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Research

Thermal Inactivation D- and z-Values of *Salmonella* Enteritidis and *Salmonella* Typhimurium in Whole Muscle Beef

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ABSTRACT

Salmonella spp. is a significant foodborne pathogen present in raw meat products and in the processing environment. It can be eliminated by thermal processes such as cooking. Time and temperature in the thermal process play an important role in eliminating this pathogen. The objective of this study is to determine the D- and z-values of *Salmonella* spp. in whole-muscle beef using the isothermal inactivation method in four temperatures at designated time intervals. Whole-muscle beef was inoculated with 7 to 8 $\log_{10}$ CFU/mL of *Salmonella* Typhimurium and *Salmonella* Enteritidis. The inoculated meat samples were heat treated at 58 °C, 60 °C, 62 °C, and 64 °C. At each temperature, *Salmonella* spp. survival rate was plotted and the D- and z-values were obtained by linear regression of the survival curve. The D-values for the thermal inactivation of whole-muscle beef are $D_{58\,°C}$=5.41 min, $D_{60\,°C}$=2.03 min, $D_{62\,°C}$=0.46 min, and $D_{64\,°C}$=0.18 min, while the z-value obtained was 3.94 °C. These findings will assist food processors in designing the critical limits on the critical control points of the cooking process that ensure safety against *Salmonella* spp. in cooked whole-muscle beef.

Key words: D-values, *Salmonella* Enteritidis, *Salmonella* Typhimurium, thermal inactivation, whole muscle beef

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INTRODUCTION

Salmonella spp. is the causative agent of one of the most prevalent foodborne illnesses, salmonellosis, and can readily survive over a wide range of temperatures due to the efficient expression of the heat stress response. Beef can become contaminated at any point along the supply chain including from the livestock, during slaughtering, post-harvest handling, processing, storage, and distribution. *Salmonella* spp. is associated with biological hazards in raw meat. Cooking plays an important role in beef microbiological safety and quality. Beef can be cooked using hot air or hot liquid. In preparing most Malaysian beef dishes, beef undergoes cooking in hot liquids such as water (beef soup) and coconut milk (beef curry & beef kurma). These processes serve two functions which are, to eliminate bacteria in raw beef and to tenderize it (Orta-Ramirez *et al*., 2005; Fabre *et al*., 2018).

Microbial heat resistance studies are required for the safe production of heat-processed foods. The D-value is the decimal reduction time to reduce 90% viable *Salmonella* spp. of the initial concentration. The D-value is obtained from *Salmonella* spp. thermal inactivation method and the *Salmonella* spp. survival plot. The D-value is important because it gives a control limit on the requirement of time and temperature in food processing. *Salmonella* spp. is an important foodborne pathogen. Regulation 39(2) of Food Regulations Malaysia 1985 states, that no person shall prepare or sell foods that are ready to be consumed contaminated with pathogenic microorganisms (Food Regulations Malaysia 1985). This is in line with other

international standards, which state that *Salmonella* spp. should not be present in 25 g of ready-to-eat foods tested (Food Standards Australia New Zealand, 2018; Food Safety Authority of Ireland, 2016)). Many studies have been carried out to determine the D-values of *Salmonella* spp. in meat and poultry, and most *of* these studies used only ground beef samples (Juneja & Marks, 2003; Murphy *et al*., 2004; Redemann *et al*., 2018). However, other studies have found that whole-muscle meat is more resistant to heat compared to ground meat (Orta-Ramirez *et al*., 2005; Tuntivanich *et al*., 2008; Velasquez *et al*., 2010). This suggests that sample composition such as fat content and the physical structure of the meat such as ground or whole-muscle meat can significantly affect D-value.

