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Isothermal Inactivation Studies of *Listeria monocytogenes*, *Salmonella*, and *Enterococcus faecium* NRRL B-2354 in Almond, Peanut, and Sunflower Butters

Ruo Fen Liao

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Isothermal Inactivation Studies of *Listeria monocytogenes*, *Salmonella*, and *Enterococcus faecium* NRRL B-2354 in Almond, Peanut, and Sunflower Butters

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Master of Science

Vegetative, non-sporeforming foodborne pathogens show notable survival and uncanny thermotolerance in low water activity (a_w) foods. Controlled studies on *Listeria monocytogenes*, *Salmonella* spp., and *Enterococcus faecium* NRRL B-2354 (a *Salmonella* surrogate) in a variety of food matrices support thermal process validation studies required to achieve global food safety objectives. In this study, we determined and compared thermal inactivation rates using independent six-strain cocktails of pathogens in three plant-based butters. Direct determinations of decimal reduction times (*D*-values) for *L. monocytogenes*, *Salmonella*, and *E. faecium*, in corresponding butters were inoculated using peanut oil, almond oil, or sunflower oil. Thermal Death Time (TDT) studies for the organisms were conducted in triplicate. Uniform bagged plant-based butter samples of *Salmonella* spp. or *L. monocytogenes*, or *E. faecium* alone were sandwiched in copper plates immobilized with recessed magnets. Samples underwent rapid heat treatments via water immersion under isothermal conditions ranging from 70°C to 85°C. Bacterial destruction in peanut butter (46% fat, 0.20 a_w @ 25°C), almond butter, (50% fat, 0.32 a_w @ 25°C), or sunflower butter (56% fat, 0.15 a_w @ 25°C) was determined by direct plating. The TDT studies showed *Salmonella* spp. had consistently higher *D*-values than *L. monocytogenes* in all treatments, but pair-wise comparisons found no statistical difference when assessing the thermotolerance of the two pathogens in the individual plant-based butters tested ($p > 0.005$). These data support *Salmonella* as the primary pathogen of concern in low water activity foods and show the heat resistance of *L. monocytogenes* can approximate destruction kinetics observed for *Salmonella* spp. in low a_w matrices. *E. faecium* exhibited the highest thermotolerance. This further supports the utility of this surrogate for *Salmonella* spp. and *L. monocytogenes* in high fat, low-moisture foods similar to the plant-based butters tested. Thermotolerance differences between a dry talc vs. peanut oil-based inoculation procedures in peanut butter were also evaluated. Surprisingly, the oil-based inoculations resulted in lower *D*-values ($p > 0.01$) for *Salmonella* spp. and the surrogate when compared to the dry inoculum.

Keywords: food safety, low-moisture foods, plant-based butter, *Listeria monocytogenes*, *Salmonella*, *Enterococcus faecium* NRRL B-2354

ACKNOWLEDGEMENTS

I want to thank my graduate advisor, Dr. Brad Taylor, for being my mentor and my friend since 2018. It has been a long journey and I could not do it without him. I also want to acknowledge Dr. Frost Steele and Dr. Gene Ahlborn for being on my committee. They have provided me a lot of feedback and encouragement in the last two years. Adam Quinn, a former graduate student in the lab, helped me with the training and the transition between undergraduate and graduate school. I'm also thankful for all the support, hours, and hard work of my research assistants: Brenda Villalobos, Carolyn Chen, and Jack Davis. Finally, I want to thank all of my friends, especially those in the program, who believed in me and encouraged me along the way.

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Introduction

Food manufacturers and distributors are responsible for providing safe food via the implementation of risk-based preventative controls. Low-moisture foods (LMFs) have generally been considered low risk because water activity (a_w) values do not support the outgrowth of pathogens under prescribed conditions of storage and handling. Concerns in the U.S. regarding LMFs, defined as ingredients and finished goods with $a_w < 0.85$ (Podolak & Black, 2017), have escalated since 2001 because LMFs may harbor foodborne pathogens. While low water activity values are the defining characteristic of all LMFs, their composition (fats, carbohydrates, proteins, organic acids, etc.) varies. The matrix composition alone, in addition to availability and energy state of water, a_w at an equilibrium, impacts microbial thermotolerance (Dhowlaghar et al., 2021).

Foodborne bacteria of public health concern, including *Salmonella* spp. and *Listeria monocytogenes*, exhibit increased thermal resistance at reduced water activity (Gautam et al., 2020; Tsai, Taylor, et al., 2019) and may survive for >12 months (Podolak et al., 2010). Outbreaks of salmonellosis and the associated recalls in peanut butter in 2008-2009, increased awareness of the survival of *Salmonella* in this high fat, shelf-stable $a_w < 0.60$ product. Survival and thermotolerance data on these organisms, especially *L. monocytogenes* in products with characteristics and applications analogous to peanut butter, are rare. In a recent seminal study, Yang et al. (2020) reported that the a_w of pure peanut oil decreases exponentially with increasing temperature. In the same study, thermal death rates of *Enterococcus faecium* NRRL B-2354 followed first-order kinetics and D_{80} values increased exponentially in oil with reduced a_w values. The fundamental understanding of thermal death times and destruction kinetics is a challenging area, especially in plant-based LMFs with high oil content.

Thermal death time (TDT) studies, like the original research from our laboratory, are important because they provide direct comparisons of *Salmonella*, a surrogate (*E. faecium* NRRL B-2354), and at least one other foodborne pathogen. Robust comparisons in multiple commercially produced matrices are highly beneficial for individuals assessing the risks associated with the thermal inactivation during processing (Anderson, 2019; Deng et al., 2020; Liu et al., 2021; Wason et al., 2021), packaging (Yang et al., 2022), and distribution of these shelf-stable products. TDT data and conclusions regarding bacterial thermotolerance support controls ensuring that processing conditions, such as heat distribution, time, and temperature, sufficiently reduce the likelihood of microorganisms of concern. Thermotolerance data for target pathogens serve a critical role in validations of heat inactivation steps in products with similar intrinsic properties to meet or exceed current regulatory requirements.

Though recent breakthrough studies have expanded the incomplete understanding of the food safety considerations of oil dominant LMFs, technical information and data for thermotolerance in relevant matrices remains limited. The evaluation of methodologies and approaches that reduce bias and variability in thermal resistance determinations are warranted. Though challenging, improved reproducibility of thermotolerance assessments in LMFs will enable more impactful results and add to the broader work benefiting the validation of processing schemes (Hildebrandt et al., 2020).

In support of global food safety objectives, this study characterizes the thermal resistance of *L. monocytogenes* in a direct comparison to *Salmonella* and *E. faecium* NRRL B-2354 in three plant-based butters with low water activities and high fat content. An intrinsic oil-based inoculum method was substituted for a non-food inorganic carrier talc (hydrous magnesium silicate). TDT experiments in sunflower butter used a sunflower oil-based inoculum while the

peanut butter and almond butter experiments used a peanut oil or almond oil-based inoculum, respectively, to determine and compare decimal reduction times (*D*-values) and trends. Additionally, the effect of inoculum (dried inoculated talc vs. the newly implemented intrinsic oil-based inoculum) on the thermotolerance of the three organisms was performed in peanut butter. The peanut butter results from this study were directly compared to those of isothermal TDT studies using talc powder as an inoculum carrier, which, prior to the addition of the dry inoculum at >1% wt./wt., were compositionally identical.

Materials and Methods

Experimental Design

Three lipid-based inoculum carriers (peanut oil, almond oil, and sunflower oil) for *L. monocytogenes, Salmonella*, and *E. faecium* were applied to corresponding LMFs (peanut, almond, and sunflower butters) in isothermal inactivation experiments. Nine treatment combinations were executed in a three-by-three block design, with thermal death times resulting in independent *D*-values using three isothermal conditions for each pathogen across the three plant-based butters.

Three isothermal treatment temperatures were selected for each of the 9 bacteria / matrix treatments based on preliminary data and laboratory capabilities. A limited range of temperatures (70 to 85°C) was used to maximize the number of well-controlled *D*-value comparisons and allow adequate sampling intervals depending on the individual rate of destruction. For each of the 9 treatments, *D*-values collected at 3 temperatures were used to estimate mean *z*-values. A *z*-value is defined as the increase in temperature required for the thermal destruction curve to traverse one log cycle, calculated as the inverse slope of experimentally determined linear trend lines. All TDT experiments were independent and conducted in triplicate.

Strains & Inoculum preparation

Strains of *Salmonella* spp. (*S.* Montevideo, *S.* Agona, *S.* Tennessee, *S.* Weltevreden, *S.* Senftenberg, and *S.* Typhimurium PT 42) and 6 strains of *L. monocytogenes*, were obtained as described previously by Quinn et al. (2021). In preparation for the TDTs, the organisms were grown independently on plates containing tryptic soy agar with 0.6% yeast extract (TSAYE, Difco, BD) for 24 h. An isolated colony was aseptically transferred to 10 mL of tryptic soy broth (TSB; Difco, BD) followed by incubation at 37°C for 24 h. A retransfer of 100 µL occurred to another fresh TSB tube containing 9 mL s of media. This retransfer was incubated for 24 h at 37°C. Lawns were plated on TSAYE using the retransferred and incubated tubes via an aliquot of 100 µL. After a 48 h incubation period, at 37°C, lawn cultures were harvested from individual plates by adding of 0.1% sterile buffered peptone water (PW; Difco, BD) to the plate to create a cocktail slurry as described by Quinn et al. (2021). Lawn cultures grown on 12 TSAYE plates were combined to yield the desired volume. The same techniques were applied to prepare the *E. faecium* inoculum. Each independent bacterial slurry measured 25-28 mL with a target of ~10 log CFU/mL.

A new food oil-based inoculation was adapted from the method reported by Grasso et al. (2015) and Keller et al. (2012). Peptone water (20-23 mL) was discarded after centrifugation for 1 h at 3500 RPM at 23°C followed by an addition of 0.25 mL tween 80 and 10 mL of oil (sunflower oil, almond oil, or peanut oil) to create a 15 mL inoculum. These bacterial/oil suspensions were then vortexed for 3 minutes to achieve the appropriate level of homogeneity; The target concentration of the lipid-based inoculum was ~10 log CFU/mL.

Inoculum stability

For the inoculums prepared and studied, the initial microbial count (time 0) was determined by direct plating in duplicate after serial dilutions. The inoculums were stored at 25°C and the enumeration process was repeated and recorded every seven days to plot and calculate the total microbial reduction up to 4 weeks.

Plant-based Butters & a_w measurements

Three plant-based butters included: almond butter (retail), sunflower butter (retail), and peanut butter (Welfare Services, Houston Cannery, Houston, TX, USA). The fat content and mean a_w for each of these low-moisture food matrices: peanut butter (46% fat, 0.20 a_w @ 25°C), almond butter, (50% fat, 0.32 a_w @ 25°C), or sunflower butter (56% fat, 0.15 a_w @ 25°C).

Analysis for all a_w measurements was performed using the LabMaster-a_w Neo with a_wSens-ENS (Novasina AG, Lachen, Switzerland). Triplicate samples of plant-based butters and corresponding oils were measured at 25°C and 60°C to better understand the relationship between temperature and water activity for these products and the intrinsic oils used for inoculation.

Sample Inoculation

To prepare samples across the 9 experimental conditions, plant-based butters (25 g) were randomly sampled and transferred to a sterile petri dish (92 x 16 mm). An aliquot (0.2 mL) of homogenous inoculum was added. Vigorous manual mixing with a sterile spatula for 3 minutes followed to ensure an even distribution of bacteria in each sample. After an overnight (~20 h) period of rest at 25°C, a 500 mg aliquot of this inoculated sample was placed into sterile 4-oz Whirl-Pak bags with a thickness of 2.25 mils (0.054 mm). The bagged sample was flattened to a

thickness of ~1 mm and a heat sealer was used to close the individual bags. The water activity of each plant-based butter was determined before and after inoculation.

Isothermal Inactivation & Plating

All TDT experiments were conducted within the 3 days immediately following inoculum preparation to maximize the initial count. Each isothermal inactivation treatment was performed using a water bath with six time intervals in addition to a time zero control sample (no heat treatment). Six sets of copper plates, containing duplicate side-by-side samples in sealed bags were immersed in a water bath (VWR International, Radnor, PA, USA) (Enache et al., 2015). As described previously, the strength of the imbedded magnets was sufficient to further compress and tightly hold the samples at a uniform thickness (~1 mm) without rupturing the bags. During the heat treatment, the plates were held in an upright position with the heating medium contacting the plates on all sides. Copper plate sets were removed at different time intervals based on prework with the specific microorganisms studied. For all *E. faecium* experiments, the time intervals ranged from 11 to 14, 6 to 7, and 3 to 3.5 minutes at 75°C, 80°C, and 85°C respectively, while in *Salmonella* and *L. monocytogenes* experiments, the copper plates were removed between 9 to 12 minutes at 70°C, 4.5 to 6 minutes at 75°C, and 2 to 3 minutes at 80°C. The copper plates holding the treated samples were immediately immersed in ice water to terminate heat treatment of the organisms. For each experimental *D*-value, a total of 36 bagged sample preparations were inoculated, treated, and directly plated. These subsequent enumerations were plotted and used in the *D*-value calculations.

