



The effect of dry headspace on the thermal resistance of bacteria in peanut oil and peanut butter in thermal treatments

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ABSTRACT

The objective of this study was to investigate the effect of a dry headspace on the thermal resistance of bacteria in lipid oil and peanut butter. Our recent study found *Enterococcus faecium* to be extremely resistant to heat in a thin oil layer when exposing to a dry environment. Thus, we hypothesized that peanut oil or peanut butter that is in contact with dry air during a thermal process may cause surface drying and, therefore, protect bacteria from heat inactivation. In this research, we treated peanut oil ($a_w = 0.31$ at 80°C) and peanut butter ($a_w = 0.38$ at 80°C) inoculated with *Salmonella* Enteritidis PT30 (*S. Enteritidis*) or *Enterococcus faecium* NRRL B-2354 (*E. faecium*) at 80°C using two types of hermetically sealed sample holders, one with negligible headspace and the other with a large headspace. The results indicated that *S. Enteritidis* and *E. faecium* in peanut oil and peanut butter became significantly ($P < 0.05$) more resistant to heat when treated in sample holders with large headspace compared to in fully filled sample holders with negligible headspace. When the sample holder head space was switched from "Negligible" to "Large", the thermal death time (D_{80} -value) of *E. faecium* increased 16.7 times in oil and 4.3 times in peanut butter; the D_{80} -value of *S. Enteritidis* increased 4.6 times in peanut oil and 2.6 times in peanut butter. When plotted vs. the material water activity measured at the treatment temperature (80°C), the D_{80} -value of both bacteria agreed well with reported data obtained from other low-moisture foods, indicating that the local water activity of the sample was the dominating factor and surface drying was the cause to the enhanced thermal resistance. This study illustrates the importance of selecting appropriate test cells in isothermal studies on thermal resistance of bacteria in high fat low moisture foods. It also suggests that food industry should be cautious about surface drying in the pasteurization of low-moisture products.

1. Introduction

Widespread outbreaks of foodborne salmonellosis between 2007 and 2009 (Cavallaro et al., 2011; Sheth et al., 2011; U.S. CDC, 2007; U.S. CDC, 2009) have raised the concern over possible contamination of peanut butter and the products that include peanuts as ingredients. In response to the concern, the U.S. Food and Drug Administration recommends a 5-log reduction in *Salmonella* spp. for peanut-derived ingredients (USDA, 2009). To comply with the 2011 Food Safety Modernization Act, food manufacturers start to focus on preventing food contamination rather than responding to foodborne illness events (USDA, 2020). Several investigations on early cases of outbreaks found that contamination might have occurred from postharvest environments or even in the production line due to flawed hygiene practices (Borrell,

2009; Kase et al., 2017; Sheth et al., 2011). Therefore, a valid heat pasteurization step for the end-product could be an ultimate guarantee for the safety of peanut butter as well as other high-fat low-moisture foods like nuts butter and sesame paste.

The design of a pasteurization process requires knowledge of the thermal death kinetics of the target pathogen(s) in the product (Silva & Gibbs, 2012). For low-moisture foods, isothermal studies on a product need to be conducted at controlled levels of water activity (a_w) to obtain the corresponding D -values (decimal reduction time) of the target pathogen(s) (Jin et al., 2020) for each treatment temperature, because different levels of dehydration of bacteria cells (including *Salmonella*, *Enterococcus*, *Listeria*, *Escherichia*, and the spores of *Bacillus* and *Clostridium*) in low-moisture environments would show different thermal resistance (Ballom et al., 2020; Gaillard et al., 1998; He et al., 2011,

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2013; Liu et al., 2018; Murrell & Scott, 1966). However, in the reported studies on peanut butter, the survival curves of *Salmonella* at a constant lethal temperature were often found to be concave, likely due to a lack of tight control of product water activity. This makes it difficult directly use the kinetic model to design proper pasteurization processes (Li et al., 2014; Ma et al., 2009; Shachar & Yaron, 2006).

Considering that peanut butter contains about 50% oil, earlier studies attributed the concave thermal death curves of *Salmonella* in peanut butter to the protective effect of oil (Li et al., 2014; Ma et al., 2009; Shachar & Yaron, 2006). Recent investigations indicated that oil protects bacteria from heat inactivation through its sharply decreased a_w at elevated temperatures which caused desiccation of bacterial cells (Yang et al., 2020a, 2020b, 2021). But this effect may not occur in peanut butter which is a mixture of oils, carbohydrates, and proteins. The a_w of peanut butter is more likely to be dominated by the a_w of starch and protein granules that generally increase with temperature (Tadapaneni et al., 2018).

Another possible explanation for the concaving death curves of *Salmonella* in peanut butter is the drying effect from the headspace of the sample holders that were used for thermal treatments. It's long been discovered that an aqueous suspension of bacteria treated in open tubes may give "erroneously high values" of thermal resistance compared to what is treated in closed capillary tubes because the evaporation of water would lower the surface temperature of the sample (Roberts et al., 1965). In low-moisture foods, like peanut butter, the water evaporation may not be large enough to cause a significant temperature reduction. But exposure to a large headspace above the sample could cause surface drying and consequently reduce the moisture content of the bacteria cells in the surface food matrix, thus making the bacteria more resistant to heat.

The objective of this research was to investigate the influence of dry gaseous space in test sample holders on the thermal resistance of bacteria in isothermal treatments. In this study, we selected a Gram-positive strain, *Enterococcus faecium* NRRL B-2354, and a Gram-negative strain, *Salmonella* Enteritidis PT 30, as the test bacteria, and peanut oil and peanut butter as the test materials. Inoculated peanut oil and peanut butter samples were treated in two different types of sample holders: one fully packed to minimize the headspace, and another enclosed a large headspace over a thin layer of sample. These data were analyzed to show if surface drying was the cause of the enhanced thermal resistance of *Salmonella* in peanut butter.

2. Material and methods

2.1. Experimental design

The experiments were conducted in two phases: 1) comparing the thermal death curves of *Enterococcus faecium* NRRL B-2354 (ATCC 8459) (*E. faecium*) in peanut oil ($a_w = 0.75$) at 80 °C in four different sample holders, three in this work: Thermal Death Time (TDT) cells, Thermal a_w Control (TAC) cells, and large test tubes (Fig. 1), and one from the literature (capillary tubes) (Yang, Xu, et al., 2020); 2) using TDT and TAC cells to study the thermal resistance of two bacterial strains, *E. faecium*, and *Salmonella enterica* Enteritidis PT 30 (ATCC BAA-1045) (*S. Enteritidis*) in peanut oil and peanut butter ($a_w \sim 0.30$) at 80 °C. The first phase experiments used one combination of bacterium and food matrix to see if the sample holder with a large headspace (TAC cells or large test tubes) would yield a greater thermal resistance of bacteria compared to fully filled sample holders (capillary tubes and TDT cells) having negligible headspace. It might also show if the results from two traditional samples holders (capillary tube and test tube) were comparable with the results when using two customized aluminum sample holders (TDT and TAC cells). The second phase experiments only used TDT and TAC cells, for the convenience of handling, to determine the thermal resistance of two different bacteria, *E. faecium* in peanut butter and *S. Enteritidis*, in peanut oil and peanut butter. The results were used

