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Effects of Inoculation Procedures on Variability and Repeatability of *Salmonella* Thermal Resistance in Wheat Flour

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ABSTRACT

Limited prior research has shown that inoculation methods affect thermal resistance of *Salmonella* in low-moisture foods; however, these effects and their repeatability have not been systematically quantified. Consequently, method variability across studies limits utility of individual data sets and cross-study comparisons. Therefore, the objective was to evaluate the effects of inoculation methodologies on stability and thermal resistance of *Salmonella* in a low-moisture food (wheat flour), and the repeatability of those results, based on data generated by two independent laboratories. The experimental design consisted of a cross-laboratory comparison, both conducting isothermal *Salmonella* inactivation studies in wheat flour (~0.45 water activity, 80°C), utilizing five different inoculation methods: (i) broth-based liquid inoculum, (ii) lawn-based liquid inoculum, (iii) lawn-based pelletized inoculum, (iv) direct harvest of lawn culture with wheat flour, and (v) fomite transfer of a lawn culture. Inoculated wheat flour was equilibrated ~5 days to ~0.45 water activity and then was subjected to isothermal treatment (80°C) in aluminum test cells. Results indicated that inoculation method impacted repeatability, population stability, and inactivation kinetics ($\alpha = 0.05$), regardless of laboratory. *Salmonella* inoculated with the broth-based liquid inoculum method and the fomite transfer of a lawn culture method exhibited instability during equilibration. Lawn-based cultures resulted in stable populations prior to thermal treatment; however, the method using direct harvest of lawn culture with wheat flour yielded different *D*-values across the laboratories ($\alpha = 0.05$), which was attributed to larger potential impact of operator variability. The lawn-based liquid inoculum and the lawn-based pelletized inoculum methods yielded stable inoculation levels and repeatable *D*-values (~250 and ~285 s, respectively). Also, inoculation level (3 to 8 log CFU/g) did not affect *D*-values (using the lawn-based liquid inoculum method). Overall, the results demonstrate that inoculation methods significantly affect *Salmonella* population kinetics and subsequent interpretation of thermal inactivation data for low-moisture foods.

Key words: Error; Flour; Low moisture; Pathogen; Thermal resistance; Water activity

In 2011, the Food Safety Modernization Act was enacted to limit the potential of foodborne outbreaks and recalls by instituting requirements for preventative controls for high-risk hazards *(32)*. One such hazard is the potential for contamination and survival of *Salmonella* in low-moisture foods. Among the possible preventative controls, thermal pasteurization processes are probably the most common solution. As a result, there have been a growing number of studies investigating effects of product characteristics (e.g., fat content, formulation, water activity, product structure), processing environment (e.g., air humidity, temperature, submersion fluid), and strain selection on the thermal resistance of *Salmonella* in low-moisture products *(1–3, 8, 11–13, 15, 20, 21, 26, 27, 34)*; however, few studies have investigated how the experimental methodologies influence *Salmonella* thermal resistance in low-moisture foods *(18, 19, 22, 31)*.

The studies that investigated the effects of experimental methodologies on the survival *(19, 31)* and thermal resistance *(18)* of *Salmonella* indicated that the means of inoculation impacts the stability and thermal inactivation kinetics of *Salmonella* in low-moisture products. However, these studies were limited to evaluating a broth-based culture against an agar lawn–based culture. Li et al. *(22)* examined thermal inactivation kinetics of *Salmonella* in peanut butter inoculated with a peptone water or peanut oil–based inoculum and reported faster *Salmonella* inactivation using peptone water. Overall, the inoculation methods have a significant influence on *Salmonella* thermal resistance in low-moisture foods but are poorly understood.

Unfortunately, the lack of standardized inoculation methodologies makes it difficult to estimate the true effect of experimental methodologies. For example, studies investigating the effect of water activity (a_w), temperature, strain selection, and product composition on thermal resistance of *Salmonella* in peanut butter yielded 5-log reduction times ranging from 4.5 to 67 min at 90°C *(13, 18,*

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22, 23, 29); however, each study used different inoculation methodologies. None of the treatment conditions tested in the aforementioned studies caused a 10-fold difference in thermal resistance, even though differences in methodologies may be causing effects nearly that large. In a meta-analysis of *Salmonella* survival in low-moisture foods, Santillana Farakos et al. *(28)* reported large thermal resistance discrepancies in peanut butter data. However, the published literature quantifying the effects of inoculation methodologies remains too limited in scope to explain variability in reported *Salmonella* thermal resistance measurements.

