Mitochondrial DNA Fragmentation to Monitor Processing Parameters in High Acid, Plant-Derived Foods

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Abstract: Mitochondrial DNA (mtDNA) fragmentation was assessed in acidified foods. Using quantitative polymerase chain reaction, C_t values measured from fresh, fermented, pasteurized, and stored cucumber mtDNA were determined to be significantly different ($P > 0.05$) based on processing and shelf-life. This indicated that the combination of lower temperature thermal processes (hot-fill at 75 °C for 15 min) and acidified conditions (pH = 3.8) was sufficient to cause mtDNA fragmentation. In studies modeling high acid juices, pasteurization (96 °C, 0 to 24 min) of tomato serum produced C_t values which had high correlation to time-temperature treatment. Primers producing longer amplicons (approximately 1 kb) targeting the same mitochondrial gene gave greater sensitivity in correlating time-temperature treatments to C_t values. Lab-scale pasteurization studies using C_t values derived from the longer amplicon differentiated between heat treatments of tomato serum (95 °C for <2 min). MtDNA fragmentation was shown to be a potential new tool to characterize low temperature (<100 °C) high acid processes (pH < 4.6), nonthermal processes such as vegetable fermentation and holding times of acidified, plant-derived products.

Keywords: high-acid foods, mitochondrial DNA, quantitative PCR, thermal processing

Practical Application: This method could be used as a tool for hot-fill process validation, monitoring of vegetable fermentations, and holding times of acidic plant-derived products. Mitochondrial DNA is universally found and easily extracted from all plant products. Processors will be able to validate processes and track process deviations using rapid molecular methods. Processors can use this presumptive test prior to shipping out a product.

Introduction

High acid foods such as fruit and some vegetable juices require a 5-log reduction of the pertinent pathogen (US FDA 2001). This is defined as the most resistant microorganism of public health significance that is likely to occur in the juice. Furthermore, this reduction should remain in effect for a period at least as long as the shelf-life of the product when stored under normal and moderately abusive conditions (US FDA 2001). *Escherichia coli* O157:H7 is widely considered as the target pathogen in high acid foods such as fruits and acidified vegetables, due to its acid-resistance, tolerance of organic acids, and ability to survive under refrigerated storage conditions for extended periods (Zhao and others 1993; Miller and Kaspar 1994; Koodie and Dhople 2001; Mak and others 2001). Many producers utilize thermal processing as a critical control point to assure a 5-log pathogen reduction. Acid and acidified foods do not require intensive heat treatment (>100 °C) since the combination of heat and low pH are lethal to target pathogens (US FDA 2001). Some examples of high-acid, low temperature thermal processes that have been reported to achieve a 5-log reduction of *E. coli* O157:H7 in acidified foods include the following: pasteurization of apple cider at 71.1 °C for 6 s for most apple varieties (New York State Department of Agriculture and Markets 1998); pasteurization of Wisconsin apple cider at 68.1 °C to 80.9 °C for 14 s (Mak and others 2001); pasteurization of acidified cucumber products at 65 °C, for <1.7 min at pH 4.1 (Breidt and others 2005). Although, Breidt and others (2005) determined that a 5-log pathogen reduction of *E. coli* O157:H7 may be achieved in acidified vegetable brines at 65 °C for a maximum of 1.7 min, at pH 4.1, typical commercial processing times are between 70 °C and 80 °C for up to 15 min. The end result is that acidified vegetables are thermally overprocessed. Linear models defined for acidified vegetable products (4.1 > pH > 3.3) were used to determine that a 5-log pathogen reduction of *E. coli* O157:H7 may be achieved by processing above 83 °C for a maximum of 6 s (Breidt and others 2010).