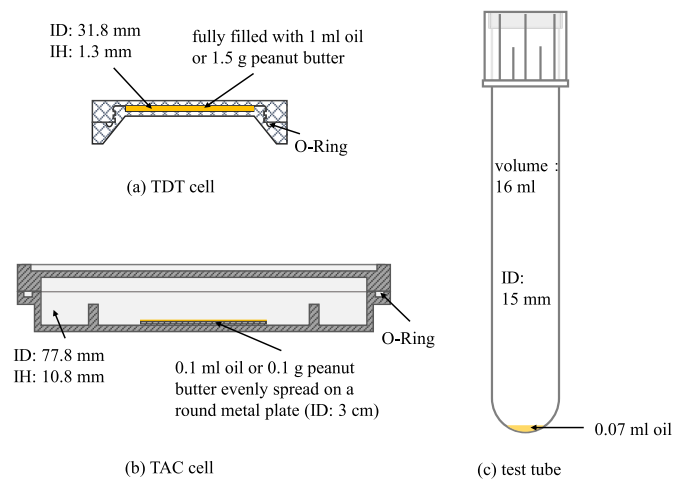


Fig. 1. Schematic representation of sample holders used in isothermal treatments (a) TDT cell, (b) TAC cell, and (c) large test tube. The abbreviation, ID, is for inner diameter and IH for inner height. Food samples (peanut oil or peanut butter) are printed in yellow.

to investigate the effect of a dry gaseous headspace on bacterial thermal resistance in two different food matrices.

E. faecium is a Gram-positive bacterium normally used as a surrogate of *Salmonella*, while *S. Enteritidis* is a Gram-negative bacterium. The strain of *S. Enteritidis* was a human isolate sampled from the 2000–2001 almonds outbreak (Isaacs et al., 2005). Both strains were obtained from Dr. Linda Harris (University of California, Davis, CA). Fig. 1 shows the sample holders used in this study. A TDT (thermal death time) cell (a) (Washington State University, Pullman, WA) had an internal cavity of 1.33 mm height and 31.8 mm diameter (Jin & Tang, 2019). It was modified from an early version of TDT cells (Chung et al., 2008) with the same volume (1 ml) but only 1/3 of sample thickness to reduce the come-up time in low-moisture foods during thermal treatments. TAC cells (b) were designed by Tadapaneni et al. (2018) to study the thermal death rate of bacteria in low-moisture food when exposed to a relative humidity controlled by salt solutions. In this study, the TAC cells did not contain a solution, so that the headspace relative humidity was not controlled during thermal treatments. The test tubes (c) used in this study were made of polypropylene (17 × 100 mm) with snap caps (Corning Falcon, Tewksbury, MA). The total volume in a test tube was about 16 ml which provided a large headspace when only filled with a small amount of sample (70 µl). The sample holders represented two extreme conditions (with or without headspace) during isothermal heat treatments.

2.2. Sample preparation

Purified peanut oil (Ventura Foods, LLC, Brea, CA) was purchased from a local grocery store. Organic peanut butter (creamy) (365, Whole Foods Market, Austin, TX) was purchased through E-commerce. This peanut butter used peanuts, palm oil, and sea salt as ingredients and its total fat content was about 56%.

Peanut oil in test tubes (10 ml each) was subjected to autoclaving for 20 min at 121 °C to eliminate background microorganisms. Then the tubes were loosely capped and stored at 23 °C for one week to allow the excess moisture, which was gained from the steam treatment, to evaporate.

The background bacteria in peanut butter used in the experiment was checked using a standard aerobic plate count method described in (U.S. FDA, 2001). In brief, two 1-g samples were taken from each batch of peanut butter from the blender jar and mixed with 9 ml of sterile 2% buffered peptone water (BPW, BD Difco, Franklin Lakes, NJ) using hand massage, followed by homogenization in a stomacher (Stomacher 400,

Seward Laboratory Systems Inc., Bohemia, NY, USA) at 260 rpm for 5 min. The diluted sample was then plated on M124 agar, 0.5% Tryptone (BD Difco) supplemented with 0.25% (w/w) yeast extract (BD Difco), and 0.1% dextrose (BD Difco). The plates were incubated at 37 °C for 24 h, all colonies were counted.

2.3. Inoculation

The inoculation procedure followed a lawn-based method (Hildebrandt et al., 2016; Yang, Xu, et al., 2020). *E. faecium* and *S. Enteritidis* were stored as stock culture in a refrigerator on streak plates of trypticase soy agar (BD Difco) with 0.6% yeast extract (BD Difco) (TSAYE). For each biological independent inoculation, a single colony of *E. faecium* or *S. Enteritidis* was subjected to two consecutive transfers of 9 ml trypticase soy broth (TSB, BD Difco) with 0.6% yeast extract (TSBYE) and each incubated at 37 °C for 24 h. Then 1 ml of culture was spread on each 150 × 15 mm plate of TSAYE and incubated for 24 h at 37 °C. Bacteria lawn was harvested with two consecutive 9 ml of sterile 2% buffered peptone water (Difco, BD) using an L-spreader, transferred into a 50 ml centrifugal tube, and vortexed for 1 min. Bacteria suspension was obtained from three plates of the lawn of *E. faecium* or one plate of the lawn of *S. Enteritidis*, then subjected to centrifugation at 3,000×g for 15 min and discarded the supernatant to obtain a pellet.

For peanut oil, the pellet of *E. faecium* or *S. Enteritidis* was first suspended in 10 ml of oil with a high shear vortex mixer for 5 min and then mixed into 20 ml of oil with another 5 min vortex. For peanut butter, the bacteria pellet was scraped out from the centrifugal tube with a sterile inoculation loop and mixed thoroughly into 50 g of peanut butter inside a stomacher bag (18 oz, WHIRL-PAK, Madison, WI) using hand massage with the help of a stainless-steel dough scraper.

2.4. a_w adjustment

Based on earlier studies (Yang et al., 2020a, 2020b) and preliminary experiments, the water activity of oil decreases sharply with increasing temperature, while the water activity of peanut remains almost the same. Thus, to compare the thermal resistance of bacteria in peanut oil and peanut butter at a similar level of about 0.3 at 80 °C, the initial water activity of the inoculated peanut oil samples and peanut butter were conditioned to 0.75 and 0.3, respectively, at room temperature.

Due to the very small moisture capacity of oil, an oil sample could easily get saturated with water when introducing a wet inoculum. Thus, the oil samples needed to be conditioned again after inoculation. In this study, inoculated peanut oil was conditioned to a_w of 0.75 following the method described in Yang, Xu, et al. (2020). In brief, the oil sample was transferred into a 100 ml sterile flask and conditioned for 4–5 days inside a 1-L glass jar at 75% relative humidity controlled by saturated sodium chloride solution. A 5-W fan located above the flask and a magnetic stirrer (3 cm long) in the oil spinning at approximately 800 rpm was used to facilitate moisture diffusion.