This study aimed to inactivate *Salmonella* spp. in whole-muscle beef using the isothermal inactivation method. The effect of time on the thermal resistance of *Salmonella* spp. was tested. The D-value for *Salmonella* spp. cocktail was determined in isothermal conditions at four different temperatures (58 °C, 60 °C, 62 °C, & 64 °C). This method was widely used to ensure the survival of *Salmonella* spp. at the designated sample core temperature at different time intervals. The D-values obtained in this study will enable the food service industries to establish heat treatments suitable to inactivate pathogens in beef products.

MATERIALS AND METHODS

Meat sample preparation

Frozen whole-muscle beef cubes were obtained from a local supplier and packed aseptically in a 384 mL Whirl-pack® Sample Bag (9.5 cm × 18 cm × 0.076 mm) individually. The air was removed from the bag before freezing it at -20 °C. Each beef cube weighed approximately 50 to 60 g, with an approximate dimension of 2.0 × 2.0 × 2.0 ± 0.5 inches (length × width × height). The high bacterial count in raw beef may hinder the growth of inoculated *Salmonella* spp. due to competitiveness between microorganisms. Frozen raw whole-muscle beef cubes were given gamma irradiation treatment which was carried out at the Malaysia Nuclear Agency. The frozen samples were placed inside polystyrene cooler boxes (43 × 30 × 29.5 cm) filled with ice packs and irradiated at a gamma dose of 7 kGy by using an irradiator with a ^{60}Cobalt source to eliminate indigenous microflora (Rajkowski, 2012). The effectiveness of the irradiation was confirmed by sterility tests. The irradiated samples were kept frozen at -20 °C until needed and random samples were tested for sterility before conducting each experiment.

Proximate analysis was performed because *Salmonella* spp. heat inactivation was found to be correlated to the physical and chemical composition of food (Murphy *et al*., 2004; Orta-Ramirez *et al*., 2005; Velasquez *et al*., 2010). A 10 g sample with 90 mL distilled water was homogenized for 2 min using a stomacher (Interscience, France) to determine the sample's pH value (Mettler Toledo, Switzerland). All analysis was conducted in triplicate.

Preparation of inoculum

Cultures of *Salmonella enterica* serovar Typhimurium *(*ATCC 14028) and *Salmonella enterica* serovar Enteritidis (ATCC 13076) were used to produce the inoculum cocktail. All serovars were stored at <-18 °C in tryptic soy broth (TSB) (Oxoid, England) containing 20% (v/v) glycerol. Single isolated *S.* Typhimurium and *S.* Enteritidis colonies on tryptic soy agar (TSA) plate (Oxoid, England) were transferred into 9 mL TSB and incubated at 37 °C for 24 hr under static conditions. The culture was then centrifuged separately at 3400 × *g* for 10 min at 4 °C (Eppendorf Centrifuge 5804R, Germany). The pellets were resuspended and washed twice in a sterile phosphate buffer solution (Oxoid, England). The two strains were mixed in equal volumes (0.5 mL) each in 9 mL of 0.1% peptone water (PW) (Oxoid, England) and serially diluted to a target *Salmonella* spp. concentration of 10^7 to 10^8 CFU/mL. This inoculum level was used to inoculate the samples. Initial counts of bacterial suspension were determined by spread plating appropriate dilutions on xylose lysine deoxycholate (XLD) agar (Oxoid, England) (Orta-Ramirez *et al*., 1997; Tuntivanich *et al*., 2008; Osaili *et al*., 2013).

Exposure to inoculation culture

Frozen whole-muscle beef cubes were thawed overnight at 1-4 °C in the original packages. Samples were inoculated with 1ml fresh *Salmonella* spp. cocktail to obtain an initial bacterial concentration of 10^7 to 10^8 CFU/g. The inoculated beef was hand-rubbed to evenly spread the culture. Each inoculated sample was stored at 1-4 °C for 60 min before treatment. This allows for the inoculation to be absorbed into the samples and attachment of bacterial cells to the meat tissues (Murphy *et al*., 2002; Osaili *et al*., 2013). Negative and positive controls were prepared where negative control is the non-inoculated sample while positive control is the inoculated sample. The function of the negative control is to ensure the sample is not contaminated with *Salmonella* while the positive control is to ensure the initial *Salmonella* spp. concentration is maintained. Both controls were kept chilled (1-4 °C) until all treatments on inoculated samples were carried out.

