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D- and z- values of *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in Heated Orange Juice

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This study was conducted to determine the decimal reduction times (D) and the thermal resistance parameter (z) values of relevant foodborne pathogens linked with fruit juice consumption – namely *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes.* Cocktails of different strains of the same bacterial species were prepared and thereafter subjected to thermal inactivation studies at 50, 53, 55, 57, and 60 °C heating temperatures in orange juice. Survivor populations were determined by surface-plating onto a non-selective medium, after which the inactivation behavior and kinetic parameters were determined. Results showed that for all tested organisms, the D-values significantly ($P < 0.05$) decreased with increasing heating temperature. In all heating temperatures, *E. coli* O157:H7 exhibited the significantly greatest heat resistance, with D-values ranging from 324.60 s to 95.12 s. At 50 °C, *L. monocytogenes* (D-values 152.32 s to 11.84 s) was significantly more resistant than *S. enterica* (D-values 106.55 s to 19.29 s). At higher heating temperatures, the D-values of *S. enterica* and *L. monocytogenes* were not significantly different ($P > 0.05$). The calculated z-values significantly varied across the test organisms, which ranged from 9.40 °C (*L. monocytogenes*) to 18.78 °C (*E. coli* O157:H7). These results may be used in the establishment of new, and validation of existing, thermal process schedules to ensure safety against the test pathogens.

Keywords: D-value, orange juice, pathogens, thermal inactivation, thermal processing, z-value

INTRODUCTION

Non-pasteurized or minimally processed juices have become popular among consumers because of the perceived health benefits and fresh-like quality characteristics. However, one of the concerns in unheated juice is the rapid deterioration of microbial, enzymatic, chemical, and physical quality attributes (Falguera and Ibarz 2014). Raujaria and Tiwari (2018) further explained that a particular public health concern is that pathogenic microorganisms can contaminate fruit juices and survive under such an acidic condition. Foodborne outbreaks have been implicated with unpasteurized juices and juice products (Stier and Nagle 2003).

Salmonella enterica is the primary pathogen associated with illnesses due to unpasteurized juices (Raujaria and Tiwari 2018). Aside from its ubiquity in nature, including the fruit juice processing environment, *Listeria*

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monocytogenes pose risks to pregnant women that could lead to abortion, fetal death, or neonatal morbidity in the form of septicemia and meningitis (Schoder 2016). Even if juices are processed, these commodities can still become vectors of diseases if processing conditions do not achieve the intended inactivation levels of pertinent pathogens. In some studies, *Escherichia coli* O157:H7 has been shown to exhibit heat and acid resistance in fruit juices (Mazzotta 2001; Topalcengiz 2019). As a result, the United States Food and Drug Administration (USFDA) ratified the Juice Hazard Analysis and Critical Control Point, which compels manufacturers to attain a 5-log reduction in the pertinent reference pathogen and ensure the safety of juices (USFDA 2001).

Fruit juice processing is commonly done by exposure to high temperatures (Kotzekidou 2016). Heating is the oldest preservation technique (Hogg 2005), which is based on its destructive effects on microorganisms (Jay *et al.* 2005). Aside from being traditional, this technology is considered simple and cheap (Simpson 2009). However, the heating of raw materials such as fruit juices can adversely affect the quality of finished products by reducing their nutritive value or altering the color and flavor (Kotzekidou 2016). Detrimental effects of heating on juice quality have been reported in cashew apple juice, grape juice, lime juice, tomato juice, pineapple juice, and orange juice (Biasoto *et al.* 2015; Jittanit *et al.* 2013; Hsu 2008; Rattanathanalerk *et al.* 2005; Polydera *et al.* 2005; Gabriel and Azanza 2009).