The a_w of peanut butter was adjusted before inoculation by mixing a pre-determined amount of distilled water with the peanut sample. Specifically, the peanut butter in a jar was mixed thoroughly using a sterile mud-mixer shaft driven by a hand drill. Then 400 g of peanut butter was transferred into a glass home-blender jar (3-in-1 Multifunctional 6-speed Tilt-head Food Stand Mixer, Costway, Fontana, CA) (sterilized with 76% ethanol and dried with hot air). A calculated amount of sterile de-ionized water (2–6 g, depending on the original a_w of the peanut butter sample) was added into peanut butter to adjust its a_w to 0.30. The sample was then homogenized thoroughly by running the blender for 1 min. To remove the heat generated in blending which caused the sample temperature to increase to 35 °C, the sample was stored hermetically in the jar for more than 12 h to allow the sample's temperature to return to ~22 °C. During 12 h of holding, a 5-W fan was used inside the jar to accelerate the thermal dynamic equilibration between air and the sample; the relative humidity in the jar was monitored

by a humidity sensor (Honeywell HumidIcon™ HIH8000 Series, Morristown, NJ). After 12 h, the steady-state relative humidity (i.e., when the readings were constant for at least 2 h) was recorded which was equal to the a_w of the peanut butter. Then the 50 g of peanut butter with a water activity of 0.30 was inoculated by mixing with 0.2 g of bacterial pellet. The inoculated peanut butter samples were sealed in a jar for 4–5 days before thermal treatments.

2.5. Isothermal treatments

Before each test, TDT cells and TAC cells were sterilized at 121 °C for 45 min, then washed, packed with kraft paper, sterilized again at 121 °C for 15 min, and dried in an oven at 60 °C for at least 5 h. Sterile and disposable test tubes were used thus required no preparation.

Samples were loaded into different sample holders for thermal treatment as illustrated in Fig. 1. For the test tubes, 70 µl of inoculated peanut oil was transferred carefully with a pipet to the bottom of each tube. The tubes were then hermetically sealed with the snap caps. For TDT cells, each cell was positioned horizontally and loaded with 1 ml of peanut oil, or 1.5 g of peanut butter. The TDT cells were sealed tightly by screwing down the lid to impose pressure on the rubber O-ring. For TAC cells, 0.1 g of peanut butter or 0.1 ml of peanut oil was spread on an aluminum metal plate (3 cm in diameter × 0.64 mm in thickness) for the convenience of sample collection after treatment (Yang et al., 2021), which gave a thin layer of sample of thickness approx. 0.1 mm. Then the plate was positioned at the center of a TAC cell and the cell was sealed hermetically by forcing the lid to press against the O-ring with three screws. The very small amount of sample imitates the extreme condition that the surface material may experience in a sealed container in which the relative humidity of the headspace drops sharply due to heating.

Duplicated samples were treated at 80 °C in an oil bath (Neslab GP-400, Newington, NH) filled with ethylene glycol (VWR International, Radnor, PA) for designated lengths of time. During the treatments, the TDT cells were tied to metal wires for the convenience to take samples in and out. The test tubes were positioned vertically in a tube rack and fixed to their positions with rubber bands. The rack was placed in the oil bath on a smaller metal rack to adjust the position of the tubes where the level of ethylene glycol lays above the central line of the tube but below the bottom of the cap. For TAC cells, every two cells, as replicates, were locked on a specially designed rack to maintain the horizontal position in the oil bath. All treatments were terminated by submerging the samples into an ice water bath for more than 2 min.

The thermal treatment times were determined from preliminary experiments. For peanut oil inoculated with *E. faecium*, the samples in the test tubes were treated for 0, 5, 30, 60, 120, 180, 240, and 300 min; the sample in TDT cells were treated as for 0, 5, 60, 120, 180, 240, and 300 min; the samples in TAC cells were treated for 0, 5, 120, 240, and 360 min. For peanut oil inoculated with *S. Enteritidis*, the samples in TDT cells were treated for 0, 5, 30, 60, and 90 min; the samples in TAC cells were treated for 0, 5, 120, 240, and 360 min. The peanut butter samples inoculated with *E. faecium* were treated in TDT or TAC cells for 0, 5, 120, 240, and 360 min. The peanut butter samples inoculated with *S. Enteritidis* were treated for 0, 5, 20, 35, 50, and 65 min in TDT cells, and 0, 5, 20, 35, 50, 65, and 80 min in TAC cells. All tests were conducted in three replicates each with independent inoculum and treated on a different day from the others.

2.6. Enumeration

After thermal treatment, each sample was collected, homogenized with dilution water, and subjected to serial dilutions, plating, incubation, and colony counting, to obtain the survival population (colony forming unit) in the sample.

For oil samples in TDT cells, the lids were opened, and each sample was stirred by hand with a sterile pipet tip. Then 0.1 ml of oil was collected and added into 10 ml of sterile BPW plus 0.1% (v/v) of Tween

80 (emulsifier) (Fisher BioReagents, Fair Lawn, NJ) in a polypropylene tube (17 × 100 mm) with a snap cap, for a 1:100 dilution. The tube was sealed, vertically shook by hand, and vortexed for 10-min at 3,200 rpm (Fisherbrand mini vortexer, Waltham, MA) for homogenization. For oil samples in the test tube, 10 ml of sterile BPW plus 0.1% (v/v) of Tween 80 was added to the 0.07 ml sample for a 0.7:100 dilution, then sealed and homogenized using a vortexer for 10-min at 3,200 rpm. For oil samples or peanut butter in TAC cells, the whole metal plate with 0.1 ml of oil or 0.1 g of peanut butter was transferred into a 4-oz stomacher bag, 10 ml of BPW plus 0.1% (v/v) of Tween 80 or 10 ml of BPW were added to obtain 1:100 dilution. The mixtures were then homogenized using hand massage and a stomacher (Stomacher 400, Seward Laboratory Systems Inc.) ran at 260 rpm for 5 min. For the peanut butter in TDT cells, 1 g of sample was weighted in a 4-oz stomacher bag, then homogenized with 9 ml of BPW (1:10 dilution) with hand massage and mixing in a stomacher at 260 rpm for 5 min.

Each homogenized sample was further diluted with appropriate serial dilutions based on preliminary studies, then plated in triplication for oil and duplication for peanut butter on corresponding differential media. *E. faecium* was recovered on TSAYE plates supplemented with 0.05% ferric ammonium citrate (Sigma-Aldrich, St Louis, MO, USA) and 0.025% esculin. *S. Enteritidis* was recovered on TSAYE plates supplemented with 0.05% ferric ammonium citrate, and 0.03% (w/v) sodium thiosulfate (Sigma-Aldrich, St Louis, MO, USA). All plates were incubated at 37 °C for 48 h. Colonies with a dark center were counted as *E. faecium* or *S. Enteritidis* depending on the inoculum.