Isothermal inactivation

D-values, expressed in minutes are determined by plotting the $\log_{10}$ number of bacteria survivors against time for each test temperature. The line of best fit for survivor plots determined by regression analysis gives the D-value in minutes for the specific temperature with a correlation coefficient of R^2>0.90 (Juneja, 2007). Alternatively, a linear regression was performed on the survivor plot to determine the D-value in minutes for the specific temperature.

The heating temperatures and time intervals used in this study were adapted from several past studies which are similar to the present study, with temperatures ranging from 57.5 °C to 65 °C (Juneja & Eblen 2000; Murphy *et al*., 2004; Horn *et al*., 2015; McMinn *et al*., 2018). After preliminary studies, four heating temperatures were selected which are 58 °C, 60 °C, 62 °C, and 64 °C. Five samples with one temperature reference sample were placed in a water bath at designated heating temperatures with time intervals of 3 min (58 °C), 1 min (60 °C), 15 s (62 °C), and 7 s (64 °C), with a total cooking time of 40 s to 30 min.

The internal temperature was monitored using a 3-mm type k-thermocouple (Hanna Instruments, USA) inserted into the center of a reference sample. Thermal lag time is the time required for the internal temperature to reach within 0.5 °C of the target temperature. This was timed as time "zero" (Juneja 2007). The timer continued according to the time intervals designed. At each time interval (including time "zero"), samples were removed from the water bath, and samples were immediately plunged into the ice-water bath and chilled in a chiller (0-4 °C) before analysis.

Detection and enumeration of *Salmonella* spp.

Each heat-treated sample was aseptically transferred to a stomacher bag, weighed 25 g, diluted with 225 mL buffered peptone water (BPW) (Oxoid, England) with a 1:9 ratio, and stomached for 2 min at 230 r.p.m. to form a beef slurry. The enumeration method was used to determine the culturability of *Salmonella* spp. cocktail of the samples using isothermal inactivation. Subsequent decimal dilutions were prepared by mixing 1 mL aliquots with 9 mL of 0.1% PW and 100 µL of these dilutions were spread plate in duplicate XLD agar plates. The plates were incubated for 18±2 hr at 37 °C and typical *Salmonella* spp. with a characteristic colorless colony with black centers were counted and converted to $\log_{10}$ CFU/g for statistical analysis. The detection limit of plate counts was 2 $\log_{10}$ CFU/g. each experiment was performed in duplicates, and an average of CFU/g of each dilution was used to determine the D-values (Kang & Fung 2000; Osaili *et al*., 2013; Wang *et al*., 2017).

Data analysis

Salmonella spp. survival curves were determined for whole-muscle beef by plotting the logarithm of the survival count data ($\log_{10}$ CFU/g) versus heating times (min) at each temperature. The D-value at each temperature was calculated by taking the negative inverse of the relevant slopes and was calculated using the equation $D_t = (t_2 - t_1)/(\log CFU/g_2 - \log CFU/g_1)$. The z-values were estimated by computing the linear regression of the log of D-values with corresponding heating temperatures. The z-values were calculated as the absolute value of the reciprocal of the regression slope. For each treatment, duplicate thermal inactivation trials were performed. An analysis of variance (ANOVA) on the log-transformed data was used to assess the effect of time and temperature on *Salmonella* spp. survival.

RESULTS AND DISCUSSION

Proximate analysis

The proximate analysis of the meat sample indicated that the beef contained an average of 11.5% fats, 22.3% protein, 65% moisture, and 1.2% ash. The average sample pH measured before analysis was 5.8 ± 0.2.

