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Effect of grape seed extract on heat resistance of *Clostridium perfringens* vegetative cells in *sous vide* processed ground beef

Serap Cosansu[a], Vijay K. Juneja[b,*], Marangeli Osoria[b], Sudarsan Mukhopadhyay[b]

[a] Food Engineering Department, Engineering Faculty, Sakarya University, Esentepe Campus, Sakarya, Turkey
[b] Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Wyndmoor, PA, USA

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ABSTRACT

The heat resistance (57.5–65 °C) of a three-strain cocktail of *Clostridium perfringens* vegetative cells in *sous vide* processed ground beef supplemented with 0–3% grape seed extract (GSE) was quantified. The surviving cell population was enumerated on tryptose–sulfite–cycloserine agar. The decimal reduction (D)-values in beef that included no GSE were 67.11, 17.15, 4.02, and 1.62 min at 57.5, 60, 62.5, and 65 °C, respectively. Addition of 1.0% GSE resulted in concomitant decrease in heat resistance as evidenced by reduced bacterial *D*-values. The *D*-values in beef with added 1.0% GSE were 62.89, 13.70, 3.47 and 1.46 min at 57.5, 60, 62.5, and 65 °C, respectively. The heat resistance was further decreased when the GSE concentration in beef was increased to 2 or 3%. The *z*-values in beef with or without GSE were similar, ranging from 4.41 to 4.56 °C. The results of this study would be beneficial to the retail and institutional food service establishments in estimating re-heating time and temperature for *sous vide* processed ground beef to ensure safety against *C. perfringens*.

1. Introduction

Considerable effort has been made to find natural antimicrobials to control microbial growth in foods due to the concerns for foodborne illnesses as well as the safety of synthetic preservatives used in foods (Gyawali & Ibrahim, 2014; Perumalla & Hettiarachchy, 2011). Plant-derived compounds have been used since ancient times for different purposes such as food flavoring agent, traditional medicine or food preservative (Gyawali & Ibrahim, 2014). Grape seed extract (GSE) is a polyphenolic rich substance derived from seeds of *Vitis vinifera* (Kumar et al., 2013). It is known as the mixture of catechin monomers, procyanidin oligomers and procyanidin polymers (Clouatre, Kandaswami, & Connolly, 2005). The amounts of phenolic compounds in seeds are higher than those in the skin or pulp of grapes (Shi, Yu, Pohorly, & Kakuda, 2003). It is considered that the phenolic compounds in plant extracts are possibly responsible for the antimicrobial activity; hydroxyl (−OH) groups in phenolic compounds can cause cell death by disrupting membrane structure and leading to leakage of cellular components of bacteria (Gyawali & Ibrahim, 2014). GSE can prevent lipid oxidation and reduce rancid flavor development in various meat and meat products (Perumalla & Hettiarachchy, 2011). It has GRAS (Generally Recognized as Safe) status approved by Food and Drug Administration (FDA) and is listed on the “Everything Added to Food in the United States (EAFUS)” (Perumalla & Hettiarachchy, 2011).

Clostridium perfringens is an anaerobic endospore forming pathogenic bacterium which is ubiquitous in nature. *C. perfringens* type A toxicoinfection has been reported as the third most common foodborne disease in United States, with 289 confirmed outbreaks resulting in 15,208 illnesses and eight deaths between 1998 and 2010 (Matthews, Kniel, & Montville, 2017). The pathogen is Gram-positive, rod shaped, encapsulated, non-motile and a sulfite-reducing bacterium. Although *C. perfringens* is classified as an anaerobe, it is relatively aerotolerant compared to many other anaerobic bacteria (Matthews et al., 2017; Robertson, Li, & McClane, 2014). Due to its extremely short generation time (Robertson et al., 2014), the number of the pathogen can reach to high levels in cooked meat following spore germination at abusive temperatures. *Sous vide* cooked meats are considered as suitable environments for *C. perfringens* because the time and temperature combinations applied during *sous vide* cooking generally is not adequate to destroy *C. perfringens* spores as well as the cooking triggers the spore germination. On the other hand, as the vacuum packaging provides an anaerobic environment, the multiplication of vegetative cells of *C. perfringens* following spore germination and outgrowth is likely to occur in *sous vide* cooked meat at abusive temperatures. Therefore, the re-heating step seems as a critical step for ensuring the safety of *sous vide* cooked meat in respect to hazards associated with *C. perfringens*. The objective of this study was to quantify the efficacy of GSE in *sous vide* processed ground beef on the heat resistance of *C. perfringens* vegetative