2.7. Data analyses

The survival population, N , of *E. faecium* and *S. Enteritidis* was modeled using the first-order kinetic, or the log-linear model (Peleg, 2006):

$$\log\left(\frac{N}{N_0}\right) = -\frac{t-t_0}{D} + C \quad (1)$$

where N and N_0 are the survival populations at treatment time t and time 0, respectively. N and N_0 for peanut oil were expressed in logCFU/ml and for peanut butter in logCFU/g. C is the modeled log reduction at time t_0 (the come-up time). The temperature come-up time (the time for the cold spot in the sample to reach 79.5 °C) was normally selected as t_0 in an isothermal study using TDT cells. However, considering the longer moisture equilibration between the sample and headspace, 5 min was used as t_0 for all experimental conditions in this study. The 5-min equilibration time was selected according to Tadapaneni et al. (2018) who reported that the moisture come-up time in 1 g (10 times the amount of peanut butter used in this study) of wheat flour in TAC cells was about 4–5 min. Without the moisture diffusivity data in peanut butter, it was assumed that 5 min might be sufficient, and it was confirmed by the thermal death curve obtained from preliminary tests.

The goodness of fit was quantified for each heating method by the root mean squared error (RMSE) using all sets of data replicates (Motulsky & Christopoulos, 2004):

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^n \left[\log\left(\frac{N}{N_0}\right)_{\text{data}, i} - \log\left(\frac{N}{N_0}\right)_{\text{model}, i} \right]^2}{n-p}} \quad (2)$$

where $\log(N/N_0)_{\text{data}, i}$ is the measured log reduction, $\log(N/N_0)_{\text{model}, i}$ is the calculated log reduction from the model, n is the number of data points, and p is the degrees of freedom.

One-way ANOVA and Fisher's least significant difference (LSD) tests were used to compare the difference between the means of $\log D_{80}$ and log-reductions at 5 min. The differences were considered significant if the probability was less than 0.05 ($P < 0.05$). The statistical analysis was done using computer software, Minitab 18.1 and Microsoft Excel 16.0.

Data obtained from literature was used to generate prediction lines for $\log D$ -values at 80 °C correspondings to the change of a_w . The Z_{aw} -value (the increase in a_w that causes a decimal reduction in D -value) was calculated as the negative inverse slope of the linear regression line (Liu et al., 2018):

$$\log D = -\frac{a_w}{Z_{aw}} + b \quad (3)$$

where, $\log D$ is a function of a_w , the intercept of this equation, b , is the $\log D$ -value corresponding to the a_w of 0.

2.8. a_w of peanut oil and peanut butter at 80 °C

The temperature-dependent water activity, a_w , of peanut oil was described in Yang, Xu, et al. (2020), as:

$$a_w = x \cdot e^{\left(\frac{1714}{T} - 2.7\right)} \quad (4)$$

where x is the mole fraction of water in the water-in-oil solution, it equals 0.035 for the oil sample conditioned to a_w of 0.75 at the room temperature; T is the absolute temperature (K) of the sample.

The high-temperature a_w of the peanut butter was measured at 80 °C using High Temperature Cell (HTC), a device developed in Tadapaneni et al. (2017). Three HTCs were each filled with 6 g of the peanut butter of a_w 0.30 (at ~22 °C) without adding inoculum, leaving the headspace less than half of the sample volume to minimize the effect of the air in the headspace. Then the cells were hermetically sealed, heated in an oven at 80 °C, and held for 3 h. The sensor in each HTC (Honeywell HumidIcon™ HIH8000 Series) read the relative humidity and temperature above the sample every minute, and the readings of relative humidity upon equilibration (reached constant) were recorded as the high-temperature a_w of the sample.

2.9. Temperature-dependent relative humidity of air in the headspace

The relative humidity (RH) of the air in a closed system with constant volume was calculated using the following equation, which was derived from the Ideal Gas Law:

$$\begin{aligned} n \cdot R &= \frac{P \cdot V}{T} = \text{constant} \\ \rightarrow \text{RH} &= \frac{P}{P_s} = \frac{P_0 \cdot T}{P_s \cdot T_0} = \frac{\text{RH}_0 \cdot P_{s0} \cdot T}{P_s \cdot T_0} \end{aligned} \quad (5)$$

where, n is the mole number of water molecules in the system; R is the ideal gas constant; V is the volume of the system; RH_0 is the initial RH of air; P_{s0} and P_s are the saturated vapor pressure of water under the atmospheric pressure at absolute temperatures T_0 and T , respectively; P_s was calculated using Buck's equation (Lide, 2005; Yang, Guan, et al., 2020):

$$P_s = 611.21 \exp\left(\left(18.678 - \frac{T - 273.15}{234.5}\right)\left(\frac{T - 273.15}{T - 16.01}\right)\right)$$

For this study, the relative humidity in the lab at the room temperature (~22 °C), RH_0 , was monitored in the daytime during experiments and used for calculation in Eq. (5).

Although the sample size was very small relative to the volume of the headspace in the sample holder, moisture migration into the headspace would still increase the RH. The calculation described above does not consider moisture migration from the sample to the headspace in the thermal treatment. Thus, the calculation only gives the lowest possible RH in the headspace in the thermal treatments.

3. Results and discussion

3.1. Background microflora & inoculation level

The tests for background microflora showed that the aerobic plate counts were below 1,000 CFU/g for all the peanut butter samples used in this study, this would not affect our results of the thermal death kinetic tests due to the high inoculation level.

The tests for the control groups (with no thermal treatment) showed that the mean (\pm SD) inoculation levels were 7.6 ± 0.5 logCFU/ml for *E. faecium* in peanut oil, 8.8 ± 0.5 log CFU/g for *E. faecium* in peanut butter, 7.9 ± 0.7 logCFU/ml for *S. Enteritidis* in peanut oil, and 8.9 ± 0.3 logCFU/g for *S. Enteritidis* in peanut butter. These results were similar to what was reported in Yang, Xu, et al. (2020) and Enache et al. (2015). Even though the inoculation ratio (inoculum/sample) was similar between peanut oil and peanut butter, the inoculation levels of both bacterial strains were lower (roughly 1-log less) in the peanut oil samples compared to in the peanut butter samples. This might be due to the hydrophobicity of oil which made a noticeable group of bacterial cells clotted and formed sediments thus couldn't keep a high concentration in a suspended form in oil.

3.2. Sample water activity & headspace relative humidity

For the sample holders with negligible headspace, the a_w in the sample matrix only depends on sample temperature and moisture content (Tadapaneni et al., 2017). The calculation from Equation (4) indicates that the a_w of the oil sample decreased from 0.75 to 0.31 as the temperature increased from 22 to 80 °C. On the other hand, the a_w of peanut butter measured using HTC increased from 0.30 ± 0.01 to 0.38 ± 0.01 as the temperature raised from 22 to 80 °C. These values are listed in Table 1.

