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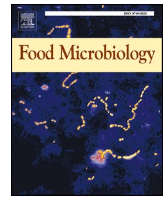

Quantification of viable protozoan parasites on leafy greens using molecular methods

Minji Kim[a,b], Karen Shapiro[a], Verónica B. Rajal[c,d], Andrea Packham[a], Beatriz Aguilar[a], Lezlie Rueda[a], Stefan Wuertz[b,d,e,*]

[a] Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, CA, 95616, USA
[b] Department of Civil and Environmental Engineering, University of California, Davis, One Shields Avenue, Davis, CA, 95616, USA
[c] Facultad de Ingeniería and Instituto de Investigaciones para la Industria Química (INIQUI), CONICET - Universidad Nacional de Salta (UNSa), Av. Bolivia 5150, Salta, 4400, Argentina
[d] Singapore Centre for Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, Singapore (NTU), 60 Nanyang Drive, 637551, Singapore
[e] School of Civil and Environmental Engineering, NTU, 50 Nanyang Avenue, 649798, Singapore

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ABSTRACT

Protozoan contamination in produce is of growing importance due to their capacity to cause illnesses in consumers of fresh leafy greens. Viability assays are essential to accurately estimate health risk caused by viable parasites that contaminate food. We evaluated the efficacy of reverse transcription quantitative PCR (RT-qPCR), propidium monoazide coupled with (q)PCR, and viability staining using propidium iodide through systematic laboratory spiking experiments for selective detection of viable *Cryptosporidium parvum*, *Giardia enterica*, and *Toxoplasma gondii*. In the presence of only viable protozoa, the RT-qPCR assays could accurately detect two to nine (oo)cysts/g spinach (in 10 g processed). When different proportions of viable and inactivated parasite were spiked, mRNA concentrations correlated with increasing proportions of viable (oo)cysts, although low levels of false-positive mRNA signals were detectable in the presence of high amounts of inactivated protozoa. Our study demonstrated that among the methods tested, RT-qPCR performed more effectively to discriminate viable from inactivated *C. parvum, G. enterica* and *T. gondii* on spinach. This application of viability methods on leafy greens can be adopted by the produce industry and regulatory agencies charged with protection of human public health to screen leafy greens for the presence of viable protozoan pathogen contamination.

1. Introduction

Over the last decades leafy greens have become more popular and important as part of a healthy diet. With the extended accessibility of ready-to-eat packaged salads the per capita availability of leafy greens such as romaine lettuce, collard greens, and spinach has also increased in the United States (Bentley, 2017). When properly handled and treated, leafy greens can be a great source of vitamins, minerals and dietary fibers. However, leafy greens as food commodity accounted for more than 20% of foodborne illnesses caused by major pathogens in the United States over the past decade (Painter et al., 2013). While norovirus, Shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* have been known to most often cause leafy green-associated outbreaks in the United States (Herman et al., 2015), recent surveillance studies showed that protozoan pathogens attributed to food-borne illnesses from fresh produce (CDC, 2019; Dixon, 2016; FAO/WHO, 2008). The etiological agents of foodborne illnesses are unknown in the majority of cases (80%) due to insufficient data on agent-specific illnesses and/or under-recognized pathogens potentially present in food commodities (Elaine et al., 2011). Protozoan contamination of produce is of growing importance, thus *Cryptosporidium parvum*, *Giardia enterica* and *Toxoplasma gondii* were targeted in this study due to their current and projected capacity to contribute to illnesses in consumers of fresh produce (Abanyie et al., 2015; Caradonna et al., 2017; Dixon et al., 2013; Lalonde and Gajadhar, 2016; McKerr et al., 2015; Scallan et al., 2011).

Symptoms of illnesses from *C. parvum* and *G. enterica* infection include diarrhea, abdominal pain, nausea/vomiting and dehydration that can last weeks to months. Symptoms of toxoplasmosis can vary depending on the stage of infection, the age and immune status of hosts as well as the parasite genotype. *T. gondii* infection can cause flu-like

* Corresponding author. Department of Civil and Environmental Engineering, University of California, Davis, One Shields Avenue, Davis, CA, 95616, USA.
E-mail address: swuertz@ntu.edu.sg (S. Wuertz).

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symptoms in immunocompetent individuals during acute infection, however serious sequelae such as severe visual impairments can also occur (Bahia-Oliveira et al., 2017). The parasite persists in the human body for life, with the potential for reactivation and severe disease when the person's immune system becomes compromised (Dubey, 2010). Congenital transmission can potentially cause a miscarriage, a stillborn child, and severe birth defects when a woman is first exposed to *T. gondii* during or just before pregnancy (Bahia-Oliveira et al., 2017).

Despite the risk of foodborne illnesses that can be acquired by protozoan pathogens, fresh vegetables are not routinely tested for the presence of protozoa, and standardized microscopy-based methods are currently available only for *Cryptosporidium* and *Giardia* (ISO, 2016; USEPA, 2012). Given the increasing needs of testing protozoan parasites in fresh produce, parasite detection methods have been developed and evaluated in protozoan spiking studies using fresh produce commodities including leafy greens, berries, mushrooms, bean sprouts, and herbs (summarized in (Shapiro et al., 2019)). In a previous study, we developed and validated a new multiplex polymerase chain reaction (PCR) assay for simultaneous detection of four key parasites that can be infectious to humans, namely, *Cryptosporidium* (*C. parvum* and *C. hominis*), *Giardia* (*G. duodenalis* and *G. enterica* (Assemblages A and B, respectively)), *Cyclospora cayetanensis* and *T. gondii,* in leafy greens (Shapiro et al., 2019). While the multiplex PCR assay can detect parasites in a rapid, efficient, and affordable manner, an important limitation of PCR is the inability to differentiate viable from dead parasites as DNA can persist long after pathogens die, which can result in an overestimation of microbial risk. Therefore, additional tests are needed to determine the viability of pathogens if protozoan contamination is found in routine screening as assessed by PCR. Reverse transcription quantitative PCR (RT-qPCR), vital dye exclusion tests using propidium iodide (PI) or propidium monoazide (PMA), and fluorescent in situ hybridization have been tested to assess the viability of *C. parvum*, *G. enterica* and *T. gondii* (oo)cysts in simple matrices such as water (summarized in (Rousseau et al., 2018)). However, applications of the assays in complex food matrices, especially in fresh produce, are limited to date. Therefore, the purpose of this study was to evaluate protozoan viability methods including RT-qPCR, PMA-(q)PCR, and viability staining with a direct fluorescent antibody together with propidium iodide (hereinafter referred to DFA/PI staining) through systematic laboratory spiking experiments using spinach as a model leafy green. In the first spiking experiment, the sensitivity of viability assays was assessed using viable protozoa spiked on spinach. The second spiking experiment included discrimination of viable (oo)cysts in the presence of inactivated parasites, which simulated real environmental conditions where live and dead parasites are present together.