* Corresponding author.
E-mail address: vijay.juneja@ars.usda.gov (V.K. Juneja).

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cells. The thermal death time values from this study could be used to reheat *sous vide* cooked ground beef, eliminate the pathogen and ensure safe consumption of the product.

2. Materials and methods

2.1. Clostridium perfringens strains and spore cocktail preparation

Three different strains of *C. perfringens* (NCTC 82238, NCTC 8239 and ATCC 10288) from the Eastern Regional Research Center culture collection were used in the experiments. From the stock culture, a 0.1-ml volume was transferred to freshly prepared Thioglycollate Broth (Difco) to prepare an active culture. Duncan Strong sporulation medium was used to produce the spores of each strain (Juneja, Call, & Miller, 1993). After harvesting by centrifugation (7212 × *g*, 20 min, 4 °C), spores of each strain were washed twice with sterile distilled water, resuspended in distilled water (Juneja & Majka, 1995) and then stored in the refrigerator (4 °C). Equal amounts from each spore suspension were combined to prepare spore cocktail immediately prior to inoculation.

2.2. Sample preparation and inoculation

Ground beef with 4% fat was purchased from a local store and kept frozen at −20 °C. A commercial grape seed extract (GSE) in liquid form (95% OPC. T.J. Clark®) sold as a dietary supplement was used in the experiments. Frozen ground beef was thawed overnight in the refrigerator, and then divided into four equal batches. One batch was separated as the control (0%) and the other three batches were individually added with 1, 2 or 3% GSE. After adding GSE, the ground beef was mixed (KitchenAid, St. Joseph, Michigan, USA) for 2 min for an even distribution of GSE. From each sample group, 5-g portions were weighed and transferred into sterile, full surface micro-perforated filter blender bags (BagPage + 100 ml [95 × 180 mm]. Ref. 122,025, Interscience, Rockland, MA). Each bag was inoculated with 100 µl of non-heat shocked spore cocktail and 2–3 log spore g^{-1} in each ground beef sample was targeted. Sample bags were massaged by hand and then mixed for 2 min in a lab blender (MiniMix 100, Interscience, Rockland, MA) for an even distribution of spores. Ground beef sample bags were compressed to a flat surface to obtain a uniform thickness of about 1 mm, subsequently, bags were evacuated to a negative pressure of 17 mbar and vacuum-sealed using a Multivac packaging machine (Model A300/16. Multivac Inc., Kansas City. MO). The vacuum sealed bags were stored at −20 °C until cooking.

2.3. Cooking, cooling and storage

The frozen samples were thawed by keeping those at ambient temperature for several minutes. Bags were immersed in a programmable water bath (VWR PolyScience, PolyScience Inc., Warrington, PA, USA) set at 23 °C and then cooked by linearly increasing temperature from 23 °C to 75 °C in a period of 1 h. When the temperature reached 75 °C, the samples were removed from the water bath. This heat process, which is simulating the conditions common in the retail food industry, was assumed to adequately heat shock *C. perfringens* spores. After rapid cooling in ice slurry, sample bags were stored in a water bath (Model RTE-17, Digital Plus, NESLAB Instruments Inc., Newington, NH, USA) at 25 °C for 48 h, to achieve growth of *C. perfringens* from spores to more than million cells required to cause foodborne illness.