The objective of this study was to evaluate the effects of cultivation and inoculation methodologies on the stability and thermal resistance of *Salmonella* in a low-moisture food (wheat flour), and the repeatability of those results, based on data generated in two independent laboratories.

MATERIALS AND METHODS

Experimental design. Five different inoculation methodologies were evaluated in a cross-laboratory comparison at two independent laboratories (Michigan State University [MSU] and Washington State University [WSU], under the direction of Drs. Bradley Marks and Juming Tang, respectively). Identical isothermal inactivation methodologies were used to quantify the thermal resistance of *Salmonella enterica* serovar Enteritidis phage type 30 at 80°C in wheat flour conditioned to ~0.45 a_w. A log-linear model was fit to the data generated by both laboratories (described below).

Wheat flour. Organic soft winter wheat flour was obtained from Eden Foods (Clinton, MI). Upon acquisition, initial a_w was measured using a a_w meter (model 3TE, Decagon Devices, Pullman, WA), and particle size distribution was determined by sieve analysis. Tyler series sieves (#30 through #200) were stacked with 100 g of wheat flour and were mechanically shaken for 30 min using a sieve shaker (H-4325, Humboldt Manufacturing, Elgin, IL). The flour remaining in each sieve after shaking was weighed and then was used to calculate the geometric mean diameter of the wheat flour (American National Standards Institute, American Society of Agricultural Engineers method S319.3, ANSI, Washington, DC). Wheat flour used in the isothermal inactivation treatment was run across a single sieve (Tyler series #120) prior to inoculation, to remove very fine particles more likely to become airborne after inoculation.

Uninoculated samples of wheat flour were periodically assessed for numbers of naturally occurring *Salmonella* pathogens by plating on tryptic soy agar (TSA; Difco, BD, Sparks, MD) supplemented with 0.6% (wt/vol) yeast extract (Difco, BD) (TSAYE) and modified with ferric citrate (0.05%; Sigma-Aldrich, St. Louis, MO) and sodium thiosulfate (0.03%; Sigma-Aldrich) (mTSA). After 48 h of incubation at 37°C, colonies with characteristic black centers were enumerated as *Salmonella*.

Bacterial strain and inoculation. *Salmonella enterica* serovar Enteritidis phage type 30 (SE PT30), previously obtained from Dr. Linda Harris (University of California, Davis), was used because this strain was previously shown to be thermally resistant in low-a_w foods *(1, 9, 11)*. The culture was maintained at –80°C in tryptic soy broth (TSB; Difco, BD) supplemented with 20% (vol/vol) glycerol.

Five inoculation methodologies for low-moisture foods were evaluated, including several based on methodologies reported in the literature *(18, 19)*: (i) broth-based liquid inoculum (BLI), broth cultivated, centrifuged, and resuspended to obtain a high concentration of liquid inoculum; (ii) lawn-based liquid inoculum (LLI), TSAYE lawn cultivated, harvested, centrifuged, and resuspended to obtain a high concentration of liquid inoculum; (iii) lawn-based pelletized inoculum (LPI), TSAYE lawn cultivated, harvested, centrifuged, and pelletized; (iv) direct-harvest method (DHM), TSAYE lawn cultivated and harvested directly into wheat flour; and (v) dry-transfer method (DTM), TSAYE lawn cultivated on filter papers for a dry inoculum. The details of each inoculation methodology are described below.

BLI. SE PT30 was subjected to two consecutive transfers (24 h, each at 37°C) in 9 ml of TSB (Difco, BD) supplemented with 0.6% (wt/vol) yeast extract (Difco, BD) (TSBYE) and then was transferred into 500 ml of TSBYE. After incubation (24 h, 37°C), the culture (400 ml) was centrifuged for 15 min at 3,000 × *g*. The supernatant was discarded, and the remaining pellet was resuspended in 3 ml of 0.1% peptone water. Next, 1 ml of this inoculum was added to a sterile plastic bag containing 10 g of wheat flour and was hand mixed for 3 min. After adding an additional 90 g of uninoculated wheat flour, the sample was mechanically homogenized for 3 min.

