Improved design of aluminum test cell to study the thermal resistance of *Salmonella enterica* and *Enterococcus faecium* in low-water activity foods

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ABSTRACT

Salmonella enterica is a major cause of foodborne pathogen contamination in the United States. The heat resistance of *Salmonella* increases as a_w decreases, leading to a significant challenge in the design of thermal processes. The aluminum thermal death time (TDT) cell is a common experimental tool to study the isothermal resistance of *Salmonella* and other pathogenic bacteria in foods. However, the TDT cell has the problem of a relatively long come-up time, and it is difficult to seal. This research improved the previous design of aluminum TDT cells and evaluated the performance of the improved design by using the thermal inactivation data of *Salmonella enterica* cocktail and *Enterococcus faecium* in brown rice flour. The come-up time of brown rice flour was reduced significantly, from 2.33 min in the previous design to 0.67 min (40 s) in the improved design of test cells when immersed in an 85 °C glycol bath. With the increase of the cavity diameter of the cell, it was easy to load and unload food samples. Additional new features of the improved design included the wrench flats on the outer surface of cell wall. The *D*-values for *Salmonella* in the a_w 0.45 (measured at 25 °C) brown rice flour were 42.70, 17.51, 8.11, and 2.27 min at 70, 75, 80, and 85 °C, respectively. The *D*-values for *Enterococcus faecium* were 65.82, 22.28, 6.05, and 1.86 min at 70, 75, 80, and 85 °C, respectively. This research shows that the improved design of test cells is a reliable experimental tool to study the thermal resistance of microorganisms in low-a_w foods. Accurate detection of microbial survival under isothermal conditions will provide reliable data for modeling and pathogen control during thermal processing to help ensure food safety and quality.

1. Introduction

Salmonella enterica is a leading cause of foodborne pathogen contamination and gastroenteritis in the United States (Juneja & Eblen, 2000). Water activity (a_w) below 0.85 is considered to be a barrier for the growth of *Salmonella* and other bacterial pathogens (Podolak, Enache, Stone, Black, & Elliot, 2010). However, *Salmonella* can still survive for years in low-a_w foods during storage and cause illness if a low number of cells exist (Hiramatsu, Matsumoto, Sakac, & Miyazaki, 2005; Jarvis et al., 2016). Recent recalls and outbreaks of *Salmonella* in low-a_w products, such as peanut butter, almonds, pistachios, rice and wheat cereals, have caused significant public health concern (CDC, 2004; CDC, 2008; CDC, 2009; CDC, 2016). The heat resistance of *Salmonella* increases as a_w decreases, leading to a significant challenge in the design of thermal processes (Jeong, Marks, & Orta-Ramirez, 2009; Jin et al., 2018; Smith, Hildebrandt, Casulli, Dolan, & Marks, 2016; Villa-Rojas et al., 2013; Xu et al., 2019).

Several studies have been published on the thermal inactivation kinetics of *Salmonella* in low-a_w foods, such as wheat flour, almond kernel flour, pet food, and other food products (Jin et al., 2018; Ma et al., 2009; Smith et al., 2016; Villa-Rojas et al., 2013). The aluminum thermal death time (TDT) cell, first reported in Chung, Birla, and Tang (2008), was proven to be reliable as a common experimental tool in previous studies (Jin et al., 2018; Liu, Xu, Xie, Zhu, & Tang, 2019; Smith et al., 2016; Taylor, Tsai, Rasco, Tang, & Zhu, 2018; Villa-Rojas et al., 2013, 2017; Xu et al., 2019). The sealed test cells containing inoculated low-a_w food samples are heated at isothermal temperatures to obtain calculable *D*-values (time needed to reduce microbial population by 10-fold at a specific temperature) from thermal kinetic models. The come-up time (time required for the sample core temperature reach 0.5 °C less than the target temperature) is subtracted from the total thermal time in the isothermal kinetic data analyses. The come-up time for the previous design of test cells usually ranges from 1.33 min to 3 min, depending upon the treatment temperature and food matrices. Xu et al. (2019) reported a come-up time of 1.5 min when heating low-moisture food powders in the previous design of test cells at 80 °C. Villa-Rojas et al. (2013) reported that the come-up time for almond kernel flour samples in the same TDT cell was 1.33 min–1.5 min

