed; they displayed a deformed cellular structure and showed no flagellum movement. Therefore,

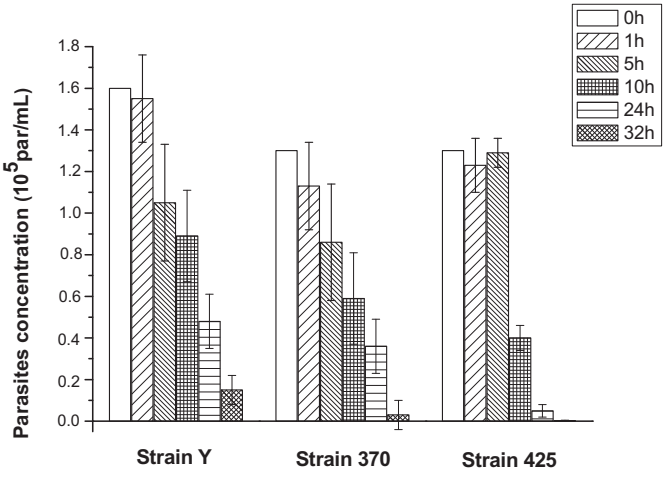

Fig. 1. Concentrations of active *Trypanosoma cruzi* from the Y, 370, and 425 strains in açai fruits at 25 °C after 0, 1, 5, 10, 24 and 32 h of experimental inoculation. The concentration at 0 h is the initial concentration used.

Table 1
Survival and viability of the *Trypanosoma cruzi* Y, 370 and 425 strains in the culture evaluation of açai juice after various time intervals at 25 °C.

Treatment	Time after experimental inoculation of açai juice (h)	Strain Y		Strain 370		Strain 425	
		ST	VB	ST	VB	ST	VB
Without treatment	Immediately after	+	+	+	+	+	+
	1	+	+	+	+	+	+
	5	+	+C	+	+C	+NA	+C
	10	+	−C	+NA	−C	+NA	−C
	24	−	−C	−	−C	−	−C
	32	−	−C	−	−C	−	−C

T. cruzi active (+); *T. cruzi* not active (+ NA); negative (−); contamination (C); survival test (ST); and viability (VB).

microscopic visualization did not confirm their survival. In the culture test, high microbial contamination was verified to be detrimental to parasite growth 10 h after inoculation. When examining viability in açai juice cultures, the presence of the parasites was not observed except in the sample corresponding to 5 h for strain 370.

Non-distilled water is the base for the preparation of açai juice, and the survival and viability of the strains studied were verified in water. Microscopic visualization showed that immediately after contact with water, the parasite initiated a process of morphological change that culminated in its disintegration and death.

3.2. Chemical and thermal treatment in physiological saline solution

To evaluate the isolated effects of sanitization with sodium hypochlorite and temperature on *T. cruzi* inactivation, experiments were performed in a 0.9% physiological saline solution, to avoid their rupture instead of water, which is a hypotonic solution and could influence the results. Aliquots of all chemical and physical treatments were analysed using survival tests, and culture viability assays and the results are shown in Tables 2 and 3, respectively.

T. cruzi was obtained from culture or inoculated acai fruits and juice served for mRNA extraction. The isolated mRNA resulted in appropriate mRNA quality and quantity with A260/A280 absorbance ratio comprised between 1.8 and 2.1. Various control treatments were carried out in order to verify for possible genomic DNA contamination of the RNA samples. One control comprised DNase control with DNase-treated RNA, another control without DNase treatment and finally a control without reverse transcriptase during cDNA synthesis step. All these

Table 2
Survival and viability of the *Trypanosoma cruzi* Y, 370 and 425 strains in culture in a 0.9% physiological saline solution after the sanitization.