D-value determination

The average range of initial concentration of *Salmonella* spp. in the beef sample after 60 minutes of inoculation was from 8.61±0.27 $\log_{10}$ CFU/g to 8.88±0.27 $\log_{10}$ CFU/g. The survival curve of *Salmonella* spp. in beef samples after each isothermal treatment was plotted and fitted for the temperatures of 58 °C, 60 °C, 62 °C, and 64 °C respectively (Figure 1 - Figure 4). No differences (p>0.05) were seen in the whole-muscle sample between the initial *Salmonella* spp. counts and *Salmonella* spp. counts at time zero, which suggested the heating of the sample to reach equilibrium temperature did not affect the concentration count. The line of best fit for survivor plots was determined by regression analysis where a regression equation of y = ax + b was derived, suggesting that *Salmonella* spp. inactivation follows first-order kinetics, which presumed log-linear inactivation of bacteria under isothermal conditions The coefficient (R^2) of the linear regression was more than 0.95 at the temperature of 58 to 64 °C. One-way ANOVA was conducted for differences in the survival count of *Salmonella* spp. from time "zero" to the last point, and the survival counts were significantly different (p<0.05) for all temperatures.

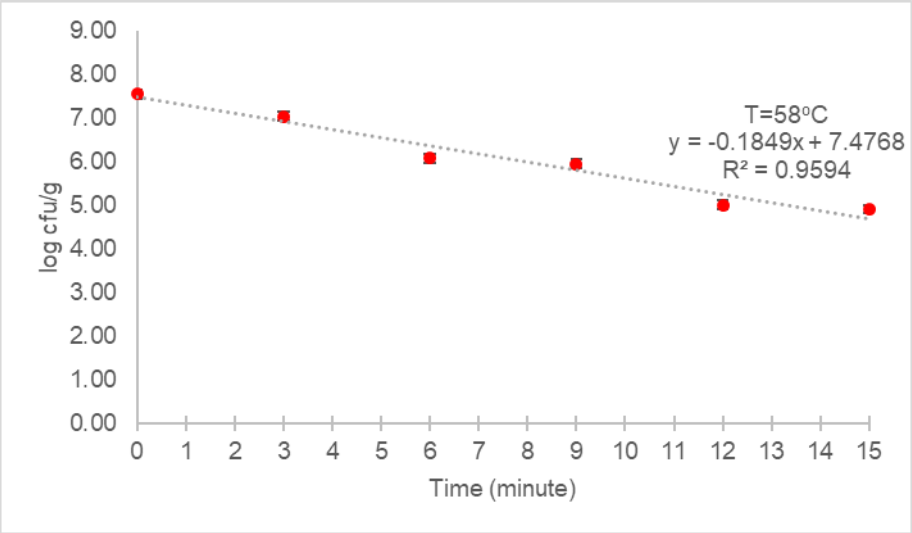

Fig. 1. Thermal inactivation of *Salmonella* spp. curve in the whole-muscle beef at 58 °C