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between 56 and 80 °C. They also reported that the *D*-value for *Salmonella* Enteritidis PT 30 in almond kernel flour samples was 0.42 min at 64 °C (Villa-Rojas et al., 2013). Thus, up to about 3 log reduction in the inoculated *Salmonella* population could occur during the come-up time. The initial microbial inoculation in food samples is usually from 7 to 9 log CFU/g (Jin et al., 2018; Smith et al., 2016; Villa-Rojas et al., 2013). Typically, in kinetic tests, at least 3 log CFU/g microbial reduction is required under an isothermal condition to provide accurate estimation of *D*-value. The lowest microbial countable level in enumeration is 25 on a standard agar (Sutton, 2011). Thus, the surviving population after come-up time may not be adequate for the isothermal tests, if the final count is less than 2500 CFU/g or 3.39 log CFU/g in a 1:100 dilution (Sutton, 2011).

The relatively large loss to the initial inoculated bacteria population during long come-up times affects the isothermal experimental design and isothermal data evaluation, especially at high temperatures. The model prediction is not persuasive if the lab-based isothermal data is inaccurate and the tested temperature range is limited. It is necessary to improve the design of test cells to shorten the come-up time and limit the microbial loss in food samples during the come-up time at elevated temperatures.

Surrogates (non-pathogenic microorganisms with attachment characteristics, growth, and inactivation behaviors similar to the target pathogen under process parameters of interest) are useful in the lethality and decontamination validation during food manufacturing operations (Bianchini et al., 2014). *Enterococcus faecium* NRRL B-2354 is commonly used as a surrogate in the thermal processing of low-a_w foods (Verma et al., 2018). A cocktail of *Salmonella* is usually utilized to perform validation studies in low-a_w foods. For example, Liu et al. (2019) selected a cocktail of *Salmonella* Agona, *Salmonella* Mbandaka, *Salmonella* Montevideo, *Salmonella* Reading, and *Salmonella* Tennessee to validate *Enterococcus faecium* as the surrogate in nonfat milk powder. Verma et al. (2018) inoculated a cocktail of *Salmonella* Agona, *Salmonella* Mbandaka, *Salmonella* Enteritidis, *Salmonella* Montevideo, and *Salmonella* Tennessee in oat flour and validated *Enterococcus faecium* as the surrogate for *Salmonella* during extrusion. These serovars were chosen for their high outbreak rate and high thermal resistance in low-a_w foods (Liu et al., 2019; Verma et al., 2018).

This research improved the previous design of aluminum TDT cells and evaluated the performance of the improved design by using the thermal inactivation data of *Salmonella enterica* cocktail and *Enterococcus faecium* in brown rice flour. Two primary models were utilized to describe *Salmonella enterica* and *Enterococcus faecium* inactivation kinetics during isothermal treatment.