LLI. SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE, after which 1 ml was spread evenly over a plate (150 by 15 mm) of TSAYE to obtain a uniform lawn. After incubation (24 h, 37°C), the bacterial lawn was harvested in 20 ml of sterile 0.1% peptone water, using a sterile plate spreader and then was collected in a sterile container. Following 15 min of centrifugation at 3,000 × *g,* the resulting pellet was resuspended in 3 ml of 0.1% peptone water. One milliliter of this inoculum was added to a sterile plastic bag containing 10 g of wheat flour and was hand mixed for 3 min. After adding an additional 90 g of uninoculated wheat flour, the sample was mechanically homogenized for 3 min.

LPI. SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE, and then 1 ml was evenly spread on a plate (150 by 15 mm) of TSAYE to obtain a uniform lawn. After incubation (24 h, 37°C), the bacterial lawn was harvested in 20 ml of sterile 0.1% peptone water, using a sterile plate spreader. Following 15 min of centrifugation at 3,000 × *g,* the resulting pellet was added to a sterile plastic bag containing 10 g of wheat flour and was hand mixed for 3 min. After adding an additional 90 g of uninoculated wheat flour, the sample was mechanically homogenized for 3 min.

DHM. SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE, after which 1 ml was evenly spread on a plate (150 by 15 mm) of TSAYE to obtain a uniform lawn. Following incubation (24 h, 37°C), 10 g of wheat flour was added to the bacterial lawn and was distributed with a sterile plate spreader to directly incorporate *Salmonella* into the flour. The inoculated wheat flour was then collected with a sterile spatula, placed in a sterile plastic bag, and hand mixed for 3 min. After adding an additional 90 g of uninoculated wheat flour, the sample was mechanically homogenized for 3 min.

DTM. SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE and then was transferred to

18 ml of TSBYE. A culture suspension (5 ml per plate) was then added to two plates (150 by 15 mm) of TSAYE, each containing four filter membranes (0.45-μm pore, 5-cm diameter; Membrane Filter, Millipore Corporation, Billerica, MA) on the surface. After incubation (24 h, 37°C), the eight filter papers were removed from the TSAYE plates and were air dried in a biosafety cabinet for 5 min. The eight filter papers were added to wheat flour (10 g) in a sterile plastic bag and were hand mixed for 3 min. After discarding the filters and adding an additional 90 g of uninoculated wheat flour, the sample was mechanically homogenized for 3 min.

Sample conditioning. All inoculated wheat flour was adjusted to a target a_w of 0.45 over a period of 4 to 6 days. The a_w conditioning system consisted of an equilibration chamber (69 by 51 by 51 cm) monitored by a custom computer-based control system composed of relative humidity sensors (Vktech DHT22/AM2302, VK Technologies, Secunderabad, India) inside the equilibration chamber, a desiccation column containing silica gel (VeriTemp, Encino, CA), and a hydration column containing deionized water, solenoid valves, and air pumps, which maintained the chamber relative humidity within ±2% of the setpoint. Prior to thermal treatment, the target a_w of the inoculated wheat flour (0.45 ± 0.02) was confirmed for each of three replicated experiments using an a_w meter.

Homogeneity of the inoculum was evaluated by randomly removing 10 1-g samples from an inoculated batch of flour (100 g) during the a_w conditioning period. *Salmonella* populations were enumerated for each inoculation methodology, and homogeneity was reported as the standard deviation of the mean log population. Similarly, repeatability of inoculation was calculated as the standard deviation of the *Salmonella* population immediately before isothermal treatment ($n \geq 9$).

Isothermal treatment. The same isothermal inactivation methodology was used to obtain inactivation curves for *Salmonella,* regardless of the laboratory or inoculation procedure. Aluminum test cells *(5)* were aseptically filled with equilibrated flour (0.5 to 0.8 g, 4 mm thick) and were immersed in a water bath (Neslab GP-400, Neslab Instruments, Newington, NH) or oil bath (Isotemp 5150 H11, Thermo Fisher Scientific, Inc., Pittsburgh, PA) at 80.5°C. Come-up time was verified using an inoculated and equilibrated sample inside a test cell with a K-type thermocouple located at the center of the test cell. The come-up time (90 to 140 s) for the sample core to reach within 0.5 of 80°C was used as time zero for the isothermal treatment. Thereafter, samples were removed at 10 uniform time intervals, starting at time zero, and were immediately placed in an ice-water bath to stop the thermal inactivation ($T < 40$°C in ~20 s).