Fine wire thermocouples (diam/gauge 0.005 in [0.13 mm]) were introduced in multiple bags to monitor the internal temperature during the heat treatment. Achieving the treatment temperature as well as the target cooling temperature (25°C) required <15 s. The cooled sample

bags were aseptically opened, and 4.5 mL of PW was added to create a 1:10 dilution. Heat treated samples were hand shaken and massaged until they were completely suspended. Serial dilution in PW and subsequent plating on TSAYE was used, and colonies were counted after incubation at 37°C for 24 to 48 h.

Model analysis

Both the log-linear and Weibull models were used in this study to analyze the appropriate fit for heat inactivation during the isothermal experiment. *D*-values, decimal reduction time (the time at a specific temperature that reduces the total bacterial load by 1 log), are calculated using the following formula:

$$\log N_t = \log \quad N_0 - \frac{t}{D}$$

where $\log N_0$ and $\log N_t$ represent the log of initial bacterial population at time 0 and the log of surviving bacteria after treatment t. The log-linear model follows the first order kinetics which assumes that each bacterium in the sample receives equal heat treatment thus results in linear correlation. However, several studies have shown that bacteria do not always follow the first order kinetics because of shouldering, tailing, and other behaviors (Bevilacqua et al., 2015; Yan et al., 2021). Therefore, Weibull model has been proposed to be an alternative approach to better fit the thermal inactivation rate. The following formula is commonly used for Weibull calculation:

$$\log N_t = \log N_0 - \left(\frac{t}{\delta}\right)^{\beta}$$

where δ stands for the time it takes to achieve the first log reduction in the heat inactivation experiment, and β represents the shape of the curve. When $\beta = 1$, the curve is a straight linear regression and non-linear when $\beta \neq 1$. The direction of the curve is determined by whether $\beta > 1$ (downward) or $\beta < 1$ (upward).

Statistical analysis

D-values determined for the pathogens and *E. faecium*, calculated as means from triplicate experimental treatments, were analyzed via pair-wise comparisons using a Student's *t*-test with a pseudo-Bonferroni adjustment and $p < 0.005$. Direct comparisons of thermal tolerance of the three microorganisms were assessed. An assessment of "goodness of fit" of the data was applied using the Weibull and linear regression models; the model resulting in the lower Akaike Information Criterion (AIC) was determined as the best fit for the data.

The peanut butter *D*-values calculated using the oil-based inoculation system were compared to an independent isothermal study conducted using talc as a dry inoculation carrier. The peanut butter matrix had the same composition and intrinsic properties (pH, a_w, fat content, etc.). However, *D*-values from a previous study in our laboratory was applied for this comparison at two temperatures (75°C and 80°C) from the TDT studies Quinn et al. (2021). These temperatures allowed pair-wise comparisons specific to the different approaches to reach uniformity prior to heat treatment in the TDTs conducted in peanut butter. Due to a lower number of multiple comparisons, $p < 0.01$ was used when assessing the difference between inoculation methods in compositionally identical peanut butter from the same source at 75°C and 80°C.

Results and Discussion

Effect of temperature on a_w of oil and plant-based butters

Water activity is a critical hurdle to microbial growth. Lower water activity environments stress cells. The osmotic stress and desiccation stress have been shown to increase the heat resistance of microorganisms which can lead to increased survival of microorganisms under specific conditions (He et al., 2013; Peña-Meléndez et al., 2014). As oil is a major intrinsic

component of plant-based butters, it is important to understand the correlation between the change of water activity in oil and temperature to explore the observed phenomenon of higher thermotolerance of microorganisms in plant-based butters. Studies have shown a protective effect of oil in a fact that foods with high lipid content and low water activity such as peanut butter increase the thermal resistance of *Salmonella* and other bacteria (Ma et al., 2009; Shachar & Yaron, 2006). However, characterization of the relationship between oil temperature and water activity of the product is limited. Previous research was reported at ambient (25°C) until Liu et al. (2018) discovered that the *D*-values of *S.* Enteritidis and *E. faecium* at 80°C increased exponentially at reduced water activity compared to 25°C which inspired research on the relationship between the change of water activity of peanut oil at elevated temperature by Yang et al. (2020). The temperature impact on the water activity of peanut oil from room temperature to 80°C was determined. The water activity decreased as the temperature increased. These findings may partially explain why pathogens are able to survive in LMFs with high-fat contents. Critically important pathogens such as *Salmonella* have shown increased thermotolerance in low-a_w environments where the food composition includes oil. The prevailing thinking on this topic highlights protective effects of food oils, or desiccation in oil, provides for this foodborne pathogen.

We hypothesized that a relationship between oil content and *D*-values could be applied across food oils. Therefore, to test our hypothesis, we performed water activity measurements on sunflower oil, peanut oil, almond oil, and their respective plant-based butters at 25°C and 60°C in triplicate (**Fig. 1 & Fig. 2)** The results showed a slight decrease in water activity in all oils with increasing temperature which agreed with the previous report from Yang et al. (2020).

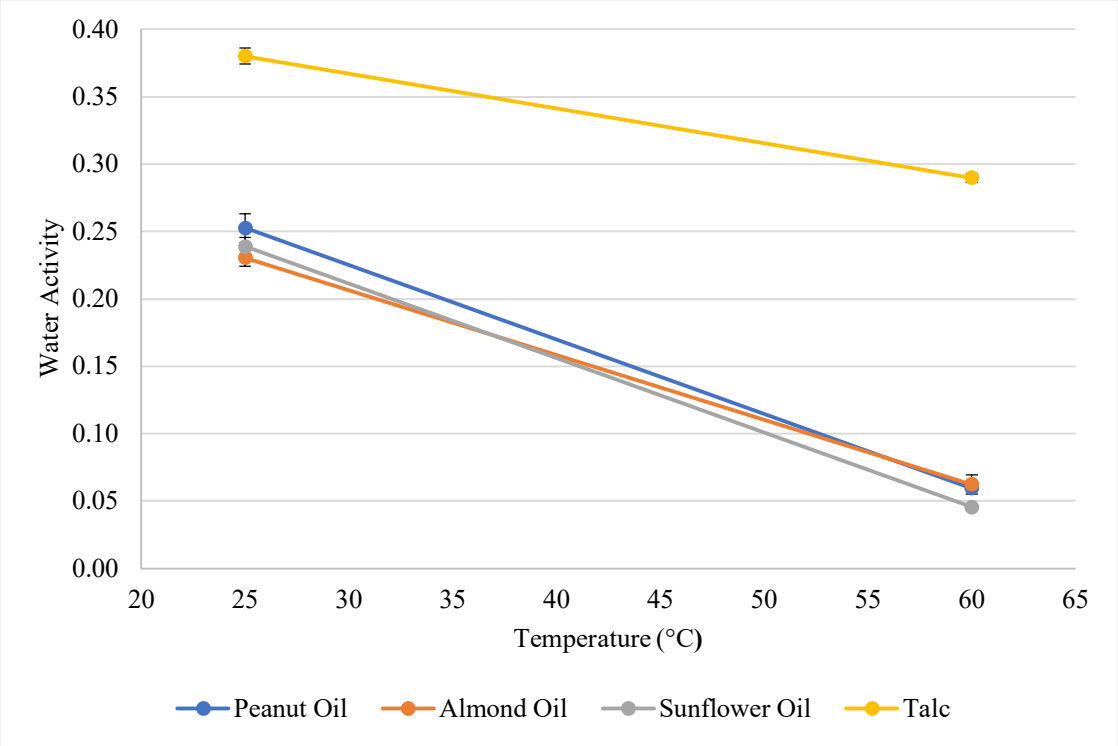

Fig. 1. The water activity of peanut oil, almond oil, sunflower oil, and talc at 25°C and 60°C (n=3).

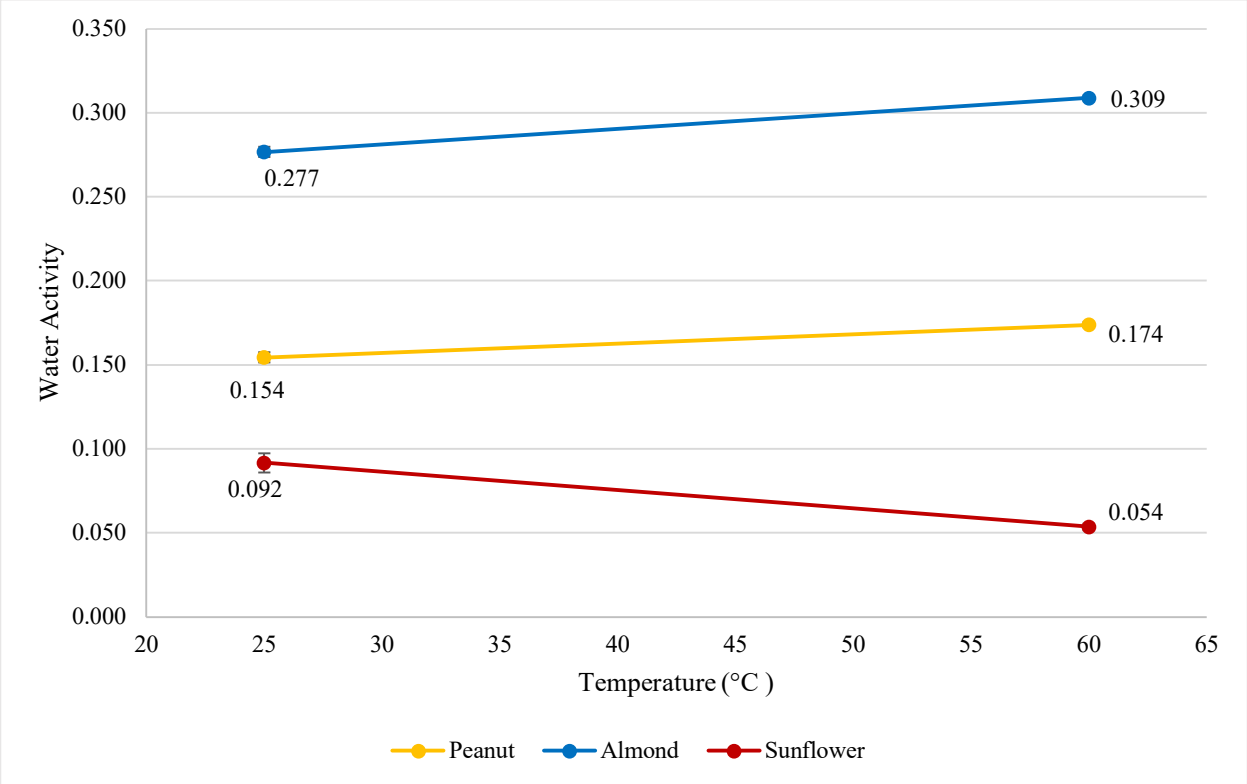

Fig. 2. The average water activity of peanut butter, almond butter, and sunflower butter at 25 and 60°C (n=3).

Statistical results

Aim 1: Total microbial reduction in nine inoculums after 4 weeks of room temperature storage

Though *Listeria* and *Salmonella* cocktails had nearly identical initial counts, *Salmonella* maintained a higher concentration (9.0-9.4 log CFU/mL) than that of *L. monocytogenes* (7.9-8.3 log CFU/mL) after 4 weeks of storage at 25°C. (**Fig. 3**). This observation indicates that high concentrations of *Salmonella* are maintained longer in food oils than high concentrations of *L. monocytogenes*. Notably, *E. faecium* did not achieve optimal cell concentrations. This surrogate strain had lower initial cell concentrations and clearly the lowest final count in all three oils after 4 weeks. Lower cell concentrations are a major disadvantage when using oil inoculation methods for TDT studies because sufficient direct log reduction data must be collected before reaching the limit of detection.

Previous work conducted in our lab, described by Quinn et al. (2021), featured dry inoculums with the same strains in low water activity TDTs. Higher initial cell counts were achieved using the dry inoculation approach compared to the oil-based inoculum. Final cell concentrations were also within 1 log of the initial counts after 4 weeks when working with a dry inoculum.

In summary, for the oil method, *Salmonella* inoculums had the most stable count after 4 weeks of storage and. *E. faecium* inoculums were the least stable due to a higher total log reduction. Although *E. faecium* inoculums were disadvantaged compared to the other two organisms, the initial cell concentration level after 7 days was sufficient to conduct robust TDT experiments using direct plating. Based on the results captured across the 4-week period and

previous experience with direct plating after heat treatment, we concluded that the oil inoculum methods, for the organisms selected for the study, were compatible with the TDT protocols as long as inoculums were applied within 1 week of preparation.