2. Materials and methods

2.1. Improved design of aluminum test cell

An improvement to the previous design of TDT cells was necessary to overcome the limitation of long come-up times. The dimension of the previous design of test cells described in Chung et al. (2008) is presented in Fig. 1A. It consists of an aluminum base and lid (Fig. 1A). Prior to heat treatments, the food sample is placed in the cavity of the base which is 3.93 mm in height and 18.00 mm in diameter (1 mL volume) (Fig. 1A). The schematic representation of improved design of the test cell is shown in Fig. 1B. To reduce the come-up time, the cavity height of the new cell was reduced to 1.30 mm and the diameter was increased to 31.18 mm to maintain the same volume holding capacity of 1 mL. With the increase of cavity diameter it was easy to load and unload food samples. The construction material was kept the same as aluminum alloy 6061 for its high thermal diffusivity, good corrosion resistance, and machinability (Chung et al., 2008). Additional new features of the improved design include the wrench flats on the outer surface of cell wall (Fig. 2). A pair of custom tightening fixtures were applied correspondingly to the structure for better opening and closing
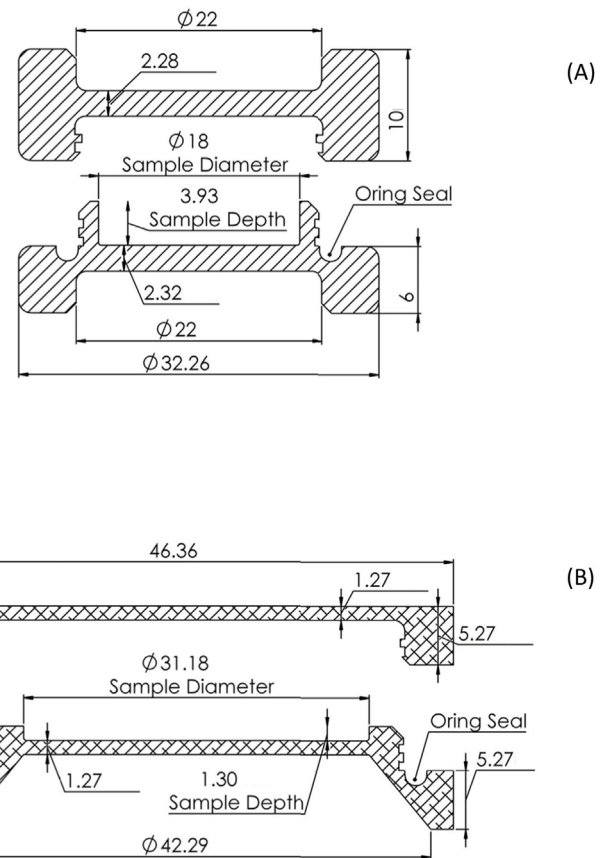

Fig. 1. Schematic representation of the previous and improved design of test cells (A) previous design of test cell (B) improved design of test cell. All dimensions are in mm.
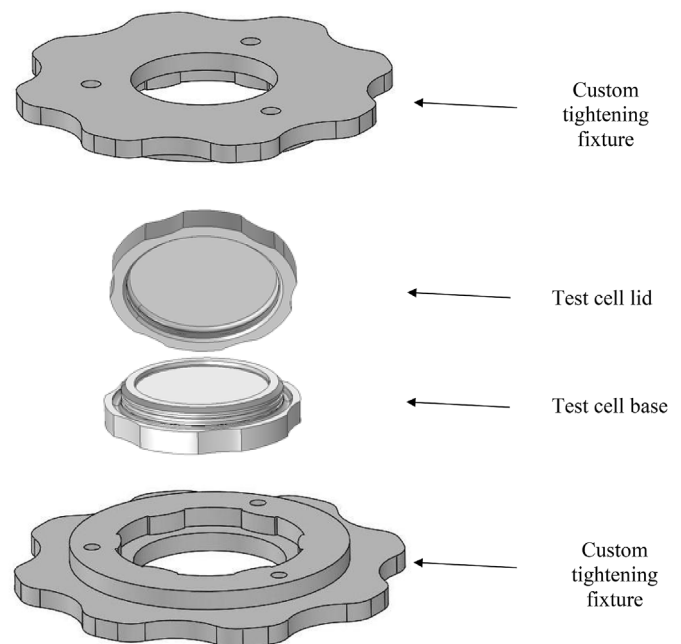

Fig. 2. 3D rendering of the improved design of test cell and custom tightening fixtures.

of the cell (Fig. 2). The surface of the improved design of the test cell was protected by tough coat anodizing of Teflon to prevent erosion.