Recovery and enumeration. To enumerate *Salmonella* survivors, thermally treated wheat flour samples were aseptically transferred from the test cells to sterile plastic bags and were diluted 1:10 in 0.1% peptone water. Appropriate serial dilutions were then plated in duplicate on mTSA. After 48 h of incubation at 37°C, all black colonies were counted as *Salmonella*. The resulting limit of detection was 1.7 log CFU/g. After converting the populations to log CFU per gram, log reductions were calculated by subtracting the survivor counts from the population at time zero for the respective replicate.

Cross-laboratory comparison. Each inoculation methodology and subsequent isothermal inactivation treatment was performed in triplicate at MSU. WSU independently repeated inoculation methodologies BLI, LLI, LPI, and DHM with subsequent isothermal inactivation treatment in triplicate, using the same materials and cultures. Materials obtained by MSU (sifted wheat flour and a_w conditioning chambers) were shared with WSU. The DTM method was not repeated at WSU due to initial inoculation levels that were too low to reliably fit the thermal inactivation model.

Effect of initial inoculation level. In addition to the cross-laboratory comparison, the effect of initial *Salmonella* inoculation levels on thermal resistance was investigated. SE PT30 was prepared using the LLI procedure before pellet resuspension. The pellet was resuspended in 2 ml of 0.1% peptone water followed by serial dilutions in 0.1% peptone water to achieve preequilibration concentrations of 8, 7, 5, and 3 log CFU/g in the flour. The inoculum (0.1 ml) was then hand mixed into separate wheat flour samples (10 g) in sterile plastic bags for 3 min. After hand mixing, this inoculated seed sample was added to 40 g of wheat flour and was stomached for 3 min.

The inoculation procedure, in addition to sample conditioning, isothermal treatment, and enumeration procedures as described earlier, were replicated twice to yield isothermal inactivation kinetics for *Salmonella* at the four different inoculum levels. Samples were enumerated immediately after inoculation and immediately before isothermal inactivation to confirm starting populations.

Model regression and statistical analysis. To quantify *Salmonella* thermal resistance, the log-linear model was applied to the inactivation data sets, where

$$\log(N) = \log(N_0) - \left(\frac{t}{D_{80°C}}\right) \quad (1)$$

and N and N_0 are the populations (CFU/g) at times t and 0, respectively; t is the time of the isothermal treatment (s) after the thermal come-up time; and $D_{80°C}$ is the decimal reduction time (s) at 80°C. Parameters for the log-linear model ($\log(N_0)$ and $D_{80°C}$) were estimated via a single regression on the aggregated replicates for each inoculation treatment within a given laboratory, using MATLAB nonlinear fitting tools (version 2014, MathWorks, Natick, MA). A nonlinear regression was used to acquire symmetrical standard error estimates for $D_{80°C}$. Postregression residual plots and normality of residuals analyses confirmed the appropriateness of the log-linear model. Parameter estimates and initial concentration levels were compared using Student's t test ($\alpha = 0.05$) calculated on Microsoft Excel (version 2013, Microsoft, Redmond, WA), with pairwise comparisons evaluating differences between inoculation methodologies and the two laboratories. Inherent error associated with the inoculation methodologies was quantified by comparing standard errors for parameter estimates and root mean squared errors of the model fits to the population data sets (equation 2).

$$\mathrm{RMSE} = \sqrt{\frac{\sum \left(\log(N)_{\mathrm{predicted}} - \log(N)_{\mathrm{observed}}\right)^2}{n - p}} \quad (2)$$

where n is the number of samples, p is the number of parameters, and N (CFU/g) is the *Salmonella* population (CFU/g) at time t.

The effect of initial inoculation levels on thermal inactivation kinetics was analyzed via analysis of covariance ($\alpha = 0.05$) using MATLAB (version 2014, MathWorks, Natick, MA) function *aoctool*.