2.3.1. Thermal inactivation

Following storage at an isothermal temperature (25 °C) for 48 h, where the *C. perfringens* cell counts reached to 7–8 log CFU g^{-1}, as determined in preliminary studies, samples were subjected to thermal inactivation. For this aim, bags were placed in a basket with enough space between two bags and fully submerged in a water bath (Model RTE-221, Digital Plus, NESLAB Instruments Inc., Newington, NH, USA) adjusted to 57.5, 60, 62.5 or 65 °C. Total heating times were 250, 90, 20 and 9 min respectively. Samples were pulled at predetermined time intervals with its frequency based on the heating temperature. In each replicate of each temperature 8–10 sampling times were planned, to achieve at least 6 $\log_{10}$ reduction. At each sampling time, the removed bags were immediately immersed in ice slurry and plated within 1 h as described below.

2.4. Enumeration of surviving bacteria

Five ml sterile peptone water (0.1% peptone) was added to the sample bag and homogenized for 2 min in a lab blender. Homogenized samples were serially diluted in peptone water. From appropriate dilutions, 0.1-ml portions were spread, in duplicate, onto Tryptose Sulfite-Cycloserine (TSC) Agar (Difco) plates. Subsequently, TSC plates were overlaid with approximately 10 ml of SFP Agar (Shahidi Ferguson Perfringens Agar, Difco) without selective supplement. After incubation in an anaerobic chamber (Bactron IV, Sheldon Laboratories, Cornelius, OR, USA) at 37 °C for 24 h, typical colonies were counted manually.

2.5. Calculation of D and z-values

Thermal inactivation experiments were conducted as two independent trials, each performed in duplicate; an average of four platings of each sampling point was used to determine the thermal death kinetics. Survival curves were obtained by plotting log numbers of survivors against time for each heating temperature. The *D*-values (time to inactivate 90% of the viable cells) were determined from the straight-line portion of the survival curves using Excel Data Tool Pack (Microsoft Corporation, Redmond, WA, USA). The survival curves used for *D*-value calculation included more than six values in the straight-line portion that descended > 5 log cycles with a correlation coefficient (R^2) of ≥ 0.94. The *D*-values were calculated using the slopes of survival curves for each heating temperature ($D = -1/\text{slope}$). The z values were estimated by computing the mean $\log_{10}D$-values against their corresponding temperatures using Excel Software. The absolute value of the inverse slope was taken to calculate the *z*-value.

2.6. Statistical analysis

Analysis of variance was applied using SPSS software (IBM SPSS Statistics for Windows. version 24.0). Duncan's multiple range test was used to determine significant differences among means ($p < .05$).

3. Results and discussion

C. perfringens vegetative cells in ground beef heated at 57.5, 60, 62.5 or 65 °C exhibited log-linear decline in the log numbers of surviving cells with time ranging from 250 min at 57.5 °C to 9 min at 65 °C. Lag periods or shoulders and tailing in any of the survivor curves of the pathogen were not evident after *sous vide* processing of ground beef at the four temperatures; thereby, suggesting that the cell population was homogeneous in heat resistance and followed first-order inactivation kinetics. This observation of microbial cells dying at a constant rate when heated at a particular temperature is in agreement with those made by other researchers. In a study by Byrne, Dunne, and Bolton (2006), when heat inactivation of *C. perfringens* vegetative cells was quantified in pork luncheon roll packaged in polyethylene bags, the thermal death time curves followed first-order kinetics. In another study, Juneja and Marmer (1998) reported log-linear decline in surviving *C. perfringens* vegetative cells in beef or turkey heated at 55 to 62.5 °C. Linear survivor curves obtained in the present study suggest that non-linear approaches to analyze thermal inactivation data were not needed.