While researchers have demonstrated that *E. coli* O157:H7 can survive in tomato products, with increased survival under refrigeration (Eribo and Ashenafi 2003), other potential health hazards exist. Spoilage organisms such as *Bacillus coagulans* and *Alicyclobacillus* spp. are frequently isolated from spoiled canned vegetables (Bevilacqua and Corbo 2011; Peng and others 2012) and may increase the pH of acidic foods, thereby creating a food safety hazard. Thermal processing treatments for 5-log reduction of pathogens are inadequate for elimination of spoilage spore-forming organisms such as *Bacillus* and *Alicyclobacillus* (ACB) spp. *Alicyclobacillus acidoterrestris* spores were able to survive hot fill protocols used to process juices (2 min at 88 °C to 96 °C; Pontius and others 1998).

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Splittstoesser and others (1994) reported *D* values of 2.4 to 2.8 min at 95 °C for *A. acidoterrestris*. Therefore, hot-fill protocols for fruit juice may produce less than l-log reduction for these thermophilic, acid-resistant spores.

Fermented foods such as cucumber pickles use salt brine (7% NaCl) to promote growth of native lactic acid bacteria (LAB), which lower pH and inhibit growth of pathogens and sporulation of toxin-producing spores. This fermentation/brining process is performed in large outdoor tanks (12500 to 38000 L) and bulk storage of the fermented goods may last from several months to over 1 y depending on production schedule. Temperatures vary by climate and season, but pH values rapidly decline to the acidic range (pH < 4.6) due to lactic acid production by native microbiota, usually *Lactobacillus plantarum* (Borg and others 1955; Costilow and others 1956). After hot-fill pasteurization, pickle products such as hamburger dill chips are stored at room temperature and have an estimated shelf-life of 2 y if unopened. At present, fermentations are monitored by determining only pH and shelf-life is estimated from these measurements.

Above 100 °C, DNA can become destabilized and permanently damaged. However, at temperatures less than 100 °C, DNA is not severely degraded (Gryson 2010). The polymerase chain reaction (PCR) cycles DNA in the range of 60 °C to 95 °C to denature, anneal, extend, and amplify the molecule with no apparent damage. However, PCR is performed at neutral pH (7.4). Earlier work from this laboratory demonstrated a high correlation between *Geobacillus stearothermophilus* spore death in hot oil bath treatments and cumulative *F* value in retort processes (Caldwell and others 2015), using mitochondrial DNA (mtDNA) fragmentation as a time-temperature integrator (TTI) for low acid, high temperature processes (121 °C). The present report examines the use of mtDNA fragmentation as a novel molecular TTI for high-acid, low-temperature (pH < 4.6; temperature <100 °C) processes for fruit juices and fermented vegetables. The utility of the protocol to characterize cucumber fermentations and tank holding times was also evaluated.

Materials and Methods

Cucumber-to-pickle processing

In order to assess mtDNA fragmentation in acidified vegetables, fermented, fermented/pasteurized, and stored pickles were taken from commercially processed jars from different lots. The cucumbers were processed as follows. Further details of the processing protocol can be found in Wolter (2013). Cucumbers of size 2B (32 to 38 mm diameter) or 3A (39 to 51 mm diameter) were fermented in 6% NaCl in open-top 38000 L plastic tanks in a commercial tank yard. Cucumbers were collected from at least 3 feet below the surface of the tank, and then sliced into 3 to 6 mm hamburger dill chip rounds. Chips were processed in 16-oz glass jars using a 58:42 cucumber to brine packout ratio, pasteurized at 75 °C for 15 min, and stored at room temperature (23 ± 2 °C) under ambient lighting for 2 or 20 mo. Untreated control samples were sliced, fresh cucumbers. Autoclaved treatments were fresh samples heated at 121 °C for 20 min in a Steris Autoclave (SG 3021) under standard conditions. All treatments were performed in triplicate; for fresh, an independent replicate was 1 cucumber; for processed pickles, a chip from 1 jar. Punch samples were taken from all cucumber/pickle products by inserting a 1000 μL pipette tip into the mesocarp to obtain a 5 to 10 mg circular slice. DNA was extracted by the MasterPure DNA extraction kit (Epicentre Biotechnologies, Madison, Wis., U.S.A.) and quantitative PCR (qPCR) run in duplicate wells as described later in the manuscript.