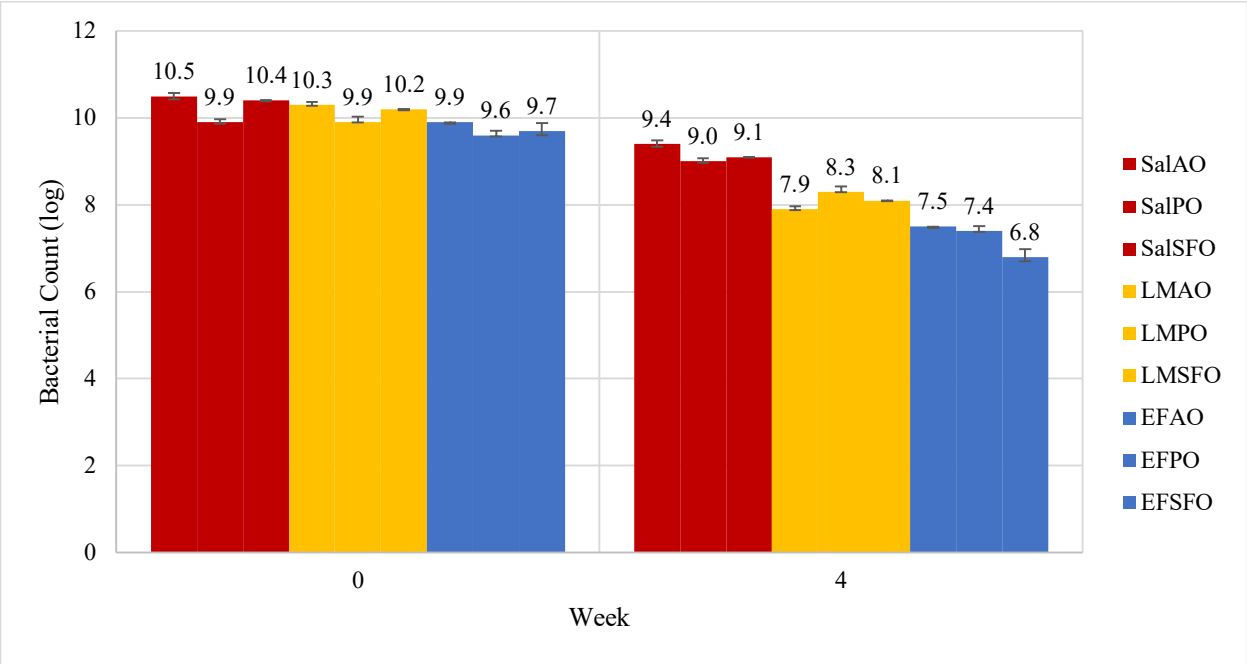

Fig. 3. The average microbial population of all *E.* faecium (EF), *Salmonella* (Sal), and *L. monocytogenes* (LM) oil inoculums at week 0 and week 4 (n=3). (AO: Almond Oil, PO: Peanut Oil, SFO: Sunflower Oil)

Aim 2.1: Thermotolerance of *Enterococcus faecium, Salmonella* spp., and *Listeria monocytogenes* in different matrices

The design enabled direct comparisons of the thermal tolerance of the microorganisms in each of the plant-based butters. In total, the data set was comprised of TDT enumerations at three isothermal settings and the associated time interval required to reduce the population by one log, known as a *D*-value. *D*-values were calculated from enumerations conducted in triplicate from 27 independent runs comprised of 42 inoculated samples.

Thermotolerance was primarily evaluated by comparing time intervals at a specific temperature required to achieve a log reduction of bacterial population. Therefore, the higher the

D-value, the greater the thermotolerance (**Table 1**). *E. faecium* in peanut butter at 75°C had a significantly higher *D*-value ($p < 0.005$) than the respective *D*-values for the same organism in almond butter and sunflower butter. At the higher temperatures, 80°C and 85°C, the pair-wise comparisons evaluating the thermotolerance of *E. faecium* yielded no differentiated effect isolated solely to the matrix for these specific conditions. Even though no differences were found between the 80°C and 85°C comparisons, the empirical values for *E. faecium* in peanut butter were higher than those in almond and sunflower butters. This was a key and unexpected finding as we expected sunflower butter, not peanut butter, to present the highest thermotolerance for the organisms. This expectation was based on prevailing theories that thermotolerance is highest at the lowest water activity of similar LMFs. As a reminder, peanut butter was 46% fat, 0.15 a_w at 25°C, almond butter 50% fat, 0.28 a_w at 25°C, and sunflower butter 56% fat, 0.09 a_w at 25°C. Therefore, it was even more surprising that this occurred at the TDT temperatures where the peanut butter would be greater and the sunflower butter similar or even lower than presented in Fig. 2.

The TDTs performed at 70°C for *L. monocytogenes* resulted in the only experimental condition where this organism was more thermotolerant in peanut butter ($p < 0.005$) when compared to almond and sunflower butters. At other temperatures tested, the thermotolerance of *L. monocytogenes* was similar across all 3 butters. Interestingly, *Salmonella* had a higher *D*-value at 75°C in sunflower butter compared to almond and peanut butters, but there was no difference in the comparisons of almond butter and sunflower. This was true for the *Salmonella* peanut butter vs. almond butter comparison under the same conditions.

We selected three plant-based butters with elevated fat contents and low but disparate water activity values. At the outset it was expected that, in a head-to-head comparison, the

thermotolerance of bacteria would be higher in any plant-based butter with a lower water activity because previous studies have found that the thermotolerance of *Salmonella* is higher in peanut butter than in wheat flour due to the increased water activity in wheat flour after the thermal inactivation treatment (Syamaladevi et al., 2016). However, previous work in our laboratory found that the $D_{80°C}$-values of *Salmonella*, *L. monocytogenes*, and *E. faecium* in peanut butter (a_w: 0.11) were lower than those in powder infant formula (a_w: 0.20) at 25°C (Quinn et al., 2021). Therefore, we hypothesized that beyond the water activity, other intrinsic properties of the food matrices impact the bacterial thermotolerance in different plant-based butters.

In addition to the statistical comparison of the *D*-values mentioned previously, we also determined the *z*-values. Estimated *z*-values are calculated to characterize the thermal destruction rate. The *z*-value is explained as the temperature increment needed for a ten-fold acceleration of the rate of thermal destruction (i.e., for shortening *D*-value by a factor of 10). Comparing the *z*-values (the estimated *z*-values are found in Appendix A, **Table S1.**), we found that sunflower butter showed a higher *z*-value than peanut butter and almond butter in the case of *E. faecium* and *Listeria*, while the *z*-value of *Salmonella* in peanut butter was greater than sunflower butter.

Overall, we were unable to conclude that a direct correlation exists between the thermotolerance of each microorganism in the three plant-based butters tested. Therefore, we attest that it is an oversimplification to assume that plant-based butters with a lower water activity at a specific treatment temperature yields a higher thermotolerance for *E. faecium*, *L. monocytogenes*, or *Salmonella*. Rather, it is more prudent to consider water activity as one of several factors that stresses cells and, in the case of bacteria in LMFs, increases heat resistance. It is suspected that cells are stressed at lower water activity environments and, as reported

previously, environmental stresses including heat, changes in osmotic conditions and desiccation (He et al., 2013; Peña-Meléndez et al., 2014).

To determine the potential contributing factors for these results, we compared the fatty acid profiles (**Table 2**) in three respective oils selected for oil-based inoculums as well as the pH (data not shown), the water activity at elevated temperatures (**Fig. 1**), and the macronutrients of the butters (**Table 3**). There were minimal differences in terms of the fatty acid profile of the oil and the pH of the butters, however the results of water activity at elevated temperatures were surprising. Both almond and peanut butters had a slight increase while sunflower butter had a constant decline as the temperature increased which was different than what was observed in the water activity experiments conducted on food oils.

We compared the nutritional content for the three butters and hypothesized that the difference in fat content was primarily responsible for the complexity of the varying thermotolerance. Jin et al. concluded that foods containing more carbohydrates and proteins might result in less thermotolerance for microorganisms than high-fat products due to the higher increase in water activity at elevated temperatures (2019). Protein content across the butters tested was uniform. Fat content varied from 47-56%. Again, there seems to be more complexity as the sunflower butter used herein contained the highest fat content and lowest total carbohydrates. The *D*-values at 4 temperatures from sunflower butter with three different bacteria showed no statistical differences. The only exceptions were *E. faecium* at 75°C, *Listeria* at 70°C, and *Salmonella* at 75°C where sunflower had lower *D*-values than peanut butter ($p < 0.005$).

Other studies also assessed the thermal resistance of *Salmonella* in peanut butter to better understand the relationship between thermal resistance and different matrices. The organic

peanut butter (sample B) selected in a study conduct by He et al. (2011) contained slightly higher fat content & protein and less carbohydrates than our peanut butter; we compared our results at 70°C with their overnight culture experiments at 72°C and found that our *D*-values were higher which also did not corroborate the conclusions drawn by Jin et al. (2019). However, several dissimilarities exist between two studies such as water activity, inoculum preparation methodology, *Salmonella* serotypes and other factors that might be correlated to the differences observed.

We also compared our results with a study on peanut butters featuring two levels of fat content and carbohydrates (He et al., 2013). Peanut butter A contained 33% fat, 42% carbohydrates, and E contained 49% fat, 24% carbohydrates. The peanut butters that the study used were conditioned to different water activities across the range (0.20, 0.40, 0.60, 0.80). We assessed the relevant data from peanut butter E at a_w 0.20 at 90°C inoculated with three-serotype cocktail for comparable water activity in our study. Though the highest temperature in our *Salmonella* experiments was 80°C, our *D*-value was 0.36 minutes lower than those reported in the study.

In a study performed by Limcharoenchat et al., the authors concluded that the inoculation protocols may exhibit an effect on the thermal tolerance of *Salmonella* (2018). Clearly the work conducted by Ma et al. (2009) represented a contrasting inoculum preparation and method implemented in comparable TDT studies. Notably, the fat content was 6% greater and the water activity was substantially higher in the peanut butters as well. The *D*-values at 71°C, 77°C, 83°C were compared to our peanut butter results at 70°C, 75°C, and 80°C. The values from their study were all surprisingly higher (> 10 minutes) than those resulting from our experimental work. It is, however, important to note that the inoculum used for their sample inoculation was cultured

in TSB for three-consecutive 24-h periods which was found to be more heat resistant than the 16-h and 24-h cultures. They concluded that culture preparation influenced the thermal resistance of *Salmonella* which agreed with Limcharoenchat et al. The intrinsic oil inoculums applied to the plant-based butters were prepared by culturing in TSB, which included two 24 incubations) followed by the creation of a lawn over 48 h, as described previously.

Table 1. *D*-values and standard deviations (min) of *E. faecium*, *L. monocytogenes*, and *Salmonella* in almond butter, peanut butter, sunflower butter at different thermal inactivation temperatures.

Temperature	Food	*E. faecium*	*L. monocytogenes*	*Salmonella* spp.
70°C	Almond Butter		11.33 ± 1.55 ^{a}	13.73 ± 2.03
	Peanut Butter		16.28 ± 1.35 ^{b}	11.13 ± 0.22
	Sunflower Butter		12.35 ± 1.63 ^{a}	19.25 ± 3.50
75°C	Almond Butter	13.76 ± 1.23 ^{Aa}	6.23 ± 1.37 ^{Ba}	7.36 ± 1.36 ^{Bab}
	Peanut Butter	19.22 ± 0.93 ^{Ab}	7.00 ± 1.13 ^{Ba}	7.28 ± 1.23 ^{Ba}
	Sunflower Butter	13.79 ± 1.44 ^{Aa}	7.96 ± 0.79 ^{Ba}	10.53 ± 0.60 ^{Bb}
80°C	Almond Butter	7.00 ± 2.10 ^{Aa}	3.11 ± 0.80 ^{Ba}	4.53 ± 1.01 ^{ABa}
	Peanut Butter	8.05 ± 0.09 ^{Aa}	4.12 ± 0.85 ^{Ba}	4.48 ± 0.29 ^{Ba}
	Sunflower Butter	7.81 ± 0.78 ^{Aa}	4.61 ± 0.65 ^{Ba}	7.01 ± 0.33 ^{ABa}
85°C	Almond Butter	3.73 ± 0.62 ^{a}		
	Peanut Butter	5.09 ± 0.85 ^{a}		
	Sunflower Butter	5.04 ± 1.20 ^{a}		

Note: Upper case letters in the same row represent the comparison of the *D*-values of the 3 microorganisms in the same plant-based butter at a specific temperature; Lower case letters in the same column represent the *D*-values of each microorganism across the 3 butters at a specific temperature. Pair-wise comparisons performed via Student's *t*-test with a pseudo-Bonferroni adjustment and a significance level of $p < 0.005$.