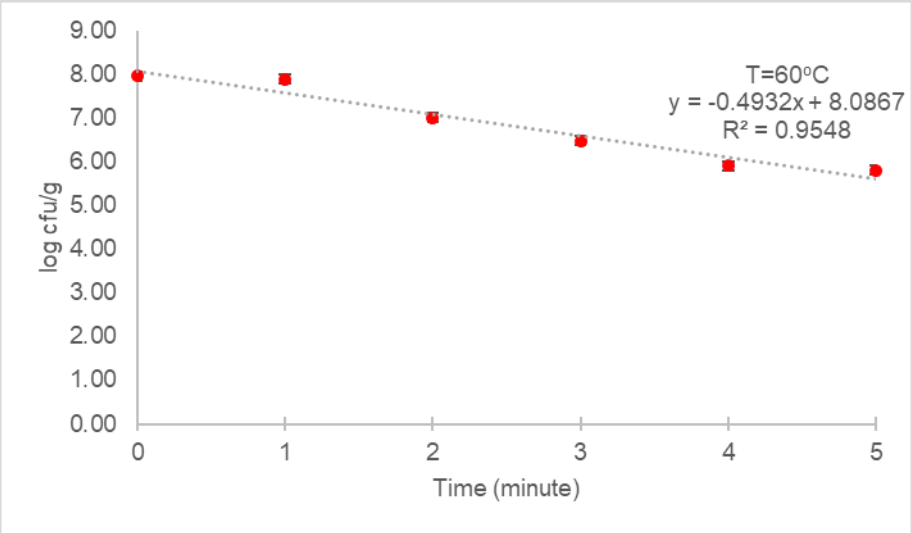

Fig. 2. Thermal inactivation of *Salmonella* spp. curve in the whole-muscle beef at 60 °C.

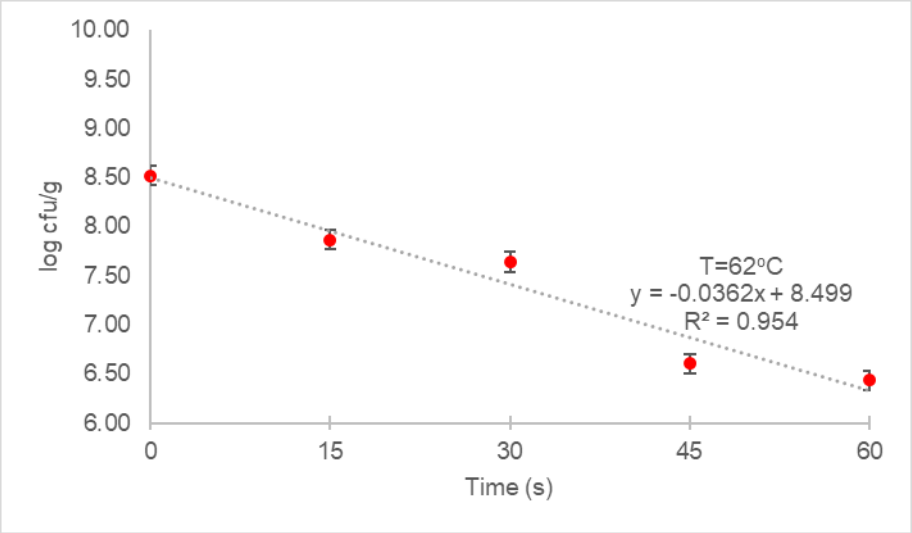

Fig. 3. Thermal inactivation of *Salmonella* spp. curve in the whole-muscle beef at 62 °C.