Pasteurization of tomato serum

This experiment compared a reduction curve of an *E. coli* O157:H7 cocktail to C_t values from intrinsic mtDNA in tomato serum at different times (0, 0.25, 0.5, 1.0, and 2.0 min) under a pasteurization-type thermal process of 95 °C. Three fresh tomatoes (Redsun variety, Mexico 4664) were processed in a blender (Waring Laboratory Science, Torrington, Conn., U.S.A.) at medium speed until homogeneous to create tomato puree. Three aliquots of puree were frozen at −20 °C for later use. The remainder of the puree was placed in 330-μm filter bags (Nasco Whirl-pak, Fisher Thermo Scientific, Atlanta, Ga., U.S.A.) and stomached (Seward Stomacher 400; Tekmar, Cincinnati, Ohio, U.S.A.) at low speed for 30 s. Aliquots of tomato serum were obtained from filtered side of bag. The pH of the filtered tomato serum was 4.26 ± 0.1 (Accumet AR25, Fisher Thermo Scientific). The serum was frozen at −20 °C until use. Five cultures of *E. coli* O157:H7 (FSRU culture collection B200-204) were cultured separately at 37 °C, at 200 rpm overnight in LB broth (Becton Dickinson, Sparks, Md., U.S.A.). Cultures were concentrated 1:10 by centrifugation (6000 rpm, 4 °C, 10 min; Sorval RC-1, Spectrofuge, Asheville, N.C., U.S.A.) and combined the day of the trial to create an inoculum cocktail pellet. A time-temperature experiment was conducted using a thermal cycler (BioRad; Heracles, Calif., U.S.A.) as a constant, programmable heat source. Come up time (CUT) to 95 °C was 36 s, determined empirically using the thermal cycler internal sensor. A total of 100 μL of tomato serum was added to cocktail pellets, providing 10^9 cells/mL. Another 100 μL tomato serum was processed separately to provide intrinsic mtDNA. Tests were conducted in 0.1 mL PCR tube strips with 3 tubes per treatment per temperature. The entire reduction curve, from 0 to 2 min, was run in triplicate on 3 different days. After the target time was reached, tubes were plunged into ice water slurry and cooled to room temperature. Tomato serum was stored at 4 °C until DNA extraction and qPCR.

Plate counts for tomato serum pasteurization

E. coli O157:H7 cells were enumerated using a simplified agar plate technique (Jett and others 1997) with square petri dishes and LB agar (Becton Dickinson; Pittsburg, Pa., U.S.A.). This simple drip-dilution method had a lower detection limit than that of automated spiral plating methods. After 24 h incubation at 37 °C, colonies were enumerated manually. The lower detection level is 10^2 CFU/mL. C_t values from tomato serum intrinsic mtDNA were compared to plate counts following exposure at 95 °C at different intervals (that is, 0, CUT, 0.25, 0.5, 1.0, and 2.0 min).

Tomato serum hot-fill process

To quantify mtDNA fragmentation in a high acid product at a relatively low temperature, we conducted a laboratory-scale hot-fill process on tomato serum and calculated its *D* value at 96 °C. Tomato puree and serum was obtained from frozen aliquots from the study above. Using a thermal cycler (BioRad) as the heating element, a time-temperature trial was conducted. A total of 200 μL filtered tomato serum was added to three 0.2 mL PCR tubes for each time point. CUT to 96 °C was 30 s, determined empirically using the thermal cycler internal sensor. Serum samples were run for 0, 2, 4, 8, 16, 24, and 48 min using 3 tubes per replicate. After the target time was reached, tubes were plunged into ice water slurry and cooled to room temperature. Tomato serum

was stored at 4 °C until DNA extraction and qPCR. qPCR was run and C_t values converted to $\log_{10}$ copy number to determine D values of tomato serum at 96 °C.

DNA extraction

DNA from cucumber and pickle mesocarp samples or tomato serum (5 to 10 mg or mL) was extracted using the MasterPure DNA purification kit (Epicentre Biotechnologies; Madison, Wis., U.S.A.) according to the manufacturer's recommendations for plant tissues. DNA samples were analyzed by spectrophotometer (Nanodrop, Wilmington, Del., U.S.A.) for quantity and quality. For qPCR, DNA was normalized by concentration, and diluted to deliver 5 to 10 ng/μL per reaction.

