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Research Note

Effect of Growth on the Thermal Resistance and Survival of *Salmonella* Tennessee and Oranienburg in Peanut Butter, Measured by a New Thin-Layer Thermal Death Time Device

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ABSTRACT

In published data the thermal destruction of *Salmonella* species in peanut butter deviates from pseudo–first-order kinetics. The reasons for such deviation are unknown. This study examined both the method used to measure the thermal destruction rate and the method of growth of the microorganisms to explain variations in destruction kinetics. Growth on a solid matrix results in a different physiological state that may provide greater resistance to adverse environments. In this study, *Salmonella* Tennessee and Oranienburg were grown for 24 h at 37°C under aerobic conditions in broth and agar media to represent planktonic and sessile cell growth, respectively. Peanut butter was held at 25°C and tested for *Salmonella* levels immediately after inoculation and at various time intervals up to 2 weeks. Thermal resistance was measured at 85°C by use of a newly developed thin-layer metal sample holder. Although thermal heat transfer through the metal device resulted in longer tau values than those obtained with plastic bags (32.5 $\pm$ 0.9 versus 12.4 $\pm$ 1.9 s), the bags have a relative variability of about 15% compared with about 3% in the plates, allowing improved uniformity of sample treatment. The two serovars tested in the thin-layer device showed similar overall thermal resistance levels in peanut butter regardless of growth in sessile or planktonic states. However, thermal destruction curves from sessile cultures exhibited greater linearity than those obtained from planktonic cells ($P = 0.0198$ and 0.0047 for *Salmonella* Oranienburg and *Salmonella* Tennessee, respectively). In addition, both *Salmonella* serovars showed significantly higher survival in peanut butter at 25°C when originally grown on solid media ($P = 0.001$) with a <1-log loss over 2 weeks as opposed to a 1- to 2-log loss when grown in liquid culture. Consequently, the use of cells grown on solid media may more accurately assess the survival of *Salmonella* at different temperatures in a low-water-activity environment such as peanut butter.

The ability of *Salmonella* to survive at low-water-activity levels ($a_w < 0.7$) has brought attention to the safety of foods formerly thought to present low risk with regard to foodborne illness *(3, 8, 12, 15, 18, 21, 23)*. In particular, low-a_w foods such as nuts and nut butters have drawn attention because of foodborne outbreaks with *Salmonella* species as the causative agent *(4–7)*. Outbreaks related to low-a_w foods are particularly troublesome, as many such foods have long shelf lives and recalls may not remove all contaminated product from the shelves *(11)*. One *Salmonella* outbreak in 2004 related to contaminated almonds continued for months and was undetected for over a year *(4)*. In response to outbreaks related to raw almonds, the Almond Board of California along with the U.S. Department of Agriculture proposed standards calling for the mandatory pasteurization of almonds to provide a 4-log reduction of *Salmonella (1, 27, 28)*. In response to outbreaks of *Salmonella* related to nuts and nut products, the U.S. Food and Drug Administration issued guidance recommending a 5-log reduction in *Salmonella* for peanuts and pistachios *(29, 30)*. To comply with such new regulations and guidelines, affected food processors must develop processes with a kill step capable of delivering the appropriate reduction in *Salmonella*. Although any type of process is allowed so long as it can provide the appropriate log reductions of *Salmonella,* in practice the most commonly used process involves the application of heat.

Application of heat to inactivate *Salmonella* in a dry or low-a_w food product requires knowledge of the behavior of that microorganism in the food matrix. In addition to its survival at low a_w, the thermal resistance of *Salmonella* species can be dramatically increased in a low-a_w environment *(9, 10)*. The thermal resistance at low a_w water has often been described as deviating from first-order kinetics, with data not log linear, thus requiring the use of nonlinear modeling to fit kinetic parameters. However, this can be explained in some cases by flawed methods used to examine the thermal resistance of *Salmonella* in a low-a_w matrix *(2, 22)*. A lack of log-linear death kinetics can also be explained as an artifact of the measurement method *(16)*. Furthermore, a clear assessment of thermal resistance in a

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low-a_w matrix may be complicated by inactivation due to the desiccation in a dry environment itself or due to the toxicity of any humectants or solutes present in the food matrix. Consequently, an appropriate assessment of the thermal resistance of any microorganism such as *Salmonella* must take into account not just the physiological state of the microorganism but also its survival in the food matrix to be tested.