Table 2. The fatty acids profile of three different oils used in inoculums. (SFA=Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids; Serving size 14g)

	SFA	MUFA	PUFA
Almond Oil	1g	10g	3g
Peanut Oil	2g	11g	1g
Sunflower Oil	1g	11g	2g

Table 3. Macronutrients and water activity of three different plant-based butters. (Serving size 32g, wt.%)

	Total Fat	Fat content (%)	Total carbohydrates	Added sugar	Protein	Aw
Almond Butter	16g	50%	7g (22%)	0g	7g (22%)	0.32
Peanut Butter	15g	47%	8g (25%)	1g	7g (22%)	0.20
Sunflower Butter	18g	56%	4g (12.5%)	0g	8g (25%)	0.15

Aim 2.2: Thermotolerance Comparisons of *E. faecium, Salmonella* spp., *and L. monocytogenes* in individual food matrixes

The thermotolerance of *E. faecium*, *L. monocytogenes*, and *Salmonella*, were determined and compared in three plant-based butters across a minimum of three temperatures (**Table 1**). At 75°C, *E. faecium* had a higher *D*-value in all three plant-based butters than *Listeria* and *Salmonella* whereas there was no statistical difference between the two pathogens ($p < 0.005$). Clearly, under extreme conditions in certain LMFs, the thermotolerance of *L. monocytogenes* can approximate that of *Salmonella*.

Again, while there was no difference between the thermotolerance of *Listeria* and *Salmonella* in three butters at 80°C, we did elucidate differences in thermotolerances between *E. faecium* and *Salmonella* in almond or sunflower butters at the same temperature using our experimental approaches. The *D*-values for *E. faecium* were significantly higher than *Listeria* across all butters. When combining the results for both 75°C and 80°C, we observed that *E. faecium* behaved consistently in peanut butter compared to almond butter and sunflower butter (**Fig. 4**) as it always had a higher thermotolerance (as measured by *D*-values) than *Listeria* and *Salmonella* ($p < 0.005$).

Historically *E. faecium* has been treated as a surrogate organism for *Salmonella* in almond products (Jeong et al., 2011; Zhu et al., 2021), but recently it has been used on other low moisture foods studies such as egg powers (Pérez-Reyes et al., 2021), wheat flour (Liu, Rojas et al., 2018), and cocoa powder (Tsai, Ballom, et al., 2019). Our results strongly indicated that it can also be used as a conservative surrogate for *Listeria* in plant-based butters. Although no significant difference was observed between *E. faecium* and *Salmonella* at 80°C in all three plant-based butters, the overall *D*-value of *E. faecium* was still greater than *Salmonella*.

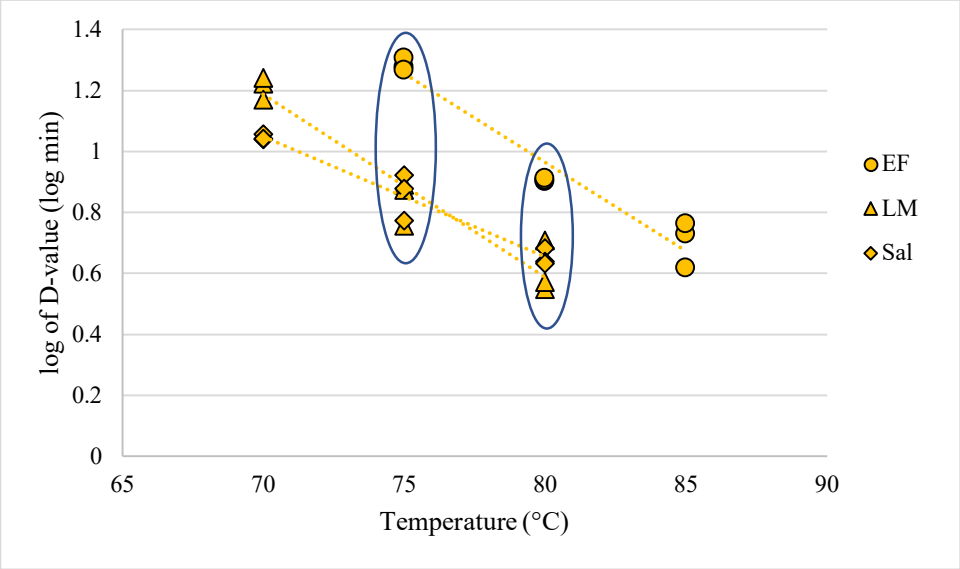

Fig. 4. The differences of thermotolerance of *E. faecium*, *L. monocytogenes*, and *Salmonella* spp. in peanut butter. The oval circles indicate the presence of significant differences ($p < 0.005$).

Aim 3: Differences of thermotolerance of three organisms between using talc and oil in inoculums in peanut butter

TDT results on the pathogens and surrogate in peanut butter were compared using the data from the dry (talc) and oil inoculation methods (**Fig. 4.1-4.3**). The purpose of this aim was to determine if the oil inoculation method could replace the use of talc inoculum in high-fat plant-based butters. In the direct side-by-side comparison conducted, *E. faecium* and *Salmonella* at 75°C & 80°C were different ($p < 0.01$) where the *D*-values of oil inoculation was lower than

the dry one. The practical importance of this finding builds primarily from the surprising result that oil-based inoculations exhibited lower thermotolerance for *Salmonella* spp. and *E. faecium* (the surrogate) when compared to the dry inoculum ($p > 0.01$). At 85°C, the results were similar but not to the level of predetermined statistical significance for the surrogate. However, in contrast to the *E. faecium* and *Salmonella* results, the analysis yielded no differences across all *Listeria* treatments. In comparing the mean *z*-values (Appendix A, **Table S2.)**, there was very little difference in *E. faecium* and *Salmonella* comparisons (< 0.5°C) compared to *Listeria* (7.5°C). *Salmonella* in oil was the only instance where the *z*-value was higher than using talc as an inoculum while the other two organisms had a higher *z*-value in dry method than in oil.

While the methodology between the two studies were nearly identical, a few differences may account for the results. First, the water activity of peanut butter after inoculation was approximately 0.11 in the previous study and 0.20 for this study. This difference could affect the heat resistance of the microorganisms which has already been extensively studied by others. Secondly, the use of dry talc as an inoculum carrier might be responsible for the change in thermal resistance. According to a study conducted by Ahmad et al., (2019), they concluded that the shielding effect from fat in almond meal accompanied with talc had increased the heat resistance of *E. faecium*. For this and other reasons, the use of talc as a carrier for paste-like products creates complexity because of the effect talc and drying of talc has on the thermotolerance of bacteria. It also is not favored because in the TDT or validation work, the researcher is introducing a non-food component to the food matrix to study the impact of the matrix. Additional contributing factors related to inoculation approaches and thermal resistance were studied in research conducted by Ahmad et al., 2019 and are acknowledged in the LMF TDT field.

In the absence of additional controlled studies with similar matrices it is difficult to provide a definitive answer as to whether the oil inoculation was more appropriate for plant-based butters than using a dry carrier. But, based on the studies conducted in our facilities, we suggest that the oil inoculation may be a more practical approach when building safety assessments because the dry inoculation potentially would lead to overestimation of lethality. Over-processing due to overly conservative *D*-values for certain products impacts food quality attributes and certainly impacts energy and time requirements in the safe production.

Having assessed the appropriateness of inoculums used in different types of LMFs and determined the suitability of the oil-based method, it is worth considering additional benefits of the wet inoculation method in TDT studies. Rather than waiting on the inoculated talc to air dry for approximately 24 h prior to sample inoculation, wet inoculums are fit for use after the harvesting step. The air-dry steps common to dry inoculation methods can add complexity and increase the risk of contamination from other organisms. Also, researchers and organizations have also voiced concerns regarding the use of talc in scientific laboratories due to human health concerns (Chang et al., 2020; Liu et al., 2019; Wild, 2006). Therefore, beyond the experimental design and results reported herein, further work is warranted to fully assess the potential benefits oil inoculums in TDT studies involving high fat foods.

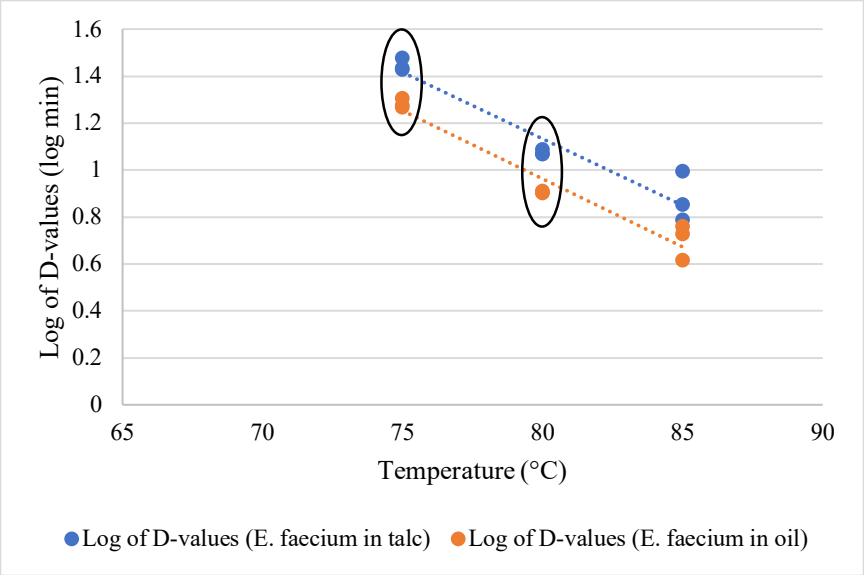

Fig. 4.1. Thermotolerance of *E. faecium* in talc vs oil as an inoculum in peanut butter. The oval circles indicate the presence significant differences ($p < 0.01$).

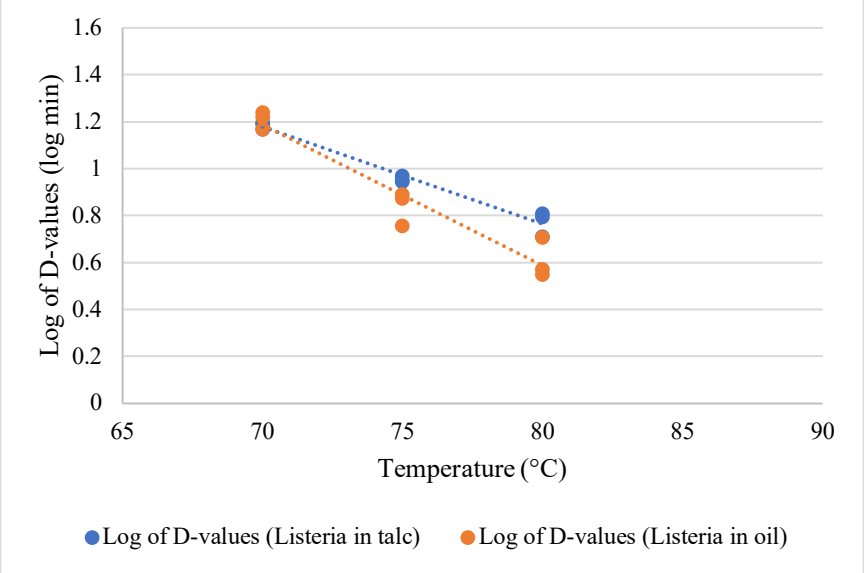

Fig. 4.2. Thermotolerance of *L. monocytogenes* in talc vs oil as an inoculum in peanut butter where no differences were found in the comparison at all temperatures.

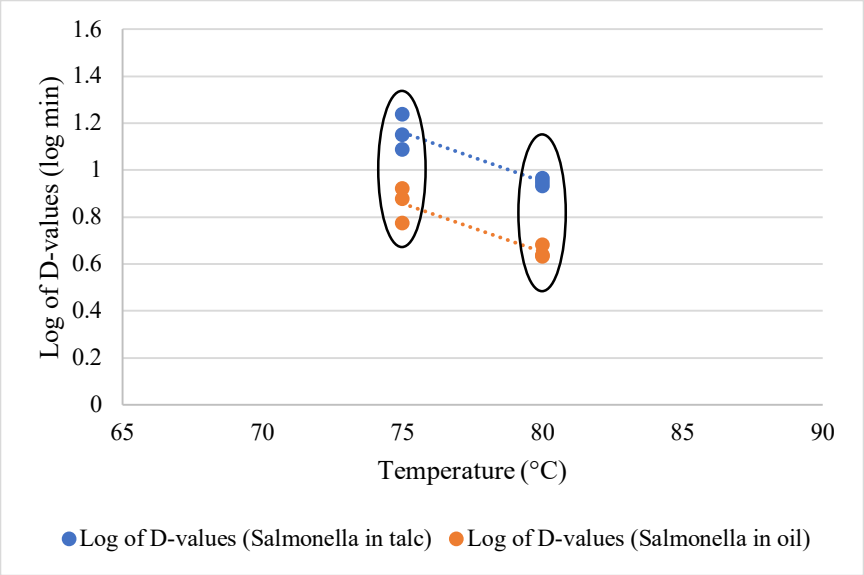

Fig. 4.3. Thermotolerance of *Salmonella* spp. in talc vs oil as an inoculum in peanut butter. The oval circles indicate the presence significant differences ($p < 0.01$)

Comparison of inactivation models

We compared the AIC between log-linear and Weibull models across all 27 treatments, and the smaller value of two indicated a better model fit (**Table 4**). We found that 18 out of 27 treatments showed that log-linear model was a better fit for the isothermal inactivation data; 5 indicated Weibull, and there were 4 that showed no differences between the two (< 0.2). However, from a practical standpoint, it is important to note that the overall AIC difference between the two models was small—the greatest being 2.78. Additionally, we did not observe shouldering/tailing in the raw data plot (Appendix A, **Fig. S1.1-S1.9)** to justify for using Weibull model**.** Therefore, we concluded that Weibull model could also be considered as a good alternative for the log-linear model in our study.