Standard curve

A standard curve for the 1016-bp amplicon was generated using gBlocks™ Gene Fragments (https://www.idtdna.com/pages/products/genes/gblocks-gene-fragments), which are double-stranded, sequence-verified oligonucleotides of the *atp*1 gene from ATP synthase subunits. Ten-fold serial dilutions of *atp*1 copies (10^9 to 10^1) were performed and PCR amplification efficiency (E) was determined using the slope of the standard curve:

$$E = (10^{-1/slope}) - 1$$

Data analysis of the qPCR standard curve was performed using goodness-of-fit linear regression correlation coefficient (R^2).

Measurement of mtDNA fragmentation (qPCR)

qPCR was performed in 25 μL total volume with 2X IQ SYBR Green supermix (SYBR Green I dye, 50 U/mL iTaq DNA polymerase, 0.4 mM each of dATP, dCTP, dGTP, and dTTP, 6 mM $MgCl_2$, 40 mM Tris-HCl, pH 8.4, 100 mM KCl, and 20 nM fluorescein (BioRad)), 300 nM final concentration each for 174F and 174R primers or 500 nM final concentration each for 1016F and 1016R primers, cucumber, pickle, or tomato serum DNA (5 to 10 ng/reaction) or 1 μL Gblock™/citric acid solution and qPCR water (Ambion, Austin, Tex., U.S.A.) to final volume (25 μL). qPCR amplifications were performed in a MyiQ (Bio-Rad) thermal cycler with the following conditions for 174 bp amplicons and Gblock™/citric acid solutions: 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; with FAM channel optics "on" during annealing stage. For longer amplicon at 1016 bp, qPCR amplifications were performed under slightly different conditions: 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 120 s; with FAM channel optics "on" during annealing stage. No template control and positive controls were used for all assays. A positive control was used to normalize data between assays and to manually adjust the threshold baseline. For a sample to be considered positive, its C_t value must be less than all negative control reactions, and the corresponding amplification curve has to exhibit the 3 distinct phases of real-time PCR: lag, linear, and plateau.

Statistical analyses

All ANOVA statistical analyses were performed using SAS (Version 9.3, SAS Institute, Inc., Cary, N.C., U.S.A.). For the tomato serum pasteurization, the MIXED procedure was used to analyze this fully randomized study with 18 reps and 6 treatment times. The treatments were considered to be statistically different if $P < 0.05$. Simple linear regressions were performed and goodness-of-fit regression correlations determined using Excel (Microsoft; Redmond, Wash., U.S.A.).

Results and Discussion

Monitoring of cucumber-to-pickle processes

Fragmentation of intrinsic cucumber mtDNA was evaluated as a method to monitor commercial cucumber-to-pickle processes. Fermented, fermented/pasteurized, and stored finished pickles were taken from jars having the same lot number. DNA was extracted and compared to fresh and fresh/autoclaved cucumber DNA (not from the same lot) by analyzing C_t values of the *atp1* gene. The C_t values of 4 processes were compared to those for fresh cucumber (Figure 1). All treatments were significantly different from the fresh cucumber control ($P < 0.05$). The fermented cucumber (process 2) had an increase of 4 C_t compared to fresh cucumbers (process 1), indicating significant mtDNA fragmentation caused by acidification of the brine and possibly other factors inherent in the growth of LAB. Process 3 (Figure 1) contained pickles, which were fermented, pasteurized, and stored for 2 mo at room temperature in 16 oz glass jars. Process 3 had C_t values, which were significantly higher than those for fresh and fermented cucumbers. However, process 3 had almost the same C_t value (*ca.* 30) as compared to process 5, the fresh autoclaved cucumber. This indicates parity between 2 very different thermal processes: an acidic hot-fill process (75 °C for 15 min) and autoclaving under pressure (121 °C). Because processes 3 and 4 differ by only time of storage, mtDNA fragmentation may be useful as a measure of shelf-life in jarred, finished, acidic vegetables. Process 4 (fermented and stored for 20 mo) had significantly higher C_t values than process 3 (fermented and stored for 2 mo); a difference of 18 mo. Twenty months is near the projected end of shelf-life (2 y) for this particular product. Only 1 study (Xu and others, 2009) was found in the literature, which considered mtDNA as a tool to quantify decomposition over time. The goal of the study was to determine the end point of carcass composting prior to its use as fertilizer on crop land. Future studies in our laboratory will examine smaller gradations in storage time of acidic vegetables to determine the usefulness of this protocol for shelf-life monitoring and end-of-shelf-life projections.