2. Materials and methods

2.1. Parasite preparation

Live *C. parvum* oocysts (Iowa isolate subtype IIa) were purchased from Sterling Parasitology Laboratory at University of Arizona (AZ, USA), and live *Giardia enterica* cysts (Human isolate H3, Assemblage B) were obtained from Waterborne™ Inc. (LA, USA). The nomenclature for *G. enterica* has been specifically proposed for the human associated Assemblage B of *G. duodenalis* (Boarato-David et al., 2017; Thompson and Monis, 2012), and therefore this taxonomy will be referred to herein. *T. gondii* oocysts (Type II strain M4) were generously donated by the Jeroen Saeij laboratory in the department of Pathology, Microbiology and Immunology at University of California, Davis (CA, USA). To obtain inactivated (oo)cysts, live (oo)cysts in 1 ml PBS were heat-inactivated by placing microcentrifuge tubes in a dry heating block in which each well was filled with DI water at 80 °C for 3 min (*C. parvum*), 70 °C for 15 min (*G. enterica*) or 80 °C for 20 min (*T. gondii*) (Shapiro et al., 2019; Travaillé et al., 2016). The heat inactivation conditions we applied were sufficient to inactivate target parasites as determined by bioassays (Travaillé et al., 2016), while (oo)cyst walls retained structural integrity as we confirmed using bright field microscopy. The numbers of parasite (oo)cysts in stock solutions were enumerated using a hemocytometer chamber under bright field microscopy. The differences in numbers of (oo)cysts before and after the heat inactivation conditions selected in our study were less than 10%. Stock solutions were diluted to targeted concentrations, and the three target parasites were mixed in individual spike dilution levels.

2.2. PMA-(q)PCR and RT-qPCR

2.2.1. Nucleic acid extraction

For PMA-(q)PCR, DNA was extracted from 100 µl suspensions using the DNeasy Blood and Tissue Kit (Qiagen, CA, USA) with slight modifications (Shapiro et al., 2019). In brief, samples were mixed with 180 µl Buffer ATL and subjected to one freeze-thaw cycle by placing samples in liquid nitrogen for 4 min and immediately transferring to boiling water for 4 min (Manore et al., 2019). After 40 µl Proteinase K was added, samples were incubated overnight at 56 °C. The nucleic acids were eluted from spin column membrane with 100 µl of 1:10 Buffer AE preheated to 95 °C. For RT-qPCR, 100 µl sample pellets were incubated at 45 °C for 20 min for messenger RNA (mRNA) induction followed by mRNA extraction using the Dynabeads® mRNA DIRECT™ Kit (Invitrogen, CA, USA) with slight modification (Travaillé et al., 2016). In brief, samples were mixed with 200 µl Lysis/Binding Buffer and followed by six freeze-thaw cycles (1 min in liquid nitrogen and 1 min in 65 °C water with 6 repeats). After 40 µl Dynabeads® Oligo(dT)$_{25}$ was added, samples were incubated at room temperature with continuous rotating at 15 rpm for 20 min. After washed with Buffer A and B, samples were mixed with 100 µl ice-cold Elution Buffer and incubated at 80 °C for 2 min. After incubation, the samples were immediately placed on the magnet, and supernatant containing mRNA was recovered.

2.2.2. PMA-(q)PCR assay optimization

The performance of PMA-qPCR assays for targeted protozoan parasites was evaluated before starting the spinach spiking experiments. After initial testing of assay sensitivity, a *Cryptosporidium* qPCR assay targeting a 159-bp region of the 18S rRNA gene (Hill et al., 2007) was chosen for PMA application. The *Giardia* PMA-qPCR assay targeting the β-giardin gene for assemblage B (Baque et al., 2011) that was used in a previous study (Alonso et al., 2014) did not sufficiently amplify *G. enterica* DNA in our study (data not shown). Thus, an alternative *Giardia* qPCR assay, the P241 primer-probe set, targeting the β-giardin gene for assemblage A and B (Guy et al., 2003) was used for PMA-qPCR application. To selectively fracture the robust oocyst wall of inactivated *T. gondii* while keeping live oocyst walls intact, a glass bead-beating method (Fritz et al., 2012) was tested with increasing beating times of 1-min intervals up to 4 min. A *T. gondii* qPCR assay targeting a 529-bp repeat element and with a target length of 162 bp (Opsteegh et al., 2010) was used to test the *T. gondii* PMA application.

PMA dye (20 mM in H_2O, Biotium, Inc., CA, USA) was diluted 10-fold with nuclease-free water, and aliquots of 25 µl were stored at −20 °C until use. Several PMA treatment conditions including PMA concentrations (25–150 µM), dark incubation time (5–60 min) and light exposure time (15 and 30 min) that have been reported in the previous studies were compared to optimize PMA application (Agulló-Barceló et al., 2014; Alonso et al., 2014; Brescia et al., 2009; Liang and Keeley, 2012; Rousseau et al., 2019; Vande Burgt et al., 2018). For *T. gondii*, bead-beating was tested to improve PMA penetration of oocyst walls of inactivated parasites. Upon addition of PMA, (oo)cysts in 100 µl suspension were incubated in the dark at room temperature followed by light exposure using the PMA-Lite™ LED Photolysis Device (Biotium, Inc., CA, USA). After PMA treatment, (oo)cysts were washed by adding 900 µl PBS followed by centrifugation at 16,000×*g* for 5 min. The supernatant was removed except for 100 µl, from which nucleic acids were extracted as described above.