The RH in the laboratory during the tests was recorded to be between 16% and 51% at 22 ± 2 °C; the average level was 34%. Using these numbers, the calculation (Equation 5) yielded RH values of air to be between 1.1% and 3.4% (2.3% on average) when the air was heated to 80 °C in a closed container. These results indicate that in a sample holder with a large headspace, the sample surfaces were exposed to a very dry environment, the sample would lose moisture to the headspace. Thus, the sample surface would be drier than the sample interior. The time to reach an equilibrium a_w between the sample and the headspace depends on the size and moisture capacity of the sample, as well as moisture diffusivity in the sample material. Since it's technically impossible to monitor the real-time a_w of the different parts of a small sample, we can only estimate a possible range between the RH of dry air and the material a_w at 80 °C as shown in Table 1.

Table 1

Thermal death parameters of *E. faecium* and *S. Enteritidis* at 80 °C in peanut oil and peanut butter obtained using different sample holders (n = 3). For sample holders, "N" stands for negligible headspace, and "L" stands for large headspace.

Food matrix	a_w at 23 °C	Sample holder	a_w at 80 °C	Bacterial strain	Reduction from the initial 5 min (log CFU per ml (or g))	D_{80} -value (min)	$\log D_{80}$ -value	RMSE (log CFU per ml (or g))
Peanut oil	0.75	Capillary tube (N)	0.31	<i>E. faecium</i>	N/A	$140 \pm 43^*$	2.21 ± 0.14^{CD}	N/A
		Test tube (L)	0.02–0.31	<i>E. faecium</i>	0.19 ± 0.17^{AB}	1949 ± 737	3.27 ± 0.16^A	0.18
		TDT cell (N)	0.31	<i>E. faecium</i>	0.41 ± 0.23^{BC}	159 ± 10	2.20 ± 0.03^C	0.32
				<i>S. Enteritidis</i>	0.73 ± 0.36^D	44.1 ± 21.8	1.61 ± 0.22^E	0.35
		TAC cell (L)	0.02–0.31	<i>E. faecium</i>	-0.02 ± 0.08^A	2668 ± 474	3.42 ± 0.08^A	0.10
				<i>S. Enteritidis</i>	1.39 ± 0.53^E	202 ± 83	2.28 ± 0.17^{BC}	0.46
Peanut butter	0.33	TDT cell (N)	0.38	<i>E. faecium</i>	0.09 ± 0.13^A	77.0 ± 3.7	1.89 ± 0.02^D	0.34
				<i>S. Enteritidis</i>	0.58 ± 0.11^{CD}	28.6 ± 10.4	1.43 ± 0.17^E	0.51
		TAC cell (L)	0.02–0.38	<i>E. faecium</i>	0.21 ± 0.09^{AB}	332 ± 86	2.51 ± 0.12^B	0.23
				<i>S. Enteritidis</i>	0.09 ± 0.18^A	74.9 ± 22.8	1.86 ± 0.13^D	0.32

* Data obtained from Yang, Xu, et al., 2020.

Differences in capital grouping letters indicate significant differences among the data within a column ($P < 0.05$, Fisher pairwise comparison).

3.3. Initial bacterial reduction in isothermal treatments

In an isothermal treatment, the temperature come-up time in the cold spot of the sample was normally counted as the initial point. However, in this study, the moisture equilibration between headspace and the sample should also be considered and it may take longer than the temperature come-up time. For practical purpose, the log reductions of bacteria of all test conditions were examined at 5 min after the sample was immersed in an oil bath. In general, the initial reductions of both strains of the inoculated bacteria (listed in Table 1) were larger in peanut oil than in peanut butter. This can be attributed to the higher initial a_w of peanut oil (0.75) compared to peanut butter (0.30) at room temperature. A higher initial a_w indicates a higher moisture content in those bacterial cells inside the oil. It has been reported by Xie et al. (2021) that the lethal effect of heating is positively related to the amount of moisture in bacterial cells. Even though the a_w of oil would decrease from 0.75 to 0.31 after being heated to 80 °C, the moisture loss from bacteria cells to surrounding oil may not be as fast as the temperature elevation. This could have led to a significant reduction in the population of the bacteria.

3.4. Thermal death kinetics of the inoculated bacteria

All the bacterial survival data, after the 5 min initial heating, were fitted using Equation (1). The fitted curves are presented in Figs. 2–4; the corresponding D -values are listed in Table 1. The root mean squared error (RMSE) calculated using D -values from three replicated data sets ranged from 0.10 to 0.51 (Table 1) which are comparable to the general range (0.30–1.13) of RMSE of a primary model (log-linear or Weibull model) in previous studies on LMFs (Smith et al., 2016; Limcharoenchat et al., 2018). Thus, both bacteria followed the first-order kinetics in the experimental conditions in this study. The D -values were used to compare the thermal resistance of the bacteria under different experimental conditions.

3.5. Comparing the thermal resistance of *E. faecium* in peanut oil in different sample holders

Fig. 2 compares the effect of sample holders on thermal inactivation of *E. faecium* in peanut oil at 80 °C. The thermal death curves for the TAC cells and test tubes (both having large headspace) are similar, less than 0.5 log reductions of *E. faecium* were observed over a 300 min heating. But more than 2 log reduction of *E. faecium* in oil was observed when heated for 300 min in TDT cells that did not provide headspace over the treated samples. A similar thermal death curve was reported by Yang, Xu, et al. (2020) for the same strain of *E. faecium* in peanut oil of 0.75 a_w (at room temperature). The inoculated samples were treated at 80 °C in