Hot-fill processing

To quantify mtDNA fragmentation in a high acid product at a relatively low temperature, we conducted a laboratory-scale hot-fill process on tomato serum to calculate its D value at 96 °C. According to Pontius and others (1998), most fruit juices are high acid (pH < 4.6) and a hot-fill and hold of 2 min at 88 °C to 96 °C is used to pasteurize them. We extended the time of the process because Pontius and others (1998) reported the D value of the spoilage organisms of concern, *Alicyclobacillus* spp. (ACB), as 8 to 9 min at 97 °C. Therefore, it was noted that commercial hot-fills would not eliminate even low concentrations of this spoilage microorganism. This thermophilic, acid-resistant spore former can not only produce off-flavors, but increase the pH of infected juices. This could cause a public health hazard by allowing toxin-producing spores, such as *Clostridium botulinum*, to grow during extended storage. *Bacillus coagulans* has a similar D value to ACB (Peng and others 2012). The goal of this experiment was to compare the D value of tomato serum mtDNA fragmentation to that of ACB and *B. coagulans* at a commercial pasteurization temperature. The tomato serum was heated to 96 °C for up to 24 min (Figure 2). This represents *ca.* 3-log reduction of ACB.

MtDNA fragmentation was measurable for the duration of the thermal treatment and C_t values increased numerically from 17 to 24 units. C_t values were converted to $\log_{10}$ copy numbers using the linear relationship determined empirically from the standard curve of the 174 bp amplicon (Caldwell and others 2015):

$$y = -3.1909x + 38.091$$

where y is the C_t value and x is the $\log_{10}$ copy number. The D_{96} value of the tomato serum mtDNA fragmentation was determined using the reciprocal slope of the line and was calculated as 11.63 min (Figure 3). This value is similar to the D_{121} for mtDNA fragmentation from sweet potato puree heated in a hot oil bath (11.3 min) using the same universal plant amplicon (Caldwell and others 2015). Since the D_{96} value exceeds that of ACB and is still measurable after at least a 3-log reduction of the spoilage organism, this molecular approach may have a place in commercial hot-fill monitoring and validation.

E. coli O157:H7 inactivation in acidic foods

This experiment compared the inactivation of *E. coli* O157:H7 seeded in tomato serum to C_t values from fragmented mtDNA, intrinsic to the same serum, at different times at a pasteurization temperature of 95 °C (Table 1). A 5-log reduction of *E. coli* O157:H7 was seen in 15 s or less (Figure 5). This reduction time was slightly longer than the predicted values published by Breidt and others (2010). In their report, 5-log reductions of *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains should occur in less than 6 s at a pH of 4.1 or below. In our study, the tomato serum pH was higher, 4.26, which could account for the small discrepancy. Looking at spoilage microorganisms, yeasts were cultured serendipitously from the tomato serum and proved very hardy. There were yeast counts (log 2.0 CFU/mL) from 0.5 to

Table 1–Tomato juice mtDNA fragmentation at 95 °C using the 174 bp amplicon protocol.

Time (min)	Mean C_t
2.0	21.20[a]
1.0	20.01[b]
0.25	20.00[b]
CUT	19.92[b]
0	19.72[b]
0.5	19.65[b]

$P < 0.05$.