Sodium hypochlorite (ppm)		100			150			200		
Strains		Y	370	425	Y	370	425	Y	370	425
VB		+	+	+	−	−	+	−	−	−
ST	5 min	8.46 ± 0.79a	6.58 ± 0.71a	7.67 ± 2.21a	−	−	0.12 ± 0.15a	−	−	−
(10^5par/mL)	10 min	3.91 ± 0.48b	2.91 ± 0.45b	6.66 ± 1.80b	−	−	0.02 ± 0.03a	−	−	−
	15 min	0.75 ± 0.21c	0.33 ± 0.22c	2.33 ± 0.29c	−	−	0.02 ± 0.02a	−	−	−
	20 min	0.23 ± 0.11c	0.16 ± 0.05c	1.49 ± 0.54c	−	−		−	−	−

T. cruzi active (+); *T. cruzi* no active (+ NA); negative (−); survival test (ST); and viability (VB). Different letters indicate significant differences by Duncan's test ($p < 0.05$).

controls were performed to ensure that the amplification was from the synthesized cDNA and not from genomic DNA. To prevent false positive RT-PCR amplification, we found that application of 2 U of DNase I is sufficient to remove any contaminating DNA.

When *T. cruzi* DNA and mRNA were extracted from live cells from pure cultures and amplified by PCR and RT-PCR, respectively, the 188-bp product was observed as expected in the positive control (data not shown). Furthermore, the 188-bp product was observed by conventional DNA-based PCR in all samples except for the negative control. The mRNA was detected by RT-PCR amplification from treatments with 50 ppm sodium hypochlorite for all three strains (45 ± 1 °C for 10 min for the 370 and 425 strains and 45 ± 1 °C for 60 min for the 425 strain). In contrast, no cDNA amplification was observed for 200 ppm sodium hypochlorite for all three strains (45 ± 1 °C for 60 min for the Y and 370 strains and 70 ± 1 °C for 10 s for the Y, 370 and 425 strains). This result confirms the non-viability of the parasites after these treatments.

The PCR and RT-PCR results agreed with the survival and viability tests evaluated by microscopy. The contact time and concentration of sodium hypochlorite were determinants of the inactivation of the *T. cruzi* strains Y, 370 and 425. The parasite load decreased with increasing contact time and sodium hypochlorite concentration. Inactivation of parasites at 125, 150 and 200 ppm was observed after 5 min for the Y, 370 and 425 strains, respectively, indicating that these concentrations are not appropriate for their survival. Interestingly, the *T. cruzi* I (425) and *T. cruzi* III (370) strains showed higher resistance to sodium hypochlorite treatment and temperature than the Y strain. Regarding temperature, live and viable *T. cruzi* were observed by microscopic observation and culture after treatment at 45 ± 1 °C for 10 min and 60 min for the 370 and 425 strains, respectively.

The isolated strain 425 from *R. pictipes* exhibited peculiar behaviour when exposed to adverse treatment, converting from the epimastigote to amastigote form during reactivation in culture. During the first two weeks of culture of the experimental samples, the amastigote forms were predominant. From the third week, epimastigote forms were observed. These changes were observed in açai juice at 5 and 10 h after experimental inoculation and in *T. cruzi* samples in 0.9% physiological saline solution subjected to heat at 45 ± 1 °C for 60 min. In addition, experiments with blanching at 70 °C for 10 s and pasteurization at 82.5 °C for 1 min were performed on açai fruits and juice, respectively. The results of the survival test, viability in culture test and RT-PCR were negative for both açai fruits and juice, while the results of PCR were positive.

4. Discussion

4.1. Açai fruits and juice

High bacterial and yeast contamination during the viability test was observed in the culture, stemming from the microbial load from the açai fruits. The microbial contamination made trypanosome growth difficult in the LIT and NNN media, most likely because of competition with these microorganisms for nutrients. Mesophilic bacteria, faecal coliforms, moulds and yeasts are naturally present on the surface of açai fruits (Rogez et al., 2012). Factors such as non-acidic pH, intense handling throughout the product chain, contaminated surfaces in contact with fruits after picking, and high local temperature and relative humidity may explain this high contamination (Aguiar et al., 2013; Bichara and Rogez, 2011). Rogez et al. (2012) observed rapid microbial growth kinetics on açai fruits due to the fruit characteristics.