The detrimental effects of thermal processing on juice quality may be minimized if the establishment of thermal process parameters considers the inactivation kinetics of a specific resistant target organism. Gabriel and Nakano (2009) previously compared the D-values of individual strains of *E. coli*, *S. enterica*, and *L. monocytogenes* in apple juice and phosphate-buffered saline (PBS) and concluded that the D-values are organism- and suspending medium-specific. The D-value is equivalent to the heating time at a specific temperature that results in a 90% or 1 logarithmic reduction in the initial population of the challenge organism in heated juice (Holdsworth and Simpson 2007). From D-values obtained at various heating temperatures, the z-value may be determined. The z-value is defined as the increase in temperature of heating necessary to reduce the D-value by 10 folds (Forsythe 2000). The D- and z-values can be used in the determination of the integrated lethality of a particular thermal process, which is denoted by the F-value (Jay *et al.* 2005). These inactivation parameters are important in coming up with process parameters to achieve food quality and safety. In this study, the thermal inactivation parameters (D- and z-values) of *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* were established to determine an appropriate target organism on which a process schedule for the test food system may be based. The efficacy of existing processes may also be evaluated using the results established in this study.

MATERIALS AND METHODS

Orange Juice

This study used commercially available 100% orange juice (Rauch Happy Day, Rauch Fruchtsäfte, Rankweil, Austria) as a suspending medium for the thermal inactivation studies. The orange juice was subjected to physicochemical analyses – including pH, titratable acidity (TA, as % citric acid), soluble solids (SS, °Brix), and insoluble suspended pulp content (IS, % wt/vol). The pH of the sample was measured using a calibrated pH meter electrode (Horiba F-70, Tokyo, Japan). The TA was determined by potentiometric titration with a previously standardized 0.1 N sodium hydroxide (NaOH, Merck, Germany) until pH 8.2. The SS content was determined using a calibrated hand-held refractometer (Atago, Tokyo, Japan). Finally, the IS level was determined gravimetrically by passing a known volume of the sample through an ultrafilter paper (Advantec MFS, Inc., Tokyo, Japan) with 0.45 um pores. The differences between the constant weights of the ultrafilter paper before and after filtration were used in the calculations of the IS content. All physicochemical analyses were done in triplicate at room temperature (25 °C).

Challenge Organisms and Culture Maintenance

The organisms used in the thermal challenge studies included seven strains of *E. coli* O157:H7, 12 strains of *Salmonella enterica* serovars, and two strains of *Listeria monocytogenes*. All challenge organisms are currently maintained in the Laboratory of Food Microbiology and Hygiene (LFMH), Department of Food Science and Nutrition, College of Home Economics, University of the Philippines Diliman. The test LFMH *E. coli* O157:H7 cultures include the LFMH EMY-10, EDT-10, EMN-10, ECR-10, ECH-10, EC-96255, and EC-96256 strains, while the strains of *Salmonella* serovars include Typhimurium (American Type Culture Collection, ATCC 14028), Abortus-Equi (ATCC 9842), Enteritidis (LFMH S1-10, S4-18, S5-18, and S6-18), Infantis (LFMH S2-10), Montevideo (LFMH S3-10) and Diarizonae (ATCC 12325 and 29934), Oranienburg (S8-18), and Senftenberg (S7-18). The *Listeria monocytogenes* strains in the study include 1/2C (LFMH L1-10) and 4b (LFMH L2-10).

A working culture was prepared for each test strain based on the protocols previously reported by Gabriel *et al.* (2015a). Briefly, a loopful of cells was obtained from

each of the stock culture slants, separately introduced into the 5-ml sterile nutrient broth (NB, Hi-Media, Mumbai, India), and incubated for 24 h at 35 °C (Memmert Incubator, Germany). The resulting 24-h cultures were further enriched by transferring a loopful of the cells into a new set of NB, which were then incubated at 35 °C for another 24 h. Cells obtained from the 2nd 24-h culture passage were finally streaked on a nutrient agar (NA, Hi-Media) slant and incubated for 24 h at 35 °C. The resulting culture slants were the working cultures that were stored at 4 °C until used in the inactivation studies. Each of the working cultures was subjected to maintenance by following the previously two-passage culture process every 14 d.