Table 4. Log-linear and Weibull model analysis of all plant-based butters inoculated with *E.* faecium, *Salmonella*, and *L. monocytogenes* at different temperatures. A lower AIC indicates the goodness of fit.

Bacteria	Temperature	Plant-based Butter	Log-linear AIC	Weibull AIC	Difference
E. faecium	75	Almond Butter	39.85	41.55	1.71
	80	Almond Butter	34.41	33.63	0.79
	85	Almond Butter	24.15	23.81	0.35
	75	Peanut Butter	38.58	40.73	2.15
	80	Peanut Butter	33.15	33.33	0.18
	85	Peanut Butter	31.32	30.04	1.29
	75	Sunflower Butter	48.11	49.71	1.60
	80	Sunflower Butter	42.79	45.03	2.24
	85	Sunflower Butter	38.75	39.48	0.73
L. monocytogenes	70	Almond Butter	31.75	32.83	1.08
	75	Almond Butter	55.52	56.81	1.29
	80	Almond Butter	19.99	22.14	2.14
	70	Peanut Butter	41.81	42.09	0.28
	75	Peanut Butter	32.36	33.59	1.23
	80	Peanut Butter	28.58	28.38	0.19
	70	Sunflower Butter	49.07	48.12	0.96
	75	Sunflower Butter	36.27	37.73	1.46
	80	Sunflower Butter	37.59	40.38	2.79
Salmonella	70	Almond Butter	43.33	42.20	1.13
	75	Almond Butter	26.62	27.14	0.52
	80	Almond Butter	27.58	29.59	2.01
	70	Peanut Butter	30.89	30.78	0.10
	75	Peanut Butter	37.76	38.30	0.54
	80	Peanut Butter	34.57	36.64	2.07
	70	Sunflower Butter	52.45	54.74	2.28
	75	Sunflower Butter	45.36	45.42	0.06
	80	Sunflower Butter	45.13	47.12	1.99

Environmental influence on water activity of plant-based butters

The results in **Fig. 5** showed that all butters performed stably in terms of water activity in 30%, 75%, and room temperature except the peanut butter at 75% which was observed to have a

much higher water activity than the starting point. Peanut butter with an extremely low water activity tends to draw and absorb water from the surrounding environment. The peanut butter used for the isothermal inactivation experiments was stored sealed at room temperature (25°C); the relative humidity ranged from 10-25%. The water activity of peanut butter used in the experiment was 0.20 ± 0.08 throughout the experiments.

The environmental influence on water activity work not only confirmed the stability of water activity of plant-based butters under different conditions but also signified the importance of proper storage. After 3 months of storage, we observed mold growth in one of the peanut butter and sunflower butter jars in the 70% relative humidity chamber; the color on the surface of those butters also significantly darkened compared to the samples in the 30% relative humidity and room temperature chambers. To avoid the unacceptable appearance and quality issues of planted-based butter, it is strongly recommended that the lids are securely tightened and stored in a controlled low relative humidity environment.

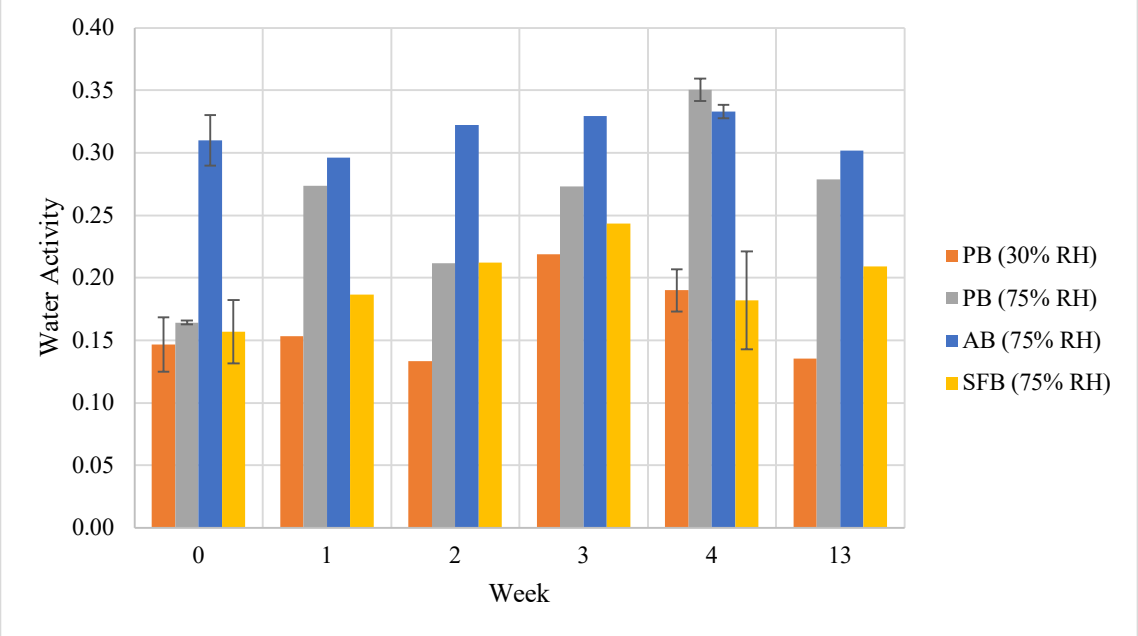

Fig. 5. Environmental influence on water activity of almond, peanut, and sunflower butters at 70% relative humidity (RH) and peanut butter at 30% RH

Conclusion

The thermotolerances of three organisms in high-fat plant-based butters were compared using an intrinsic oil-based inoculation method enabling a series of controlled TDT experiments. *D*-values were determined for *E. faecium*, *Salmonella*, and *Listeria* across peanut, almond, and sunflower butters at a minimum of three temperatures. Notably, *E. faecium* in peanut butter at 75°C and 80°C had greater *D*-values than both *Salmonella* and *Listeria monocytogenes* ($p > 0.005$). *E. faecium* also had higher *D*-values than *Salmonella* at 75°C in both almond and sunflower butters ($p > 0.005$).

Overall, this study piquantly confirmed the validity of *E. faecium* as a conservative surrogate in these matrices; All isothermal measures indicated the destruction kinetics of the surrogate required more time at a specific temperature than the pathogen strains. The utility of this surrogate for *Salmonella* and *L. monocytogenes* in high fat, exceptionally low water activity plant-based butters is highly conclusive.

The TDT studies found *Salmonella* thermotolerance was consistently greater than *L. monocytogenes* across the treatments. However, when assessing the thermotolerance of the two pathogens in the individual plant-based butters tested the *D*-values were not statistically different ($p > 0.005$). These data support *Salmonella* as the primary pathogen of concern in low water activity foods. Surprisingly however, this work definitively found the heat resistance of *L. monocytogenes* can approximate destruction kinetics observed for *Salmonella* in the plant-based butters tested.

Secondarily, a side-by-side comparison of oil vs. dry inoculation methods in peanut butters was conducted to understand the impact of the inoculum on the TDT results. The dry inoculation method had higher *D*-values in comparison to the oil inoculation in peanut butter.

This indicates a higher microbial heat resistance in peanut butter after a drying stress was applied. The magnitude of the difference was ~0.625 times between 75°C and 80°C for *Salmonella*. The increase in thermotolerance for the surrogate at these temperatures was confirmed at ~2 times that of the pathogen (*Salmonella*) under the same condition. In contrast, there were no differences between inoculation methods for *L. monocytogenes* across the three temperatures tested.

Therefore, the use of the dry inoculum provided *D*-values that are highly conservative, meaning they require more heat and/or time to deliver a log reduction of the target organism. The oil inoculum was less conservative but, under the conditions tested, suitable surrogate performance was observed. Again, this further supports its application in challenge studies and validation work. *E. faecium* exhibited the highest thermotolerance in all matrices independent of the inoculation method applied.

The model fit analysis for the massive amount of TDT data collected on plant-based butters in this study found that the log-linear model was not only suitable but, in the vast majority of comparisons, better than the Weibull model. This was unexpected and attributed to the linearity of the TDT counts and redundancy of the experimental design. It should be noted however that the AIC differences between the log-linear and Weibull models were minor. Therefore, if required, either model could be appropriately applied in this original LMF research. Our analysis mirrored approaches from the same laboratory and utilized the same organisms to allow comparisons to prior independent work on low water activity peanut butter. Thus, the consistency regarding the log-linear approach applied in both this and previous work did not require additional data transformations and minimized analytical complexity.

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APPENDIX A

Supplemental Materials

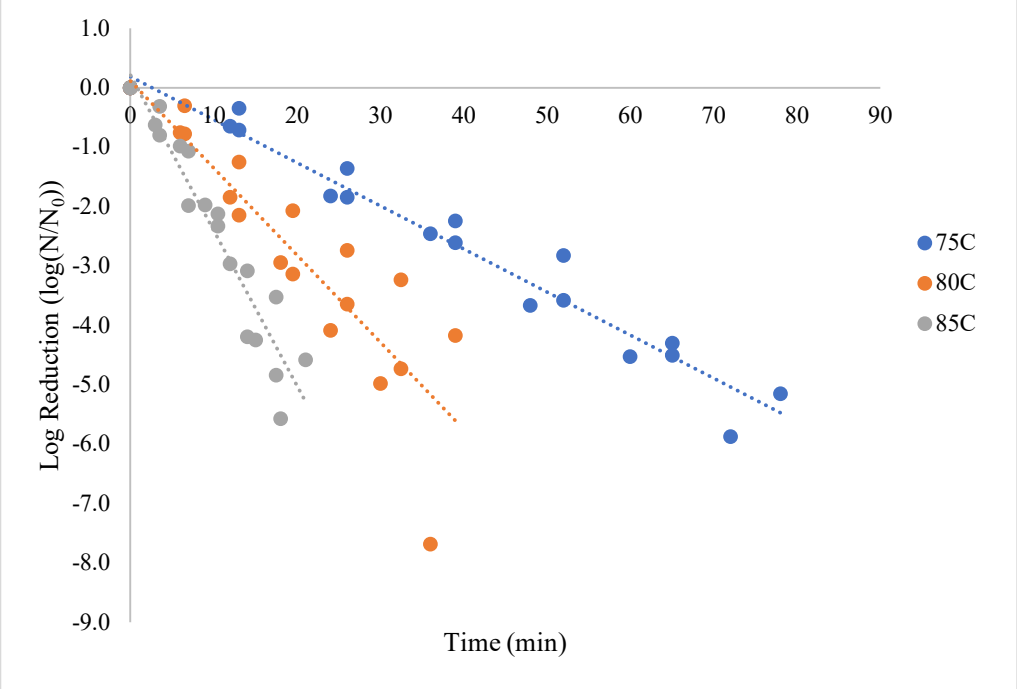


Fig. S1.1. Log reduction of *E. faecium* vs. time in almond butter at 75°C, 80°C, and 85°C.

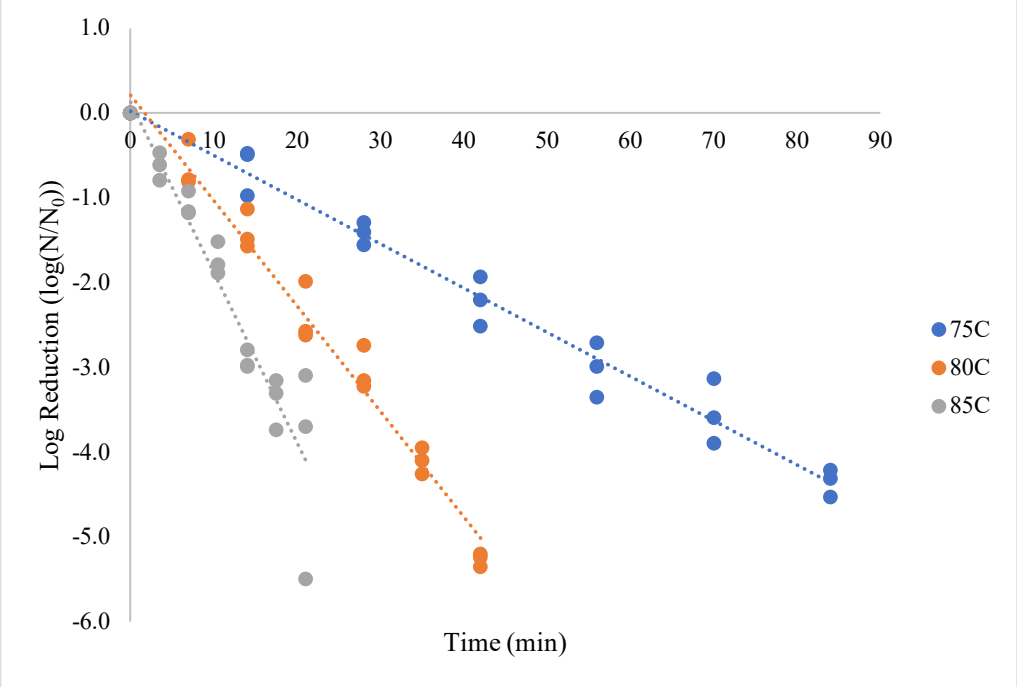


Fig. S1.2. Log reduction of *E. faecium* vs. time in peanut butter at 75°C, 80°C, and 85°C.

