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Ready-to-use qPCR for detection of *Cyclospora cayetanensis* or *Trypanosoma cruzi* in food matrices

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ABSTRACT

Foodborne outbreaks caused by parasites have long been a public health issue. Among the available contamination detection methods, qPCR is one of the most sensitive and specific. However, it can be cumbersome and error-prone, if used by unexperienced users. Moreover, qPCR reagents usually require freezer temperatures for transportation and storage. We present a gelified reaction format that allows the reagents to be stored at 2–8 °C for up to 90 days without losing performance. The gelification process eliminates most operator mistakes during reaction setup, and renders the qPCR plates ready-to-use. The new reaction makeup was evaluated using artificially contaminated samples of distinct food matrices for sensitivity, specificity, repeatability, reproducibility, and stability. Samples consisted of cilantro leaves and raspberry fruits spiked with *Cyclospora cayetanensis* oocysts, as well as açai pulp and sugarcane juice tainted with *Trypanosoma cruzi* trypomastigotes. No significant difference between the gelified and the non-gelified qPCR was found. Our results suggest that gelifying the assay may help to achieve more reproducible qPCR data across laboratories, thus supporting surveillance actions.
(170 words)

1. Introduction

Various outbreaks of foodborne illness associated with protozoan parasitic contamination of fresh produce have been reported in recent years. *Cyclospora cayetanensis* has been linked to outbreaks associated with consumption of cilantro, leafy greens, and berries (Caradonna et al., 2017; Lalonde and Gajadhar, 2016; Murphy et al., 2017). In recent years the number of cases in

Abbreviations: MGB, minor groove binding; GE, genome equivalents; LoD, limit of detection; C_p, quantification cycle.

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outbreaks of C. cayetanensis infections have significantly increased in the US. In 2018 and 2019 the number of domestically acquired cases were 2299 and 2408, respectively (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/index.html). This represented a significant increase considering that a total of 2207 had been reported through a period of 5 years from 2011 to 2015 (Casillas et al., 2019). Foodborne outbreaks of Chagas disease have been reported throughout Latin America, especially in the Brazilian Amazon region (Franco-Paredes et al., 2020). In Brazil, these outbreaks have been associated mainly with consumption of açai pulp, sugarcane juice, and babaçu wine (Ferreira et al., 2018; Franco-Paredes et al., 2020; de Mattos et al., 2017). In Venezuela, outbreaks were associated with the consumption of artisan guava juice and yucca with dips, or were clinically consistent with orally-transmitted disease (Alarcón de Noya et al., 2016; Anez et al., 2016; de Noya et al., 2017). In Colombia, acute Chagas disease has been mostly associated with consumption of palm wine or orange juice and the presence of infected opossums in the vicinity of the outbreak areas (Hernández et al., 2016; Rueda et al., 2014). Cases have also been reported in Bolivia, Ecuador, and French Guyana (Franco-Paredes et al., 2020). Indeed, FAO/WHO ranks *T. cruzi* and *C. cayetanensis* as the 10th and 13th most prevalent foodborne infections (FAO, World Health Organization, 2014). The presence of *Toxoplasma gondii* in water and meat samples is well documented (Guo et al., 2015), and detection of *T. gondii* oocysts in leafy greens has also been reported (Lass et al., 2012). Similar situations occur for *Cryptosporidium* spp., which are regularly detected in routine produce surveys and have been linked to disease outbreaks (Caradonna et al., 2017; Dixon et al., 2013; Lalonde and Gajadhar, 2016).

The detection of these parasites in implicated fresh produce is very challenging for a number of reasons. These include but are not limited to the relative low abundance of parasites per gram of produce, the short shelf life of the produce which limits the ability to investigate it, the relative slow growth rate of parasites in most common detection methods (culturing), as well as the difficulty in determining a clear history of the patient's food exposure to pinpoint the original food source of the infection. Although identification of *Cyclospora* sp. oocysts or *T. cruzi* life forms can be routinely performed by optical microscopy, limitations prevent such simple procedures to be used for detection of the parasites in foods. Limitations include the low level of sensitivity that is required for the unequivocal detection of such parasites in food matrices, the dependency of the results on experience of well-trained microscopists, and the low processivity of the technique (number of samples that can be analyzed by one operator in a given time). In addition, although *T. cruzi* infective forms can be identified by microscopy methods (de Souza and Attias, 2018), *C. cayetanensis* oocysts are difficult to be morphologically distinguished from other species that are known to infect humans (Eberhard et al., 1999; Li et al., 2015).

In the last years, even though optical microscopy examination as well as culturing remain the most common methods to identify parasites in food samples, molecular protocols such as genomic sequencing, numerous microarray designs and procedures, and quantitative polymerase chain reaction technique (qPCR) have greatly improved the identification of parasites at the species level (Umesha and Manukumar, 2018; Zeng et al., 2016). Among the molecular methods, qPCR is the most commonly used technique in foodborne pathogen detection protocols. The popularity derives mainly of its specificity and sensitivity due to the detection and amplification of specific genomic sequences, but also due to it relatively lower costs in comparison to the other molecular methods.

However, most commercially available qPCR reagents are thermolabile, thus requiring transportation and storage at temperatures around −20 °C. Before use, reagents must be thawed and mixed in a specific recipe, and then loaded into each well of a 96-well plate. The process of combining the reagents and loading onto the well plate is long, cumbersome, and error-prone, and even the most experienced user will eventually make a mistake in one of these steps. Technologies such as lyophilization (Arbefeville et al., 2014; Kamau et al., 2014) or gelification (Iglesias et al., 2014; Rampazzo et al., 2019; Rampazzo et al., 2017; Rosado et al., 2002; Sun et al., 2013) of qPCR reagents preloaded onto the reaction vessel have been shown to exhibit similar performance to their traditional frozen storage format, while allowing for faster plate setup as well as transportation and storage at higher temperatures (refrigerator or room temperature, for example). Pre-loading the reagents onto the reaction vessel also allows batch production of reactions, producing greater repeatability and reproducibility of results. Batch production, in turn, allows for reaction quality control and results in robust and comparable data across multiple operators. Altogether, these features increase the reliability of the qPCR technique by eliminating operator errors in crucial steps, and eliminate the requirements for freezing temperatures (Rampazzo et al., 2019; Rampazzo et al., 2017).

In the present work, we developed two ready-to-use duplex qPCR reactions: one for detection of *Trypanosoma cruzi* in plant-based samples, and the second for *Cyclospora cayetanensis* detection in distinct produce matrices. Reactions for *T. cruzi* detection were evaluated using DNA extracted from açai berry pulp or sugarcane juice artificially seeded with *T. cruzi* at the trypomastigote stage. Sensitivity, repeatability, and reproducibility of the *T. cruzi* duplex ready-to-use reaction were evaluated. Reactions for *C. cayetanensis* detection were evaluated using raspberry and cilantro samples seeded with *C. cayetanensis* oocysts. A small evaluation study was carried out to assess the performance of the gelified ready-to-use reaction format for the detection of *C. cayetanensis* in raspberries and cilantro samples. Stability studies of the *T. cruzi* and the *C. cayetanensis* gelified qPCR plates showed unchanged performance for at least 90 days. Our results support the possibility of using gelification of qPCR reagents as a ready-to-use tool for the detection of parasite's DNA signatures in food samples.

2. Materials and methods

2.1. C. cayetanensis-positive food samples

For the evaluation of the gelified *C. cayetanenis* assay, DNA was extracted from 14 raspberry and 20 cilantro samples seeded with different concentrations of *C. cayetanensis* oocysts (Murphy et al., 2017). Oocysts were originally recovered from human

stools, counted on a hemocytometer, and used to spike the food matrices within a short time frame of the preparation, according to protocols described in extensive details elsewhere (Murphy et al., 2017). DNA was extracted from 25 g of cilantro leaves or 50 g of raspberry fruits using the FastDNA SPIN kit for Soil in conjunction with a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, USA) and suspended in 75 μL of nuclease-free water, as previously described (Murphy et al., 2017). DNA was also extracted from non-spiked cilantro or raspberry samples, using the same amount of sample (25 or 50 g, respectively). DNA concentrations and quality were estimated by measuring the absorbance at 260 nm (A260), 280 nm (A280) and 230 nm (A230) using the Nanodrop 2000 spectrophotometer (Waltham, USA). The average concentration of the extracted DNA of the non-spiked samples was 75.4 ± 10.5 ng/μL. For the PCR-positive control standards and reaction development, a double strand 998-bp gene fragment consisting of the *C. cayetanensis* 18S rRNA gene (AF111183) containing two nucleotide substitutions was synthesized and diluted in Tris-EDTA buffer (Supplemental Table 1). This positive control molecule, Hm135m, was used to calculate the reaction's efficiency and dynamic range (Murphy et al., 2017). In all reactions targeting *C. cayetanensis*, an exogenous synthetic internal amplification control ("IAC Cyclo", described in (Murphy et al., 2017)) was used in a final concentration of 10^4 molecules/μL in the qPCR reaction.