TaqMan qPCR assays were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems™, CA, USA). Each 25-μl qPCR reaction contained 12.5 μl of TaqMan Environmental Master Mix 2.0 (Life Technologies Corporation, CA, USA), specified concentrations of primers and probes as described in Table S1, and 10 μl of nucleic acid extract, diluted from 3- to 9-fold in nuclease-free water. The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 55 °C (*C. parvum*) or 60 °C (*G. enterica* and *T. gondii*) for 60 s. The protozoa qPCR standard curves were constructed from seven 4-fold dilutions (1,000 to 0.24 (oo)cysts per reaction) of protozoa genomic DNA in four replicates. In addition, a nested *Giardia* PCR assay targeting 432 bp of the glutamate dehydrogenase (GDH) gene (Read et al., 2004) was compared to the performance of the *Giardia* qPCR assay for use with PMA.

2.2.3. RT-qPCR

To discriminate live from inactivated protozoan parasites, RT-qPCR assays targeting mRNA of heat shock protein 70 (hsp70), β-giardin and SporoSAG genes were performed for *C. parvum*, *G. enterica* and *T. gondii*, respectively (Guy et al., 2003; Travaillé et al., 2016). The oligonucleotides of primers and probes of each RT-qPCR assay and their final concentrations are listed in Table S2. The RT-qPCR was performed using the StepOnePlus Real-time PCR System (Applied Biosystems™, CA, USA). Each 25-μl mixture contained 12.5 μl Path ID Multiplex One-Step RT-PCR Buffer and 2.5 μl Multiplex Enzyme Mix (Life Technologies Corporation, CA, USA), specified amount of primers and probes (Table S2), and 5 μl of mRNA extract, diluted from 3- to 9-fold in nuclease-free water. The cycling conditions were 30 min at 50 °C and 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. To prepare standard curves, plasmids containing target DNA sequences were constructed for each assay by inserting target PCR amplicons into the PCR™4-TOPO vector of the TA Cloning® Kit for sequencing (Invitrogen, CA, USA). Plasmid DNA was extracted using the QIAprep Spin MiniPrep Kit (Qiagen, CA, USA) and then quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA). Serial dilutions of plasmid DNA were used in the range of 10^6–10^1 target gene copies per reaction in six replicates for standard curve construction (Fig. S1). The assay limits of detection (ALODs) for individual assays were determined based on a 99% confidence interval.

2.3. DFA/PI staining

The microscopic dye exclusion test using propidium iodide (PI) was evaluated to discriminate live and heat inactivated *C. parvum* and *G. enterica* (oo)cysts according to (Campbell et al., 1992), with modifications based on personal communication with Waterborne™ technical support staff. In brief, (oo)cyst suspended in 20 μl were mixed with 20 μl of the FITC antibody in the EasyStain™ kit (BTF Precise Microbiology, Inc., PA, USA) and 5 μl of 1 mg/ml PI. The mixture was vortexed for 20 s and incubated in the dark at room temperature for 20 min. After the incubation, 10 μl of stained (oo)cysts were loaded onto the 3-well SuperStick™ Slides (Waterborne™, Inc., LA, USA) without PI wash. After a cover slip was applied, the entire wet mounted well was observed using an Olympus BH-2 epifluorescence microscope equipped with a FITC and Texas Red multi-band filter set at × 200 within 2 h of slide preparation. Given that live (oo)cyst walls should be impermeable to PI, (oo)cysts appearing with green outline and red interior when stained by FITC antibody and PI, respectively, were considered dead (oo)cysts (Fig. S2).

2.4. Spinach spiking experiments

Two spiking experiments were conducted using spinach as a model leafy green to evaluate i) the limits of detection of viability assays using serial dilutions of live protozoa mixtures containing *C. parvum, G. enterica* and *T. gondii*; and ii) the ability of viability assays to discriminate live from inactivated parasites when mixed in different ratios (Fig. 1). Bagged and pre-washed spinach was purchased from a local grocery store, and we confirmed the absence of initial contamination of target parasites before the spiking experiments using multiplex PCR (Shapiro et al., 2019).

2.4.1. Sample limits of detection

Live parasite mixtures containing 10 to 1,000 (oo)cysts of *C. parvum, G. enterica,* and *T. gondii* were spiked on spinach leaves by pipetting small droplets (approximately 5 μl) about 10–20 times onto the surfaces of 10 g of spinach placed on individual weighing dishes (Fisher Scientific, CA, USA). PBS without parasites was spiked on spinach as a negative control. Five replicates were used at each spiking level. Following application of parasites, spinach samples were air-dried inside a biosafety cabinet at room temperature for 2 h. After droplets had dried, spinach leaves were placed in 18-oz Whirl-Pak bags (Whirl-Pak®, WI, USA) and rinsed with 100 ml of 0.1% Tween 80 by agitating the closed bags back and forth by hands for 2 min (Shapiro et al., 2019). The eluate solution was recovered from the Whirl-pak bag and transferred to new conical tubes. Samples were centrifuged at 900×*g* for 15 min at 4 °C to concentrate protozoa pellets, and then the supernatant was carefully removed to retain approximately 5 ml of pellet. The pellets were resuspended and aliquoted into 1 ml subsamples for each detection method including RT-qPCR, PMA-(q)PCR, DFA/PI staining and qPCR. The 1 ml aliquots were further centrifuged at 16,000×*g* for 5 min, and 100 μl pellets were retained for analysis. Five replicates were tested per method at each spike level.