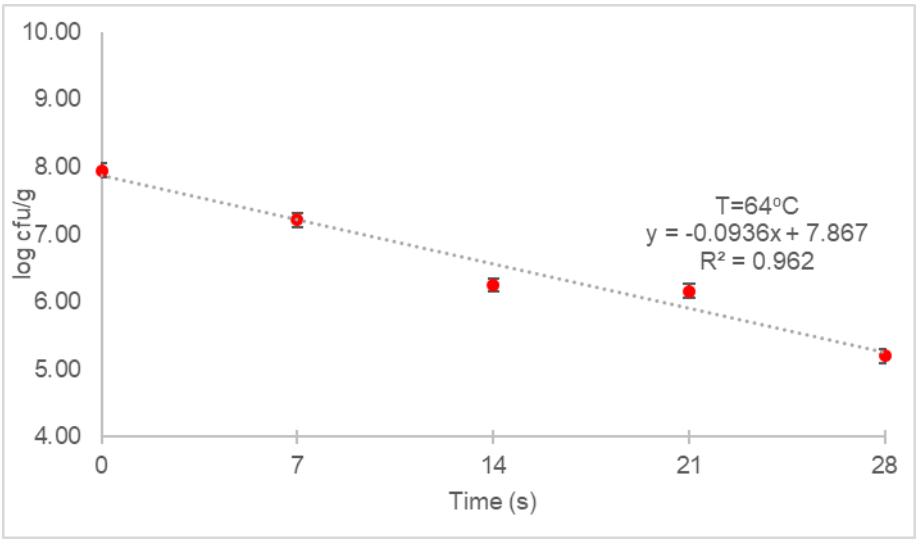

Fig. 4. Thermal inactivation of *Salmonella* spp. curve in the whole-muscle beef at 64 °C.

The survival curve presented in this study is consistent with past thermal inactivation studies (Murphy *et al*., 2002; Orta-Ramirez *et al*., 2005; Osaili *et al*., 2007). The coefficient (R^2) of the linear regression was more than 0.95 for all the temperatures that were tested. In a study by Orta-Ramirez *et al*., (2005), when heat resistance of 8 *Salmonella* serovars in whole-muscle beef was given heat treatment in a temperature-controlled water bath, the survival curve was linear with an R^2 of 0.84-0.96. The D-value for *Salmonella* spp. at each temperature was calculated from the linear regression model for the $\log_{10}$ of surviving *Salmonella* spp. and heating time. Since food standards show *Salmonella* spp. shall not be present in ready-to-eat foods, data from this study was extrapolated using the linear regression model in Table 1, and *Salmonella* spp. thermal death time was calculated.

Table 1. D-values and thermal death time of *Salmonella* spp. at 58 °C, 60 °C, 62 °C, and 64 °C

Temperature (°C)	Linear regression model	R^2	D-value (min)	Thermal death time (min)
58	y = -0.1849x + 7.4768	0.9594	5.41	40.4
60	y = -0.4932x + 8.0867	0.9548	2.03	16.4
62	y = -0.0362x + 8.499	0.9540	0.46	3.91
64	y = -0.0936x + 7.867	0.9620	0.18	1.40

The bacterial heat resistance D-values results are greatly influenced by factors such as meat species, muscle type, pH, fat content, types of *Salmonella* strains used, and method of enumeration (Juneja, Eblen, & Ransom, 2001). The type of bacteria survival curve model used for each study also influenced the overall results. These considerations must be considered when comparing the inconsistency of results obtained in the present study with those reported from past studies. Variations in bacterial strains used may account for D-value differences (Doyle & Mazzota, 2000; McMinn *et al*., 2018). The present study uses *S.* Typhimurium and *S.* Enteritidis cocktail. It was found that certain serotypes of *Salmonella* enterica are highly thermal resistant such as *Salmonella* Senftenberg and will give a higher D-value (Murphy *et al*., 2004; Osaili *et al*., 2007). This may be due to higher survivability in high moisture foods, and genotypic heat resistance islands in the serovar (Etter *et al*., 2019).

The literature contains limited information on thermal inactivations of *Salmonella* spp. in whole-muscle beef. The present study shows similar D-values of *Salmonella* spp. for whole-muscle beef and ground beef samples for certain temperatures (Table 2). Another distinct comparison is differences in sample fat content. Fats provide protective effects to bacterial cells. Together with the fats characteristic which is low heat conductivity, higher fat contents allow the microorganisms to survive higher heating temperatures, thus resulting in increased thermal resistance of microorganisms (Juneja, Eblen & Ransom 2001; Murphy *et al*., 2004; Huang *et al*. 2019).

The D-values from this study were in general in agreement in terms of higher fat concentrations resulting in higher D-values for *Salmonella* in beef.

z-value determination

The z-values were estimated by computing the linear regression of mean $\log_{10}$ D-values versus their corresponding heating temperatures (Figure 5). The regression coefficient, R^2 is 0.9919. From the D-values ranging from 40.4 to 1.4 min at 58 to 64 °C, the z-value obtained was 3.94 °C. This means

that increasing the heat treatment temperature by 3.94 °C will result in a 1 log reduction of the D-value.