Cocktail Inoculum Preparation

The study used a cocktail of challenge microorganisms based on the recommendations of the Institute of Food Technologists (IFT 2003) to account for strain-to-strain variations and to mimic the multi-strain contamination of food commodities. Strains of the same bacterial species were used to prepare cocktails used in the inactivation studies. Cells were obtained from each of the working culture slants and subjected to the previously described two-passage NB culture. TA two-milliliter (2-ml) aliquot was obtained from the 2nd passage, combined in a sterile 100-mL Erlenmeyer flask, and vortexed for 10 s for homogeneity. The cocktailed cells were then harvested by spinning 1 mL aliquots in microcentrifuge tubes for 15 min at 2400 ×*g* using a high-speed benchtop centrifuge (Cole Parmer, USA). The supernatant liquids were decanted and the pellets were resuspended in the equivalent amount of test orange juice by vortex mixing for 20 s. The cell suspensions were pooled in a sterile Erlenmeyer flask and set aside for 10 min for acclimatization. The NB residuals were not removed through the washing of pellets since this step may expose the cells to stresses that could affect inactivation kinetics. Furthermore, since the NB suspension was previously reported to maintain neutrality throughout the culture (Gabriel and Nakano 2011), the addition of the acidic orange juice was assumed to have little effects on the physicochemical properties of the suspension.

Heat Inactivation

Prior to heat inactivation studies at 50, 53, 55, 57, and 60 °C, 9.9-ml orange juice aliquots were placed in sterile glass tubes, which were immersed in a circulating water bath (Lab Companion, Batch Circulator, Jejo Tech, Korea) set to a specific test heating temperature. To monitor the orange juice temperature, a probe-type thermometer was placed through the cold point of an identical tube containing an equal amount of orange juice. Upon reaching the desired challenge temperature, around 6.0 log CFU/mL cells were introduced into the juice by adding 0.1 mL of acclimatized cocktails of strains into the heated tubes. This initial population is based on the recommendation of the IFT (2003) for microbiological challenge testing that aims to validate a process lethality step such as in thermal processing, high-pressure processing, or irradiation. The cells were then exposed to specific heating temperatures at predetermined time intervals. The addition of the inoculum did not result in the change in the juice temperature (data not presented). The tubes were immediately withdrawn from the water bath and cooled in wet ice prior to survivor enumeration. All inactivation runs per heating temperature per organism were conducted at least twice, each of which had at least two internal replicates.

Survivor Enumeration and Thermal Inactivation Kinetics Determination

Surviving cells were enumerated by subjecting the cooled juice samples to serial 10-fold dilution with sterile 0.1% peptone water (PW, Hi-Media, Mumbai, India). The 0.1 mL aliquots of appropriate dilutions were surface-plated onto NA plates, which were thereafter incubated upside-down at 35 °C for 24–48 h. Surviving populations were enumerated and reported as log CFU/mL. The D-value per organism per heating temperature was graphically determined as the negative inverse of the slope of the best-fitted regression inactivation curve. In this study, D-values were only determined from inactivation curves with the coefficient of determination (R^2) ≥ 0.90, and inactivation curves that traversed at least one logarithmic cycle. Furthermore, the thermal resistance parameter z-value was determined per organism by plotting the log D-values against heating temperatures.

Statistical Analyses

Inactivation kinetic parameters were obtained from at least two independent runs. There were two internal replications per run. All data obtained from the inactivation studies were subjected to single-factor analysis of variance using the general linear procedure (PROC GLM) of the SAS Statistical Software Package version 8.0 (Cary, NC, USA). *Post hoc* determinations of significant differences were done using Duncan's Multiple Range Test at a 95% level of significance.

RESULTS AND DISCUSSION

Orange Juice Properties

The results of pH, SS, and TA analyses (Table 1) are detailed in the following and are compared to those previously reported by Gabriel *et al.* (2015b), who did an

Table 1. Physicochemical and optical properties of commercially available orange juice used in challenge studies.