2.4.2. Discrimination between live and inactivated parasites

Mixtures with different ratios of live and inactivated protozoa were prepared to obtain proportions of live parasites representing 0%, 25%, 50%, 75% and 100% of spiked (oo)cysts. Every spike level contained a total of 5,000 (oo)cysts of each parasite. Thus, for example, the 0% live sample contained 5,000 (oo)cysts of heat-inactivated (oo)cysts for each of the target parasites, while the 50% live samples consisted of 2,500 (oo)cysts of live and 2,500 (oo)cysts of heat-inactivated parasites for each protozoan. The live and inactivated (oo)cyst mixture was spiked on spinach surfaces and processed as described above in 2.4.1. RT-qPCR, PMA-(q)PCR and DFA/PI staining were conducted for viability discrimination. qPCR was also performed on extracted DNA to confirm the number of target (oo)cysts that had been spiked (Fig. S3).

2.5. Quality control

To minimize and monitor cross-contamination during sample analysis, multiple quality control measures were applied. Blank spikes using PBS without target parasites were used in all spiking experiments to assess cross-contamination during parasite spiking and processing. Extraction and reagent negative controls were processed through the entire analytical procedure in molecular analysis. Positive controls in (q) PCR and RT-qPCR consisted of target parasite DNA from concentrated stock solutions and plasmid DNA.

2.6. Data analysis

Statistical analyses were performed using STATA software (StataCorp LLC, TX, USA) to create probability curves for estimating the likelihood of parasite detection in the spinach spiking experiments. The probability was determined for each parasite by fitting experimental data to a logistic regression model. The binomial results of PMA-PCR analyses (presence/absence) were directly used in the regression while quantitative data in RT-qPCR and PMA-qPCR were converted to a binomial format for logistic regression models. Each regression model was evaluated using the Hosmer-Lemeshow goodness-of-fit test. Regression coefficients were then used to estimate the probability of parasite detection across a range of potential (oo)cyst contamination

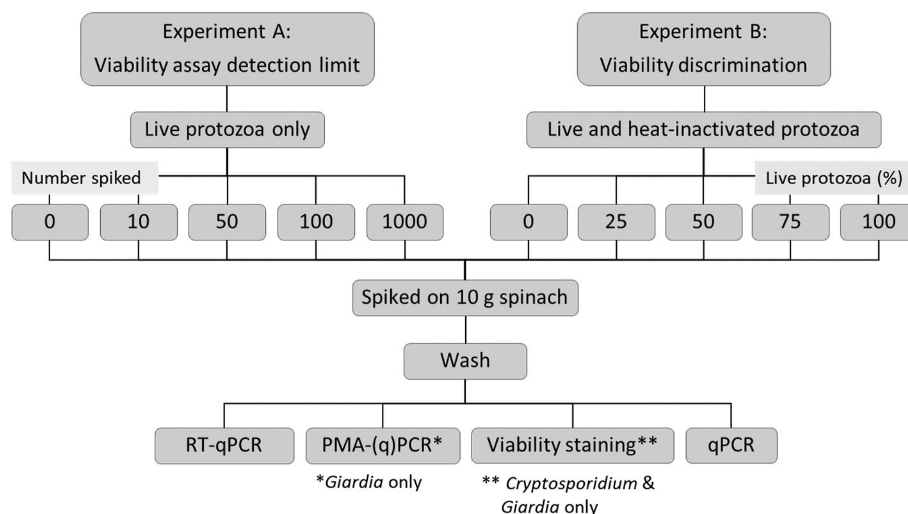

Fig. 1. Study design for the viability spiking experiments where spinach samples (n = 5) were spiked with serial dilutions of a mixture of live *Cryptosporidium parvum*, *Giardia enterica*, and *Toxoplasma gondii* (oo)cysts (Experiment A) or different ratios of live and heat-inactivated (oo)cysts (Experiment B).

levels per 10 g of spinach. Concentrations of protozoan parasites measured by RT-qPCR were compared using an ANOVA on Ranks test followed by Student-Newman-Keuls multiple comparison with significance level set at $P \leq 0.05$ in SigmaPlot (Systat Software, Inc., CA, USA).

3. Results

3.1. Evaluation of PMA-(q)PCR and RT-qPCR

We initially tested one PMA treatment condition (150 μM PMA, 5 min dark incubation, 15 min light exposure) used in the previous studies for viability discrimination of *Cryptosporidium* and *Giardia* (oo)cysts (see Section 2.2.2). Heat-inactivated *C. parvum* treated with PMA yielded 70% lower oocyst concentrations than the no-PMA control (heat-inactivated parasites amplified using qPCR without PMA treatment; data not shown). For assay optimization, several PMA concentrations (25–150 μM), dark incubation times (5–60 min) and light exposure times (15–30 min) were further tested. However, 20–30% of inactivated *C. parvum* oocysts were still amplified by PMA-qPCR. Due to relatively high false-positive amplification under the various conditions tested, PMA-qPCR for *C. parvum* was not further pursued in the spinach spiking experiments. To evaluate RT-qPCR for *C. parvum*, mRNA concentrations in serial dilutions of live and heat inactivated parasites (2–2000 oocysts in 100 μl PBS, single sample per dilution) were tested by RT-qPCR. The results showed that RT-qPCR could detect mRNA in as low as 1 oocyst per reaction and efficiently discriminate live from dead parasites; no mRNA was detected in inactivated oocysts except for the highest concentration. However, the mRNA levels detected in inactivated oocysts were about 200-fold lower than in live oocysts in these highest concentration samples. Thus, RT-qPCR was chosen as a viability method in the spinach spiking experiments. It should be noted that mRNA concentrations were calculated using plasmid-based rather than mRNA-based standard curves (Fig. S1). The average correlation coefficient (R^2) of several mRNA-based standard curves was 0.95 ± 0.03, while plasmid-based standard curves yielded $R^2 > 0.99$. Because of varying mRNA expression levels between parasite batches and low linearity of mRNA-based standard curves, plasmid-based standard curves were used for absolute quantification of target gene copies in spiking experiments.