TABLE 1. Salmonella *Enteritidis PT30 populations and resulting* D*-values in wheat flour inoculated using different methodologies and subjected to 80°C isothermal treatment*[a]

Laboratory	Inoculation methodology	Postconditioning population (log CFU/g)	Post–come-up time population (log CFU/g)	$D_{80°C}$ (s)	RMSE (log CFU/g)
MSU	BLI	6.18 (0.23) D	3.86 (0.19) E	504.9 (4.4) A	0.204
MSU	LLI	7.39 (0.50) C	6.17 (0.31) BC	250.9 (2.6) B	0.766
MSU	LPI	8.29 (0.42) B	7.81 (0.50) A	285.9 (2.1) C	0.385
MSU	DHM	8.71 (0.24) A	7.54 (0.51) A	226.7 (1.7) D	0.568
MSU	DTM	6.33 (0.70) DE	4.79 (1.07) D	NA[b]	NA[b]
WSU	BLI	5.93 (0.16) E	NA[b]	NA[b]	NA[b]
WSU	LLI	7.65 (0.16) C	5.72 (0.26) CD	256.4 (2.4) B	0.349
WSU	LPI	8.85 (0.17) A	7.28 (0.45) AB	293.6 (5.5) C	0.571
WSU	DHM	8.66 (0.43) AB	8.00 (0.39) A	318.9 (7.5) E	0.596
WSU	DTM	NA[c]	NA[c]	NA[c]	NA[c]

[a] *D*-value, thermal resistance; RMSE, root mean squared error; MSU, Michigan State University; WSU, Washington State University. Population values are reported as the mean (standard deviation). $D_{80°C}$-values are reported as the parameter estimate (standard error) yielded from regression analyses. Within columns, values sharing a common letter are not significantly different ($\alpha = 0.05$).

[b] Values could not be estimated due to low *Salmonella* population levels.

[c] Inoculation method DTM was not replicated at WSU.

RESULTS AND DISCUSSION

Wheat flour. Upon acquisition, the wheat flour had an a_w of ~0.46 and a geometric mean (±standard deviation) particle diameter of 144 ± 60 μm. The a_w values of the inoculated wheat flour immediately before thermal inactivation at MSU and WSU were 0.440 ± 0.005 and 0.460 ± 0.009, respectively. *Salmonella* was never quantifiable in the uninoculated flour by direct plating (i.e., <1.7 log CFU/g).

Inoculation repeatability. Inoculation homogeneity for BLI, LLI, LPI, DHM, and DTM methods was ±0.24, ±0.12, ±0.42, ±0.23, and ±0.36 log CFU/g, respectively. The average postconditioning *Salmonella* populations ranged from 5.93 to 8.85 log CFU/g, depending on methodology and laboratory (Table 1). Lawn-based inoculation methodologies (LLI, LPI, and DHM) yielded higher populations of *Salmonella* during the equilibration-holding period *(18, 19, 31)*. The homogeneities of inoculation levels were consistent with the limited values previously reported *(18, 19, 25, 31)*.

Although significant differences ($P < 0.05$) were observed between the MSU and WSU inoculation levels for methods BLI and LPI, the nominal differences were 0.25 and 0.56 log CFU/g, respectively. Additional replicates of the inoculation methodologies should be performed by a larger group of laboratories to further substantiate these estimates of variability. Published reports discussing the reproducibility of inoculation for low-moisture foods are limited; however, when the Danyluk et al. (2005) *(7)* almond inoculation methodology was used as a template for almond kernel inoculation, initial mean *Salmonella* populations of 7.8 to 8.9 log CFU/g were previously reported from multiple studies *(1, 7, 9, 11, 16, 31)*.

Impact of inoculation level on thermal resistance. With the nominal preequilibration populations of 8, 7, 5, and 3 log CFU/g, the achieved postequilibration inoculation levels were 7.75, 6.51, 4.59, and 2.78 log CFU/g, respectively. The thermal inactivation rate was not significantly impacted by the initial inoculation level ($P > 0.05$; Fig. 1).

Impact of inoculation methodology on thermal resistance. Inoculation methodology significantly impacted the thermal resistance of *Salmonella* (Table 1 and Fig. 2). Broth-based inoculation method BLI yielded the highest *D*-value (~505 s) ($P < 0.05$) but also yielded the largest decrease in population during equilibration (~4 log). Consequently, owing to the low postconditioning populations, WSU was unable to reliably estimate thermal resistance. The higher thermal resistance of broth-based cultures contradicts trends presented by Keller et al. *(18)*; however, they investigated the effects of growth media on thermal resistance of *Salmonella* in a different food matrix (peanut butter emulsion).