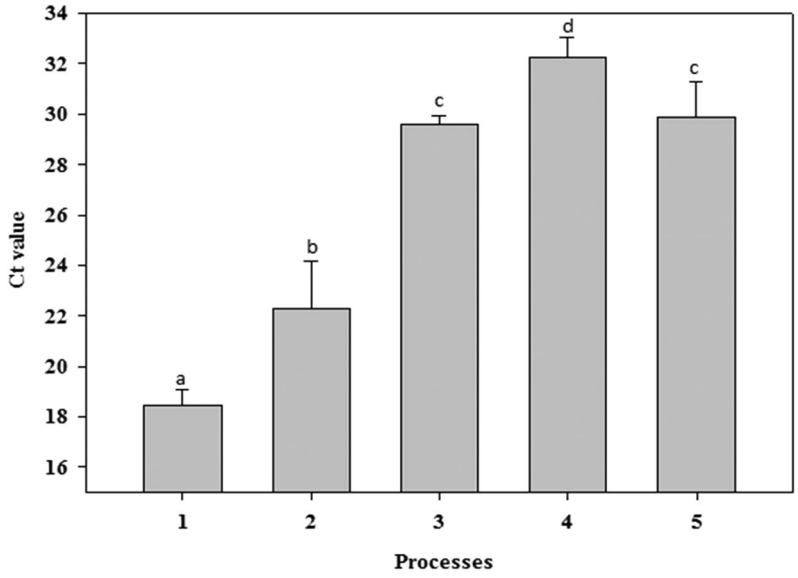

Figure 1–Comparison of mtDNA fragmentation as measured by C_t value during pickle processing and storage. 1, fresh cucumber; 2, fermented cucumber (6% NaCl), 3, fermented (6% NaCl), pasteurized (75 °C; 15 min), 2 mo storage at room temperature (RT) with ambient lighting; 4, fermented (6% NaCl), pasteurized (75 °C; 15 min), 20 mo storage at RT with ambient lighting; 5, fresh, autoclaved cucumber (121 °C). $P < 0.05$.

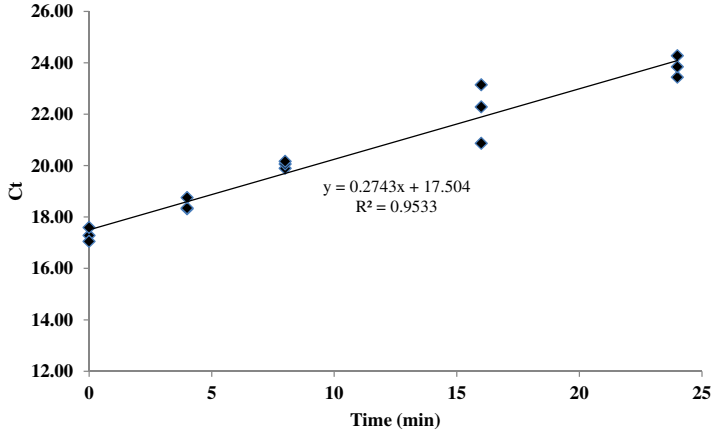

Figure 2–Tomato juice mtDNA fragmentation as measured by C_t value at 96 °C.

2 min at 95 °C; long after pathogenic *E. coli* had been eliminated (data not shown).

When C_t values were statistically analyzed, there was no significant difference in C_t values for 0, CUT, 0.25, 0.5 and 1 min ($P < 0.05$; Table 1). The mean C_t values for 2 min were significantly higher than that for other times. We observed that the C_t values were not sensitive enough to enumerate the 5-log reduction of pathogens in the serum. However, it was indicative of total pathogen kill (9-log reduction), which was achieved at 2 min (Figure 5). The previous hot-fill assay with tomato serum exhibited a high correlation with C_t values and time-temperature treatment at the same pH at 96 °C ($R^2 = 0.95$; Figure 2). However, the time points of this previous assay were much greater than 2 min: 4, 8, 16, and 24 min.