2.2. Brown rice flour

Whole grain organic brown rice flour (Bob's Red Mill Company, Milwaukie, OR) was obtained from a local grocery store in Pullman, WA. The rice flour samples were used to assess the performance of the new TDT cells in comparison with that of the previous design. The absence of *Salmonella* in the source brown rice flour was confirmed by soaking 10 g of the sample into 90 mL tryptic soy broth with 0.6% yeast extract (TSBYE; Difco, BD, Franklin Lakes, NJ) for 1 h, and then plating onto tryptic soy agar with 0.6% yeast extract (TSAYE; Difco, BD) supplemented with 0.05% ammonium ferric citrate (Sigma-Aldrich, St. Louis, MO) and 0.03% sodium thiosulfate pentahydrate (Sigma-Aldrich). This differential media (mTSAYE) was able to separate *Salmonella* from other background microflora by the characteristic black precipitate in the center of colonies (Smith et al., 2016). The absence of *Enterococcus faecium* in the source brown rice flour was confirmed by TSAYE supplemented with 0.05% ammonium ferric citrate and 0.025% esculin hydrate (Sigma-Aldrich). This eTSAYE media differentiated *Enterococcus faecium* as a black precipitate in the center of colonies.

2.3. Heat transfer performance

The come-up time was measured following the procedure described in Chung et al. (2008). A 0.5 mm diameter thermocouple (Type T, OMEGA Engineering, INC., Stamford, CT) was secured to the center of a custom test cell through the lid with a Swagelok fitting compression to prevent any glycol leakage between the thermocouple and cell wall. Around 0.7 g (~1 mL) of the brown rice flour sample was filled into the cavity. The sealed test cell was immersed in ice water to achieve an equilibrium initial temperature of 0 °C before immersing into a 85 °C glycol bath (Isotemp 5150 H11, Fisher Scientific, Waltham, MA). The core temperature of the brown rice flour sample was recorded by a data logger (LR8402-20, HIOKI E. E. Corporation, Nagano, Japan) at a time interval of 0.02 s. The come-up time for the rice flour sample heated in the previous design of test cell was also recorded using the same procedures described above.

2.4. Microbial serovar

A cocktail of five *Salmonella enterica* serovars—*Salmonella enterica* Agona 447967, *Salmonella enterica* Mbandaka 698538, *Salmonella enterica* Montevideo 488275, *Salmonella enterica* Tennessee K4643, and *Salmonella enterica* Reading ATCC 6967—were chosen based on their high thermal resistance and frequent outbreak in low-a_w foods in previous reports (Liu et al., 2019; Verma et al., 2018). The non-pathogenic *Enterococcus faecium* NRRL B-2354 was obtained from the Agricultural Research Service at the U.S. Department of Agriculture.

2.5. Inoculum preparation

The inoculum preparation method followed that in Keller et al. (2012). Each isolated colony from working cultures was inoculated into 10 mL of TSBYE, respectively, and grown for 24 h at 37 °C. A 100 μL aliquot from each incubated cultures was transferred onto TSAYE to produce a bacterial lawn at 37 °C for 24 h. Each inoculum of *Salmonella enterica* and *Enterococcus faecium* was harvested by adding 1 mL of buffered peptone water (BPW; Difco, BD) per plate and scraped gently with a sterile spreader. Each serovar of *Salmonella* was mixed in equal proportions to make a cocktail.

2.6. Sample preparation

Salmonella cocktail was inoculated into brown rice flour at a ratio of 1 mL (cocktail): 10 g (flour). The inoculated brown rice flour was mixed by hand massage for approximately 1 min, followed by a stomaching blender (Stomacher 400 Circulator, Seward Laboratory System Inc., Norfolk, UK) for 3 min with 230 strokes/min, alternatively repeated three times. To prepare the *Enterococcus faecium* inoculated samples, the *Enterococcus faecium* cell suspension was mixed with brown rice flour following the same procedure described above. The inoculated brown rice flour was conditioned for 2 days in a humidity controlled glove box (Smith et al., 2016) pre-equilibrated to a_w 0.45 at room temperature. The brown rice flour could reach an equilibrium a_w of 0.45 in 2 days, and the final a_w was validated by a water activity meter (Aqualab, Meter Group, Inc., Pullman, WA) at 25 °C. A homogeneity study was performed by randomly taking a 1 g sample from 10 locations in the inoculated flour for both *Salmonella* and *Enterococcus faecium*, serially diluted to detect the plate count.