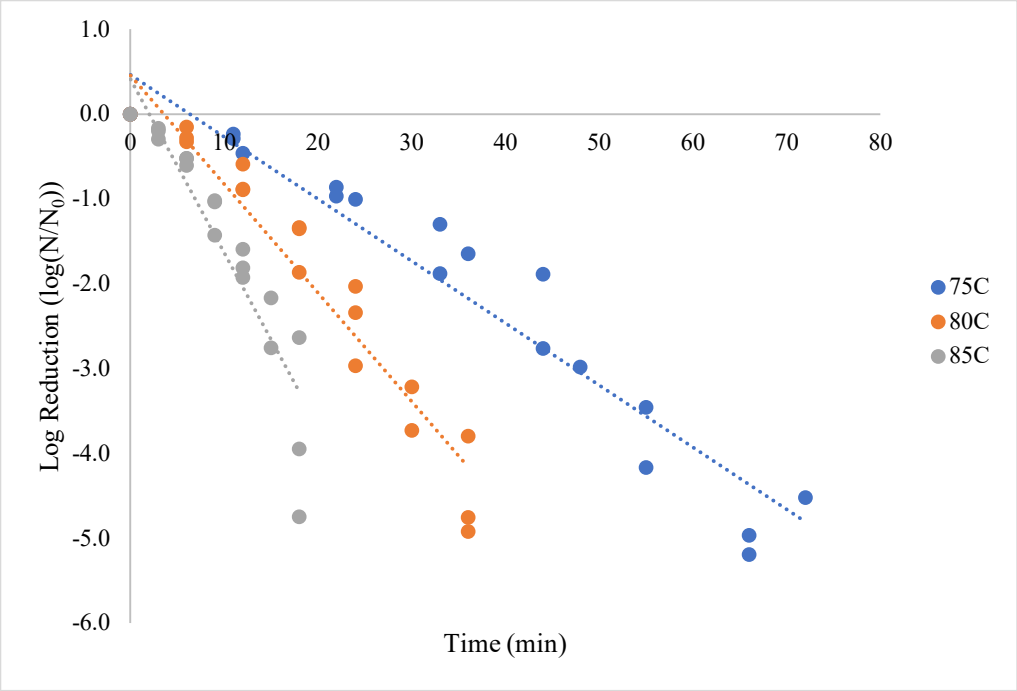


Fig. S1.3. Log reduction of *E. faecium* in vs. time sunflower butter at 75°C, 80°C, and 85°C.

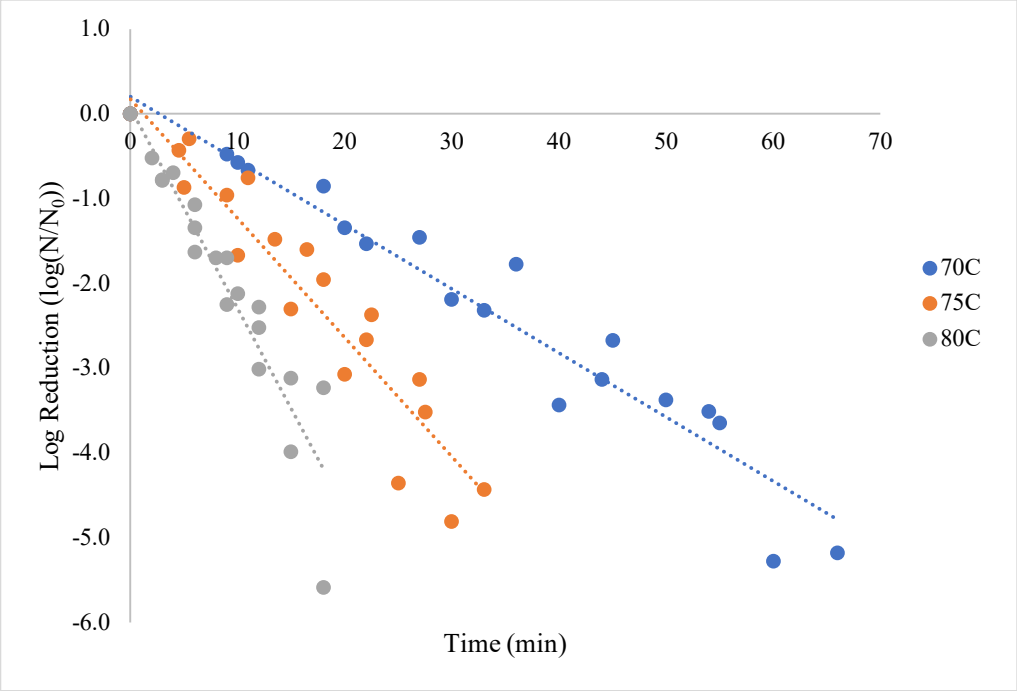


Fig. S1.4. Log reduction of *Salmonella* spp. vs. time in almond butter at 70°C, 85°C, and 80°C.

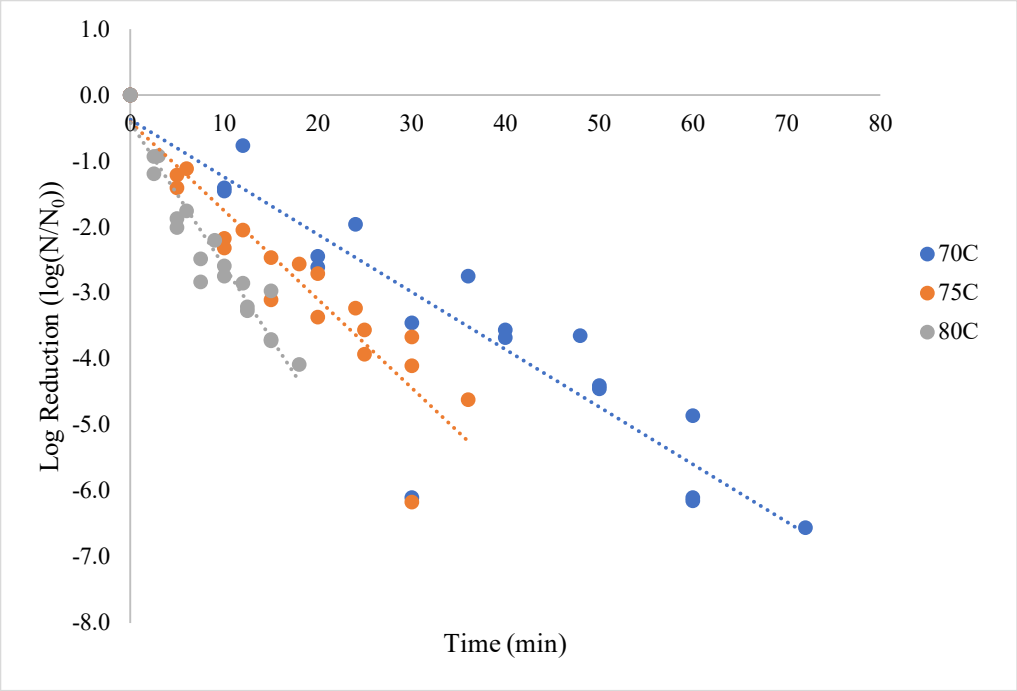


Fig. S1.5. Log reduction of *Salmonella* spp. vs. time in peanut butter at 70°C, 85°C, and 80°C.

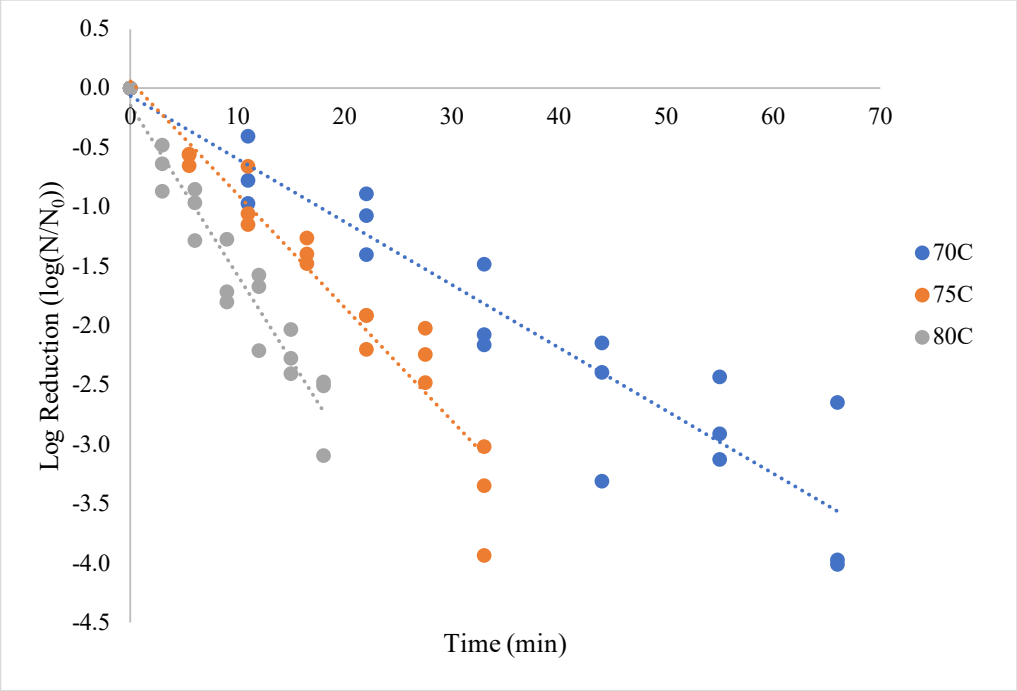


Fig. S1.6. Log reduction of *Salmonella* spp. vs. time in sunflower butter at 70°C, 85°C, and 80°C.

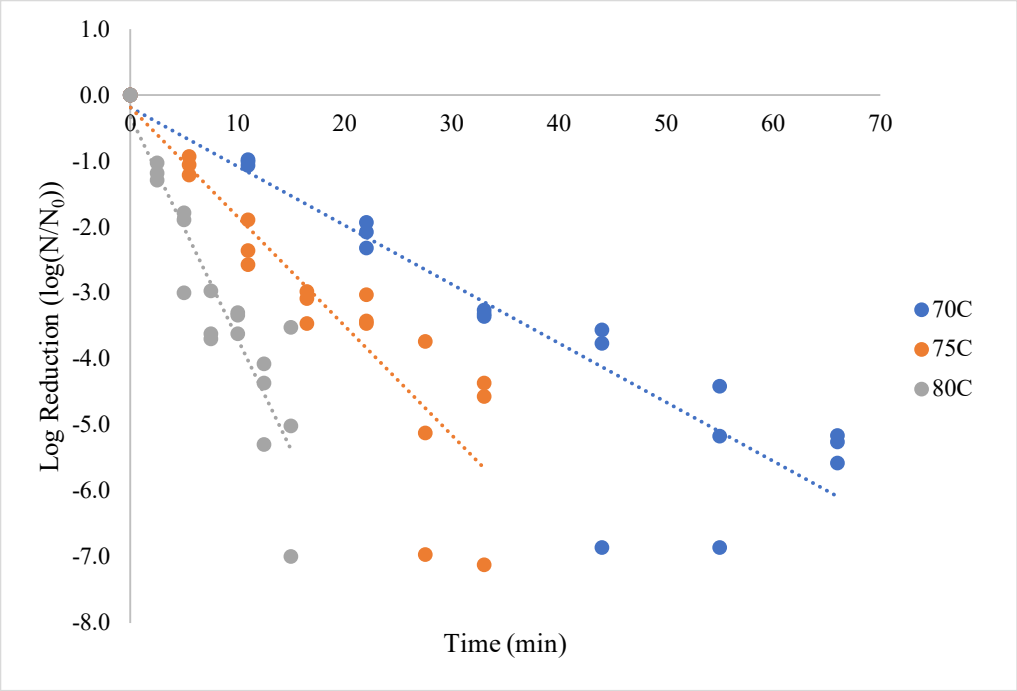


Fig. S1.7. Log reduction of *L. monocytogenes* vs. time in almond butter at 70°C, 85°C, and 80°C.