Longer qPCR amplicons might yield more sensitive results as they have a higher probability of fragmentation. The amplicon length used for the initial assay was 174 bp (Table 1). Primers which produced a 1016 bp amplicon from the same mitochondrial gene were tested to see if increased length would also increase sensitivity. The longer amplicon did increase the sensitivity from 2 min to 0.5 min ($P < 0.05$; Table 2). However, there was no significant difference between the C_t values of unheated tomato serum and the 0.25 min heated product, nor the one held for CUT. There was a significant difference between the C_t values of the 0.5 and 2 min treatment, but no difference between the treatments for 0.5 and 1 min at 95 °C.

Table 2–Tomato juice mtDNA fragmentation at 95 °C using the 1016 bp amplicon protocol.

Time (min)	Mean C_t
2.0	25.54[a]
1.0	21.28[b]
0.5	21.17[b]
CUT	21.04[bc]
0.25	20.82[bc]
0	20.25[ca]

$P < 0.05$.

Protocols for qPCR generally use amplicons less than 200 bp due to the kinetic limitations of the rapid process. An amplicon of over 1000 bp approaches the limits of the assay. The standard curve for the 1016 bp amplicon had a linear range of 10^4 to 10^9 $\log_{10}$ copy numbers, a limit of detection of 10^4,

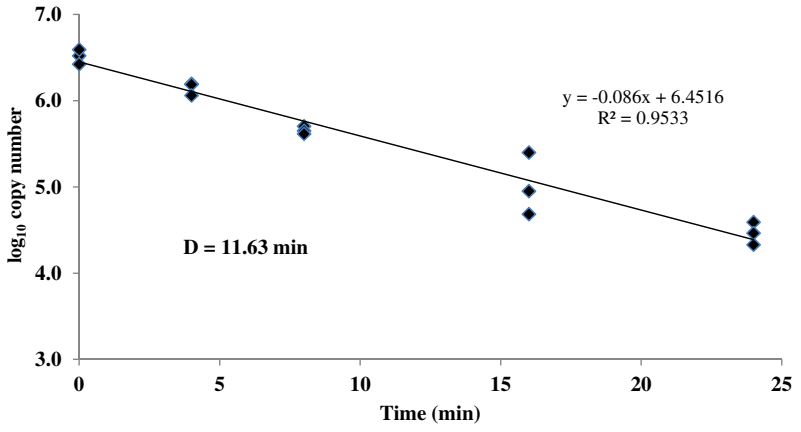

Figure 3–Calculation of tomato serum D_{96} value.

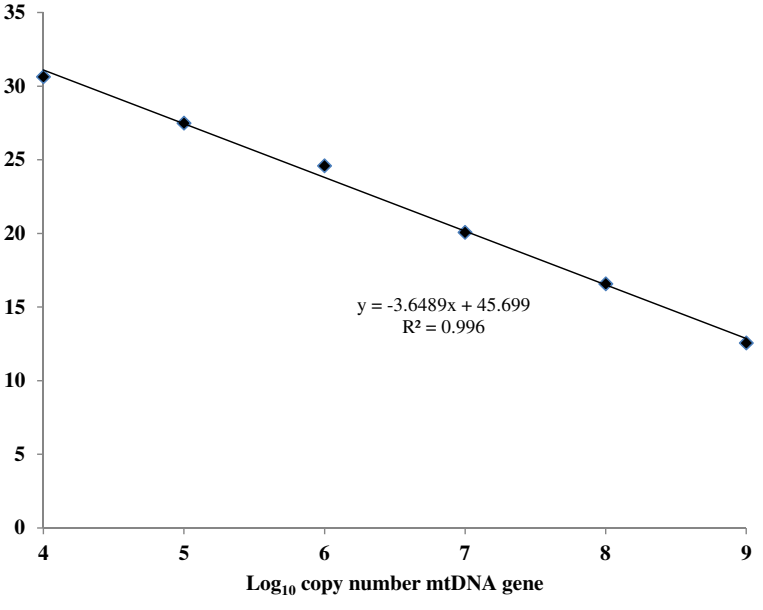

Figure 4–Mitochondrial DNA standard curve for 1016 bp amplicon.