Physicochemical and optical property	Average ± SD
pH	3.91 ± 0.04
TA (% citric acid)	0.08 ± 0.00
SS (°Brix)	11.33 ± 0.35
IS (% wt/vol)	2.38 ± 0.07

Values were obtained from at least three independent runs.

extensive survey of these physicochemical properties on freshly squeezed and processed citrus juices. The orange juice suspending medium used in the thermal inactivation studies had a pH of 3.91 ± 0.04, which is near the pH range of commercially available processed orange juices (pH 2.85–3.87, n =17) and within the pH range of freshly-squeezed orange juices (pH 2.65–4.73, n =30). The test orange juice had SS of 11.33 ± 0.35 °Brix, which is within the reported ranges of freshly-squeezed (8.73–14.53 °Brix) and processed (2.40–12.90 °Brix) orange juices. The TA of the test orange juice of 0.083 ± 0.003% citric acid falls within the range reported for processed orange juices (0.08–0.41% citric acid) but is lower than those of freshly-squeezed oranges (0.23–2.41% citric acid). The IS suspended in the juice was determined to be at 2.38 ± 0.07 % wt/vol. This value is within the range tested by Estilo and Gabriel (2018) for UV-C irradiation of simulated juices.

Thermal Inactivation Behavior

Results of the thermal inactivation studies summarized in Figures 1–3 indicate that in all heating temperatures, the challenge organisms exhibited first-order inactivation kinetics with logarithmic-linear inactivation curves ($R^2 \geq 0.90$). According to Moats (1971), this inactivation behavior is common for microorganisms in heated food suspending medium, in which a steady population decline was observed with increasing heating time. The inactivation behavior observed in this study may be a hallmark of the homogeneity in the thermal susceptibility of the tested organisms despite the use of cocktails of strains to attribute for inter-strain variations that could occur in nature. Linear inactivation curves are also an indicator of the efficacy of the inactivating factor on the treated cells. Non-linearities in the inactivation curves have been explained to be due to the presence of resistant fractions in the challenge population and accumulation of injury prior to cell inactivation (Peleg 2006). In a previous study conducted by Gabriel *et al.* (2018) for *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in coconut liquid endosperm (pH 5.15, 0.06% malic acid, 4.4 °Brix) heated at 55–63 °C, the same linear inactivation behavior was observed.

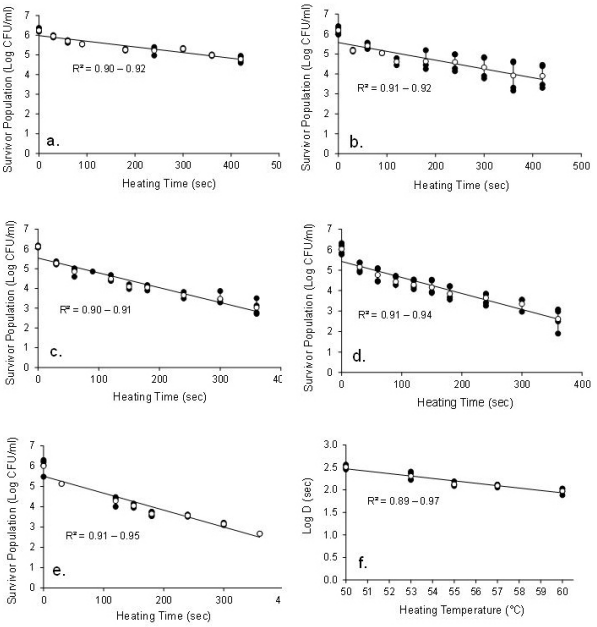

Figure 3. Thermal inactivation curves of *Listeria monocytogenes* cocktail in orange juice heated at (a.) 50 °C, (b.) 53 °C, (c.) 55 °C, (d.) 57 °C, and (e.) 60 °C, from which the D-values were determined. The Log D *vs.* Heating Temperature curve (f.) was also generated to determine the z-value. Filled markers represent data points obtained from independently replicated experiments. Empty markers represent average values.