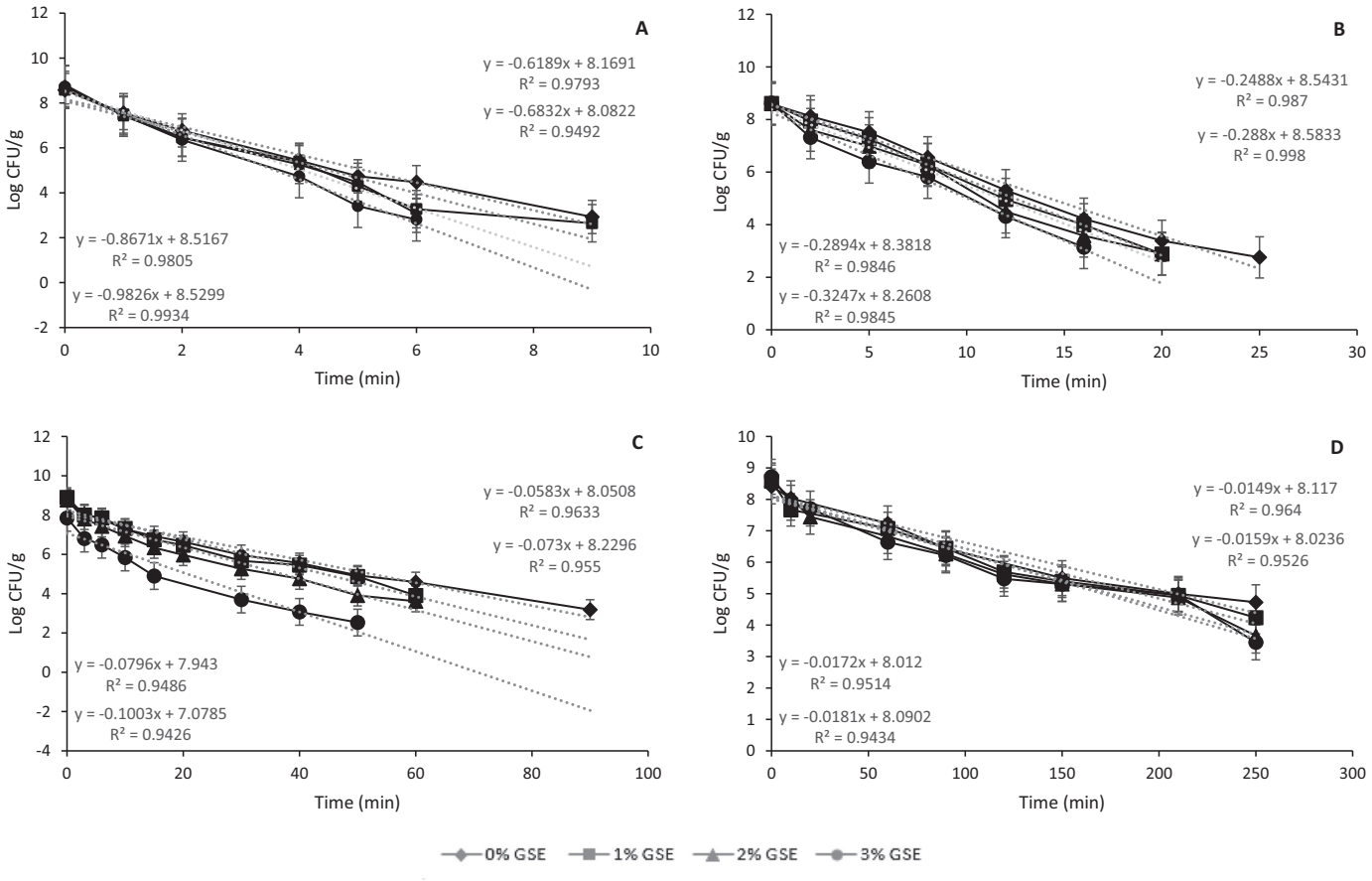

Fig. 1. Survival curves of *C. perfringens* vegetative cells in *sous vide* processed ground beef (96% lean) supplemented with 0–3% grape seed extract (GSE); (A) 65 °C, (B) 62.5 °C, (C) 60 °C, (D) 57.5 °C.

Table 1
Heat resistance (expressed as *D*-values) for cocktail of *C. perfringens* vegetative cells in *sous vide* processed ground beef with 0–3% grape seed extract (GSE).