2.2. T. cruzi-positive food samples

Metacyclic trypomastigotes were obtained as described in details elsewhere (de Mattos et al., 2017). In brief, *T. cruzi* (Y strain) trypomastigotes were grown and maintained in MK2 cells at 37 °C, 5% CO2, in RPMI medium containing 2.2 g/L sodium bicarbonate, 5 mg/mL gentamicin 10% fetal bovine serum at the Centro de Parasitologia e Micologia (Instituto Adolfo Lutz, SP, Brazil). Parasites were collected from supernatants of cell cultures after 4–5 days and suspended in phosphate-buffered saline pH 7.2 after being buffer-washed and counted in a Neubauer chamber. A stock solution of 1×10^7 trypomastigotes was used to prepare the spiking experiments. Açai pulp and sugarcane juice were purchased from local stores (açai pulp) or street vendors (sugarcane juice) in Para State (northern Brazil) or in São Paulo State (southeastern Brazil), respectively. To produce the standard curves, aliquots of açai pulp or sugarcane juice (50 g each) were artificially contaminated with different amounts of *T. cruzi* trypomastigotes (1×10^5, 1×10^4, 1×10^3, 1×10^2 and 10 trypomastigotes each), centrifuged and washed with PBS. DNA extraction is described in detail elsewhere (de Mattos et al., 2017). In a different set of experiments, purified DNA from *T. cruzi* trypomastigotes (5×10^1 to 10^2 GE/reaction) was diluted in DNA extracted from açai pulp. DNA extraction was performed with the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) or the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) using 500 μL of each sample (sediment or supernatant) and 50 μL of final eluate volume. DNA was also extracted from non-spiked açai pulp or sugarcane juice, using the same amount of sample (50 g). DNA concentrations and quality were estimated by measuring the absorbance at 260 nm (A260), 280 nm (A280) and 230 nm (A230) using the Nanodrop 2000 spectrophotometer (Waltham, USA). The average concentration of the extracted DNA of the non-spiked samples was 62.3 ± 12.9 ng/μL, similar to the concentration reported elsewhere (Ferreira et al., 2018). A synthetic positive control (IDT, Coralville, USA) consisting of a double-strand DNA containing one copy of the genomic target for *T. cruzi* as well as one copy of the genomic target for *Euterpe oleracea* (açai) was used to evaluate the effect of gelification on the repeatability and reproducibility of the duplex qPCR (Supplemental Table 1).

2.3. Specificity controls

The specificity of the ready-to-use qPCR for detection of *C. cayetanensis* was assessed by testing DNA extracted from the following microorganisms: *Trypanosoma cruzi* trypomastigotes (10^7 cells/mL), *Trypanosoma rangeli* (10^7 cells/mL), *Leishmania amazonensis* (10^7 cells/mL), *Leishmania braziliensis* (10^7 cells/mL), *Leishmania guyanensis* (10^7 cells/mL), *Leishmania infantum* (10^7 cells/mL), *Plasmodium falciparum* (17,000 cells/mL), *Plasmodium vivax*, *Escherichia coli* (89 ng/μL), *Toxoplasma gondii* (177 ng/μL), *Eimeria tenella* (approximately 725 oocysts/μL; i.e. 145,000 total oocysts in 200 μL of sample), *Eimeria acervulina* (approximately 1450 oocysts/μL; i.e. 290,000 total oocysts in 200 μL of sample), *Eimeria faecalis*, *Eimeria cloaceae*, *Eimeria faecium*. Special emphasis was given to DNA from *Eimeria* spp. due to the close phylogenetic relationship between *Cyclospora* and *Eimeria* at the level of the 18sRNA gene. Negative results were validated with parallel reactions using specific oligonucleotides for each target organism.

2.4. Gelification protocol

Gelification of the reagents was performed by adding the gelification solution in substitution for water in all qPCR reactions, according to published protocols (Iglesias et al., 2014; Rampazzo et al., 2019; Rampazzo et al., 2017; Sun et al., 2013). There are several options for the composition of gelification solution, and each must be evaluated against pre-determined reaction parameters (Rosado et al., 2002). The gelification solution used in the present study consisted of 1 volume of melezitose (400 mg/mL), 1 volume of lysine (0.75 mg/mL), 1 volume of glycogen (200 mg/mL), and 2 volumes of trehalose (400 mg/mL). The gelification solution was kept in the dark, under controlled temperature (2–8 °C), for up to three months. Each target-specific qPCR mix containing the gelification solution was manually aliquoted to each reaction well, and the ABI7500 regular or FAST® plastic 96-well plates were submitted to vacuum (30 ± 5 mbar) under controlled temperature (30 ± 1 °C) to gelify the reagents. For the long-term storage stability experiments, reaction vessels with gelified reactions were stored in refrigerator (2–8 °C) for up to 90 days and were evaluated every fifteen days. For all other experiments, gelified reactions were stored at

2–8 °C for a maximum of 12–48 h. Strips for qPCR (8- or 12- tubes) were also evaluated as storage vessel for the gelified reaction with identical results to the 96-well plates.

2.5. qPCR protocols

Two separate reactions were used for the detection of *T. cruzi* and *C. cayetanensis*. Detection of *T. cruzi* DNA was performed using the Universal PCR Mastermix (IBMP – Curitiba, Brazil) supplemented with 8 mM $MgCl_2$ and 0.2 mg/mL BSA in a final volume of 50 μL, using 20 μL of extracted DNA as sample volume. For the *T. cruzi* internal control assay we used primers and probes designed for the detection of conserved genes in sugarcane and acai genomes. Detection of açai or sugarcane DNA was performed simultaneously with the detection of *T. cruzi* DNA in a qPCR duplex reaction. Reactions were performed on the ABI7500 Standard instrument (Thermo Fisher Scientific – Waltham, USA), with the following cycling conditions: 50 °C/2 min, 95 °C/10 min, and 45 cycles of 95 °C/15 s and 60 °C/1 min (fluorescence detection at 60 °C). Detection of *C. cayetanensis* DNA was performed using the Multiplex PCR Mastermix (IBMP – Curitiba, Brazil) containing 9 mM magnesium acetate and 2 mg/mL BSA in a final volume of 50 μL, using 20 μL of sample volume. Detection of the *C. cayetanensis* target and a synthetic internal amplification control were performed in duplex format in the same reaction (Murphy et al., 2017). Reactions were performed on the ABI7500 FAST® instrument (Thermo Fisher Scientific – Waltham, USA), with the following cycling conditions: 95 °C/5 min, and 40 cycles of 95 °C/30 s and 68 °C/30 s (fluorescence detection at 68 °C). All baseline levels were set to automatic and thresholds were defined as follows: for *T. cruzi* DNA, 0.25; for açai or sugarcane DNA, 0.05; for *C. cayetanensis* DNA, 0.35; and for IAC Cyclo, 0.35. Sequences and final reaction concentration of all oligonucleotides used in the present study are described in Table 1. Each reaction is species-specific, with robust analytical and validation studies supporting the choice of each genomic target for the detection of *T. cruzi* or *C. cayetanensis* DNA (Murphy et al., 2017; Ramírez et al., 2015). Probes with a BHQ quencher were purchased from IDT (Coralville, IA, USA), while probes with a MGB quencher were purchase from IDT or Thermo Fisher (USA). DNA extracted from non-spiked açai pulp, sugarcane juice, cilantro leaves or raspberry fruits, as well as nuclease-free water were used as negative controls in the corresponding qPCR runs.