For *Giardia*, PMA treatments showed sufficient suppression of DNA amplification from inactivated cysts, while live *G. enterica* amplification were not affected by PMA treatments (Fig. S4). Different PMA concentrations, dark incubation times and light exposure times did not show significant differences in assay performance. Therefore, 150 μM of PMA, 5 min of dark incubation and 15 min of light exposure time were chosen as the optimized condition for the spinach spiking experiments. When serial dilutions of live and inactivated *G. enterica* cysts in PBS were tested, RT-qPCR had relatively low sensitivity.

Different PMA concentrations were applied to suppress the amplification of inactivated *T. gondii* oocyst DNA; however, no significant reduction in DNA amplification was found with any PMA treatment (data not shown). The glass bead-beating method to open oocyst walls did not improve PMA penetration; and no considerable difference in threshold cycle (Ct) number was found between live and inactivated *T. gondii* oocysts when using PMA-qPCR regardless of bead-beating time (data not shown). One minute of bead-beating time was sufficient to fracture *T. gondii* oocysts as observed by microscopy; however, a majority of sporocysts inside the oocysts remained intact even after 4 min of bead-beating, presumably preventing PMA penetration into sporozoites. Thus, PMA-qPCR on *T. gondii* was not pursued in the spinach spiking experiments. RT-qPCR successfully detected mRNA from live *T. gondii* oocysts but not from inactivated oocysts when serial dilutions of parasites in PBS were tested. For live *T. gondii*, mRNA was detected in as low as 10 oocysts per reaction. Therefore, RT-qPCR was further applied in the systematic spinach spiking experiments.

3.2. Spiking experiment A – sample limits of detection

The first set of experiments served to evaluate the sensitivity of viability assays when applied to spinach spiked with serial dilutions of viable *C. parvum, G. enterica,* and *T. gondii* mixtures. RT-qPCR results showed that mRNA of *C. parvum* and *G. enterica* was amplified in two of five replicates when 10 (oo)cysts were spiked on 10 g spinach. However, *G. enterica* mRNA was detected in less than half of replicates across all dilutions except for the highest spike level (Fig. 2). *T. gondii* was consistently detected in more than half of the replicates at 50 oocysts per 10 g spinach or higher. No targeted mRNA was detected on spinach spiked with PBS as a negative control. Logistic regression applied to the RT-qPCR data indicated that viable protozoa could be detected 90% of the time when 16, 21 and 93 (oo)cysts of viable *C. parvum*, *T. gondii* and *G. enterica*, respectively, were present on 10 g spinach (Fig. 3). PMA-qPCR did not result in the detection of *G. enterica* DNA even at the highest spike level during 40 cycles of qPCR thermal cycles. In the nested PMA-PCR assay targeting the GDH gene, *G. enterica* DNA was detected in 3 of 5 replicates spiked with a 1000 (oo)cyst mixture (Table S3). DFA/PI staining showed that *C. parvum* and *G. enterica* were only detected on spinach spiked with the highest concentration of 1000 (oo)cysts per 10 g

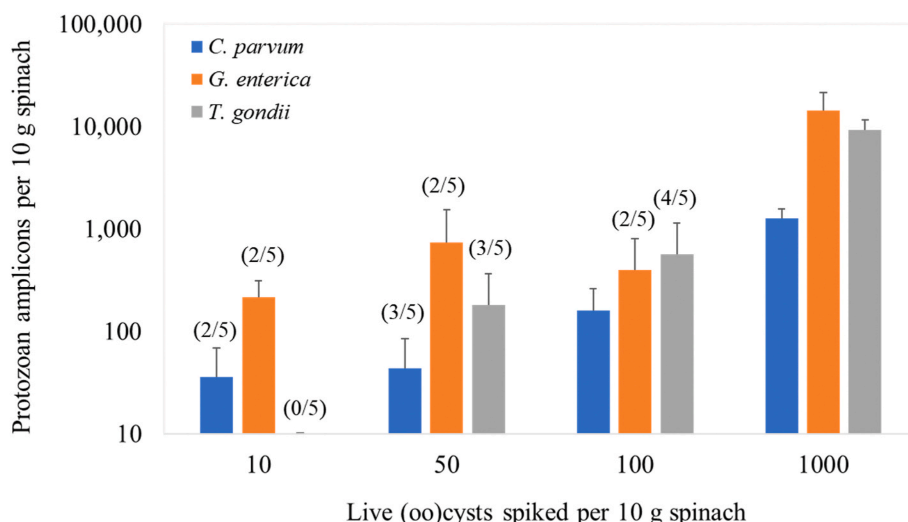

Fig. 2. Detection of serially diluted viable *Cryptosporidium parvum*, *Giardia enterica* and *Toxoplasma gondii* (oo)cysts spiked on spinach and measured by RT-qPCR (see Experiment A, Fig. 1). Non-detect values were assumed to be one-half of the sample limit of detection. Error bars represent the standard deviation of five replicates. For those cases where not all replicates were amplified, the number of detects/replicates is shown in parenthesis above each standard deviation bar.