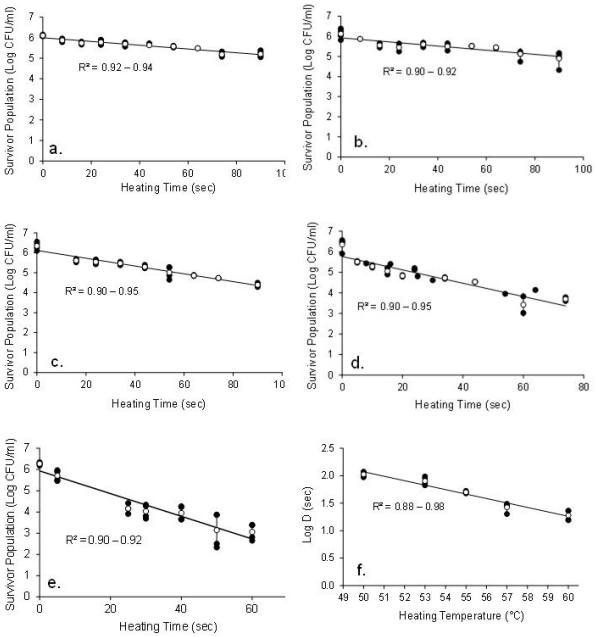

Figure 2. Thermal inactivation curves of *Salmonella enterica* cocktail in orange juice heated at (a.) 50 °C, (b.) 53 °C, (c.) 55 °C, (d.) 57 °C, and (e.) 60 °C, from which the D-values were determined. The Log D *vs.* Heating Temperature curve (f.) was also generated to determine the z-value. Filled markers represent data points obtained from independently replicated experiments. Empty markers represent average values.

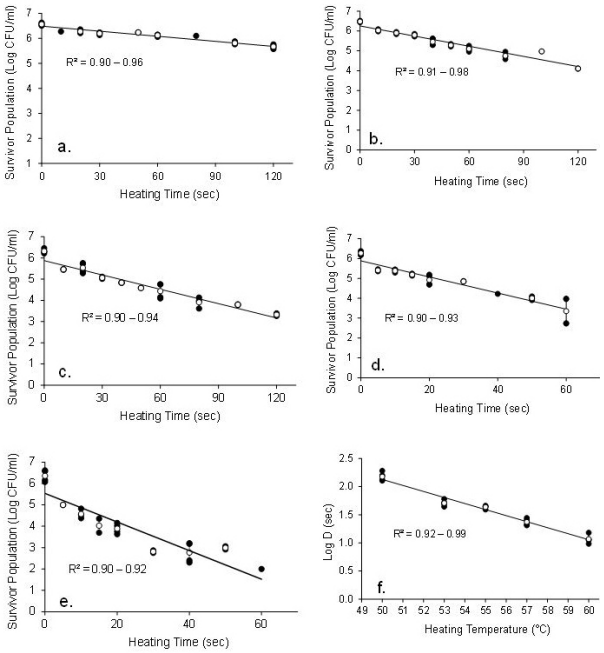

Figure 1. Thermal inactivation curves of *Escherichia coli* O157:H7 in orange juice heated at (a.) 50 °C, (b.) 53 °C, (c.) 55 °C, (d.) 57 °C, and (e.) 60 °C, from which the D-values were determined. The Log D *vs.* Heating Temperature curve (f.) was also generated to determine the z-value. Filled markers represent data points obtained from independently replicated experiments. Empty markers represent average values.

Thermal Inactivation Kinetic Parameters

As expected in all organisms, the D-values decreased with increasing heating temperature. The average and standard deviation values for the calculated thermal resistance parameters for all organisms in all tested temperatures are summarized in Table 2. Among the challenged organisms, *E. coli* O157:H7 exhibited the significantly ($P < 0.05$) greatest thermal resistance in the heated orange juice. At 50 °C, the calculated D-values for *E. coli* O157:H7 ranged from 302.61–365.35 s. Increasing the heating to 55 °C, resulted in > 50% reduction in the D-values, which ranged from 131.73–155.28 s. As much as > 50% decrease in the D-values were further observed in *E. coli* O157:H7 upon the increase of heating temperature to 60 °C. At the highest heating temperature, the D-values ranged from 76.37–107.46 s. A similar trend was observed for *S. enterica* and *L. monocytogenes*. In the lowest heating temperature, *S. enterica* – with a D-value range of 93.53–118.26 s – was significantly less resistant than *L. monocytogenes*, which had D-values ranging from 127.61–190.94 s. In all other heating temperatures, the D-values exhibited by *S. enterica* and *L. monocytogenes* were no longer significantly different ($P > 0.05$). In the middle heating temperature test setting of 55 C, a > 50% decrease in the resistance indicators was observed for *S. enterica*, with D-values ranging from 47.56–53.03 s. For *L. monocytogenes*, an almost four-fold reduction in the D-values was observed, which ranged from 39.05–45.75 s. In the highest heating temperature of 60 °C, *S. enterica* had D-values ranging from 15.48–22.98, while *L. monocytogenes* had D-values ranging from 9.60–15.16 s.