The protozoan *T. cruzi* cannot multiply independent of its hosts (vectors or mammals) (Kessler et al., 2017). *T. cruzi* can survive on various fruits and vegetables maintained at room temperature for many hours, such as up to 10 h on melon and tomato, up to 18 h on papaya and banana, up to 24 h on apple and up to 48 h on potato and carrot (Añez and Crisante, 2008; Añez et al., 2009). The measured survival time of *T. cruzi* in açai juice is shorter for all strains than the results recorded by Passos et al. (2012), who demonstrated that 100% of active parasites remained present in açai juice supernatants for up to 6 h at room temperature, and 100% of living parasites showed slow motility after 48 h. However, the survival time obtained in this study is sufficient to allow oral contamination.

Barbosa et al. (2012) reported that parasites were not visible in açai juice during microscopic inspection due to its characteristic dark colour, which is because of its high organic matter and anthocyanin contents. The triple centrifugation methodology employed in this work efficiently recovered the parasite in inoculated açai juice, allowing microscopic visualization of all samples. The methodology is more efficient and has been able to solve this problem. After the triple centrifugation the parasites were washed twice with Tris-HCl buffer prior to DNA and mRNA extraction. The DNA and mRNA extraction directly from the samples without washing with buffer did not produce amplification.

Up to now the survival of *T. cruzi* in various fruit and vegetable

Table 3
Survival and viability of the *Trypanosoma cruzi* Y, 370 and 425 strains after thermal treatments.

Temperature/time	45 °C/10 min			45 °C/60 min			70 °C/10 s		
Strains	Y	370	425	Y	370	425	Y	425	370
VB	−	+	+	−	−	+	−	−	−
ST (10^5par/mL)	−	0.21 ± 0.32	0.34 ± 0.13	−	−	+NA	−	−	−

T. cruzi active (+); *T. cruzi* no active (+ NA); negative (−); survival test (ST); and viability (VB).

juices has been investigated in laboratory studies. The results indicated that for most (73%) of these fresh products, parasite survival ranged from 6 to 72 h, with the largest numbers of live parasites detected up to 18 h after inoculation (Pinto et al., 1990; Cardoso et al., 2006; Añez and Crisante, 2008; Suárez et al., 2012). However, on acidic fruits with a pH of approximately 3, such as pineapple, survival was not observed (Añez et al., 2009). Although the ideal pH for the parasite is approximately 7.2, however, Toso et al. (2011) showed that they can crosses through the gastric barrier, tolerating low pH values. In our study, the parasite survived in açai with a pH of 4.74 ± 0.11. Therefore, the elimination of the parasite in fruits such as pineapple may be related to the activity of proteases that could hydrolyse *T. cruzi*, as they do bacteria, and not due to acidic pH.

The survival of *T. cruzi* in açai juice has been demonstrated after 144 h in a sample inoculated with the Y strain maintained under refrigeration at 4 °C, after 26 h of freezing (−20 °C), and after 24 h at room temperature (Barbosa et al., 2012). In addition, the in vivo infective capacity and virulence of açai juice contaminated with the trypomastigote form of *T. cruzi* isolated from *R. pictipes* of the Amazon region was confirmed, which reinforces the attribution of Chagas disease outbreaks by oral transmission (Barbosa et al., 2018). Therefore, we concluded that açai provides pH, osmosis and minimal ionic strength conditions that allow for *T. cruzi* survival. The ability of *T. cruzi* to survive in food is considered a risk for infection in humans.

4.2. Chemical and thermal treatment in physiological saline solution

To simulate açai post-harvest conditions, the inoculum was subjected to heat treatment according to the softening (45 °C for 10 and 60 min) and blanching (70 °C for 10 s) steps in the production chain, according to Bichara and Rogez (2011). Prior to the depulping process, the fruits are immersed in water to soften their epicarp and mesocarp (variables in this process are water temperature and immersion time). The water used is usually at room temperature or from 30 to 45 °C. The softening time varies from 10 to 60 min and is shorter for ripe fruits. Blanching, a thermal process in which the fruits are plunged into water at 80 °C for 10 s, is recommended to reduce the microbial load and avoid enzymatic browning caused by enzymes such as polyphenoloxidase through the elimination of oxygen (Rogez and Aguiar, 2012). The blanching temperature tested was 70 °C and not 80 °C, as recommended to allow a safety margin, as the temperature is not strictly measured for small equipment given the lack of appropriate infrastructure in some traditional shops and residences in Amazonian cities.