Cross-laboratory comparisons of *D*-values yielded differences within 2, 3, and 41% for lawn-based inoculation methods LLI, LPI, and DHM, respectively (Table 1). The larger cross-laboratory difference in estimated *D*-values for the direct-harvest method (DHM) may have resulted, in part, from nutrient uptake from the solid medium when the inoculum was incorporated into the wheat flour with a plate spreader. The level of incidental nutrient or media removal with the harvested *Salmonella* would depend heavily on the individual performing the procedure, which could have altered the bacterial response in wheat flour during thermal inactivation; therefore, based on this observation and the resulting difference in *D*-values, DHM was not considered sufficiently repeatable.

The inoculation methods resulting from lawn cultures harvested with a peptone buffer (LLI and LPI) yielded thermal resistance parameter estimates that were statistically different across inoculation methodologies and laboratories ($P < 0.05$); however, the nominal differences

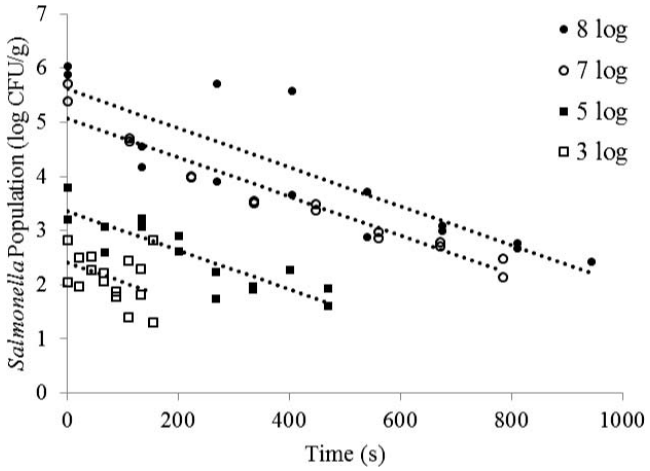

FIGURE 1. Salmonella *Enteritidis PT30 survivors (and the best-fit inactivation curves) during isothermal treatment (80°C) of inoculated wheat flour with four different initial inoculation levels.*

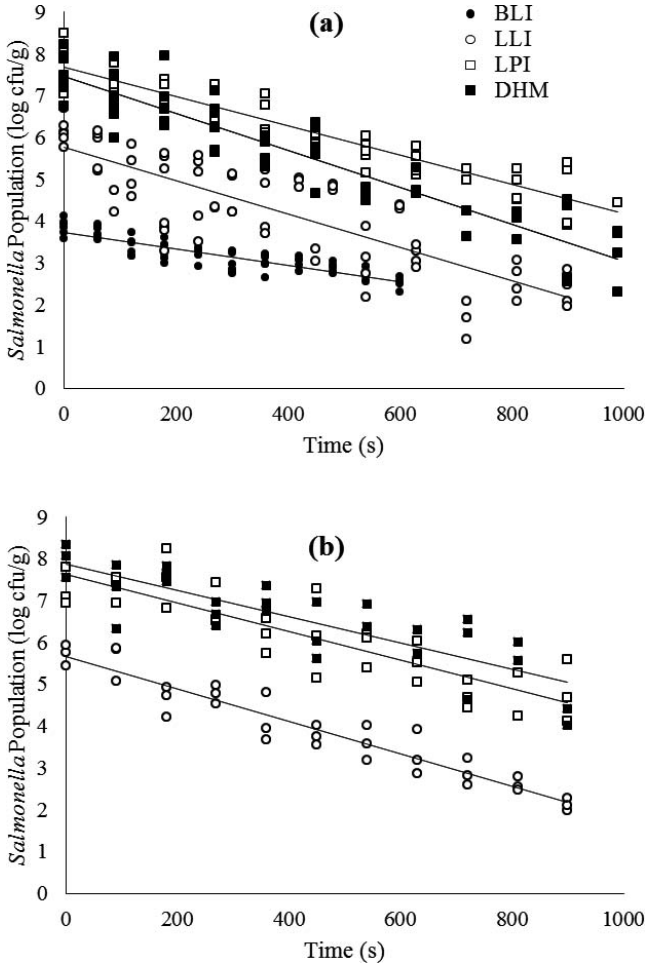

FIGURE 2. Salmonella *Enteritidis PT30 survivors during isothermal treatment (80°C) of inoculated wheat flour from (a) MSU and (b) WSU laboratories, using inoculation methods BLI, LLI, LPI, and DHM.*