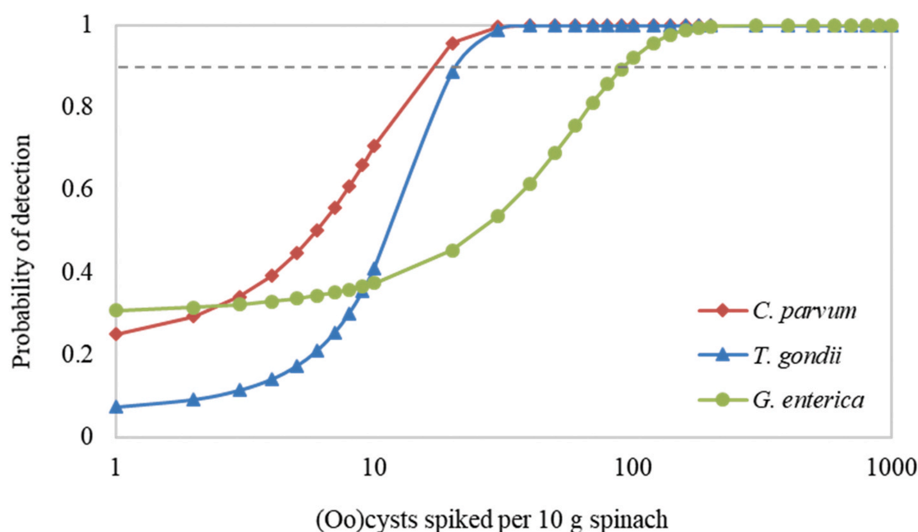

Fig. 3. Probabilities of detecting viable *Cryptosporidium parvum*, *Giardia enterica* and *Toxoplasma gondii* (oo)cysts in the absence of heat-inactivated protozoa via RT-qPCR assays (see Experiment A, Fig. 1). Probability curves were fitted using logistic regression coefficients obtained from the binarized RT-qPCR data. The dashed line indicates the 90% probability of viable parasite detection. Per 10 g of spinach, viable parasites could be detected with 90% likelihood at the following contamination levels: *C. parvum*, 16 oocysts; *T. gondii*, 21 oocysts; and *G. enterica*, 93 cysts.

(Table S4).

3.3. Spiking experiment B – discrimination between live and inactivated parasites

In the second experiment, spinach was spiked with mixtures of live and heat-inactivated parasites to evaluate the effect of different proportions of live and inactivated (oo)cysts on the performance of viability assays. RT-qPCR results showed false-positive mRNA amplification in three (*G. enterica* and *T. gondii*) and four (*C. parvum*) of five replicates when no viable protozoa were present and against a background of 5000 inactivated target (oo)cysts per 10 g spinach (Fig. 4). However, the concentrations of mRNA amplicons measured in samples containing 0% live (oo)cysts were significantly lower from those spiked with 25% or higher amounts of live (oo)cysts. The concentrations of mRNA amplicons measured in the samples containing 100% inactivated (oo)cysts were equivalent to 0.3–3% of the corresponding 100% live (oo)cyst samples. The mRNA amplicons of live *T. gondii* increased as the proportions of live oocysts increased while the trend was relatively less apparent for *C. parvum* and *G. enterica.* In PMA-qPCR analysis, no *G. enterica* DNA was amplified from the spinach spiked with 100% inactivated cysts, and three and four samples were amplified among five replicates tested in the spinach spiked with 75% and 100% live cysts, respectively (Table S5). Compared to PMA-qPCR, PMA-PCR results showed higher sensitivity with the detection of live *G. enterica* DNA. PCR amplification was observed in the samples spiked with 25% live (oo) cysts, while no amplification was found in samples containing no live parasites. In qPCR and PCR without PMA treatment, *G. enterica* DNA was equally amplified from all samples tested regardless of live and inactivated proportions. Detection of viable *G. enterica* on spinach in two viability spiking experiments demonstrated that RT-qPCR and PMA-PCR methods were more sensitive (110 and 220 cysts per 10 g of spinach, respectively) than PMA-qPCR (810 cysts per 10 g of spinach) (Fig. 5). DFA/PI staining showed that *C. parvum* oocysts stained with PI in spinach wash decreased as proportions of spiked inactivated parasites decreased (Fig. S5). However, up to 15% of PI negative oocysts were detected even when no live oocysts were spiked on spinach, resulting in overestimation of *C. parvum* viability. DFA/PI staining of *G. enterica* cysts was unsuccessful; the red PI staining was difficult to visualize or very faint in most spinach wash samples, thus hindering discrimination

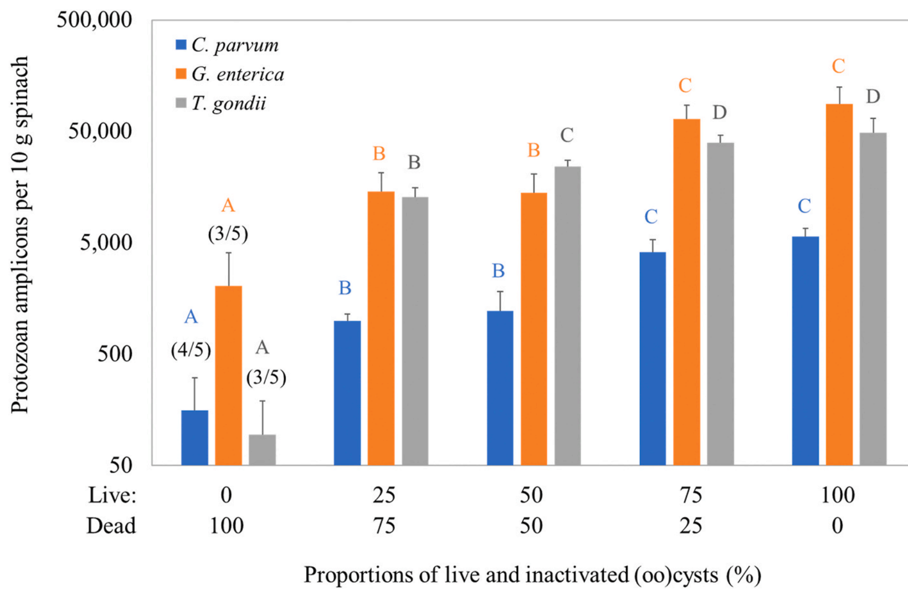

Fig. 4. Ability to discriminate live from inactivated (oo)cysts spiked on spinach and measured by RT-qPCR (see Experiment B, Fig. 1). Each mixture contained 5,000 (oo)cysts of *Cryptosporidium parvum*, *Giardia enterica*, and *Toxoplasma gondii* with defined ratios of live to inactivated (oo)cysts. Error bars represent the standard deviation of five replicates. Non-detect values were assumed to be one-half of the respective sample limits of detection. For those cases where not all replicates were amplified, the number of detects in replicates is shown in parenthesis above each standard deviation bar. Different letters above individual bars of the same color indicate significant statistical differences. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