2.7. Thermal treatment and microbial enumeration

Four temperatures and a minimum of 5 time intervals were used for each inoculated (*Salmonella* cocktail and *Enterococcus faecium*) brown rice flour sample. The improved TDT cells were loaded with inoculated flour (0.7 g per cell) and sealed by the custom tightening fixtures in the humidity controlled glove box. The test cells were heated in the glycol bath at isothermal temperatures of 70, 75, 80, and 85 °C to obtain *D*-values. The heating temperature was measured by glycol bath internal thermometer, and monitored by a hand-held thermometer (Type 52 II, Fluke Corporation, Everett, WA). Three test cells were removed from the bath at each dwell time. The test cells were immediately cooled in ice water. The survival of *Enterococcus faecium* after come-up time in both the previous design and improved design of test cells was characterized at 85 °C as a comparison following the above procedures.

Flour samples were transferred into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) and diluted with 6.3 mL BPW to create an initial 1:10 dilution. The mixture was stomached for 3 min with 230 strokes/min then serially diluted with BPW. Spread plates (0.1 mL in duplicate) were made on either mTSAYE or eTSAYE to detect *Salmonella* or *Enterococcus faecium* survival, respectively. Agar plates were incubated at 37 °C for 24 h.

2.8. Model fitting

The microbial counts were converted to log CFU/g. The come-up time was subtracted from the total thermal time. Log-linear and Weibull models were applied to both *Salmonella enterica* and *Enterococcus faecium* inactivation data. The log-linear model is (Peleg, 2006):

$$\log\left(\frac{N}{N_o}\right) = \frac{-t}{D} \tag{3}$$

where N is surviving population (CFU/g) at time t (min), N_o is the microbial population after come-up time (t = 0), D is the time (min) needed to reduce microbial population by 10-fold at a specific temperature (°C).

The equation for Weibull distribution (Peleg, 2006) is as follows:

$$\log\left(\frac{N}{N_o}\right) = \left(-\frac{t}{d}\right)^b \tag{4}$$

where N and N_o (CFU/g) are microbial population at time t (min) and 0 (min), d is the scale factor (min), b is shape factor, and describes whether the inactivation curve is linear ($b = 1$), upward ($b < 1$), or downward ($b > 1$).

The z-value was calculated by using the Bigelow secondary model. The Bigelow model (Bigelow, 1921) is:

$$D = D_{ref} 10^{\left(\frac{T_{ref} - T}{z}\right)} \tag{5}$$

where D_{ref} is the time required to obtain a 10-fold reduction of microbial population at reference temperature T_{ref}, z is temperature change required to obtain a 10-fold change in D-value.

The log-logistic equation (Mendenhall, Beaver, & Beaver, 2003) for Weibull distribution was applied as the secondary model:

$$\frac{1}{d_T} = \ln\{1 + exp[k(T - T_c)]\} \tag{6}$$

where d_T is the scale factor d (min) at temperature T (°C), T_c is the critical temperature at which d_T starts an exponential growth with respect to temperature T, and k is the slope of linear relation between d_T and T.

The root mean square error (RMSE) was used to estimate model error:

$$RMSE = \sqrt{\frac{\sum\left(\log\left(N/N_0\right)_{predicted} - \log\left(N/N_0\right)_{observed}\right)^2}{n - p}} \tag{7}$$

where n is the total number of observations, N and N_0 (CFU/g) are microbial populations at times t and 0, and p is the number of model parameters.

Statistical analysis and model estimations were performed in Microsoft Excel (16.10, Microsoft, WA, USA). The statistical difference between model parameters was tested, and differences were considered significant at a $p < 0.05$.