2.6. Statistical analysis

All qPCR reactions were performed in triplicate, at a minimum. For the two lowermost concentrations of each target (*T. cruzi* DNA or synthetic templates), 8–12 replicates were performed. Measured C_p values were analyzed using custom scripts in R (v. 3.5.1). The Limit of Detection at 95% tolerance interval ($LoD_{95\%}$) or at 75% tolerance interval ($LoD_{75\%}$) were calculated by fitting a Probit model to the pooled data obtained from the stability study. Inter-assay (reproducibility) and intra-assay (repeatability) variances were calculated using the percentage of the coefficient of variation of the C_p values as a proxy for robustness of the method because the reported method is qualitative and its interpretation is based on C_p values. The Kappa coefficient was calculated between the duplex qPCR in the ready-to-use (this work) and in the non-gelified formats (published by (Murphy et al., 2017)). The coefficient was used to test agreement between the methods, and Kappa results were interpreted according to (Landis and Koch, 1977): 1.00–0.81 almost perfect, 0.80–0.61 substantial, 0.60–0.41 moderate, 0.40–0.21 fair and ≤ 0.20 slight agreement. Additionally, a Bland-Altman (Bland and Altman, 1986; Giavarina, 2015) analysis was used to evaluate the existence of any bias between data obtained with the gelified, ready-to-use reaction format versus the regular, freezer-stored reaction format previously published. Bland-Altman analyses quantify the agreement between two quantitative measurements (each reagent storage format, in the case of this study) by determining the mean differences for specific samples between each condition and constructing limits of agreement within which 95% of the differences between conditions fall when analyzing a given sample. Bland-Altman analyses were performed on data for cilantro and raspberry sample classifications (positive or negative) based on detection of *C. cayetanensis* DNA by each qPCR (this study and (Murphy et al., 2017)). It should be emphasized that acceptable limits of agreement must be defined a priori, based on the clinical relevance or biological characteristics of the samples and/or techniques under evaluation. In the present study, a bias of ±3.33 would correspond to a 10-fold variation in target detection because of the technique used (qPCR). All reported biases should be analyzed with this correspondence in mind.

3. Results

3.1. Evaluation of endogenous controls for açai or sugarcane samples in a ready-to-use duplex qPCR

As a first step towards the development of a ready-to-use qPCR for screening food matrices, we developed a reliable internal control for reactions containing açai or sugarcane genetic material. Four sets of oligonucleotides (*Cob1*, *Cob2*, *RbcL2*, and *RbcL3*) were evaluated for robust detection of açai or sugarcane DNA as well as for lowest interference on the detection of *T. cruzi* DNA. The concentration of each oligonucleotide used in the PCR is shown in Table I.

Detection of açai pulp DNA as well as *T. cruzi* DNA using the ready-to-use duplex qPCR is shown in Fig. 1A. The upper panel shows that all four sets of oligonucleotides display robust performance for detection of açai DNA (gray lines). *Cob1* showed a C_p of 23.77 ± 0.37, *Cob2* of 23.47 ± 0.24, *RbcL2* of 19.63 ± 0.09, and *RbcL3* of 20.59 ± 0.15. The data clearly show that (i) *RbcL2* and *RbcL3* gave lower C_p values with the açai pulp target than *Cob1* and *Cob2*, and (ii) *RbcL2* and *RbcL3* displayed a smaller deviation of the mean. Fig. 1 middle panel shows the concomitant detection of *T. cruzi* DNA within the same qPCR reaction. There was no difference in the detection of the higher concentrations of parasite's DNA. At the lower end, only *Cob2* and *RbcL2* were able to

Table 1
Sequence, genomic target, concentration and reference of the oligonucleotides used in this study. Genbank number is shown for each genomic target. Primers were purified by reverse phase chromatography and probes by HPLC.

Name	Genomic target (GenBank no.)	Sequence (5′-3′)	Concentration	Tm (°C)	Reference
Cob1_F	Apocytochrome b (DQ916690.1)	ACGTTATGAGAGATGTTGAAGG	100 nM	51.8	This study
Cob1_R		CGCATGATATAGACCACGAAA	100 nM	52.0	
Cob1_P		HEX- GAACCACAATGAGAAACATACTTGCCC-BHQ1	50 nM	57.6	
Cob2_F	Apocytochrome b (DQ916690.1)	CCTCATGTGGATCTAGCTTTC	100 nM	51.9	This study
Cob2_R		CCACAATGAGAAACATACTTGC	100 nM	51.9	
Cob2_P		HEX- TAGCATGCATATAACGGAGCAACCAGC-BHQ1	50 nM	60.3	
RbcL2_F	Ribulose biphosphate carboxylase Large	CAGAGAATTGGGAGTTCCTATC	100 nM	51.6	This study
RbcL2_R	chain 2 (JQ626233.1)	GTGAAGAAGTAGGCCGTTATC	100 nM	52.2	
RbcL2_P		HEX- AGCCAAGCTAGTATTTGCAGTGAATCCC-BHQ1	50 nM	59.9	
RbcL3_F	Ribulose biphosphate carboxylase Large	CATTCCGAGTAACTCCTCAAC	100 nM	52.2	This study
RbcL3_R	chain 3 (AJ404802.1)	CAAGACTGGTAAGTCCATCAG	100 nM	51.7	
RbcL3_P		HEX- CCATGTACCAGTAGAAGATTCGGCAGC-BHQ1	50 nM	60.0	
cruzi1	*T. cruzi* satellite DNA (AY520036)	ASTCGGCTGATCGTTTTCGA	750 nM	58.7	(Ramírez et al.,
cruzi2		AATTCCTCCAAGCAGCGGATA	750 nM	58.1	2015)
cruzi3		FAM- CACACACTGGACACCAA-NFQ-MGB	100 nM	52.2	
Cyclo250F	18S rRNA (CSU40261) and synthetic positive	TAGTAACCGAACGGATCGCATT	750 nM	58.3	(Murphy et al.,
Cyclo350RN	control Hm135m	AATGCCACGGTAGGCCAATA	750 nM	58.3	2017)
Cyclo281T		FAM-CCGGCGATAGATCATTCAAGTTTCTGACC-BHQ1	250 nM	61.8	
dd_IAC_F	Cyclospora synthetic internal amplification	CTAACCTTCGTGATGAGCAATCG	100 nM	55.6	(Murphy et al.,
dd_IAC_R	control target	GATCAGCTACGTGAGGTCCTAC	100 nM	55.6	2017)
dd_IAC_Cy5		Cy5- AGCTAGTCGATGCACTCCAGTCCTCCT-BHQ2	200 nM	64.3	

detect the most diluted samples tested (5×10^{-3} genome equivalents/reaction), even though these detections were out of the linear range (open symbols, Fig. 1B). This lower limit of detection is similar to the values reported in the literature for detection of *T. cruzi* DNA in food (de Mattos et al., 2017) or human samples (Ramírez et al., 2015). However, the amplification efficiency for *T. cruzi* DNA detection in reactions containing oligonucleotides for the target *Cob2* was better when compared to reactions containing oligonucleotides for the target *RbcL2*. *T. cruzi* DNA was detected with a R^2 of 0.995 and efficiency of 95.0% versus a R^2 of 0.997 and efficiency of 86.8% for duplex reactions containing *Cob2* and *RbcL2*, respectively. Therefore, we chose to use *Cob2* for the remainder of this study.

A linear regression curve for the detection dynamic range of purified DNA from *T. cruzi* trypomastigotes (5×10^1 to 10^2 GE/reaction) diluted in DNA extracted from açai pulp, obtained using a duplex qPCR consisting of the *T. cruzi* and the *Cob2* set of oligonucleotides (black dots), is shown in Fig. 2A. It should be noted that, although the determined $LOD_{95\%}$ was of 0.168 GE/reaction, the duplex reaction was able to detect *T. cruzi* DNA amounts as low as 5×10^{-3} GE/reaction (when using 20 μL per reaction), even though these detections are out of the linear range. Fig. 2B shows that the gelified duplex qPCR also detects DNA from *T. cruzi* trypomastigotes cells (10^1 to 10^5 cells/mL) diluted in sugarcane juice (gray triangles and squares) or açai pulp (black triangles and squares) with similar efficiency and with a detection limit of 10 cells/mL. Our results agrees with previous data (de Mattos et al., 2017) showing that detection of *T. cruzi* DNA is more efficient in the sediments of a 2500 *g*/10 min centrifugation of an açai pulp suspension in comparison with the supernatant (squares versus triangles in Fig. 2B).