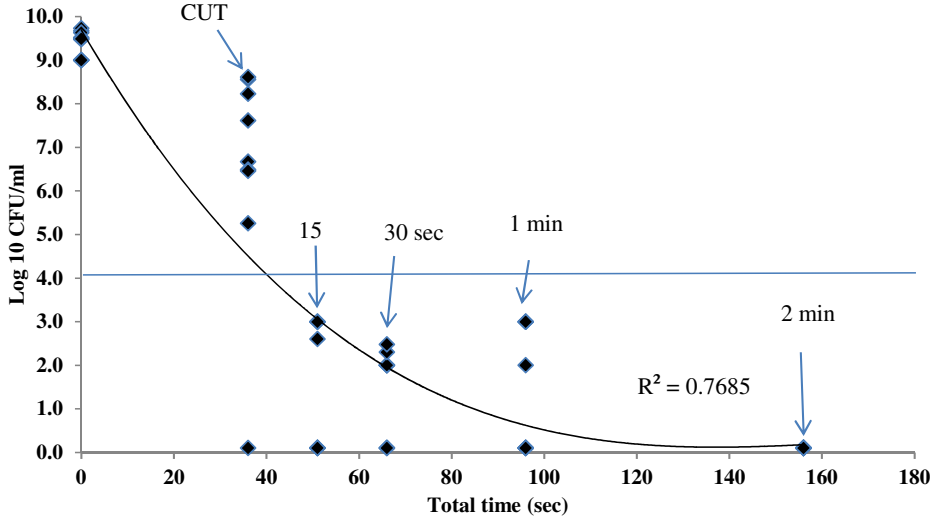

Figure 5–*E. coli* O157:H7 reduction at 95 °C in tomato juice.

and an amplification efficiency of 88% (Figure 4). The amplification efficiency was too low to meet MIQE standards of 90% to 110% for qPCR (Bustin and others 2009), but data can be used if the C_t values are in the empirically determined linear range. The limit of detection (10^4 copy numbers) was high and could limit the usefulness of this assay. Researchers in past studies have used longer qPCR amplicons in heat treatments and have reached conclusions without validating with Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) standards (Hird and others 2006). These researchers measured the effect of amplicon length during thermal treatments and observed an increase in C_t value with increasing amplicon size. They concluded that designing longer amplicons might improve specificity while still providing sufficient measurable signal after thermal treatment of meats. However, this article did not provide standard curves for each amplicon size, showing PCR amplification efficiency, linear range of detection, and lower limits of detection levels. The increases in C_t value may have been partly or fully due to lack of assay optimization. This was not an isolated incident as there are many peer-reviewed articles, which use qPCR without MIQE validation (Bustin and others 2009).

Noting the caveats in the paragraph above, a longer amplicon (1016 bp) with a lower amplification efficiency (88%) and smaller range of detection for the same mitochondrial gene (Figure 4) was shown to increase the sensitivity of the mtDNA time-temperature integration in thermal protocols requiring rapid heat treatments at lower temperatures such as 5-log reduction of *E. coli* O157:H7 in high acid foods (tomato serum, pH = 4.26; processed at 95 °C for 30 s).

Conclusions

MtDNA fragmentation as a molecular TTI was assessed in high-acid, low-temperature thermal processes, cucumber fermentations, holding and storage times of acidified vegetables. Kinetic studies indicate that *D* values for high-acid, low-temperature processes have numerically similar *D* values to those obtained in similar studies on low-acid, high-temperature processes. Furthermore, *D* values from mtDNA fragmentation in tomato serum studies are similar to those for spoilage microorganisms of concern. Fermentation times in cucumbers could be monitored and optimized using mtDNA fragmentation in comparison with sensory evaluation. Shelf-life evaluation and ultimately, prediction, could rely on quantification of decomposition of mtDNA at room temperatures. Finally, mtDNA fragmentation protocols could be adjusted and optimized to target desired *D*- and *z*-values by changes in oligonucleotide parameters such as amplicon size.

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