between the parameter estimates using the same inoculation methodology across the two laboratories were less than 3%. Inoculation method LPI, which pelletized the *Salmonella* inoculum prior to inoculation, consistently yielded higher *D*-values than LLI (at both laboratories), despite an identical *Salmonella* strain, heat treatment, a_w, and food matrix. The addition of water to the wheat flour from the resuspended *Salmonella* inoculum used in method LLI may result in increased thermal sensitivity of *Salmonella* in low-a_w environments. This small difference in inoculation methodology resulted in ~35 s (~14%) difference in *D*-values. Although inoculation methods LLI and LPI are considered to be repeatable, further research, including replication across additional independent laboratories, is needed to more fully characterize the repeatability of results generated by each inoculation methodology.

The method of inoculation clearly influences the observed thermal resistance of *Salmonella*. Prior to this study, the literature investigating the effect of different inoculation methods on *Salmonella* survival *(4, 19, 31)* and thermal resistance *(18)* in low-moisture foods was limited to two inoculation methods each and two specific products (nuts and peanut butter). For most low-moisture products, the impact of inoculation methodologies on thermal resistance has not been evaluated *(8, 10, 17, 24, 26)*; for the few products with evaluated inoculation methodologies (almond kernels, peanut butter, and flour), there are other inoculation methodologies used in the literature that have not been investigated, in terms of repeatability and inherent uncertainty *(13, 14, 22, 23, 29, 34)*. Of the five inoculation methods investigated in this study, only the methods involving lawn cultures harvested with a peptone buffer (LLI and LPI) produced results that were deemed sufficiently stable and repeatable to be used reliably when comparing or aggregating data across multiple studies or laboratories. Inoculation methods LLI and LPI, which were very similar, yielded $D_{80°C}$-values that differed by ~14%. This result suggested that, beyond having unknown stability and repeatability, other unevaluated inoculation methodologies for low-moisture foods may have important impacts on *Salmonella* thermal resistance.

In addition to the inoculation method, the isothermal inactivation method also may influence observed thermal resistance *(6, 30, 35),* adding additional uncertainty when comparing results. Zimmermann et al. *(35)* reported that "test methods are at least as important in determining thermal processes as the micro-organisms and media used." Unfortunately, the inoculation and isothermal inactivation methods used in studies quantifying *Salmonella* thermal resistance are highly variable. Consequently, combining results from multiple laboratories (e.g., in reviews, meta-analyses, industry processes) may propagate errors due to variability across methods. For example, in their analysis of *D*-values for *Salmonella,* van Asselt and Zwietering *(33)* observed that most factors reported to influence thermal resistance are insignificant when compared with the large overall variability in reported *D*-values. Although error is inherent to every experiment, variability among future studies can be reduced by

incorporating preevaluated experimental methodologies with known reproducibility and minimal artifacts influencing the resulting thermal resistance. Future studies involving similar low-moisture products can use the recommended inoculation methodologies evaluated here to reduce variability.

Overall, inoculation method significantly influenced repeatability, survival, and thermal resistance of *Salmonella* in wheat flour during isothermal treatment. Of the five inoculation methods investigated, only methods LLI and LPI yielded both a stable *Salmonella* population in wheat flour prior to isothermal treatment and consistent *D*-value estimates resulting from tests conducted at both MSU and WSU. Despite similar inoculation methods, the $D_{80°C}$-value for *Salmonella* was longer using method LPI (~285 s) than LLI (~250 s). Additionally, inoculation level (3 to 8 log CFU/g) did not affect *D*-values (using LLI). Overall, the results of this study suggest that inoculation methodology can meaningfully impact the accuracy, precision, and overall results of experiments quantifying the thermal resistance of *Salmonella* in low-moisture products; therefore, variability among studies could be reduced with improved standardization of inoculation methodologies.

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