3. Results and discussion

3.1. Improved design of aluminum test cell

Typical temperature-time histories at the center of a brown rice flour sample in the previous and the improved test cells are presented in Fig. 3. The come-up time was reduced significantly, from 2.33 min in the previous test cell to 0.67 min (40 s) in the improved test cell design when immersed in 85 °C glycol bath (Fig. 3). Besides the reduction of come-up time, additional new features also performed well. It was easy to load and unload low-a_w food samples with the increase of test cell diameter. The improved design of the test cell allowed it to be sealed and opened conveniently by the custom tightening fixtures, avoiding the discomfort of fingers. The previous design of the test cell, on the other hand, needed to be sealed and opened by hand twisting which was difficult. Also, the airtightness of the test cell was guaranteed, as a larger torque was applied by the tightening fixtures.
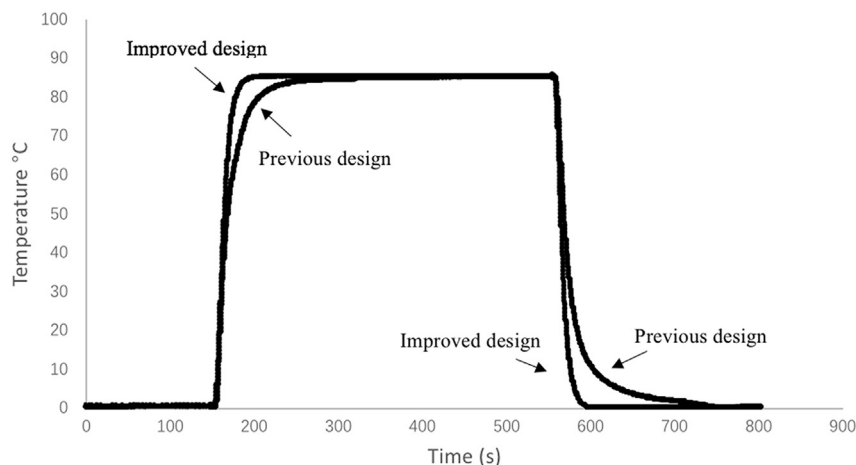

Fig. 4. Survival of *Enterococcus faecium* in brown rice flour equilibrated to a_w 0.45 at 25 °C, and treated at 85 °C in 1 min in the improved TDT cell, and 3 min in the previous TDT cell (n = 3).

Fig. 5. Thermal inactivation of *Salmonella enterica* cocktail in brown rice flour equilibrated to a_w 0.45 at 25 °C, and treated at 70, 75, 80, and 85 °C in the improved TDT cells.

3.2. Comparison with previous design

No *Salmonella* or *Enterococcus faecium* was detected in the source flour. The post inoculation population were > 8 log CFU/g for both *Salmonella* and *Enterococcus faecium*. Homogeneity was achieved, as