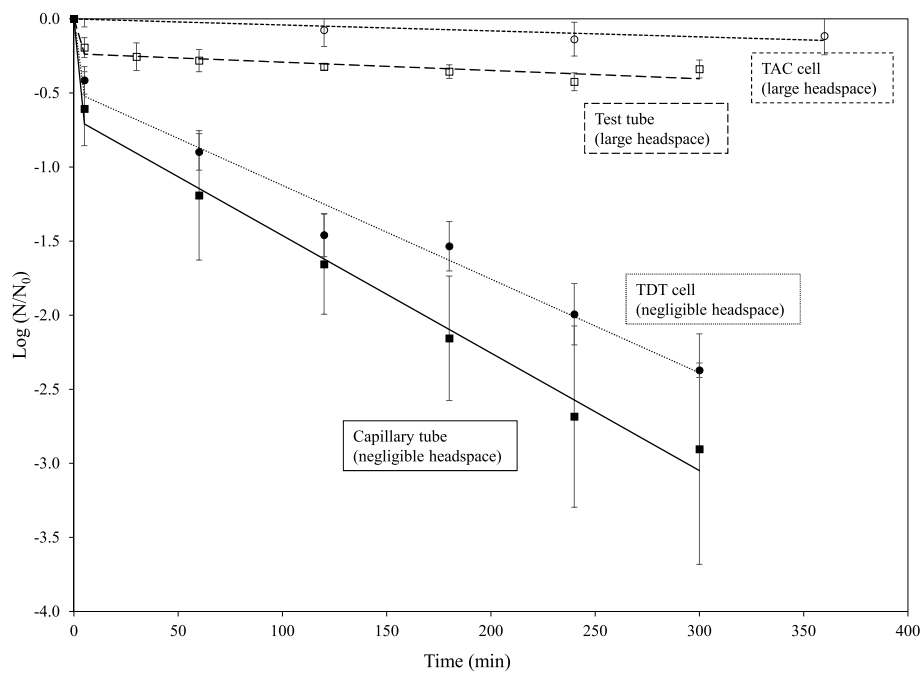


Fig. 2. Thermal death (mean CFU/ml \pm standard error) of *E. faecium* in peanut oil ($a_w = 0.75$) isothermally treated at 80 °C in four different sample holders ($n = 6$). Prediction lines were drawn using fitted Equation (1). Data of capillary tube were obtained from Yang, Xu, et al. (2020).

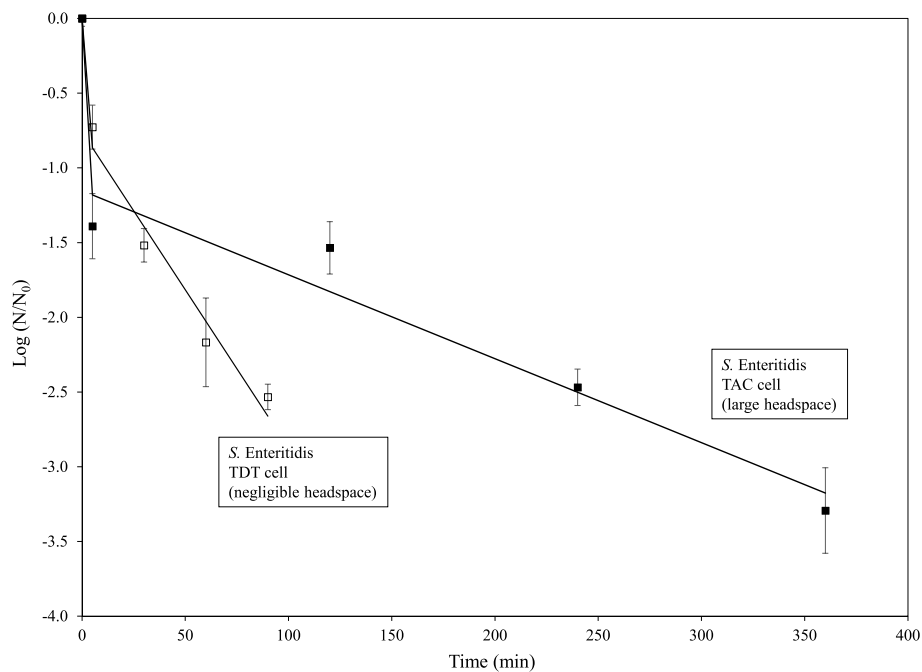


Fig. 3. Thermal death (mean CFU/g \pm standard error) of *S. Enteritidis* in peanut oil ($a_{w,22^\circ\text{C}} = 0.75$, $a_{w,80^\circ\text{C}} = 0.31$) from isothermal treatment (80 °C) in TDT and TAC cells ($n = 6$). Prediction lines were drawn using fitted Equation (1).

sealed capillary tubes (with negligible headspace). This curve is added to Fig. 2 for comparison. The D -value of *E. faecium* in oil from each sample holder is presented in Table 1. The D -values were statistically compared in their transformed form ($\log D$) due to unequal variances. The D -values from the large test tubes (1949 ± 737 min) and TAC cells (2668 ± 474 min), both having large headspace, were significantly larger than what in capillary tubes (140 ± 43 min) and TDT cells (159 ± 10 min), both having negligible headspace. But the difference was not significant between capillary tubes and TDT cells, or between large test tubes and TAC cells ($P > 0.05$).

The results clearly indicate that the large headspace in the large test tubes and TAC cells enhanced the thermal resistance of *E. faecium* in peanut oil. This part of study indicated that the two customized sample holders (TDT cell and TAC cell) can be used to generate similar thermal resistance data comparing with the two traditional sample holders (capillary tube and large test tube).

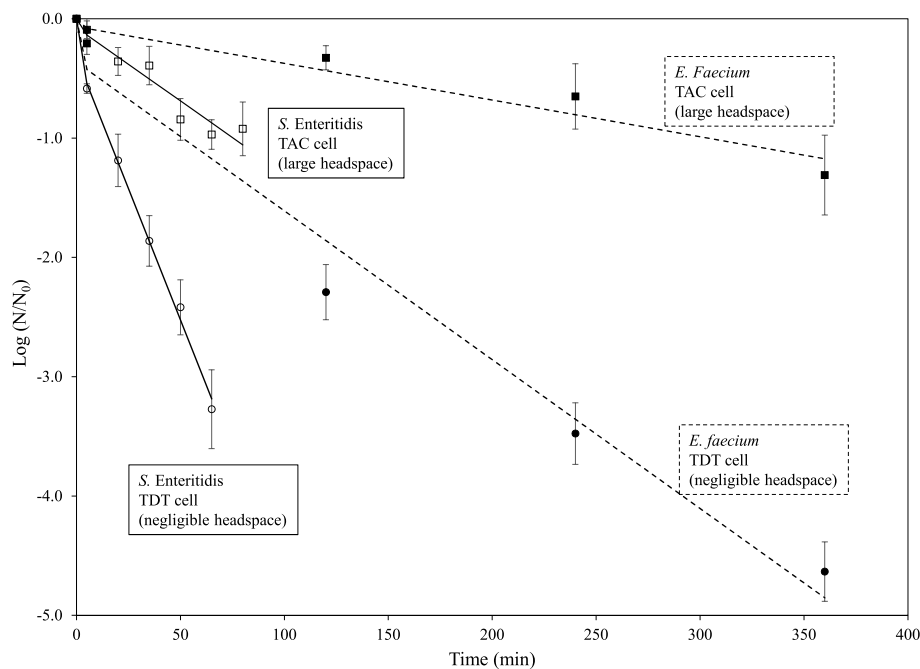


Fig. 4. Thermal death (mean CFU/g \pm standard error) of *E. faecium* and *S. Enteritidis* in peanut butter ($a_{w,22^\circ\text{C}} = 0.30$, $a_{w,80^\circ\text{C}} = 0.38$) from isothermal treatment (80°C) in TDT and TAC cells ($n = 6$). Prediction lines were drawn using fitted Equation (1).