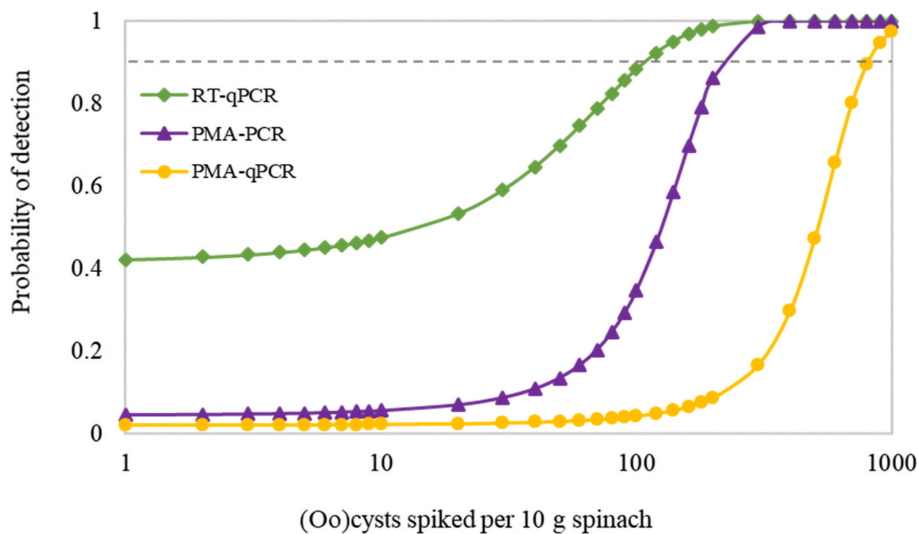

Fig. 5. Comparison of probability curves for detection of viable *Giardia enterica* in the presence of viable and inactivated cysts using viability assays based on RT-qPCR, PMA-PCR and PMA-qPCR. Data from serial dilutions of live cysts (see Experiment A, Fig. 1) and different ratios in mixtures of live/inactivated cysts (see Experiment B, Fig. 1) were combined for the regression to increase statistical power. The dashed line indicates the 90% probability of live cyst detection. Per 10 g of spinach, the estimated viable *G. enterica* cyst concentrations detected with 90% likelihood are: RT-qPCR, 110 cysts; PMA-PCR, 220 cysts; and PMA-qPCR, 810 cysts.

between live and dead cysts. Since these *G. enterica* data were difficult to interpret, the results are not reported.

4. Discussion

The application of viability assays for discriminating live from dead parasites on produce presents a fundamental advance in the field of produce safety. Detection of parasite DNA alone is not sufficient for making appropriate management and/or policy decisions that affect public health due to prolonged persistence of DNA in pathogens that are no longer viable. Insight on whether positive detection via screening tests is attributed to the presence of viable agents is imperative. We assessed three viability assays that have been applied to differentiate between live and inactivated protozoa for their application in leafy green commodities.

PMA application for viability discrimination of protozoan parasites including *Cryptosporidium, Giardia* and *Toxoplasma* has been reported in PBS and environmental waters (Agulló-Barceló et al., 2014; Alonso et al., 2014; Brescia et al., 2009; Liang and Keeley, 2012; Rousseau et al., 2019; Vande Burgt et al., 2018). In our study, tested conditions such as PMA concentration, incubation time, light exposure time, and/or bead-beating pretreatment did not significantly reduce DNA amplification from inactivated *C. parvum* and *T. gondii* oocysts. Approximately 20–40% of qPCR signals were still amplified from inactivated oocysts following PMA treatment, leading to overestimation of the viability of *C. parvum* and *T. gondii* by PMA-qPCR. It has been reported that amplicon size is an important factor affecting PMA-qPCR performance (Agulló-Barceló et al., 2014). The amplicon length used in our *Cryptosporidium* PMA-qPCR assay (159 bp) was comparable or longer to those (108–150 bp) of other assays (Agulló-Barceló et al., 2014; Alonso et al., 2014; Liang and Keeley, 2012). While previous studies on PMA-qPCR application for *C. parvum* showed about or over 90% reduction in DNA amplification in inactivated oocysts, the high DNA suppression might have resulted from relatively long protozoa inactivation time at high temperatures such as 30 min at 60–85 °C (Agulló-Barceló et al., 2014; Alonso et al., 2014; Liang and Keeley, 2012). It has been reported that DNA amplification of PMA treated oocysts decreased as heat inactivation temperature increased (Agulló-Barceló et al., 2014; Vande Burgt et al., 2018). During our pilot investigation, we found that large numbers of *C. parvum* oocysts were disintegrated and rarely visible after

heat inactivation at 80 °C for 20 min as counted by a hemocytometer. We surmise that intense heat inactivation conditions may have enhanced binding of the PMA dye to DNA in the previous studies. However, the goal of our study was to evaluate viability methods that can be applied for differentiating viable from dead parasites in contaminated leafy greens. Given that intact *C. parvum*, *G. enterica* and *T. gondii* (oo)cysts can remain as non-infectious particles in the environment, the heat inactivation methods least likely to cause (oo)cyst degradation were chosen in the current study to simulate as real conditions as possible. Previous studies on the effect of UV or ammonia on *C. parvum* viability also revealed that the viability of oocysts can be overestimated by PMA-qPCR when the inactivation methods do not induce membrane damage (Agulló-Barceló et al., 2014; Liang and Keeley, 2012). Our results supported the notion that PMA does not reliably suppress DNA amplification of inactivated *C. parvum* when oocysts are inactivated without direct membrane damage. For *T. gondii*, recent studies that evaluated the utility of PMA for viability discrimination showed that PCR signals were not sufficiently reduced under several tested conditions (Opsteegh et al., 2020; Rousseau et al., 2019), which is consistent with our finding. Therefore, PMA-qPCR was not further considered for *C. parvum* and *T. gondii* in the spinach spiking experiments, and viability discrimination by PMA-(q)PCR was only applied for *G. enterica.* In the present study, we used heat treatment to inactivate viable (oo)cysts under conditions described in the literature (Travaillé et al., 2016). The temperature and time chosen for inactivating our target pathogens met or exceeded those reported to reliably render these pathogens non-infectious. While heat inactivation is one of the most commonly used methods to inactivate protozoa in the laboratories, the heat inactivation conditions that we applied did not appear to damage the (oo)cyst walls. Hence, PMA application to (oo)cysts treated by other forms of inactivation methods such as intense heat treatment, irradiation and chemicals could yield different results depending on their effect on (oo)cysts wall integrity.