Barbosa et al. (2016) reported that heating açai juice above 43 °C for 20 min is a practical and effective measure to eliminate *T. cruzi* and prevent Chagas disease. However, in contrast to widespread literature reports about *T. cruzi*, viable and live parasites of the Amazonian strains 370 and 425 were observed after incubation at 45 ± 1 °C for 10 min and 60 min, respectively. This fact reinforces the possibility of oral transmission and explains the large number of cases related to this pathway. When strain 425 was subjected to treatment at 45 ± 1 °C for 60 min, parasites were still observed. However, these parasites showed no activity or flagellar movement. Therefore, it was not possible to confirm their survival by microscopic visualization. The viability of the parasite was instead confirmed by growth in culture medium and the detection of mRNA by RT-PCR amplification.

Considering the increased number of Chagas disease cases by oral transmission, methods for the detection for *T. cruzi* DNA in food have been developed. Godoi et al. (2017) and Mattos et al. (2017) optimized a real-time PCR technique to detect DNA from *T. cruzi* in contaminated açai juice. Ferreira et al. (2016) compared the quantity and quality of *T. cruzi* DNA isolated by four different methods from açai that was artificially contaminated with *T. cruzi* cells. To overcome potential false-positive viability results via DNA-based PCR amplification assays, a method detecting *T. cruzi* based on the RT-PCR amplification of cDNA was developed in this study. The ability of an RT-PCR assay to distinguish between viable and non-viable *T. cruzi* cells was examined and compared with PCR assays of genomic DNA extracted from the samples.

The DNA from non-viable parasites was quite stable and capable of being amplified for hours following the loss of viability. In contrast, mRNA from dead cells could not be amplified by RT-PCR at any time following the treatment due to the rapid degradation of mRNA after the loss of cell viability. Thus, the RT-PCR amplification can provide an indicator of cell viability, whereas the detection of gene sequences by DNA-based PCR amplification cannot (Postollec et al., 2011; Filion, 2012). The identification of viable and non-viable pathogens has been determined by the RT-PCR amplification of mRNA in foods previously (Vaitilingom et al., 1998). The authors observed that when all cells had died, the RT-PCR detection was negative. Supporting our results and validating our methodology.

We were unable to detect the amplification of *T. cruzi* mRNA by RT-PCR after exposing to 200 ppm sodium hypochlorite or to 70 °C for 10 s or in açai juice pasteurized after the experimental inoculation. This result indicates the sensitivity of mRNA to rapid degradation as well as the close association between cellular mRNA and cell viability. Additionally, traditional parasite detection methods by culture are labour and time-intensive, in many cases requiring four weeks to complete (Pereira et al., 2012). In contrast, the RT-PCR method developed required only one day to complete. To our knowledge, this work is the first report of a method for the detection of viable *T. cruzi* based on the RT-PCR amplification of mRNA. Other method for detecting the *T. cruzi* viability in LIT medium by the use of propidium monoazide as a sample pretreatment have been investigated (Cancino-Faure et al., 2016). However, this method failed when a low concentration of live parasites are combined with a high concentration of dead parasites. Moreover, the solids content and turbidity of food samples could adversely affect the DNA-based viability assay results (Liang and Keeley, 2012). The information available to date on the viability of *T. cruzi* has mainly been related to reference strains, such as the Y strain. Little is known about lineages from regions where oral transmission is endemic. Thus, the *T. cruzi* I (425) and *T. cruzi* TcIII (370) strains were selected for study due to the occurrence of orally transmitted cases related to both genotypes in the Latin America. Interestingly, strain 425, isolated from the Amazon region, exhibited peculiar behaviour during reactivation in culture after inoculation in açai juice and adverse heat treatment.