Fig. 3. Temperature-time history at the center of brown rice flour sealed in the improved TDT cell and previous design of TDT cell, which were immersed in a 85 °C glycol bath.
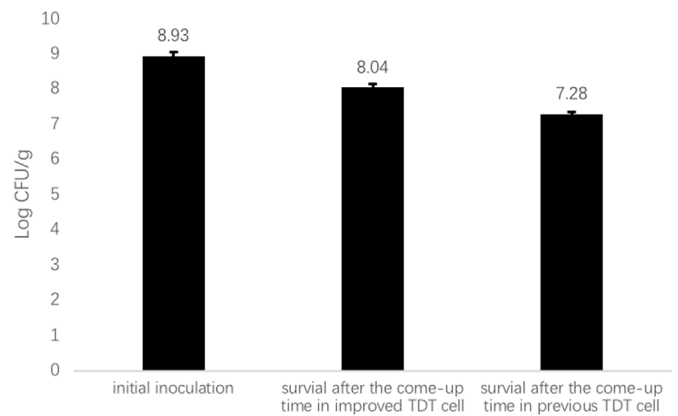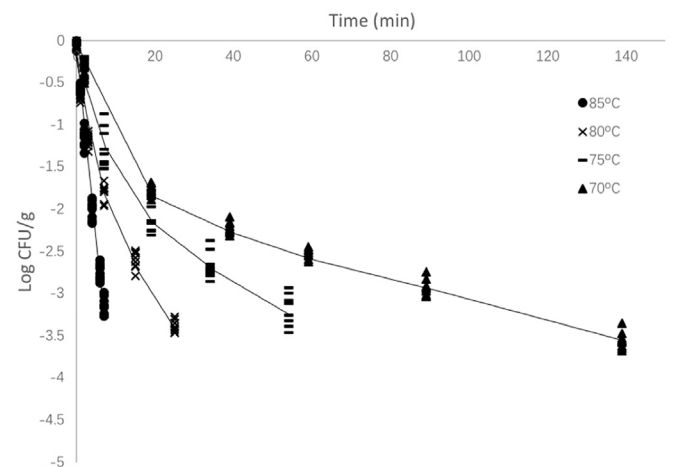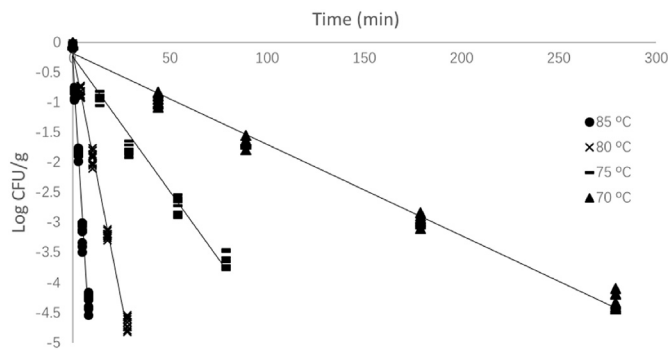
Fig. 6. Thermal inactivation of *Enterococcus faecium* in brown rice flour equilibrated to a_w 0.45 at 25 °C, and treated at 70, 75, 80, and 85 °C in the improved TDT cells.

Table 1
Fitted *D*-values of first-order kinetic model and Weibull model for *Salmonella enterica* and *Enterococcus faecium* in brown rice flour pre-conditioned to a_w 0.45 at room temperature.

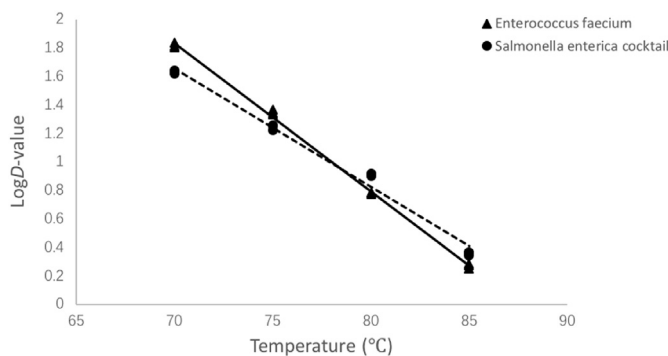| Culture	Temperature (°C)	First-order kinetics		Weibull model		
		D-value (min)	RMSE	*d*	*b*	RMSE
Salmonella	85	2.27	0.18	1.75	0.83	0.09
enterica	80	8.11	0.64	2.21	0.50	0.07
	75	17.51	0.65	5.22	0.51	0.23
	70	42.70	0.90	6.00	0.41	0.14
Enterococcus	85	1.86	0.30	1.27	0.80	0.15
faecium	80	6.05	0.20	4.71	0.87	0.08
	75	22.28	0.30	14.20	0.75	0.10
	70	65.82	0.23	48.60	0.84	0.08

Fig. 7. Comparison of log *D*-values between *Salmonella enterica* cocktail and *Enterococcus faecium* in the samples with a_w 0.45 at 25 °C.

Table 2
Calculated *z*-values, T_c, *k* values on thermal inactivation of *Salmonella enterica* and *Enterococcus faecium*.