Temperature (°C)	GSE Concentration (%)	*D*-value (min)*	R^{2**}
57.5	0	67.11 ± 2.62^{a}	0.96
	1	62.89 ± 0.40^{ab}	0.95
	2	58.14 ± 2.50^{bc}	0.95
	3	55.25 ± 3.42^{c}	0.94
60	0	17.15 ± 0.00^{a}	0.96
	1	13.70 ± 0.48^{b}	0.96
	2	12.56 ± 0.08^{c}	0.95
	3	9.97 ± 0.07^{d}	0.94
62.5	0	4.02 ± 0.00^{a}	0.99
	1	3.47 ± 0.17^{b}	0.95
	2	3.46 ± 0.07^{b}	0.94
	3	3.08 ± 0.13^{c}	0.95
65	0	1.62 ± 0.02^{a}	0.98
	1	1.46 ± 0.01^{b}	0.99
	2	1.15 ± 0.00^{c}	0.98
	3	1.02 ± 0.07^{d}	0.98

* *D*-values are the mean ± standard deviation of two replicates and were obtained by linear regression. Means followed by different letters are significantly different ($p < .05$).

** Correlation coefficient in each column within each temperature.

For the rate of thermal inactivation of *C. perfringens* vegetative cells in ground beef 57.5, 60, 62.5 or 65 °C, the population densities are being discussed as log of the ratio of microbial count at time *t* and initial count. The resulting data gave the log numbers of the pathogen per gram of beef destroyed at a particular sampling time during cooking. For beef without GSE heated at 57.5 °C, *C. perfringens* vegetative cells population densities decreased by 3.68 log CFU (8.41 to 4.73 log CFU g^{-1}) after 250 min of cooking (Fig. 1). In contrast, beef with added 1% GSE and heated at 57.5 °C resulted in a 4.34 log CFU (8.58 to 4.24 log CFU g^{-1}) reduction in the pathogen counts. Increasing the levels of GSE in ground beef to 2 and 3% and heating at 57.5 °C for 250 min yielded 4.95 log CFU (8.61 to 3.66 log CFU g^{-1}) and 5.27 log CFU (8.72 to 3.45 log CFU g^{-1}) reduction (Fig. 1). At higher temperatures, similar increased rates of *C. perfringens* vegetative cells inactivation were observed in ground beef supplemented with 1, 2 or 3% GSE. Thus, heat resistance of *C. perfringens* vegetative cells was temperature and GSE dependent. Since sensitivity of the pathogen to heat was increased in the presence of GSE, lower re-heating temperatures prior to consumption would be sufficient to inactivate high numbers of *C. perfringens* vegetative cells and prevent food poisoning.

The thermal resistance (*D*-values in min) of *C. perfringens* vegetative cells in ground beef with or without added 1–3% GSE at 57.5 to 65 °C, obtained by linear regression of the data are presented in Table 1. Regression curves for inactivation of the pathogen at all four temperatures fitted with an R^2 value of ≥ 0.94. The *D*-values obtained in control beef ranged from 67.11 at 57.5 °C to 1.62 min at 65 °C. Supplementing ground beef with GSE increased the sensitivity of *C. perfringens* vegetative cells in ground beef to heat lethality as indicated by lower recovery of heated cells. Except for 57.5 °C, addition of 1% GSE significantly decreased ($p < .05$) *D*-values at 60, 62.5 and 65 °C to 13.70, 3.47 and 1.46 min, respectively. Parallel significant decrease ($p < .05$) in *D*-values were obtained at all temperatures as the level of GSE in ground beef was increased to 2 or 3%. For example, addition of 2% GSE significantly decreased ($p < .05$) *D*-values at 57.5, 60, 62.5 and 65 °C by 8.97, 4.59, 0.56 and 0.47 min, respectively. With 3% added GSE in ground beef, reduction in *D*-values ranged from 11.86 min