As with other low-a_w foods, the thermal destruction kinetics of *Salmonella* species in peanut butter was reported as deviating from traditional first-order behavior by Ma et al. *(19)* and by Shachar and Yaron *(25)*. Their studies relied on the use of plastic Whirl-Pak bags for thermal treatment of peanut butter. The reasons for deviations from first-order kinetics are unknown, but they could be a result of artifacts of the method utilized to determine thermal resistance or from the physiological state of the organism as influenced by state or type of growth *(10, 14)*. One means by which the physiological state of a microorganism can be influenced is by growth in liquid versus growth on a solid substrate. Both methods have been used in the literature for the growth of inoculum in the assessment of survival in peanut butter or other low-moisture products *(3, 17, 20, 25, 26)*. In growth in a liquid matrix, cells are in a planktonic state, whereas when attached to a surface the cells should be in a sessile form. Growth in liquid versus growth on solid agar has been used in the literature as one method to examine the differences between these cells *(24)*. In this study, reasons for deviation from expected first-order kinetics displayed by *Salmonella* were explored, including the type of method utilized for measurement of the thermal destruction rate and the possible influence of physiological state of growth on that rate. The survival of *Salmonella* in the peanut butter at a typical storage temperature (25°C) was also determined.

MATERIALS AND METHODS

Preparation of bacterial inoculum. *Salmonella enterica* serovars Tennessee and Oranienburg (donated by L. Beuchat, University of Georgia) were stored as stock cultures on Trypticase soy agar with 0.6% yeast extract (TSAYE; BD, Franklin Lakes, NJ) added. Stock cultures were transferred to fresh TSAYE media on a monthly basis. Initial transfers for experiments were made from isolated single colonies to 10 ml of Trypticase soy broth with 0.6% yeast extract (TSBYE; BD). Tubes were incubated 24 h at 37°C.

For planktonic cells, 1 ml of culture was transferred to 100 ml of TSBYE in a 500-ml baffled flask and grown aerobically with agitation (150 rpm; Innova 4430, New Brunswick Scientific, Edison, NJ) for 24 h at 37°C. The cells were then harvested by centrifugation (model 5804, Eppendorf, Hamburg, Germany) at approximately 3,300 × *g* for 5 min. Cell pellets were washed one time in buffered peptone water (BPW; BD) and then resuspended in approximately 1.5 ml of peanut oil with 2 or 3 drops of Tween 80 (Fisher Scientific, Fair Lawn, NJ) added.

For sessile cells, 100 μl of the initial 10-ml 24-h culture was spread onto the surface of TSAYE plates. The plates were incubated 24 h at 37°C. Cells were harvested by adding 1 ml of a mixture of peanut oil–Trypticase soy broth (2:1) with 2 or 3 drops of Tween 80 to the agar surface and mixing the cells with a sterile glass plate spreader. After mixing, the cell suspension was removed and collected in a sterile 50-ml conical tube (Fisher Scientific). Each plate yielded approximately 1 ml of cell suspension.