Despite the statistical similarities in the D-values of *S. enterica* and *L. monocytogenes* in most of the heating temperatures, the rate of change of the thermal resistance with increasing challenge temperature significantly varied, as also shown in Table 1, in the thermal resistance parameters (z-values). The tested cocktail of *E. coli* O157:H7 exhibited the significantly greatest z-value of 18.78 °C. The cocktail of *S. enterica* had a z-value of 12.35 °C, which is about > 30% lower than *E. coli* O157:H7. The tested cocktail of *L. monocytogenes* was found to be least thermal resistant with a z-value of 9.40 °C, which is > 20% lower than that of *S. enterica* and > 50% lower than that of *E. coli* O157:H7.

Gabriel and Nakano (2009) similarly challenged individual strains of *E. coli* K-12, *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in clear apple juice (pH 3.68) and PBS(pH 6.77), and reported that thermal resistance of microorganisms is dependent on variables such as those related to the implicit or inherent characteristics of the test organisms, and the intrinsic properties of the suspending medium. In a similarly acidic apple juice heated to 55 °C, a lone strain of *E. coli* O157:H7 (Hiroshima City Institute of Public Health Strain 96055) was significantly more resistant (D-value = 265.8 s) than the non-pathogenic *E. coli* K-12 (57.6 s) and more resistant than any of the

Table 2. Thermal resistance parameters of *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in orange juice.

Organisms	D-values (s) per heating temperature[1]					z-value
	50 °C	53 °C	55 °C	57 °C	60 °C	
E. coli O157:H7	$324.60 \pm 37.97^{a, x}$	$206.24 \pm 44.11^{b, x}$	$133.85 \pm 14.84^{c, x}$	$124.16 \pm 8.78^{c, x}$	$95.12 \pm 14.16^{c, x}$	18.78 ± 1.44^{x}
S. enterica	$106.55 \pm 12.67^{a, z}$	$81.42 \pm 14.28^{b, y}$	$50.06 \pm 2.41^{c, y}$	$26.94 \pm 4.71^{d, y}$	$19.29 \pm 4.24^{d, y}$	12.35 ± 0.49^{y}
L. monocytogenes	$152.32 \pm 28.95^{a, y}$	$51.23 \pm 6.88^{b, y}$	$43.30 \pm 2.94^{cb, y}$	$23.73 \pm 3.50^{cd, y}$	$11.84 \pm 2.35^{d, y}$	9.40 ± 0.96^{z}

[1]Values are reported as averages ± SD obtained from at least four raw values obtained from at least two independent runs.
[a,b,c] Values on the same row followed by the same letter are not significantly different ($P < 0.05$).
[x,y,z] Values on the same column followed by the same letter are not significantly different ($P < 0.05$).

tested *S. enterica* serovars Enteritidis (D-value = 31.8 s) and Typhimurium (D-value = 29.4 s); and individually tested *L. monocytogenes* 4b (D-value = 79.2 s), and *L. monocytogenes* 1/2c (104.4 s).

In a more recent study conducted by Gabriel *et al.* (2018) in heated coconut liquid endosperm, a cocktail of five strains of *E. coli* O157:H7 consistently exhibited greater resistance towards heat at 55–63 °C (D-value range of 27.6–1185 s) than cocktails of seven strains of *S. enterica* (D-value range of 15–184.8 s) and a cocktail of two strains of *L. monocytogenes* (D-value range of 15.6–700.8 s). It was very interesting to note that despite the consistently highest D-values of *E. coli* O157:H7 in coconut liquid endosperm, the rate of change in the D-values with increasing heating temperature was faster than that of *S. enterica*, which exhibited the consistently lowest D-values. Hence, with respect to the z-values, *E. coli* O157:H7 was deemed less resistant (z-value = 4.95 °C) than *S. enterica* (z-value =7.10 °C). These results showed that the thermal resistance of a specific organism is also temperature-dependent and, therefore, should be evaluated at varying challenge temperatures.