3.2. Evaluation of an exogenous synthetic control for raspberry or cilantro samples in a ready-to-use duplex qPCR

The same ready-to-use qPCR technology can be used to detect the synthetic control molecule HM135m when diluted in DNA extracted from fresh raspberry (black dots) or cilantro (gray triangles (Fig. 3).. To allow a direct comparison with published data (Murphy et al., 2017), a Probit analysis was used to calculate the LoD in which 75% of the samples were positive. The $LoD_{75\%}$ was determined to be 11.2 and 17.2 copies/reaction for cilantro and raspberry, respectively, which is in agreement with previously published data obtained using the non-gelified format reactions (Murphy et al., 2017). The $LoD_{95\%}$ was also calculated and it was determined to be 17.54 and 37.72 copies/reaction for cilantro and raspberry, respectively (Supplemental Fig. 1).

A pilot study to evaluate the detection of *C. cayetanensis* using the ready-to-use qPCR was performed using DNA extracted from 20 cilantro and 14 raspberry samples which were used in a previous study (Murphy et al., 2017). Table 3 shows that the ready-to-use reaction exhibited a similar performance as the non-gelified qPCR, correctly identifying 91.2% of the samples. No false-positives were observed: all nine negative samples (5 for cilantro and 4 for raspberry) were found to be negative by the gelified qPCR. Most of the discrepant results were at the lower oocyst concentrations (5 and 10 oocysts/μL), with 3 wrong classifications out of 16 determinations: the gelified qPCR identified as negative two 5 oocysts/μL samples and one 10 oocysts/μL sample. For the highest concentration evaluated (200 oocysts/μL), the ready-to-use reaction correctly identified all samples (Table 2). Considering all samples, Kappa coefficient analysis found a substantial agreement between the qPCR assay formats, with 0.72 ($p < 0.001$) and 0.73 ($p < 0.005$) for cilantro and raspberry samples, respectively. Considering only the 5 and 10 oocysts/μL samples, the Kappa coefficient analysis found a moderate agreement between the qPCR assay formats, with 0.54 ($p < 0.005$) for both

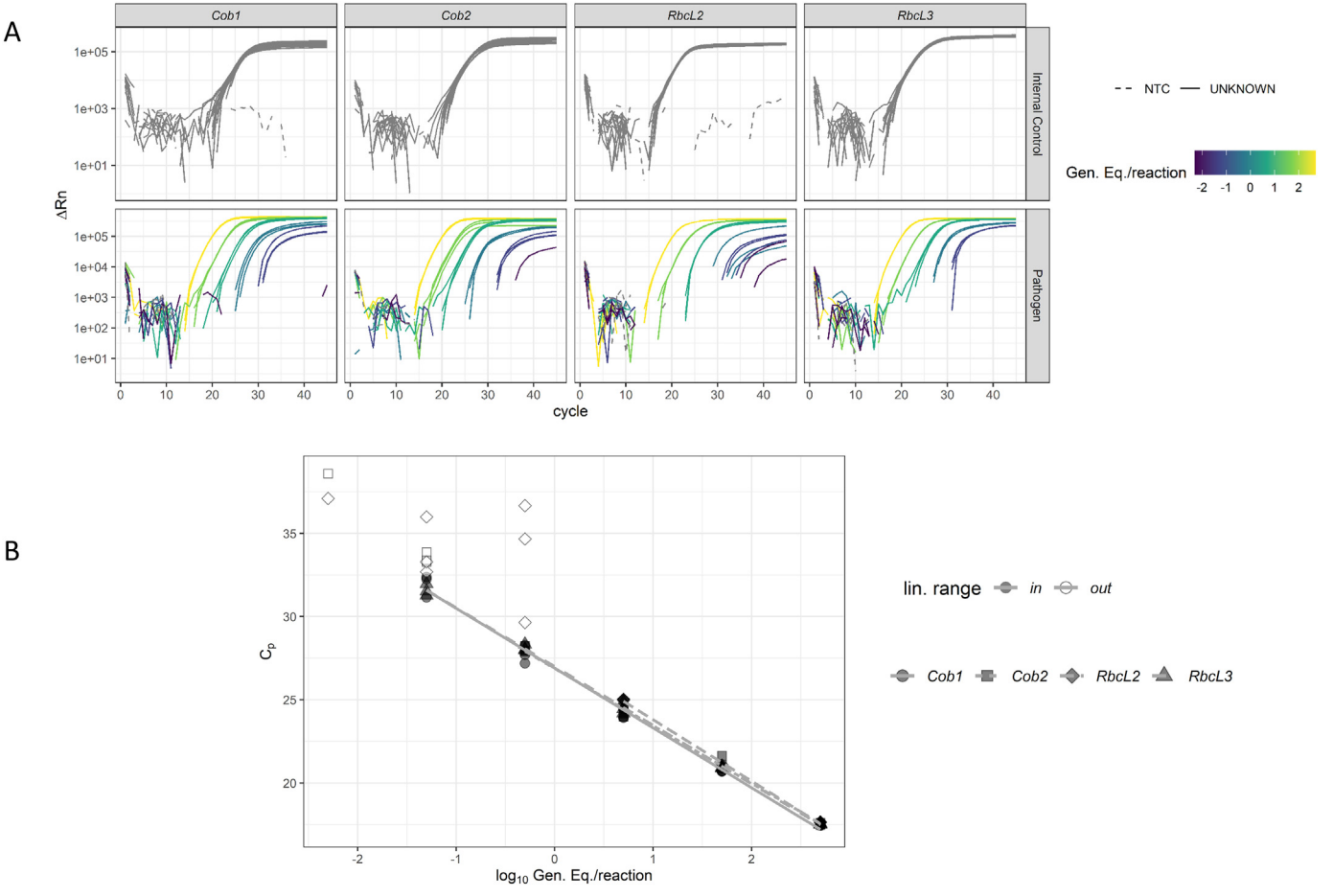

Fig. 1. Selection of an endogenous reaction target for açai DNA concomitantly to *T. cruzi* DNA detection. Panel A: Representative curves for the detection of açai pulp DNA (upper panel) and *T. cruzi* DNA (lower panel). Four sets of oligonucleotides (*Cob1*, *Cob2*, *RbcL2*, and *RbcL3*) were evaluated for robustness in the detection of açai DNA and least interference with the detection of different concentrations of *T. cruzi* DNA (5×10^{-2} to 5×10^{-3} GE/reaction). Curves are representative of at least two independent experiments. Panel B: Linear regression of the detection of *T. cruzi* DNA when each of the four sets of oligonucleotides were used to detect açai pulp DNA. Data are shown as mean ± SD for each parasite concentration obtained from at least two independent experiments. Open symbols are detections out of the linear range for that target.

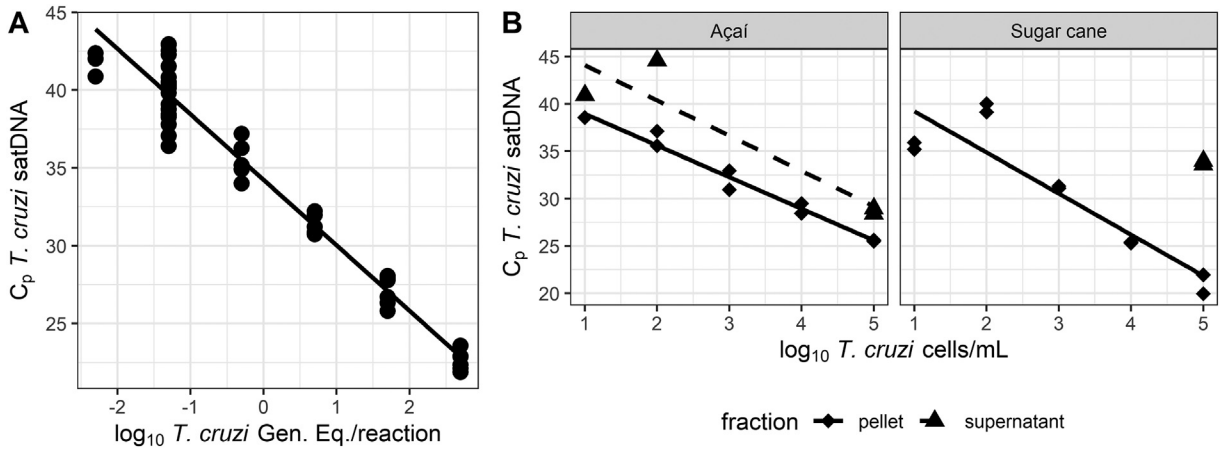

Fig. 2. Linear regression for the detection of *T. cruzi* DNA in açaí pulp (black) or sugarcane (gray) matrices. Panel A: Purified *T. cruzi* DNA with concentrations ranging from 5×10^{-3} to 5×10^{2} genome equivalents/reaction were diluted in purified açai pulp DNA and evaluated with the duplex qPCR. The obtained C_p are shown as black circles and the linear regression is shown as a black line. Panel B: *T. cruzi* trypomastigotes (10^{-1} to 10^{4} cells/mL) were diluted in crude açai pulp or sugarcane extracts and centrifuged as described in Methods, producing pellet (triangles) and supernatant (squares) fractions from which total DNA was extracted and probed for *T. cruzi* DNA. Results were obtained from at least two independent experiments.