According to Mello et al. (1980), the in vitro behaviour in LIT medium of *T. cruzi* strains isolated from reservoir hosts or human cases varied as a function of the strain studied. Some strains have numerous agglomerates of amastigote forms in young cultures, which progressively decrease in successive sub-cultures to epimastigote and trypomastigote forms. In strains of human origin, such as the Y strain, the epimastigote forms or rosettes predominate. The presence or absence of amastigotes is directly related to the intrinsic characteristics of each strain. In many trypanosomes, temperature and chemicals are known to induce cyclic differentiation. However, the underlying molecular mechanisms causing this process are not yet understood (Hashimoto et al., 2015). More recently, Kessler et al. (2017) showed that several forms exist in the three intestinal segments of the vectors. The elucidation of the presence this form contributes to understanding why *T. cruzi* oral infection, through the ingestion of food containing these parasites (crushed during juice preparation), usually presents a more intense acute phase.

Experiments with blanching at 70 °C for 10 s and pasteurization at 82.5 °C for 1 min were performed on açai fruits and juice, respectively. The results were positive for PCR and negative for the survival test, viability in culture test and RT-PCR for both açai fruits and juice, confirming the efficiency of the treatments to eliminate *T. cruzi* in açai.

The effective control of *T. cruzi* in food products occurs by preventing triatomine contact and contamination at all steps along the productive chain. This goal can be better achieved via the implementation of good manufacturing practices (GMP) and hazard

analysis critical control points (HACCP). The prevention procedures include GMP during picking as well as surfaces in contact with the fruits during storage, transportation and processing (Aguiar et al., 2013; Pereira et al., 2012). According to the WHO A63.20 resolution "Chagas disease: control and elimination", the main objectives and strategies for the next ten years are the implementation of measures to reduce the risk of oral transmission through good hygiene practices at the domicile level and good manufacturing practices in the food industry (WHO, 2010).

Appropriate prevention technologies to eliminate contamination include inactivation by washing in a disinfectant solution, blanching and pasteurization, which can eliminate the possibility of infectious parasitic ingestion. The application of sodium hypochlorite at 200 ppm for > 5 min may potentially be used for preventive disinfection purposes. According to the FAO/WHO (2008), the concentration of active chlorine used for the disinfection of fruits and vegetables ranges from 25 to 200 ppm with a contact time of < 2 min. For periods longer than 3 min, water tends to penetrate the fruit, affecting its sensorial quality. Rogez et al. (2012) showed that the pasteurization presents a good overall shelf-life acceptability for açai juice over 5 months. Therefore, we recommend the blanching (70 ± 1 °C for 10 s) of fruits before juice preparation and pasteurization (82.5 °C for 1 min) of the juice for the preventive disinfection and effective inactivation of *T. cruzi* in foods.

5. Conclusion

The results from this work provide a better understanding of the behaviours of Amazonian *T. cruzi* strains in relation to optimized prevention technologies for orally transmitted Chagas disease. The Amazonian strains *T. cruzi* I (425) and *T. cruzi* III (370) showed higher resistance to sodium hypochlorite treatment and heat temperature than did the reference strain Y. Appropriate prevention technologies to eliminate the possibility of the ingestion of infectious parasites include the application of GMP and HACCP with inactivation by blanching fruits (70 ± 1 °C for 10 s) or pasteurizing juice (82.5 °C for 1 min). Additionally, we develop a mRNA-based RT-PCR amplification method for the detection of viable *T. cruzi* in foods. The RT-PCR assay requires less time than traditional, culture-based testing methods for the *T. cruzi* viability study. Thus, this work contributes to the improvement of critical control points in food processing and to the assurance of food safety. Besides may aid in implementing preventive actions to prevent the occurrence of new acute cases by oral transmission.

Conflicts of interest

The authors have no conflicts of interest to declare.

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