3.6. The effect of headspace on thermal resistance of *E. faecium* and *S. Enteritidis* in peanut oil and peanut butter

The thermal death curves of *S. Enteritidis* in peanut oil, treated in TDT and TAC cells, are compared in Fig. 3. Similarly, the death curves for *E. faecium* and *S. Enteritidis* in peanut butter are presented in Fig. 4. Thermal death parameters at each condition are listed in Table 1. In peanut butter (Fig. 3), the thermal resistance of *S. Enteritidis* was lower when tested in TDT cells compared to in TAC cells. The D -value of *S. Enteritidis* at 80°C increased from 44.1 ± 21.8 min to 202 ± 83 min (4.6 times), as the sample holder was changed from the TDT cells (negligible headspace) to the TAC cells (large headspace). Similarly, the thermal death curves for both *E. faecium* and *S. Enteritidis* in peanut butter (see Fig. 4) were also steeper when tested in TDT cells compared to in TAC cells. The D -values at 80°C were 77.0 ± 3.7 min and 28.6 ± 10.4 min for *E. faecium* and *S. Enteritidis*, respectively, in peanut butter treated with TDT cells. While the D -values in TAC cells were 4.3 (332 ± 86 min) and 2.6 (74.9 ± 22.8 min) times larger for *E. faecium* and *S. Enteritidis*, respectively. These results showed a significant impact of a large headspace in the sample holder on the thermal resistance of bacteria in both peanut butter and peanut oil.

The thermal death curves from TDT and TAC cells may provide a good explanation to the concaving curves observed from previous studies (Li et al., 2014; Limcharoenchat et al., 2018; Ma et al., 2009; Shachar & Yaron, 2006). In the study of Limcharoenchat et al. (2018), a 1 mm peanut butter sample layer was heated in a 4 mm deep sample holder, dehydration would occur to the sample which could cause the thermal death curve of *S. Enteritidis* to lay between the two curves (solid lines) in Fig. 4. In the studies on the thermal resistance of *Salmonella* in peanut butter, Shachar & Yaron. (2006), Ma et al. (2009), and Li et al. (2014) used stomacher bags, a plastic container that was designed to homogenize samples in a stomacher. The stomacher bags are normally closed by rolling the metal wire located at the opening, but the closure is meant to be non-hermetic so that the bag would not burst when subject to the pressure from stomacher movement. In a stomacher bag, the level of dehydration is likely determined by the way the bag was sealed and positioned in the oil bath, so it would be difficult to quantify. This explains why the results from those studies were not consistent. In a more

recent study, Park et al. (2021) reported concaving thermal death curves for *E. faecium* in 0.6-mm peanut butter ($a_w = 0.80$) samples that were fully exposed to superheated steams. The authors noted moisture exchange between superheated steam and the peanut butter samples.

3.7. Local a_w of food as a key factor for the thermal resistance

Water activity (a_w) of food matrices at the treatment temperature has been identified as a key parameter determining the D -values of bacteria in low moisture foods. This conclusion was made by studying the relationship between the a_w of the matrices and the thermal resistance of *S. Enteritidis* and *E. faecium* in different matrices, including honey powder, silicone dioxide granules, egg powder, wheat flour, almond flour, milk powder, and peanut oil, all treated with minimum headspace or in humidity-controlled conditions (Alshammari et al., 2020; Liu et al., 2018; Pérez-Reyes et al., 2021; Xu et al., 2019; Yang, Xu, et al., 2020; Yang et al., 2021). From a microscopic perspective, the water activity in bacterial cells changes readily with the RH (or a_w) of the surrounding food matrix through water vapor diffusion. The change in a_w indicates a change in the moisture content of the bacterial cells, which directly influences the thermal inactivation of the bacterial cells (Syamaladevi et al., 2016; Xie et al., 2021).

To examine the role a_w played in our experimental conditions, the D -values of *E. faecium* and *S. Enteritidis* in peanut oil and peanut butter from this work were compared in Fig. 5 and Fig. 6, respectively, with published data for the same bacterial strains in different matrices treated at 80°C in sample holders with no headspace. As discussed in Section 3.2, the a_w of the samples in the sample holders with negligible headspace had uniform a_w during heat treatment, while the samples in sample holders with large headspace had nonuniform a_w which may range between the RH of dry air and the material a_w when heated without headspace. Thus, in Figs. 5 and 6, the data points from capillary tubes and TDT cells are presented in solid dots, while the data from the large test tubes and TAC cells are presented in horizontal bars covering the possible ranges of the local a_w that the inoculum could have been exposed to during the treatments.

In Fig. 5, a trend line with 95% confidential interval was made using the data for *E. faecium* in different matrices from the literature (Liu et al.,

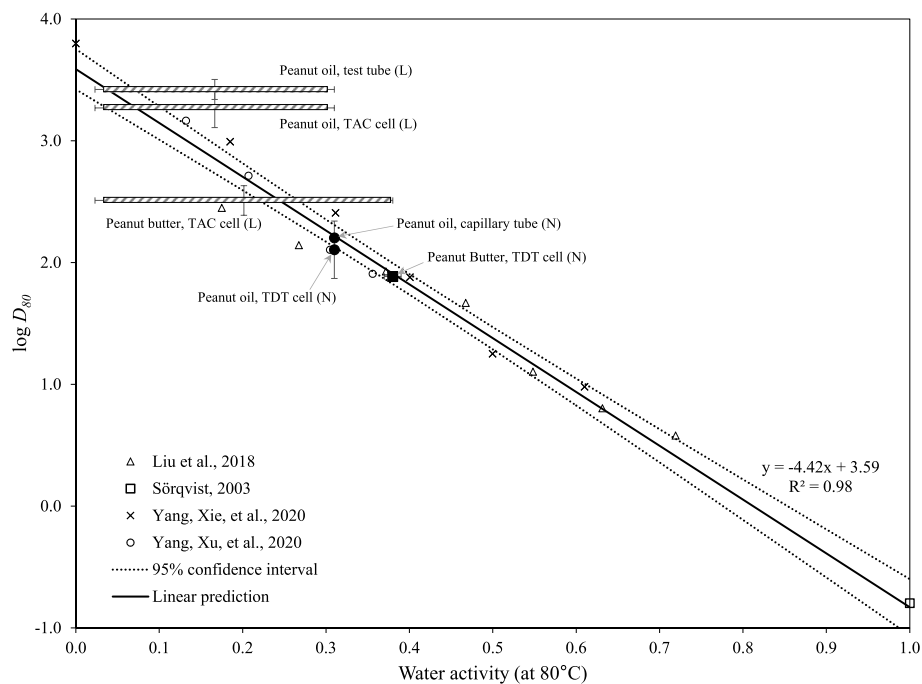


Fig. 5. Comparison of the thermal resistance ($\log D_{80} \pm$ standard deviation) of *E. faecium* in peanut oil and peanut butter with published data as influenced by a_w of sample at 80 °C ($n = 3$). Capital letters N & L indicate “negligible headspace” and “large headspace”, respectively.