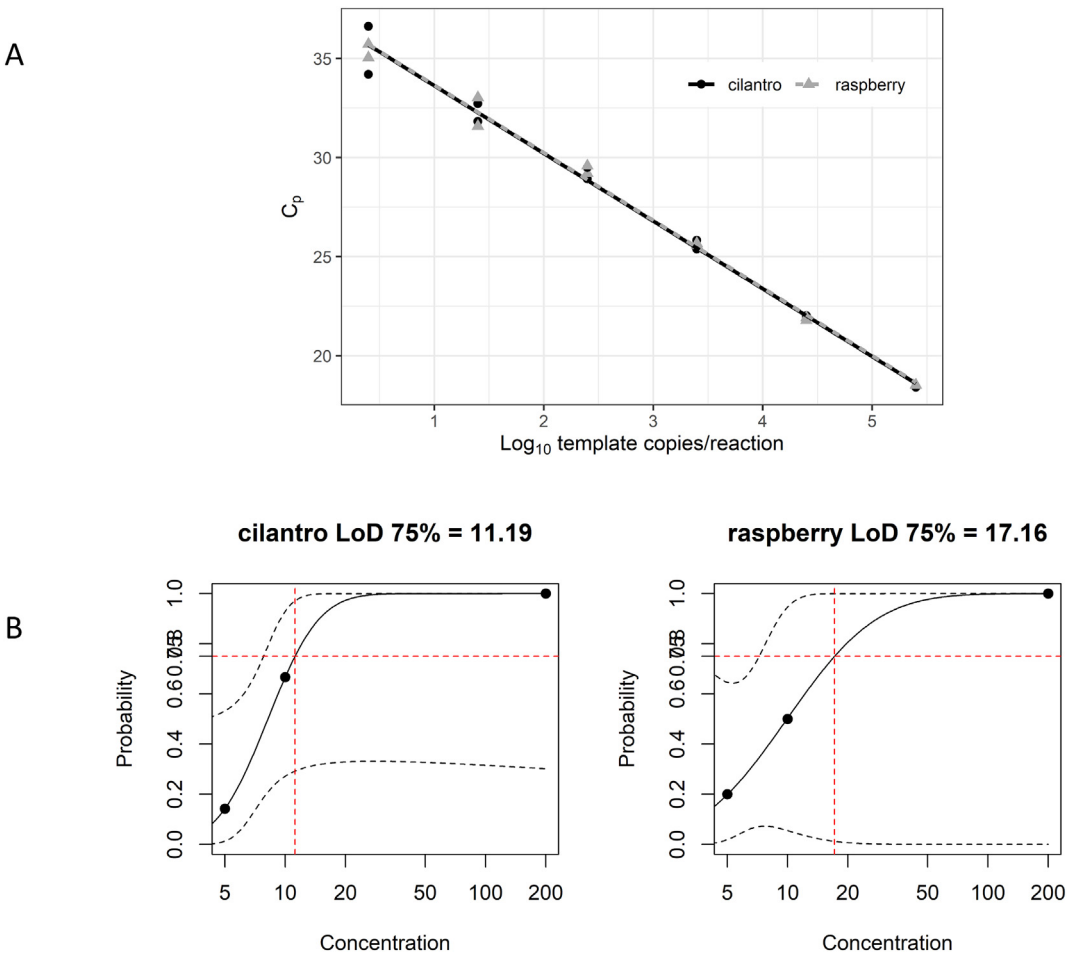

Fig. 3. Determination of the limit of detection of the ready-to-use reaction for detection of *C. cayetanensis* synthetic control. Panel A: Linear regression for the detection of *C. cayetanensis* synthetic control DNA (Hm135m) diluted in DNA from raspberry (gray triangles) or cilantro leaves (black circles). Panel B: Probit analysis to calculate the $LOD_{75\%}$ for the detection of the Hm135m control DNA diluted in raspberry or cilantro leaves DNA. Data obtained from two independent experiments.

Table 2
Evaluation of *C. cayetanensis* detection in artificially spiked raspberry fruits and cilantro leaves using the ready-to-use qPCR. Shown are sample's classification status relative to the presence of *C. cayetanensis* DNA as obtained using the ready-to-use format and a direct comparison with results published by (Murphy et al., 2017).

Matrix	Oocysts concentration	Gelified qPCR (this study)	Regular qPCR (Murphy et al., 2017)
cilantro	0	Negative	Negative
cilantro	0	Negative	Negative
cilantro	0	Negative	Negative
cilantro	0	Negative	Negative
cilantro	0	Negative	Negative
cilantro	5	Negative	Negative
cilantro	5	Negative	Negative
cilantro	5	Negative	Negative
cilantro	5	Negative	Positive
cilantro	5	Negative	Negative
cilantro	5	Positive	Positive
cilantro	10	Positive	Positive
cilantro	10	Negative	Positive
cilantro	10	Positive	Positive
cilantro	10	Positive	Positive
cilantro	200	Positive	Positive
cilantro	200	Positive	Positive
cilantro	200	Positive	Positive
cilantro	200	Positive	Positive
cilantro	200	Positive	Positive
raspberry	0	Negative	Negative
raspberry	0	Negative	Negative
raspberry	0	Negative	Negative
raspberry	0	Negative	Negative
raspberry	5	Positive	Positive
raspberry	5	Negative	Positive
raspberry	5	Negative	Negative
raspberry	5	Negative	Negative
raspberry	10	Positive	Positive
raspberry	10	Negative	Negative
raspberry	200	Positive	Positive
raspberry	200	Positive	Positive
raspberry	200	Positive	Positive
raspberry	200	Positive	Positive

sets of samples. The Bland-Altman analysis show a bias of −0.82 C_p for the gelified versus the non-gelified qPCR, with lower and higher limits of −4.94 and 3.32 C_p, respectively.

3.3. Specificity, repeatability, reproducibility, and stability studies

The specificity of the ready-to-use qPCR assays was evaluated against DNA from organisms that (i) are closely related to *T. cruzi* or *C. cayetanensis*; or (ii) might produce similar symptoms in case of acute infections. The ready-to-use duplex qPCR for *C. cayetanesis* DNA detection showed no amplification when tested against DNA from *Eimeria acervulina*, *Eimeria cloaceae*, *Eimeria faecalis*, *Eimeria faecium, Escherichia coli*, *Eimeria tenella*, *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania infantum*, *Plasmodium falciparum*, *Plasmodium vivax*, *Trypanosoma cruzi* or *Toxoplasma gondii* (Supplemental Fig. 2). Similarly, the ready-to-use duplex qPCR for *T. cruzi* DNA detection showed no amplification when tested against DNA from *C. cayetanensis*, *Eimeria acervulina*, *Eimeria tenella*, *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania infantum*, *Plasmodium falciparum*, *Plasmodium vivax*, or *Trypanosoma rangeli* (Supplemental Fig. 3). Reactions aimed to detect *T. cruzi* DNA containing *T. gondii* DNA yielded inconclusive results. The oligonucleotides that detect *T. cruzi* or *C. cayetanensis* were also evaluated for their specificity using non-spiked samples of each food matrix (açai pulp and sugarcane juice for *T. cruzi* and cilantro leaves and raspberry fruits for *C. cayetanensis*) and no cross reactivity was observed. Positive and negative controls were included in each run to validate the results.

The oligonucleotides designed to detect conserved regions of the genes that code for the ubiquitous proteins apocytochrome b and ribulose biphosphate carboxylase "large chain 2" (Fig. 1) were also evaluated in silico for their specificity. A primerBLAST search using the sequences of the *Cob2* and *RbcL2* oligonucleotides and allowing up to 4 mismatches (none in the 3´ end) in the "Higher Plants" set of the NCBI database resulted in a list of 696 species with 960 hits to *Cob2* set, and of 601 species with 996 hits to *RbcL2* oligonucleotides (Supplemental Table 2).