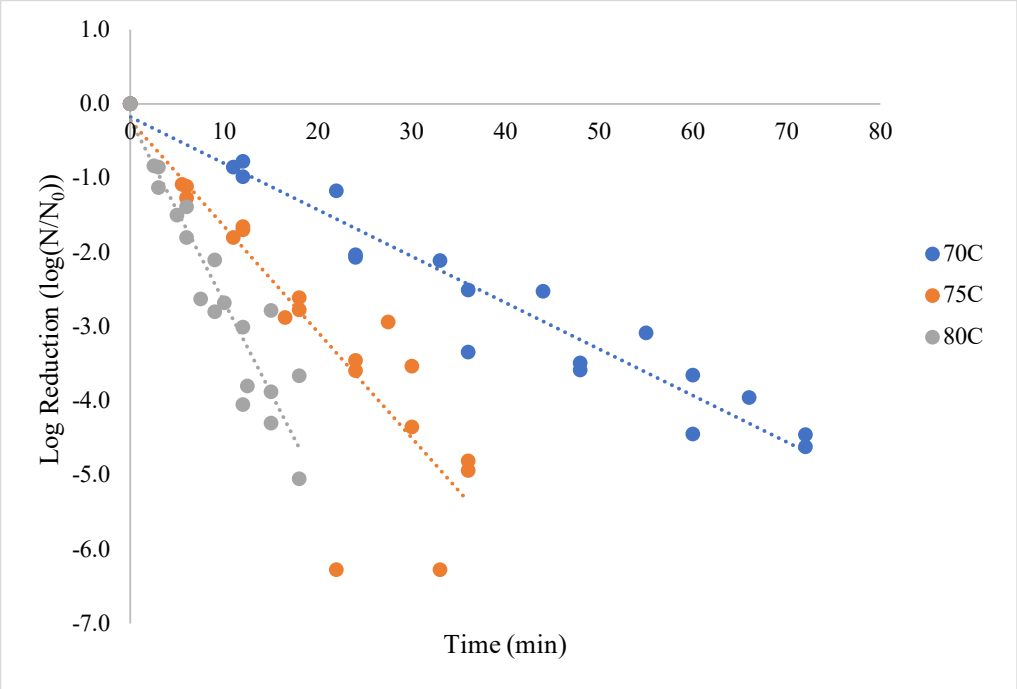


Fig. S1.8. Log reduction of *L. monocytogenes* vs. time in peanut butter at 70°C, 85°C, and 80°C.

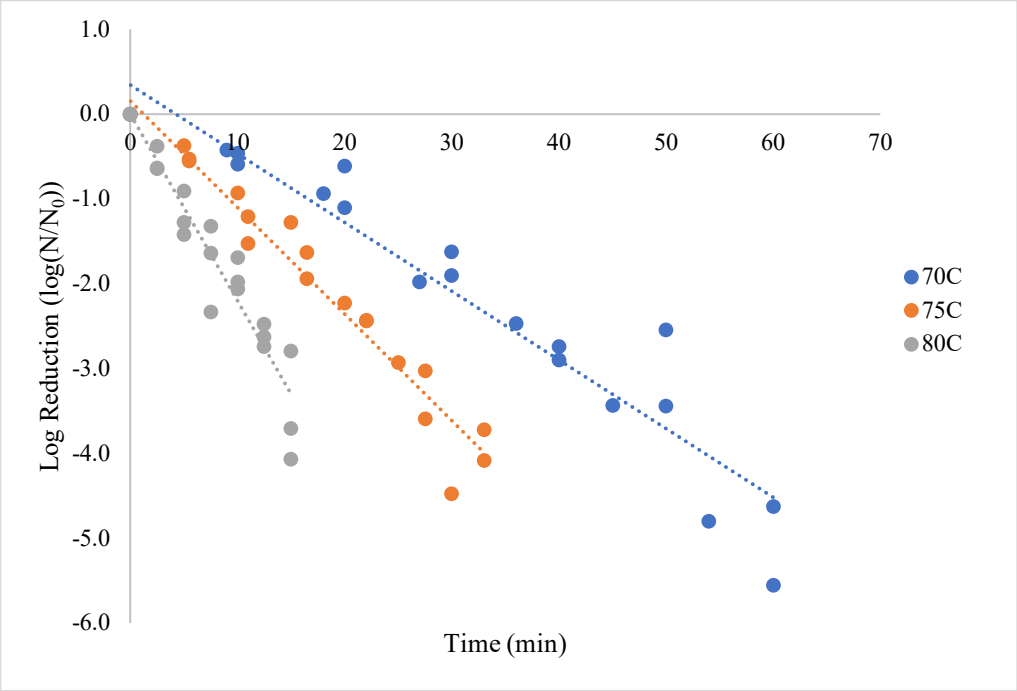


Fig. S1.9. Log reduction of *L. monocytogenes* vs. time in sunflower butter at 70°C, 85°C, and 80°C.

Table S1. *z*-values (°C) of *E. faecium, Listeria*, and *Salmonella* in three butters

Matrix	*E. faecium*	*Listeria*	*Salmonella*
Almond Butter	17.6	17.6	20.6
Peanut Butter	17.2	16.6	25.3
Sunflower Butter	22.5	23.3	23.0

Table S2. *z*-values (°C) of *E. faecium, Listeria*, and *Salmonella* in talc vs oil

Carrier	*E. faecium*	*Listeria*	*Salmonella*
Talc	17.6	24.2	23.8
Oil	17.2	16.6	24.2

APPENDIX B

Prospectus

Specific Aims

Prevention of foodborne illness, though not new, is a relevant topic in the food industry. To provide general welfare for the society, it is necessary to ensure the safety of the foods we consume. The US food industry operates under the Food Safety Modernization Act (FSMA) which encourages or requires firms to identify, implement, monitor, and enhance risk-based preventative controls. Currently, the most common bacterial food pathogens of concern are pathogenic strains/serovars of *E. coli*, *Salmonella*, *Listeria monocytogenes*, and *Campylobacter*, based on the severity of the disease, frequency of outbreaks, and the food product matrix itself. The availability of "unbound water," more appropriately known as water activity (a_w), is a crucial product attribute as part of understanding the requirements for the growth of microorganisms. Low-moisture foods (LMFs), also known as low- a_w foods, generally have a lower risk of causing a major outbreak due to the low-a_w environment. It is well established that his characteristic directly limits the growth of bacterial pathogens. However, in recent years outbreaks due to *Salmonella* spp. in LMFs such as peanut butter, wheat flour, dry milk powder, and other matrices have refocused specific attention to the survival and thermotolerance of pathogens in products commonly stored for extended periods in the home pantry. No outbreaks of *Listeria monocytogenes* in LMFs have been reported but several recalls occurred due to contamination and the absence of information regarding this organism. Recent work at Brigham Young University and Washington State University indicate that both *Salmonella* and *Listeria monocytogenes* have the ability to survive and resist at high temperature in low-a_w environments such as powder infant formula, wheat flour, or peanut butter.

Peanut butter is one of the more interesting model foods in terms of the safety of LMFs. Its matrix is notably different from other LMFs, such as wheat products including flours and traditional pastas, because of its higher fat content. The Food and Drug Administration in cooperation with the Center for Disease control have tracked and reported *Salmonella* outbreaks in peanut butter. In a new prevention-based food safety environment, even in matrices that do not support growth, the thermotolerance of *Salmonella* in these matrices has become an important component of food safety research. Similarly, a few outbreaks of *Salmonella* in almond butter have been reported. Therefore, the characteristics of *Salmonella* in LMFs in model nut butters were identified as a need and are currently under investigation in multiple applied microbiology laboratories in academia, government, and industrial settings.

Although no outbreaks of *Listeria monocytogenes* in nut butters have been observed, several precautionary recalls have been issued due to the detection of *Listeria monocytogenes* in the products. *Listeria monocytogenes* is a pathogen that can cause severe illness and mortality. In a review published in 2019, Taylor, Kataoka and Quinn, a student at Brigham Young University, identified a gap in the technical literature specific to this organism in critical matrices. Initial studies in the Taylor lab resulted in a comparison of isothermal inactivation of *Salmonella*, *Listeria monocytogenes*, and *Enterococcus faecium* NRRL B-2354 in peanut butter, powder infant formula, and wheat flour using a dry inoculum (accepted in August 2020 and subsequently published in 2021). This work builds on the foundation of this prior experimental work and will generate new data towards a deeper understanding of the thermotolerance of *Listeria monocytogenes* in nut butters. Again, the goal is to reduce the probability of an outbreak in the future.

The inoculation method in LMFs differs from foods that have a higher water activity for practical reasons. The ideal inoculation method for LMFs uses inoculum carriers that minimize or eliminate the change in food matrices, especially 1) a_w, 2) composition, and 3) viscosity after being added into the foods. Talc (magnesium silicate) powder has been widely used in the food industry as an inoculum carrier; it is an approved food additive in Europe and the United States (as an anticaking or flow agent). However, some potential health risks related to inhalation have been identified for scientists and technicians conducting experiments with this matrix. Additionally, talc powder is not part of the food matrix, and adding it can alter the composition of the food. Therefore, we plan to investigate the use of plant-based food oils as carriers in nut butters. This work will assess the fitness of oil inoculums in substitution for talc. A full substitution would eliminate the potential concerns associated with talc. On paper this appears straightforward, however, it is known that oil provides a protective effect for the bacteria which may be one of the contributors to the survival of pathogens in LMFs that contain high-fat content. We will address data gaps in the literature including the comparison of *D*-values of organisms studied using talc powder or food oils as an inoculum in nut butters.

Aim 1: Determine the stability of microorganisms suspended in peanut oil, sunflower oil, and almond oil inoculums stored at 24°C for 4 weeks.

Aim 2: Compare *D*-values of *L. monocytogenes*, *Salmonella*, and *E. faecium* in peanut oil, sunflower oil, and almond oil inoculums and corresponding nut butters.

Aim 3: Conduct a side-by-side comparison of thermotolerance using dry talc versus peanut oil inoculum for *Listeria, Salmonella*, and *E. faecium* in low-a_w peanut butter.

Aim 1 null hypothesis: The microorganism concentration in respective oil inoculums is not reduced before 4 weeks at ambient conditions.

Aim 2 null hypothesis: The paired oil inoculum and corresponding food matrix of three nut butters does not affect the thermotolerance of *Listeria, Salmonella*, and *E. faecium.*

Aim 3 null hypothesis: The thermotolerances of *Listeria, Salmonella*, and *E. faecium* in peanut butter (at the same low-a_w) are not different when comparing dry talc vs. oil inoculation methodologies.

Introduction

Foods are sources of enjoyment, nutrients, and energy; nutrition is essential for human health. Food manufacturers and distributors are responsible for providing safe food by controlling the presence of microorganisms and meeting (or exceeding) regulatory requirements. LMFs have generally been considered "low risk" because the water activity (a_w) values prohibit the growth of pathogens. Concerns regarding "no growth" LMFs, defined as ingredients and finished goods with $a_w < 0.85$, have escalated because LMFs may harbor foodborne pathogens, albeit in low numbers, and result in illness. While bacteria are not able to replicate in LMFs, they have marked increases in thermotolerance and may survive for >12 months. Outbreaks and recalls in peanut butter, a LMF with high oil content, raised awareness of the survival and thermotolerance of *Salmonella* in similar low-a_w matrices. However, until recently, little information was publicly available on the thermotolerance of *Listeria* in nut butters and less is known about its stability in plant-based food oils.

To date, dry inoculums using talc or a similar carrier were the most common and primary means of conducting thermotolerance studies in LMFs including peanut butter. Previous work in

our laboratory has shown that talc has an advantage of maintaining high counts of bacteria of interest for more than 4 weeks. Also, when mixed with a variety of LMFs, talc does not alter the a_w of the food matrices prior to the determination of the thermotolerance of organisms of concern or potential surrogates. Due to potential concerns regarding the health of technicians handling talc, there is a need to characterize suitable alternative carriers for thermal death time studies in high fat LMFs.

The objective of this manuscript is to gather the publicly available technical information regarding this recently expanded field of study. The augmented understanding of the thermotolerance and survival of *Salmonella* and Listeria will be used to prevent future outbreaks and recalls in nut butters. Additionally, it is necessary to compare and characterize the implications for pathogens and surrogates if laboratories of move away from previous methods reliant on talc as an inoculum or carrier in food safety validations conducted to meet newly enforced regulations in the United States.

Common foodborne pathogens and the establishment of food safety plan

The food safety arena has gained attention from the general population due to past outbreaks, new regulations. CDC estimates that each year there are about 48 million people who get sick from a foodborne illness and approximately 128,000 of them get admitted into the hospital; unfortunately, around 3,000 patients don't survive (4). Food safety issues have driven the food industry to put more effort on ensuring the safety of food. According to the CDC, there are five common foodborne pathogens which are *Norovirus*, *Salmonella*, *Clostridium perfringens*, *Campylobacter*, and *Staphylococcus aureus*. There are also 4 other food pathogens—*Clostridium botulinum*, *Listeria monocytogenes*, *E. coli*, and *Vibrio*—even though the infections or intoxication do not happen as frequently, they may require hospitalization

because of the acute symptoms that may potentially develop after consumption *(8).* High water activity foods are generally considered the highest risk because they provide nutrients and available "unbound" water meeting or exceeding growth requirements for microorganisms. In 2011, Food Safety Modernization Act (FSMA) was signed into law; its purpose is to prevent foodborne illness from happening rather than responding to post-event *(7).* Therefore, a food safety plan (FSP) is currently required for all foods including those that were previously considered as low risk *(9).* Moisture content is common but water activity is the critical measure associated with survival during and after LMF processing; an FSP is designed to manage risks in the transformation of products and ingredients. It is based on prevention and relies on science-based preventative controls.