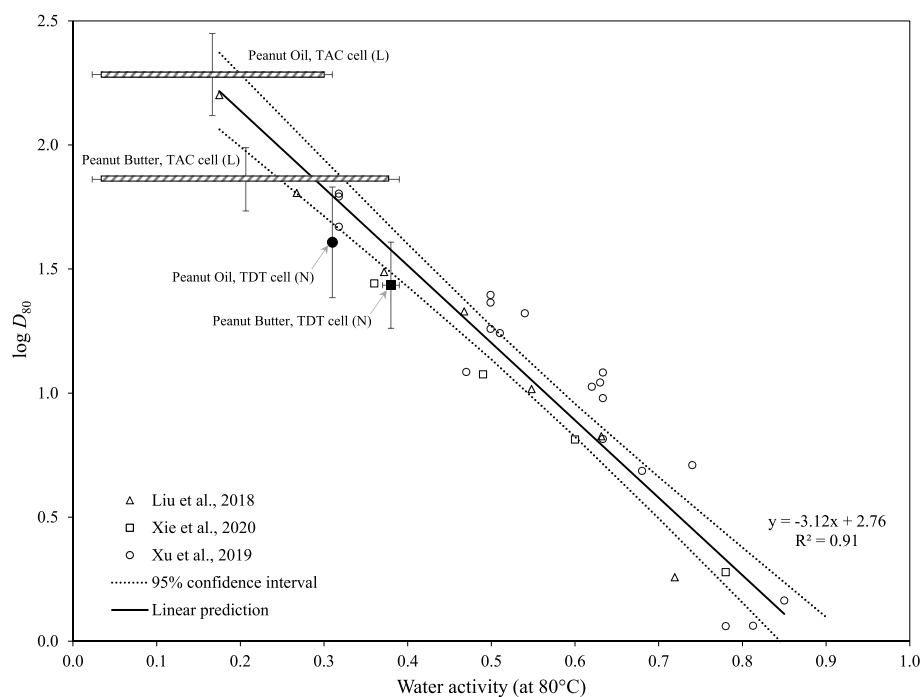


Fig. 6. Comparison of the thermal resistance ($\log D_{80} \pm$ standard deviation) of *S. Enteritidis* in peanut oil and peanut butter with published data as influenced by a_w of sample at 80 °C ($n = 3$). Capital letters N & L indicate “negligible headspace” and “large headspace”, respectively.

2018; Sörqvist, 2003; Yang, Xu, et al., 2020; Yang et al., 2021) to compare with the data from this work. The three data points (solid) from the sample holders with negligible headspace (i.e., capillary tubes and TDT cells) fall within the 95% confidential interval, indicating that the thermal resistance of *E. faecium* was indeed governed by the a_w of the food matrix. The horizontal bar for peanut butter in the test cells with large headspace (TAC cells) is positioned lower to the horizontal bars for peanut oil in TAC cells and in large test tubes. This indicates that the

actual a_w of peanut butter samples might be higher than that of the peanut oil samples, which can be attributed to the slower a_w reduction in the peanut butter samples during the thermal treatments. Peanut butter is a mixture of protein and starch granules in oil (Young & Schadel, 1990), the hydrophilic particles could largely enhance the moisture capacity (Table 1). The a_w of peanut butter samples was higher than that of the oil samples (0.38 vs 0.31) when heated to 80 °C in the test cells without headspace (Table 1). In addition, peanut paste is highly viscous.

This would make it difficult for moisture migration within the sample so that the a_w of a peanut butter layer within the sample would change slowly to reach that of the dry air in the headspace during the thermal treatments.

Fig. 6 presents the $\log D_{80}$ for *S. Enteritidis* in peanut oil and peanut butter treated in TDT and TAC cells at 80 °C. The data from the literature (Liu et al., 2018; Xie et al., 2021; Xu et al., 2019) were also included in Fig. 6 as a trend line with 95% confidence interval for comparison. Similar to Fig. 5, the data from TDT cells are in solid dots and the data from TAC cells are in bars over the possible a_w range of the sample. The solid dots from TDT cells fall very close to the lower 95% confidential line. Considering the relatively wide standard deviations from typical microbial thermal inactivation tests, it is fair to say that the thermal resistance of *S. Enteritidis* in peanut oil and peanut butter followed the same trend as in many other low-moisture foods when tested without headspace, and the D -values were determined by the a_w of the sample matrix. The two horizontal bars from the TAC cells crossed the prediction line, and the horizontal bar for peanut butter was positioned lower, for the same reason explained above for Fig. 5.

Overall, Figs. 5 and 6 suggest that the thermal resistance of *E. faecium* and *S. Enteritidis* in peanut oil and peanut butter were governed by the local a_w of the samples, which agrees with our previous studies in which the inoculated samples were treated in sample holders with no headspace (Xie et al., 2021; Yang, Xu, et al., 2020). It can be concluded that the surface drying was the cause of the enhanced thermal resistance of bacteria when the samples were exposed to a large headspace. Based on the more accurate measurement of D values using TDT cells, the D -values for *E. faecium* were 2.7 and 3.6 times the D -values for *S. Enteritidis* in peanut butter ($a_w = 0.38$) and peanut oil ($a_w = 0.31$), respectively (see Table 1). The strain *E. faecium* can, therefore potentially be a valid surrogate of *Salmonella* in peanut butter in the tested water activity range.

Surface dehydration is common in a pasteurization process of low-moisture food in an open system. This study suggests that the dry spot in the product with the lowest a_w or the least moisture content may receive less lethality from the process and, thus, should be considered as one of the control points. For example, Song and Kang (2021) noted that the thermal death of *Salmonella* on almonds was significantly smaller in an open pouch than in a sealed pouch (with some headspace) and even less than in a vacuum sealed pouch (no headspace). In a mixing tank for high temperature holding to pasteurize peanut butter, the openings on the tank should be closed because they may cause surface drying of the food, especially for those residues attached to the wall of the tank.

4. Conclusion

This study investigated the effect of a large headspace on the thermal death behavior of *E. faecium* and *S. Enteritidis* in peanut oil and peanut butter. We found that a large headspace in the sample holder caused surface drying of the sample during thermal treatments and greatly increased the thermal resistance of both bacteria at 80 °C. The thermal death of *E. faecium* and *S. Enteritidis* followed the first-order kinetics and their thermal resistance had a strong correlation with the water activity of the food matrix at the treatment temperature (80 °C), with or without the influence of a headspace. This finding may partially explain the discrepancies in the thermal resistance results for *Salmonella* spp. in peanut butter among different research laboratories, thus will guide future research about how to produce accurate thermal resistance data and help the industry to develop valid pasteurization processes for high-fat low-moisture food products. It suggests that the dry spots of the product should be considered as control points in a thermal pasteurization process.

CRedit authorship contribution statement

Ren Yang: Methodology, Conceptualization, Investigation, Formal

analysis, Writing – original draft, Project administration. **Teng Cheng:** Investigation, Writing – review & editing. **Yoonki Hong:** Investigation, Writing – review & editing. **Lina Wei:** Investigation, Writing – review & editing. **Juming Tang:** Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

No conflict of interest.

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