The effect of the gelification technology and its reagents on the qPCR was further analyzed by studying the repeatability and reproducibility of the "*T. cruzi* – *Cob2*" reaction. The detection of a synthetic positive control was performed by three independent operators in three consecutive days and the relative standard deviation (RSD) calculation was based on the C_p values

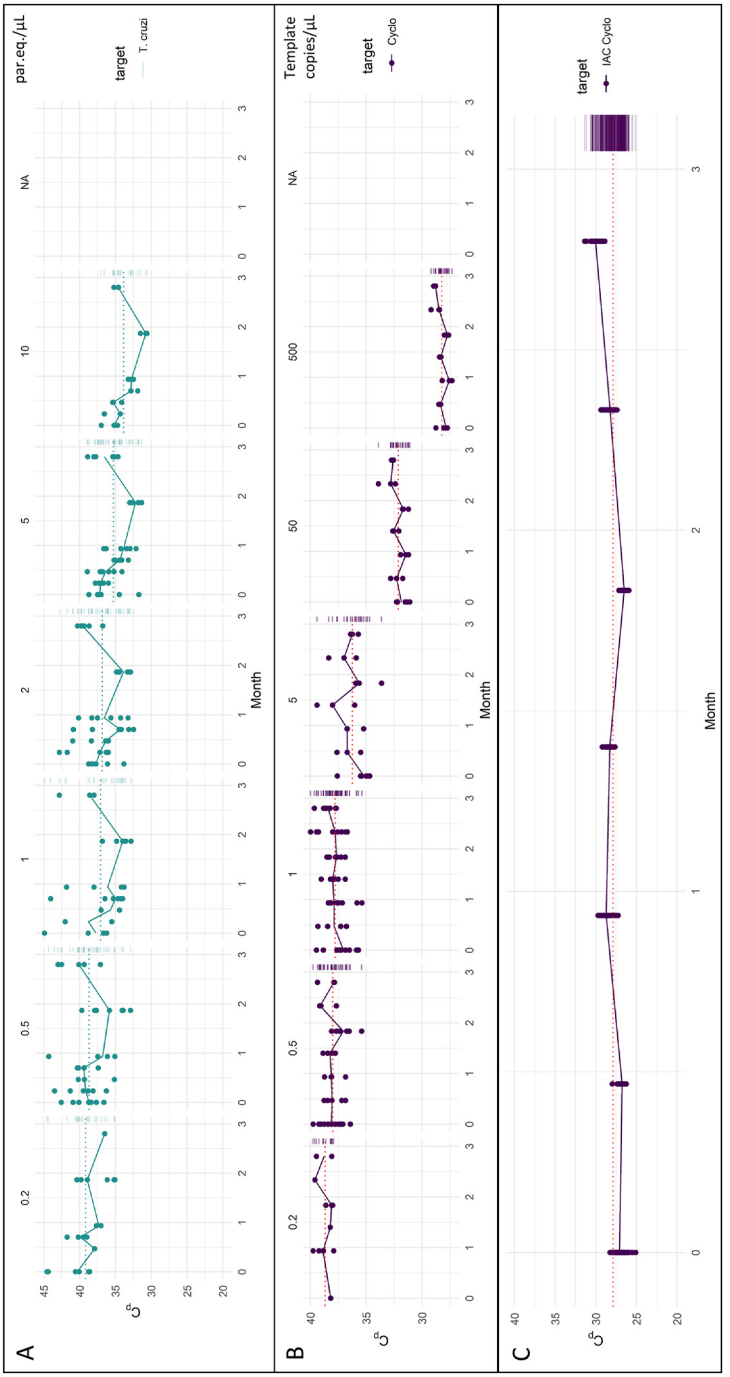

Fig. 4. Stability studies of the ready-to-use reactions. Medium-term stability of the ready-to-use duplex qPCR for detection of *T. cruzi* DNA in açai pulp (panel A) and detection of *C. cayetanensis* (panel B) synthetic DNA (Hm135m). Panel C shows the detection of the synthetic internal amplification control IAC Cyclo over the same period of time as panel B.

(Supplemental Table 3). Intra-operator RSD for the C_p (repeatability) was not higher than 5.3% (Supplemental Table 4), and the inter-operator RSD (reproducibility) was not higher than 2.6% (Supplemental Table 5).

Finally, we conducted a medium-term stability study to observe how the gelified qPCR reagents would perform over time when stored in the 96-well plate at 2–8 °C (refrigerator) instead of stored in 1.5-mL tubes at −20 °C (freezer). Fig. 4 shows that both the duplex qPCR for detection of *T. cruzi* DNA in açai pulp (upper panel) and the duplex qPCR for detection of *C. cayetanensis* (middle panel) are stable for up to 90 days. The detection of the lyophilized synthetic positive control Hm135m (lower panel) or *Cob2* (data not shown) also remained stable over the same period. An analysis of the $LoD_{95\%}$ at each time point of both reactions shows the reaction's stability over a 3-month period, evidenced by the linear regression on each plot (gray lines on Supplemental Fig. 3, panel A for *T. cruzi* DNA detection and panel B for the *C. cayetanensis* synthetic positive control).

4. Discussion

PCR has long been used to determine the presence of pathogens in different food matrices (Umesha and Manukumar, 2018; Zeng et al., 2016). However, there are a large number of food commodities that could be prone to contamination by microorganisms which creates challenges to establishment of general protocols which could work with different categories of foods. In this regard, Murphy and colleagues developed and validated a method for detection of *C. cayetanensis* in fresh produce. This method includes a wash step using Alconox® to recover the parasite's oocysts from produce, a robust DNA extraction based on disruption of the oocyst's wall, and qPCR that detects a fragment of the *C. cayetanensis* 18SrRNA gene (Murphy et al., 2017). Mattos and colleagues evaluated a protocol for treating samples and extracting DNA for subsequent detection of *T. cruzi* by qPCR in açai pulp and sugarcane juice (de Mattos et al., 2017). Such protocols should help harmonize the procedures among different laboratories, especially regarding sample size and preparation, facilitating the comparison of results among different studies.

Several authors have shown the use of PCR for the detection of *C. cayetanensis* or *T. cruzi* in food products, either as a diagnostic or as a quality control tool (de Oliveira et al., 2019; de Souza Godoi et al., 2017; Dixon et al., 2013; Ferreira et al., 2018; Lalonde and Gajadhar, 2016). These protocols required reagents to be stored at freezing temperatures and the reaction mastermix to be prepared for every experiment, increasing the likelihood of operator mistakes. These errors include inaccurate pipetting of reagents into wells and incorrect calculations of reagent's concentrations when preparing the qPCR mastermix. Moreover, qPCR reagents are thermolabile and degrade quickly when not stored properly, hindering their use in routine food screening services.

In the present work, we developed and evaluated two ready-to-use qPCR with all reagents pre-loaded into reaction wells of 96-well qPCR plates or tube strips. By using this format, lab analysts only need to add the DNA template in the wells and start the run in the instrument. Our estimates indicate a reduction of up to 60% in the operator's hand-on time due to the elimination of the steps of preparation and loading the mastermix onto the 96-well plate. When analyzing few samples, 8- or 12-tube strips can also be used with the same performance and ease of use. Furthermore, because molecular tests should be produced in highly regulated facilities, ideally under Good Manufacturing Practices, there are numerous quality control procedures that guarantee the performance between batches.

Importantly, ease of use was not achieved at the expense of other qPCR performance parameters. The ready-to-use format did not significantly affect the LoD of either reaction (Figs. 1-3) when compared to published results using non-gelified PCR reagents for detection of *T. cruzi* or *C. cayetanensis* in food samples (Caradonna et al., 2017; Lalonde and Gajadhar, 2016; de Mattos et al., 2017; Murphy et al., 2017; Ramírez et al., 2015; Rampazzo et al., 2019; Rampazzo et al., 2017). Indeed, the re-evaluation of 34 samples previously characterized by Murphy and collaborators (Murphy et al., 2017) using the new ready-to-use qPCR showed a substantial agreement between the results. It must be noted that the ready-to-use qPCR produced three false negative results, which could indicate a lower sensitivity when compared to regular assay (Murphy et al., 2017). However, these could be the result of the current integrity conditions of the samples, which were used to evaluate the liquid format several months before the evaluation by the ready-to-use reaction. Although this result could indeed indicate a lower sensitivity when compared to the regular assay (Murphy et al., 2017), a more robust evaluation in a multi laboratory strategy must be conducted to ascertain the analytical sensitivity of the ready-to-use-assay. The repeatability (intra-operator relative standard deviation) and reproducibility (the inter-operator relative standard deviation) were found to be within acceptable ranges for qPCR methods (Broeders et al., 2014), showing the robustness of the technique. Furthermore, an evaluation against organisms that might cause similar symptoms, or are phylogenetically related, or have sympatric occurrence showed no amplification, attesting the specificity of the proposed assays. It is important to highlight that the assay targeting *T. cruzi* DNA yielded inconclusive results when tested against samples containing *T. gondii* DNA. Although the epidemiology of *T. gondii* and *T. cruzi* and their foodborne transmission profiles are quite distinct, further investigation of the specificity of the *T. cruzi* reaction towards *T. gondii* DNA is currently underway.