The z-value for this study was relatively low as compared to previous studies. When heat resistance of *S.* Senftenberg in roast beef was determined, the D-values ranged from 53.0 to 0.22 min at 53 °C to 68 °C, with a z-value of 5.64 °C (Orta-Ramirez *et al*., 1997). A higher z-value (6.60°C) was also observed with D-values ranging from 49.2 to 0.3 min at 55 °C to 70 °C (Horn, 2015). The D-value for the present study is relatively high due to the factors discussed, and this contributes to low z-values.

Table 2. D-values of *Salmonella* spp. in whole muscle and ground beef

Reference	Type of sample	Fat (%)	Temperature (˚C)	D-value (min)
McMinn *et al*., (2018)	Roast beef	<3	60	0.70
			65	0.14
Horn *et al*., (2015)	Beef	NA	58	17.3
			60	8.60
			62	4.30
			64	2.20
Murphy *et al*., (2004)	Ground beef	34.4	57.5	18.35
			60	6.90
			62.5	2.62
Juneja and Eblen (2000)	Ground beef	12.5	58	8.65
			60	5.48
			62.5	1.50
			65	0.67

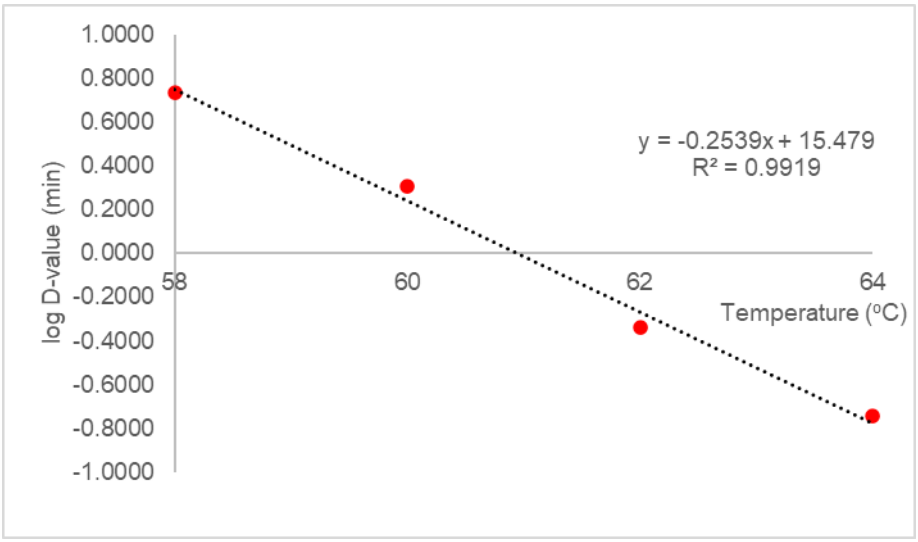

Fig. 5. z-value of thermal inactivation of *Salmonella* spp. for 58 °C, 60 °C, 62 °C, and 64 °C.

CONCLUSION

Based on the D-value and the thermal death time determined in this study, contaminated whole-muscle beef should be heated to an internal temperature of 64 °C for at least 1.40 min. This is designed to achieve an 8D process for *Salmonella* spp. which is based on the initial concentration of *Salmonella* spp. studied in this study (8 $\log_{10}$ CFU/g). This also aligns with standards in the regulations that *Salmonella* spp. shall not be present in 25 g ready-to-eat food. Findings from this study will assist the food service industries in designing the critical limits on the critical control points that ensure safety against *Salmonella* spp. in cooked whole-muscle beef and will be used to predict the time required for specific temperatures to achieve specific targeted $\log_{10}$ reductions when heat treatment is introduced in whole-muscle beef products.

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ETHICAL STATEMENT
Not applicable.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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