Foodborne outbreaks and recalls in LMFs

LMFs, defined as foods that contained a water activity that is below 0.85 (*26)*, were historically considered "low risk" because they do not support for the growth of pathogens or the majority of spoilage microorganisms. However, there have been multiple outbreaks in the US in LMFs such as wheat flour, peanut butter, nuts, raw almond, almond butter, etc. linked to various *Salmonella* spp *(22, 25).*

The process of making peanut butter includes roasting, blanching, grinding, and tempering. Roasting is the most important step in peanut butter processing because it brings out the flavor, aroma, and texture, and it also aids in the reduction of water content to approximately 1% *(10).* Moreover, it is the only step that provides sufficient inactivation of pathogens that are introduced in the pre-processing step due to the high temperature and time. It has been suggested that improper handling of food during or post-process may be one of the potential causes of the

contamination as the later steps do not cause significant reduction of bacteria due to the food matrix of peanut butter that does not allow enough bacterial reduction.

Although no outbreaks of *Listeria monocytogenes* in LMFs have been reported, there have been some recalls due to the contamination of *Listeria monocytogenes*. The recalls include several categories including nuts, nut butters, protein bars, etc., and therefore it is vital to investigate the potential causes of the contamination as *Listeria monocytogenes* is a pathogen that may result in hospitalization and spontaneous abortion *(18)*.

Nonthermal inactivation methods in LMFs

It is important to implement a kill step to inactivate pathogens based on a hazard analysis and risk assessment(s). The intrinsic properties of many LMFs fundamentally differ from the majority of ready-to-eat human foods and limit palatability. Nonthermal processing technologies are widely used in foods that contain elevated a_w but are not as feasible or common in LMFs. Methods that have been studied in LMFs are high-pressure processing, nonthermal plasma, UV light, pulsed light, irradiation, ozone, etc *(22).* While they are considered novel methods and do provide some advantages, clear disadvantages limit the effectiveness of the key outcomes such as bacterial inactivation. For example, the protective effect of oil and the proteins present in peanut butter prevent HPP (high-pressure processing) from effectively killing Salmonella Typhimurium *(12);* Hvizdzak, Beamer, Jaczynski, and Matak *(14)* also reported that the low- a_w and high fat content environments may affect the bacterial reduction of *Salmonella* Typhimurium and *Salmonella* Tennessee in irradiation. Ban and Kang *(2)* concluded that water activity may be the most important contributor when it comes to bacterial inactivation, specifically *Salmonella* Typhimurium, by irradiation. It is clear that more research needs to be performed to increase the

effectiveness of non-thermal inactivation methods on peanut butter products before these applications will become more suitable to LMFs including nut butters.

Impact of water activity and food matrices on the thermal resistance of *Salmonella*, *Enterococcus faecium*, and *Listeria monocytogenes*

Water activity modulation in LMFs is related to the composition of the food matrices themselves and, like all chemical equilibriums, is impacted by temperature. Different foods exhibit unique characteristics, therefore, foods that have varied matrices such as physiochemical properties are expected to behave distinctively in the aspect of water activity *(26).* For instance, when all-purpose flour and peanut butter were treated with the same initial water activity (0.45) and temperature (20°C), all-purpose flour had an increase in water activity whereas the water activity of peanut butter decreased as the treatment temperature was elevated from 20°C to 80°C (*23*).

Water activity not only is critical to microbial growth but also to heat resistance (thermal tolerance) of microorganisms which leads to the survival of microorganisms. Recently, new sensors at Washington State University were successfully designed to measure the water activity at temperatures above 60°C. Previous research was limited to mainly ambient or slightly above ambient (30°C) conditions. Liu et al *(16)* discovered that the *D*-values (the time it takes to reduce bacterial load by 1 log) of *S.* Enteritidis and *Enterococcus faecium* at 80°C increased exponentially at reduced water activity compared to 25°C which inspired research on the relationship between the change of water activity of peanut oil at elevated temperature done by Yang et al *(27)* later on. Interestingly, foods that have the lowest water activity do not always exhibit the highest thermal tolerance as food matrices may also affect the thermal resistance of microorganisms. Previous work in our laboratory found that the *D*-values of *Salmonella*, *Listeria*

monocytogenes, and *Enterococcus faecium* (a surrogate for *Salmonella* and *Listeria monocytogenes*) in peanut butter are lower than those in power infant formula with a water activity of 0.11 and 0.20, respectively at ambient conditions *(21)*.

Impact of oil temperature on water activity

It is known that water activity has an impact on the survival of pathogens as well as the protective effect of oil. Previous studies support the hypothesis that it plays an important role in reduced bacterial inactivation in peanut butter *(19)*. However, not much is known about the relationship between oil temperature and water activity of the product. In recent work, Yang et al *(27)* conducted a research on the trend of water activity of peanut oil ranged from room temperature to 80°C, and found that water activity decreased as the temperature used for isothermal inactivation in several other studies increased. The results from this research may help explain why pathogens are able to survive in LMFs that contain a high-fat content. Critically important pathogens such as Salmonella tend to have a higher thermal tolerance in a low-a_w environment in addition to the protective effect that oil provides the organism.

Microbiological challenge testing of foods

Microbiological challenge testing (MCT) is a validation method used in the food industry to ensure the adequacy of a process. Simply put, it is a challenge test for a process conducted by intentionally introducing pathogens or a conservative surrogate into the food product. The inoculated product then goes through the intended treatment simulating the conditions of contamination of potential pathogens. These tests are also called process MCTs, and their purpose is to prevent harmful microorganisms from being able to survive after being treated with a preferred process. Setting Preventative Controls (formerly known as “Critical Control Points”) is the most important step when creating a HACCP plan, and MCT can provide constructive

information to the food processors since it is conducted as a simulation of the lowest setpoint to capture the worst scenario in the proposed processing environment. The inoculation methods including the inoculation level or bacteria harvesting techniques are also critical in MCTs because it can affect the accuracy of the results. The study must be designed and executed with preliminary data and calibrated surrogates to fully characterize and study the desired inactivation of pathogens in the process that will preventively control the hazard and therefore reduce or eliminate the risk of outbreaks or recalls if foods are stored appropriately *(15, 20)*.

Purpose of using inoculum carriers and concerns of using talc

A dry inoculation method is often used in MCT in LMFs because wet inoculation will create physical irregularities (clumps or caking) which significantly changes the nature of the original food matrices (*1, 17*). An ideal carrier needs to be able to retain a considerable amount of target microorganisms and later be completely removed after the organisms are transferred to the foods.

Ahmad et al *(1)* stated that such an ideal carrier does not exist. However, it is worth noting that dry inoculum carriers such as silica beads *(13),* sand (3), SiO2 *(16),* and talc powder (*6, 21*) have been used in their respective research to introduce microorganisms into LMFs. The research conducted by Ahmad and the team *(1)* focused on the validation of the effectiveness of talc powder as a dry inoculum carrier. They found that it affected the thermal resistance of *E. faecium*. Therefore, they suggested that using talc powder may over or underestimate the thermal resistance of target microorganisms. Moreover, studies have shown that talc powder may be carcinogenic. A meta-analysis done by Chang et al *(5)* suggested that there was an association between occupational talc exposure and stomach cancer, but they could not make the same conclusion with talc that did not contain asbestiform fibers. The American Cancer Society stated

that asbestos-containing talc can cause cancer if inhaled, but there is not enough evidence on asbestos-free talc *(24)*. In summary, it is important to find an alternative for talc to reduce the exposure and concern that lab technicians may inhale talc when doing experiments.

Experimental Design

In this phase of our research, 6 strains of *Salmonella* and *Listeria monocytogenes*, and 1 single strain of *Enterococcus faecium* NRRLB-2354 are inoculated into 3 different nut butters: peanut butter (produced by Welfare Services at the Houston Cannery), sunflower butter, and almond butter (purchased at a local grocery store), as these categories of nut butters have had at least one *Salmonella* outbreak or *Listeria monocytogenes* recall in the past. Since there is a need to explore an alternative inoculum carrier, the oil that is naturally present in each nut butter, peanut oil, sunflower oil, and almond oil, have been selected to serve as an inoculum carrier in order to incorporate bacteria into nut butters. The entire study consists of 9 complete isothermal inactivation experiments. Each experiment includes 3 independent treatments, and every treatment is performed in duplicate.

The purpose of this study is broken out in three phases. First, we will determine the stability of selected microorganisms in oil inoculums at room temperature for 4 weeks. Then, we will compare the *D*-values of selected microorganisms among 3 nut butters using their corresponding oil as an inoculum. Finally, we will compare the thermotolerance of selected microorganisms using a high-count oil inoculation versus a dry inoculation method (talc powder) in peanut butter based on recent findings reported by Quinn et al *(21)*.

Inoculation preparation

Oil inoculation method was adopted and modified from research conducted by Grasso et al (1). Each strain of *Salmonella* spp (*Salmonella* Montevideo, *Salmonella* Agona, *Salmonella*

Tennessee, *Salmonella* Weltevreden, *Salmonella* Senftenberg, and *Salmonella* Typhimurium PT 42) and *Listeria monocytogenes* (6 strains total), obtained from Utah State University's culture collection, will be grown on TSAYE (24 hours) and harvested separately from an individual lawn culture (48 hours) to form a cocktail slurry. The same technique also applies to *E. faecium*. Each bacterial slurry contains approximately 28 ml of bacteria cells and peptone water (0.1%), and 18 ml of peptone water is discarded after being centrifuged for 20 min at 5000 RPM at 25°C. 10 ml of oil will then be pipetted into the slurry after the 0.5 ml tween 80 addition. The final inoculum will achieve homogeneity after vortexing for 3 minutes. The concentration of the mixture is about 10 log CFU/ml.

Sample inoculation

Place 25 grams of desired nut butter on an unused sterile petri dish and add approximately 0.375 grams of homogenous final inoculum; a 3-minute manual mixing with a spatula is needed to ensure an even distribution of bacteria in the sample. The inoculated nut butter is then left to incubate at room temperature overnight (~24 hours). After incubation, 0.5 gram of the inoculated sample is placed and sealed in a 4 oz. Whirl-Pak bag and flattened to a thickness of 1 mm.

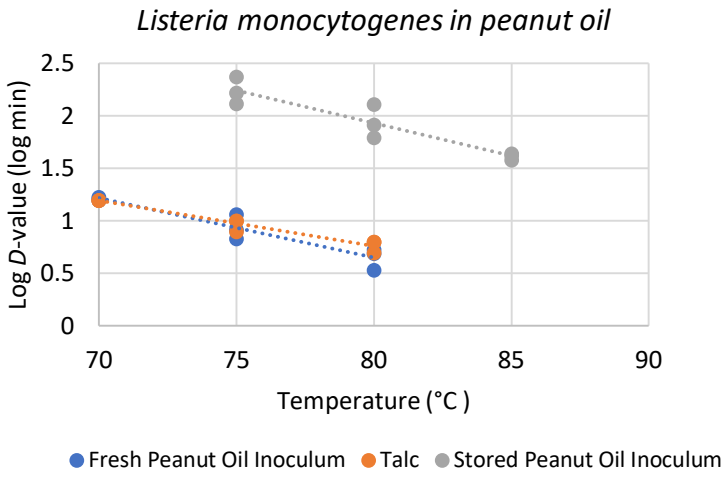
Isothermal inactivation treatment

Each isothermal inactivation treatment is done at 3 different temperatures by using a water bath, and each temperature includes 6-time intervals in addition to a control sample (time 0) without any treatment. The flattened sample bag and its duplicate are placed in between 2 magnetic copper plates for each time interval, so in other words, a total of 42 bags will be used to complete a treatment. The use of magnetic copper plates is to ensure uniformity in thickness and

a shorter come up time during the isothermal treatments. We will also explore the *D*-value of pure oil by using a capillary tube in the same isothermal treatment method.

The magnetic copper plates are placed in a rack and then immersed in the water bath to initiate heat inactivation of the microbes. After each time interval, a set of copper plates and the associated experimental materials will be removed from the water bath and cooled immediately. This is followed by an addition of 4.5 ml of peptone water into each bag (1:10 dilution). The mixture of nut butter and peptone water is then hand massaged until it's fully suspended, and the sample is enumerated on TSAYE and incubated at 37°C for 24 hours.

Preliminary Results



An isothermal inactivation experiment (3 treatments) was completed on *Listeria monocytogenes* in peanut oil. The figure shows the comparison of *D*-values of *Listeria monocytogenes* between fresh peanut oil inoculum (week 0) and talc powder in peanut butter. It was observed that both curves were similar and overlapped at 75°C which initially indicated that peanut oil could serve as a substitute for talc powder (comparing blue and orange plots). However, it was noted in the same experiment that the *D*-value of *Listeria* in peanut oil increased as the storage time lengthened (shown in grey). Therefore, during the execution of our experimental plan, we will gather and analyze data to

statistically confirm the initial observation and somewhat surprising results related directly to the storage time.

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