Culture	Bigelow model		Log-logistic model		
	z-value (°C)	RMSE	T_c (°C)	*k*	RMSE
Salmonella enterica	12.02	0.61	89.39	0.09	0.73
Enterococcus faecium	9.59	0.23	85.49	0.25	0.21

reflected by a standard deviation for the bacteria population of less than 0.3 log CFU/g in both *Salmonella* and *Enterococcus faecium* inoculated samples. The come-up time for heating brown rice flour sample in the improved design and in the previous design of test cells were about 0.67 min and 2.33 min, respectively. It could be conservatively assumed as 1 min and 3 min in this research. The survival of *Enterococcus faecium*

after the come-up time in the previous design and the improved design of test cells is shown in Fig. 4. The initial inoculation of *Enterococcus faecium* was 8.93 log CFU/g. There was a 0.89 log CFU/g reduction over the come-up time in the improved test cell at 85 °C, whereas, a 1.65 log reduction was observed in the previous design. A high survival of *Enterococcus faecium* over the come-up time was necessary to meet the experimental requirements and obtain complete inactivation data. The improved test cell showed its advantage compared with the previous design when performing isothermal studies.

3.3. Thermal resistance of Salmonella and Enterococcus faecium

The log reduction of *Salmonella enterica* cocktail and *Enterococcus faecium* at 70, 75, 80, and 85 °C are presented in Figs. 5 and 6. Two primary models were utilized to describe microbial inactivation during isothermal treatment (Table 1). Higher temperatures led to smaller *D*-values for *Salmonella enterica* cocktail and *Enterococcus faecium*. Results were consistent with the reports from others (Jin et al., 2018; Ma et al., 2009), exhibiting the temperature influence on thermal resistance. *Enterococcus faecium* tended to show a more semi-log trend compared with that of *Salmonella enterica* cocktail in brown rice flour, with shape factor *b* close to 1 indicating a linear relationship between log reduction and time (Table 1). The non-linear upward concavity was found in *Salmonella enterica* cocktail, suggesting a tailing effect as the treatment time was prolonged. Lower temperature led to a more pronounced tailing effect in this research. Similar trends were also reported in other research (Rachon, Peñaloza, & Gibbs, 2016; Villa-Rojas et al., 2013). One possible cause of the tailing might be that the majority of *Salmonella enterica* cocktail was relatively heat sensitive, whereas the minority was heat resistant (Smelt & Brul, 2014), the heat-sensitive microbial cells were rapidly inactivated at the beginning of the thermal treatment while more-resistant cells survived over longer time (Villa-Rojas et al., 2013). Also, it was reported that vegetative cells could adapt to stress conditions leading to a lower inactivation rate, especially in long heating time at low temperatures (Smelt & Brul, 2014).

The *D*-values of *Salmonella enterica* cocktail were smaller than the *D*-values of *Enterococcus faecium* at 70 and 75 °C ($p < 0.05$), while larger at 85 °C ($p < 0.05$). An intersecting temperature in which the *D*-values were same was observed at around 78 °C (Fig. 7), and the *D*-values at 80 °C were not significantly different ($p > 0.05$).

The *z*-value of *Salmonella enterica* cocktail was larger than the *z*-value of *Enterococcus faecium* from the secondary Bigelow model (Table 2). *Salmonella enterica* cocktail has a larger T_c than *Enterococcus faecium* in the log-logistics model, indicating that a higher critical temperature was required for the shape factor *b* of *Salmonella enterica* cocktail in the primary Weibull model to start an exponential increase with respect to temperature.

4. Conclusion

Our results show that the improved design of test cells is a useful experimental tool to study the thermal inactivation of microorganisms in low-a_w foods. Accurate assessment of microbial survival is achieved by shortening the come-up time at elevated temperatures. This improved design will facilitate the microbial inactivation study in low-a_w foods, and provide reliable data for modeling and pathogen control during high temperature thermal processing.

Acknowledgements

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