When serial dilutions of viable *C. parvum*, *G. enterica,* and *T. gondii* (oo)cyst mixtures were spiked on spinach, RT-qPCR showed higher sensitivity for parasite detection as compared to PMA-(q)PCR and DFA/PI staining. *C. parvum* and *T. gondii* oocysts could be detected with 90% likelihood at 2 oocysts/g spinach (in 10-g samples), and *G. enterica* cysts could be detected at 9 cysts/g spinach when measured by RT-qPCR. A previous study using RT-qPCR for the detection of *C. parvum, G. enterica* (called *G. intestinalis* in the reference) and *T. gondii* from experimentally contaminated basil reported 3 (oo)cysts/g basil (Travaillé et al., 2016), which yielded comparable results with the present study. The relatively low sensitivity of DFA/PI staining in the present study can be attributed to a maximum volume of suspension that could be fit per slide well. Only 10 μl of spinach wash/fluorescent dye mixture (representing ca. 5% of the total spinach wash) could be loaded without spillage when a cover slip was applied. As DFA/PI staining was observed immediately without further drying, it was impractical to analyze entire pellets in multiple wells given the number of replicates (5) and spike levels (4) in the spiking experiment.

When both viable and heat-inactivated protozoa (oo)cysts were spiked on spinach, low levels of mRNA were detected from the 0% live (oo)cyst samples in the presence of high concentrations of inactivated (oo)cysts. Similar results were obtained by Travaillé et al. (2016) when quantifying mRNA of heat-inactivated *C. parvum*, *G. enterica* (called *G. intestinalis* in the study) and *T. gondii*. While 10^4 heat-inactivated (oo) cysts were not infectious as determined via mouse bioassays, mRNA signals were still detected. Our results could also reflect remnants of mRNA detected in dead parasites. Although PMA-PCR provided relatively lower sensitivity than RT-qPCR in our study, the PMA-PCR assay for the detection of live *Giardia* can be useful, especially when a simpler and more affordable method is preferred in viable *Giardia* monitoring. DFA/PI staining had lower sensitivity than the molecular assays, and the discrimination of live from inactivated *C. parvum* and *G. enterica* (oo) cysts in spinach wash was less clearly visualized compared to those in simple matrix such as PBS. Thus, this microscopy-based approach may not be as efficient for viability determination of protozoa on leafy greens. To the best of our knowledge, our study is the first to compare the application of molecular viability assays including RT-qPCR and PMA-(q)PCR and DFA/PI staining for detection of viable protozoan pathogens in a fresh produce commodity. Our results demonstrate that RT-qPCR can effectively discriminate viable from inactivated *C. parvum, G. enterica* and *T. gondii* (oo)cysts on leafy greens. In our previous study, a multiplex PCR could simultaneously detect 1–10 (oo)cysts of *C. parvum*, *G. enterica*, *T. gondii* and *C. cayetanensis* per g of spinach (Shapiro et al., 2019). The multiplex PCR approach can serve as a rapid and sensitive screening tool to determine if protozoan contamination is present. We propose a monitoring strategy that combines the multiplex PCR assay with viability assays to improve detection of potentially infectious pathogens on produce.

One limitation of our study is that the viability assays were applied using a relatively small quantity of spinach (10 g) due to limited space available for conducting studies on organisms considered as biosafety level 2 pathogens. In our investigation, priority was given to performing the spike experiment at the same time across multiple concentration levels (4–5 dilutions) and replicates (n = 5) to avoid variables caused by time differences between samples. Most prior spiking studies for detection of protozoan pathogens in produce used larger amounts ranging from 25 to 250 g (summarized in Shapiro et al., 2019). Assuming the same (oo)cyst recovery efficiency when larger quantities of produce are processed than the 10 g used in our experiments, limits of detection per g of produce would likely decrease. For example, there was no significant difference in protozoan recovery efficiency from 1 g versus 25 g of seeded leafy greens (Shields et al., 2012), and hence the probability of detection should be higher in the larger sample size at low overall (oo)cyst numbers. However, an increase in sample size may also result in more inhibitors being present in nucleic acid extracts, leading to an increase in limits of detection of parasites per g of leafy greens. Further studies are needed for validation of viable pathogen detection methods on larger volumes of produce that represent a consumed meal or purchased product.

5. Conclusions

Contamination of fresh produce with disease causing microorganisms is a significant and global health problem. Optimizing molecular methods for detection of key protozoan pathogens on produce is an essential component for developing a comprehensive toolkit with the aim of identifying and managing health risks associated with consumption of fresh produce. The application of RT-qPCR assay can detect 2–9 live parasites per gram of artificially-contaminated spinach and discriminate live from dead protozoan (oo)cysts. Applying viability assays in field surveillance investigations is a prerequisite for estimating contamination due to live versus dead parasites and, thus, can aid accurate modeling efforts to predict the risk of illness to consumers and to guide more effective risk management practices. Application of our monitoring strategy is not limited to leafy green commodities, and the total number of potential beneficiaries extends to growers, harvesters and processors of other specialty crops potentially contaminated with protozoan pathogens and associated health risks to consumers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103816.

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