Additionally, as a quality control feature, the reactions presented here used two different internal amplification controls, one was an exogenous synthetic DNA ultramer oligo (for *C. cayetanensis* detection in fresh produce samples) and the other was the detection of the matrix's DNA (as for *T. cruzi* detection in açai or sugarcane samples). While the exogenous synthetic DNA for *C. cayetanensis* detection has been described elsewhere (Murphy et al., 2017), the detection of plant-based DNA for this purpose has not. In order to show reliable performance as an internal control for the reaction, the target must yield a strong signal in different food matrices without interfering with the detection of the pathogen's target at the lowest concentration. Oligonucleotides sets for the detection of sequences of the matrix target genes *Cob2* (apocytochrome b) and *RbcL2* (ribulose biphosphate carboxylase “large chain 2”) were the most efficient of those evaluated. Interestingly, *Cob2* is a single copy gene in both the açai and sugarcane genomes, while *RbcL2* is a chloroplast gene, present in multiple copies per cell, which should result in stronger

amplification. However, *Cob2* detection yielded the most efficient reaction and, although not perfect, was the chosen internal amplification control target for *T. cruzi* detection for the remainder of the present study. Although we did not test other food matrices, we propose that these oligonucleotides would be able to detect genomic DNA from other plant species and, hence, be used as general markers for the presence of the plant-based DNA because the chosen genes (apocytochrome b and ribulose biphosphate carboxylase "large chain 2") are ubiquitous and the targeted regions are well conserved.

Another interesting feature is that the reaction remained stable for up to 90 days when stored at 2–8 °C, eliminating the need for −20 °C storage and transportation. Indeed, ready-to-use PCR reactions have been shown to be stable at above-freezing temperatures without any significant loss of performance. Rampazzo and collaborators showed the performance of ready-to-use qPCR assays for detection of *T. cruzi* or *Plasmodium* spp. DNA remained stable for up to 4 weeks when stored at 2–8 °C or 21–23 °C (Rampazzo et al., 2019). Kamau and coworkers reported a lyophylized qPCR assay for detection of *Plasmodium* spp. that was stable for 42 days at 37 °C (Kamau et al., 2014). Sun and collaborators reported that a gelified PCR assay for detection of *Campylobacter* spp. remained stable for 90 days (Sun et al., 2013). When all steps of production, transportation, storage, and effective use are accounted for, our estimates suggest that elimination of the −20 °C cold chain could result in an overall reduction of the cost of a qPCR test of up to 20%, either directly through cheaper operations or in through decreased operator's hands on time (which ultimately relates to cheaper operation) (Rampazzo et al., 2019).

5. Conclusions

In summary, the gelification process described in the present study could be valuable for development of detection assays to streamline the use of qPCR for screening large number of samples for presence of microorganisms. We successfully demonstrated the application of this technology for the detection of *C. cayetanensis* and *T. cruzi* in four distinct food matrices. When compared to the regular frozen storage format, the ready-to-use PCR exhibited an overall similar performance, suggesting that the gelification technology is robust and can be reliably implemented for the diagnostic detection of microorganisms, either in routine quality control industrial processes, or to support different types of investigations including those triggered by outbreaks.

Data statement

All data related to the results presented in this work is available upon request.

Disclaimers

The views expressed are those of the authors and should not be construed as the U.S. Food and Drug Administration views or policies.

The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the Department of Health and Human Services.

Declaration of Competing Interest

Instituto de Biologia Molecular do Paraná (IBMP) produces both qPCR mastermixes used in the present study.

BNDES or IBMP had no participation in the study's design, data collection, analysis, interpretation, or writing of the report, and decision to submit for publication.

The authors declare no further competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2021.e00111.

References

Alarcón de Noya, B., Colmenares, C., Díaz-Bello, Z., Ruiz-Guevara, R., Medina, K., Muñoz-Calderón, A., Mauriello, L., Cabrera, E., Montiel, L., Losada, S., Martínez, J., Espinosa, R., Abate, T., 2016. Orally-transmitted Chagas disease: epidemiological, clinical, serological and molecular outcomes of a school microepidemic in Chichiriviche de la Costa, Venezuela. Parasite Epidemiol. Control 1, 188–198. https://doi.org/10.1016/j.parepi.2016.02.005.