Li and Gänzle (2016) and Gayán *et al.* (2016) comprehensively discussed the various mechanisms behind the thermal resistance of *E. coli* in food. Aside from the commonly reported effects of the variability in strain characteristics as well as food formulations, thermal resistance in *E. coli* was attributed to several molecular mechanisms that minimize the detrimental effect of high temperature on the cell membrane, cytoplasm, ribosomes, DNA, and essential proteins. Specific gene regulatory mechanisms that result in the expression of heat-shock proteins that re-fold misfolded proteins and eventually result in stability during heat stress were highlighted. Other mechanisms attributed to the thermal resistance of the bacterium was attributed to its ability to express key proteins that stabilize membrane fluidity during such a stressful condition.

Furthermore, the previous study of Mazzotta (2001) showed that previous acid adaptation of *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* resulted in thermal cross-protection in heated fruit juices. This was similarly observed by Gabriel (2012b) for *E. coli* O157:H7 in liquid heating medium with varying pH and SS heated to different temperatures. Topalcengiz and Danyluk (2017) observed an increase in thermal resistance in *E. coli* (STEC) in orange juice after acid adaptation. The same was observed in some heated *S. enterica*, while *L. monocytogenes* did not exhibit an increased in thermal resistance after acid adaptation. These varying results show the complexity of the relationships of microbial, food, and process characteristics and thermal resistance.

The disparities between the results being reported in this study and that of previous works could be attributed to differences in the organisms challenged. In this study, seven strains of *E. coli* O157:H7 composed the challenged cocktail, as opposed to the individual strains challenged by Gabriel and Nakano (2009) and Topalcengiz and Danyluk (2019), and the five-strain cocktail challenged in Gabriel *et al.* (2018). This study also challenged a cocktail of 12 *S. enterica* isolates while Gabriel and Nakano (2009) and Gabriel *et al.* (2018) challenged one and seven-strain cocktail, respectively. The IFT (2003) explained that it is typical to include multiple specific strains of target pathogens in challenge studies in order to account for potential strain-to-strain variation. In fact, it is not unusual to have a cocktail of five or more strains as challenge organisms in studies like this. When Gabriel (2012a) compared the individual thermal resistance of three individual strains of *E. coli* O157:H7 and that of their composited inoculum, the mean of D-values of the individual strains was very close to that of the composited inoculum. This observation was attributed to the possible distribution of the resistances within the heterogeneous cell mixture. While choosing the inactivation kinetic parameter of the most resistant strain as the basis for establishing thermal processes may result in a safe commodity, this may similarly result in overestimation of process schedules and could compromise food quality.

The significant influence of suspending medium properties on thermal inactivation could also be emphasized as Gabriel *et al.* (2018) challenged the same two-strain cocktail of *L. monocytogenes* used in this study. The apparent higher resistance of *L. monocytogenes* in coconut liquid endosperm in the same challenge temperatures could be attributed to differences in the intrinsic properties of the suspending medium – such as pH, SS, and TA. Po *et al.* (2002) characterized and quantified the individual and interaction effects of these three juice properties on the thermal resistance of *E. coli* O157:H7. In their study, the thermal resistance of the challenge organism decreased with decreasing pH. The sugar content of the juice was found to have a protective effect on *E. coli* O157:H7, while increasing TA decreased thermal resistance.

CONCLUSION

The results in the study showed that *E. coli* O157:H7 exhibited the significantly greatest heat resistance among the tested pathogens. Significant variations in the calculated z- values were observed across test organisms and *E. coli* O157:H7 is the most heat resistant microorganism with the greatest z-value. The results established in this study highlight the importance of

identifying the most heat resistant organism, inactivation behavior, and kinetics as the basis for process calculations. These results may be used in the establishment of new, and validation of existing, thermal process schedules to ensure safety against the test pathogens.

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STATEMENT ON CONFLICT OF INTEREST

The authors whose names are listed above certify that they have no affiliations and relationships with any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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