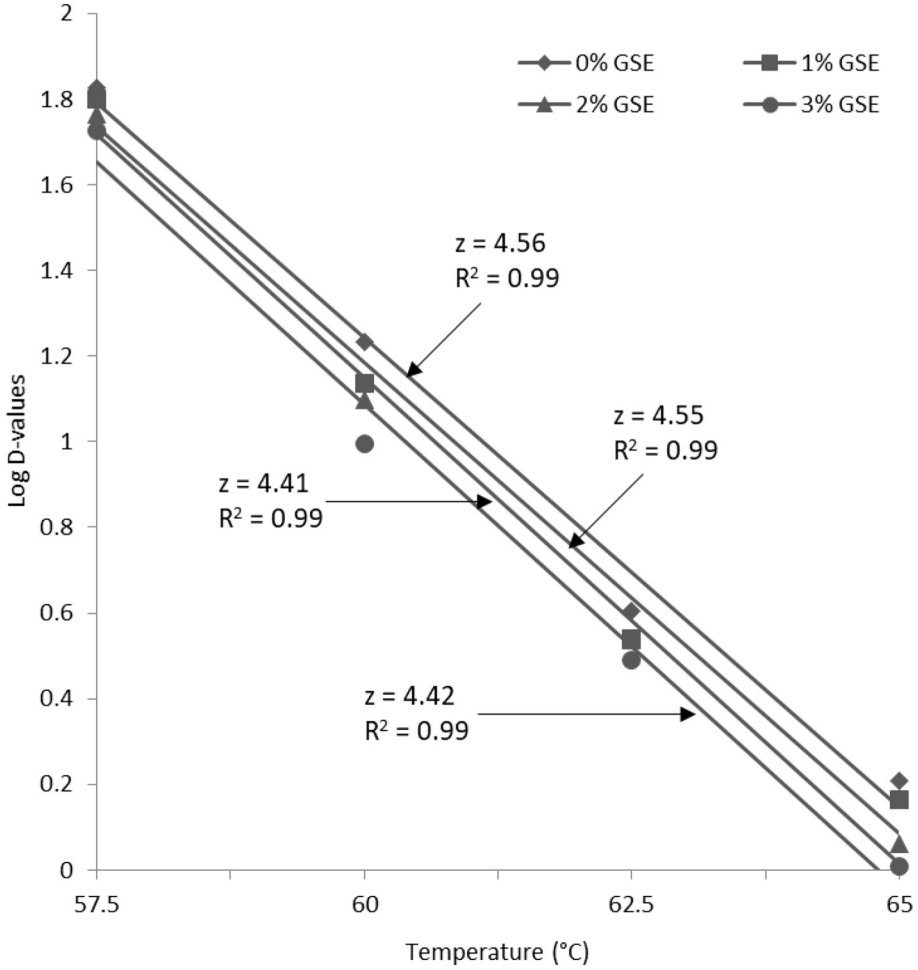

Fig. 2. Thermal death time curves (*z*-values) for *C. perfringens* vegetative cells in ground beef with GSE (0–3%) over a temperature range 57.5–65 °C. To calculate the *z*-values, the mean *D*-values of two replicates for each temperature were used.

(55.25 min, 3% GSE vs 67.11 min, control) at 57.5 °C to 0.6 min (1.02 min, 3% GSE vs 1.62 min, control) at 65 °C, respectively.

To place the *D*-values obtained in the present study into context with previous research, the heat inactivation rates at common test temperatures were compared with similar data published in the literature on heat resistance of *C. perfringens* vegetative cells in meat. Juneja and Marmer (1998) quantified heat resistance of a mixture of three *C. perfringens* vegetative cells inoculated into ground beef and reported *D*-values at 57.5, 60 and 62.5 °C of 10.2, 5.3 and 1.6 min, respectively; *D*-values in ground turkey ranged from 9.1 min at 57.5 to 1.3 min at 62.5 °C. Byrne et al. (2006) determined thermal resistance of three *C. perfringens* vegetative cells in pork luncheon meat in polyethylene bags at 60 °C and reported *D*-value of 8.5 min. In these studies (Byrne et al., 2006; Juneja & Marmer, 1998), meat samples inoculated with *C. perfringens* vegetative cells were heated in a temperature-controlled water bath stabilized at test temperatures. In contrast, meat samples, in the present study, inoculated with the pathogen were heated in a water bath at a slow, linearly increasing heating rate to achieve the final pre-determined target temperature in a period of 1 h. These differences in methodologies used to treat inoculated meat samples may have attributed to higher *D*-values observed in the present study. Therefore, data in the published literature are not in agreement with those reported in the present study.