Anez, N., Crisante, G., Rojas, A., Rojas, R.O., Bastidas, J., 2016. A new acute Oral Chagas disease outbreak in Merida, Venezuela: a comprehensive study. Int. J. Clin. Med. Res. 3, 29–37.
Arbefeville, S., Levi, M.H., Ferrieri, P., 2014. Development of a multiplex real-time PCR assay for the detection of Bordetella pertussis and Bordetella parapertussis in a single tube reaction. J. Microbiol. Methods 97, 15–19. https://doi.org/10.1016/j.mimet.2013.12.003.
Bland, J.M., Altman, D.G., 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet (London, England) 1, 307–310. https://doi.org/10.1016/S0140-6736(86)90837-8.
Broeders, S., Huber, I., Grohmann, L., Berben, G., Taverniers, I., Mazzara, M., Roosens, N., Morisset, D., 2014. Guidelines for validation of qualitative real-time PCR methods. Trends Food Sci. Technol. https://doi.org/10.1016/j.tifs.2014.03.008.
Caradonna, T., Marangi, M., Del Chierico, F., Ferrari, N., Reddel, S., Bracaglia, G., Normanno, G., Putignani, L., Giangaspero, A., 2017. Detection and prevalence of protozoan parasites in ready-to-eat packaged salads on sale in Italy. Food Microbiol. 67, 67–75. https://doi.org/10.1016/j.fm.2017.06.006.
Casillas, S.M., Hall, R.L., Herwaldt, B.L., 2019. Cyclosporiasis surveillance — United States, 2011–2015. MMWR Surveill. Summ. 68, 1–16. https://doi.org/10.15585/mmwr.ss6803a1.
de Mattos, E.C., da Meira-Strejevitch, C.S., Marciano, M.A.M., Faccini, C.C., Lourenço, A.M., Pereira-Chioccola, V.L., 2017. Molecular detection of Trypanosoma cruzi in acai pulp and sugarcane juice. Acta Trop. 176, 311–315. https://doi.org/10.1016/j.actatropica.2017.08.025.
de Noya, B.A., Pérez-Chacón, G., Díaz-Bello, Z., Dickson, S., Muñoz-Calderón, A., Hernández, C., Pérez, Y., Mauriello, L., Moronta, E., 2017. Description of an oral Chagas disease outbreak in Venezuela, including a vertically transmitted case. Mem. Inst. Oswaldo Cruz 112, 569–571. https://doi.org/10.1590/0074-02760170009.
de Oliveira, A.C., Soccol, V.T., Rogez, H., 2019. Prevention methods of foodborne Chagas disease: disinfection, heat treatment and quality control by RT-PCR. Int. J. Food Microbiol. 301, 34–40. https://doi.org/10.1016/j.ijfoodmicro.2019.04.009.
de Souza Godoi, P.A., Piechnik, C.A., de Oliveira, A.C., Sfeir, M.Z., de Souza, E.M., Rogez, H., Thomaz Soccol, V., 2017. qPCR for the detection of foodborne Trypanosoma cruzi. Parasitol. Int. 66, 563–566. https://doi.org/10.1016/j.parint.2017.06.001.
de Souza, W., Attias, M., 2018. New advances in scanning microscopy and its application to study parasitic protozoa. Exp. Parasitol. 190, 10–33. https://doi.org/10.1016/j.exppara.2018.04.018.
Dixon, B., Parrington, L., Cook, A., Pollari, F., Farber, J., 2013. Detection of Cyclospora, Cryptosporidium, and Giardia in ready-to-eat packaged leafy greens in Ontario, Canada. J. Food Prot. 76, 307–313. https://doi.org/10.4315/0362-028X.JFP-12-282.
Eberhard, M.L., da Silva, A.J., Lilley, B.G., Pieniazek, N.J., 1999. Morphologic and molecular characterization of new Cyclospora species from Ethiopian monkeys: C. cercopitheci sp.n., C. colobi sp.n., and C. papionis sp.n. Emerg. Infect. Dis. 5, 651–658. https://doi.org/10.3201/eid0505.990506.
FAO, World Health Organization, 2014. Multicriteria-Based Ranking for Risk Management of Food-Borne Parasites. World Health Organization, FAO.
Ferreira, R.T.B., Cabral, M.L., Martins, R.S., Araujo, P.F., Da Silva, S.A., Britto, C., Branquinho, M.R., Cardarelli-Leite, P., Moreira, O.C., 2018. Detection and genotyping of Trypanosoma cruzi from açai products commercialized in Rio de Janeiro and Pará, Brazil. Parasit. Vectors 11, 1–11. https://doi.org/10.1186/s13071-018-2699-6.
Franco-Paredes, C., Villamil-Gómez, W.E., Schultz, J., Henao-Martínez, A.F., Parra-Henao, G., Rassi, A., Rodríguez-Morales, A.J., Suarez, J.A., 2020. A deadly feast: Elucidating the burden of orally acquired acute Chagas disease in Latin America – Public health and travel medicine importance. Travel Med. Infect. Dis., 101565 https://doi.org/10.1016/j.tmaid.2020.101565.
Giavarina, D., 2015. Understanding Bland Altman analysis. Biochem. Medica 25, 141–151. https://doi.org/10.11613/BM.2015.015.
Guo, M., Dubey, J.P., Hill, D., Buchanan, R.L., Gamble, H.R., Jones, J.L., Pradhan, A.K., 2015. Prevalence and risk factors for toxoplasma gondii infection in meat animals and meat products destined for human consumption. J. Food Prot. 78, 457–476. https://doi.org/10.4315/0362-028X.JFP-14-328.
Hernández, C., Vera, M.J., Cucunubá, Z., Flórez, C., Cantillo, O., Buitrago, L.S., González, M.S., Ardila, S., Dueñas, L.Z., Tovar, R., Forero, L.F., Ramírez, J.D., 2016. High-resolution molecular typing of Trypanosoma cruzi in 2 large outbreaks of acute Chagas disease in Colombia. J. Infect. Dis. 214, 1252–1255. https://doi.org/10.1093/infdis/jiw360.
Iglesias, N., Subirats, M., Trevisi, P., Ramírez-Olivencia, G., Castán, P., Puente, S., Toro, C., 2014. Performance of a new gelled nested PCR test for the diagnosis of imported malaria: comparison with microscopy, rapid diagnostic test, and real-time PCR. Parasitol. Res. 113, 2587–2591. https://doi.org/10.1007/s00436-014-3911-z.
Kamau, E., Alemayehu, S., Feghali, K.C., Juma, D.W., Blackstone, G.M., Marion, W.R., Obare, P., Ogutu, B., Ockenhouse, C.F., 2014. Sample-ready multiplex qPCR assay for detection of malaria. Malar. J. 13, 158. https://doi.org/10.1186/1475-2875-13-158.
Lalonde, L.F., Gajadhar, A.A., 2016. Detection of Cyclospora cayetanensis, Cryptosporidium spp., and toxoplasma gondii on imported leafy green vegetables in Canadian survey. Food Waterborne Parasitol. 2, 8–14. https://doi.org/10.1016/j.fawpar.2016.01.001.
Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159. https://doi.org/10.2307/2529310.
Lass, A., Pietkiewicz, H., Szostakowska, B., Myjak, P., 2012. The first detection of toxoplasma gondii DNA in environmental fruits and vegetables samples. Eur. J. Clin. Microbiol. Infect. Dis. 31, 1101–1108. https://doi.org/10.1007/s10096-011-1414-8.
Li, N., Ye, J., Arrowood, M.J., Ma, J., Wang, L., Xu, H., Feng, Y., Xiao, L., 2015. Identification and morphologic and molecular characterization of Cyclospora macacae n. sp. from rhesus monkeys in China. Parasitol. Res. 114, 1811–1816. https://doi.org/10.1007/s00436-015-4367-5.
Murphy, H.R., Lee, S., Da Silva, A.J., 2017. Evaluation of an improved U.S. food and drug administration method for the detection of cyclospora cayetanensis in produce using real-time PCR. J. Food Prot. 80, 1133–1144. https://doi.org/10.4315/0362-028X.JFP-16-492.
Ramírez, J.C., Cura, C.I., Da Cruz Moreira, O., Lages-Silva, E., Juiz, N., Velázquez, E., Ramírez, J.D., Alberti, A., Pavia, P., Flores-Chávez, M.D., Muñoz-Calderón, A., Pérez-Morales, D., Santalla, J., Marcos Da Matta Guedes, P., Peneau, J., Marcet, P., Padilla, C., Cruz-Robles, D., Valencia, E., Crisante, G.E., Greif, G., Zulantay, I., Costales, J.A., Alvarez-Martínez, M., Martínez, N.E., Villarroel, R., Villarroel, S., Sánchez, Z., Bisio, M., Parrado, R., Maria Da Cunha Galvão, L., Da Câmara, A.C.J., Espinoza, B., De Noya, B.A., Puerta, C., Riarte, A., Diosque, P., Sosa-Estani, S., Guhl, F., Ribeiro, I., Aznar, C., Britto, C., Yadón, Z.E., Schijman, A.G., 2015. Analytical validation of quantitative real-time PCR methods for quantification of trypanosoma cruzi DNA in blood samples from chagas disease patients. J. Mol. Diagnost. 17, 605–615. https://doi.org/10.1016/j.jmoldx.2015.04.010.
Rampazzo, R.C.P., Graziani, A.C., Leite, K.K., Surdi, J.A., Biondo, C.A., Costa, M.L.N., Jacomasso, T., Cereda, M., De Fazio, M., Bianchessi, M.A., Moreira, O.C., Britto, C., Costa, J.D.N., Góes, V.M., da Silva, A.J., Krieger, M.A., Costa, A.D.T., 2019. Proof of concept for a portable platform for molecular diagnosis of tropical diseases. J. Mol. Diagnost. 21, 839–851. https://doi.org/10.1016/j.jmoldx.2019.04.008.
Rampazzo, R.D.C.P., Solcà, M.D.S., Santos, L.C.S., Pereira, L.D.N., Guedes, J.C.O., Veras, P.S.T., Fraga, D.B.M., Krieger, M.A., Costa, A.D.T., 2017. A ready-to-use duplex qPCR to detect Leishmania infantum DNA in naturally infected dogs. Vet. Parasitol. 246. https://doi.org/10.1016/j.vetpar.2017.09.009.
Rosado, P.M.F.S., López, G.L., Seiz, A.M., Alberdi, M.M., 2002. Method for preparing stabilised reaction mixtures, which are totally or partially dried, comprising at least one enzyme, reaction mixtures and kits containing said mixtures WO 02/072002A3.
Rueda, K., Trujillo, J.E., Carranza, J.C., Vallejo, G.A., 2014. Transmisión oral de Trypanosoma cruzi: un nuevo escenario epidemiológico de la enfermedad de Chagas en Colombia y otros países suramericanos. Biomédica 34. https://doi.org/10.7705/biomedica.v34i4.2204.
Sun, Y., Høgberg, J., Christine, T., Florian, L., Monsalve, L.G., Rodriguez, S., Cao, C., Wolff, A., Ruano-Lopez, J.M., Bang, D.D., 2013. Pre-storage of gelified reagents in a lab-on-a-foil system for rapid nucleic acid analysis. Lab Chip 13, 1509–1514. https://doi.org/10.1039/c2lc41386h.
Umesha, S., Manukumar, H.M., 2018. Advanced molecular diagnostic techniques for detection of food-borne pathogens: current applications and future challenges. Crit. Rev. Food Sci. Nutr. 58, 84–104. https://doi.org/10.1080/10408398.2015.1126701.
Zeng, D., Chen, Z., Jiang, Y., Xue, F., Li, B., 2016. Advances and challenges in viability detection of foodborne pathogens. Front. Microbiol. 7, 1833. https://doi.org/10.3389/fmicb.2016.01833.