The microbiological safety of cooked foods depends on ensuring that the time and temperature employed are adequate to destroy the contaminating pathogens. However, certain conditions render the pathogen more resistant to the lethal effect of heat. Such conditions prior to cooking include sublethal environmental stresses encountered by the pathogen such as mild temperature abuse of products, e.g., products displayed on warming trays, partially cooked foods that are re-heated prior to consumption, thermal processing equipment failure/interrupted cooking cycle, etc. Slow heating rate to the final test temperatures employed in the production of *sous vide* cooked foods, in the present study, exposed the microbial cells to conductions similar to heat shock stresses. In a study by Juneja, Novak, Eblen, and McClane (2001), when beef gravy inoculated with *C. perfringens* vegetative cells were subjected to sublethal heating at 48 °C for 10 min, the pathogen survived longer at 58 °C and this increase in heat resistance was as high as 1.5-fold depending upon the strain. In another study (Heredia, García, Luévanos, Labbé, & García-Alvarado, 1997), sub-lethal heat shock of *C. perfringens* vegetative cells at 50 °C for 30 min resulted in at least 2–3-fold increase in heat resistance at 58 °C. This induced thermotolerance of the pathogen after heat shocking, an environmental stress, is a significant public health concern and must be considered while designing the cooking time/temperature to guard against the pathogens and produce microbiologically safety products. Higher *D*-values observed in the present study as compared to previous studies (Byrne et al., 2006; Juneja & Marmer, 1998) may also be attributed to heat-shock response and induced thermotolerance of *C. perfringens* vegetative cells.

To calculate *z*-values, thermal death time curves were plotted from *D*-values obtained in control beef as well as in beef supplemented with 1–3% GSE (Fig. 2). The *z*-values in beef, with and without added GSE, calculated from the curve between 57.5 and 65 °C ranged from 4.41 to 4.56 °C. No changes in *z*-values in beef with or without GSE, obtained in

this study, were in agreement with those reported in the literature. In a study by Juneja and Marmer (1998), addition of sodium pyrophosphate in beef did not alter the *z*-values when thermal death time curves were plotted from *D*-values obtained at 55–62.5 °C. Similar slopes of the thermal death time curves suggest uniform heat inactivation kinetics for *C. perfringens* vegetative cells in beef with and without additives.

4. Conclusions

To protect against *C. perfringens* food poisoning, *sous vide* processed ground beef must be thoroughly re-heated prior to consumption to inactivate large numbers of vegetative cells produced due to germination and outgrowth of surviving spores. The findings presented in this study suggest that GSE addition in *sous vide* processed ground beef renders *C. perfringens* vegetative cells more sensitive to heat lethality, i.e., GSE influenced (increased) the slopes of thermal inactivation curves, by decreasing the respective *D*-values. When ground beef is supplemented with 1–3% GSE, the time needed at a particular temperature to achieve a specific reduction of the pathogen in *sous vide* cooked beef can be predicted from the data presented in Table 1 and Fig. 2. Based on the thermal death time values calculated in this study, *sous vide* cooked ground beef in which growth of *C. perfringens* vegetative cells have occurred from spores should be re-heated to an internal temperature of 62.5 °C for at least 24 min to achieve a 6 log CFU g^{-1} reduction of the pathogen. The re-heating time at 62.5 °C required to achieve the same level of reduction can be reduced to 18.5 min in the presence of 3% GSE in ground beef. The thermal death time values obtained in the present study will assist restaurants and food service establishments in estimating re-heating time and temperature for *sous vide* cooked ground beef before consumption to ensure elimination of the contaminated pathogen and ensure microbiological safety of the products. Impact on sensory attributes *sous vide* processed ground beef with added GSE warrant further study.

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