Inoculation of peanut butter and recovery of *Salmonella*. Each new lot of peanut butter (creamy style, purchased from a retail store in Chicago, IL) with a_w of 0.3337 and fat content of 50% was sampled for background populations prior to inoculation with *Salmonella*. Initial background populations on TSAYE at 37°C were typically <30 CFU/g. After harvesting *Salmonella* from either liquid culture or solid surfaces, approximately 1.5 g of cell-oil-TSBYE suspension was added to 100 g of peanut butter in a Whirl-Pak bag (7.5 by 12 in., 1.6-liter capacity; Nasco, Fort Atkinson, WI) and then thoroughly mixed by alternating between a 1-min hand massage and 1 min in a stomacher (Seward Stomacher 400 Circulator, Seward, Bohemia, NY) at 250 rpm, approximately three times each. *Salmonella* cells were recovered from triplicate peanut butter samples, by diluting 1 g of peanut butter in 9 g of BPW, stomaching at 230 rpm for 1 min, performing serial 10-fold dilutions, and then spread plating on TSAYE. Duplicate agar plates were spread from every sample plated. The starting population in peanut butter for all studies was approximately 8 to 9 log CFU/g of peanut butter. Plates were examined for typical *Salmonella* colonies after 24 h of incubation at 37°C. It was noted that when peanut butter was not sufficiently diluted prior to plating on TSAYE, colonies were difficult to enumerate due to observable morphological changes in the shape and size of *Salmonella* colonies. Isolated colonies were typically smaller and flatter with irregular edges, with occasional spreading. As the cause for the morphological changes was unknown, most *Salmonella* enumeration was carried out at dilutions equal to or greater than 1:100, where isolated colonies were typically 1 to 2 mm in diameter, smooth, circular, and raised with entire edges.

***Salmonella* survival study.** For experiments determining the survival of *Salmonella* at 25°C, three 1-g aliquots were removed from different locations in the bag holding the inoculated peanut butter immediately after mixing and at each time point thereafter, diluted, spread plated in duplicate on TSAYE, and incubated at 37°C for 24 h to enumerate populations. Standard deviations were <0.2 log CFU/g for the triplicate samples. Inoculated peanut butter was stored at 25°C for up to 2 weeks. Starting *Salmonella* populations were approximately 8 to 9 log CFU/g of peanut butter. Each survival experiment was conducted a minimum of three times.

Thermal inactivation study. Thermal resistance of *Salmonella* in peanut butter was measured in a thin metal plate thermal death time device designed to hold the inoculated food paste. Each thermal inactivation test for each serovar and growth condition was completed a minimum of three times. Separately grown culture, either in liquid broth or on solid agar, was used for each trial.

The thin-layer device used for all thermal resistance studies with *Salmonella* consisted of an aluminum square plate, a 430 stainless steel lid, a 410 stainless steel square ''ring,'' and 16 N42SH neodymium disc magnets. The 2.36-mm-thick square plate measured 55 by 55 mm and had a square depression that accommodated up to 1.5 g of paste held in a uniform 1-mm-thick layer. The underside of the plate also had a depression that made the plate wall 0.3 mm thick. The neodymium magnets measured 3.2 mm in diameter and 1.6 mm in thickness and were fitted into holes on the underside of the plate. The magnets were held in place by the square ring that was glued onto the underside of the plate. The lid also measured 55 by 55 mm and was 0.3 mm thick. Small

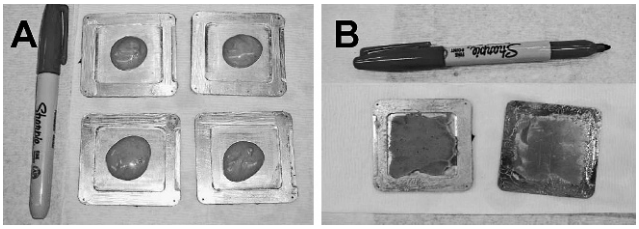

FIGURE 1. *Appearance of metal plates with peanut butter before and after heat treatment. (A) Open plates, prior to heat treatment, showing appearance and placement of peanut butter; (B) plates opened after heat treatment at 121°C for 2 min.*

holes were cut through the device to aid in hanging it in a temperature-controlled oil bath. In each experiment, approximately 1.5 g of peanut butter was placed in the plate depression (Fig. 1A). Vacuum grease was applied to the surface where the plate and lid meet to form a seal between the two components. The plates were weighed before and after thermal treatment and cooling to ensure that no leakage of the peanut butter or gain from the silicon oil bath or from the cooling water occurred during treatment.

To assess the performance of the new thin-layer device prior to use in the *Salmonella* thermal inactivation studies, its thermal properties were compared with those determined by an established method utilizing Whirl-Pak bags *(19)*. Whirl-Pak bags (Nasco) were made of low-density polyethylene with the bag material measuring 2.25 mil (0.057 mm) in thickness. In each experiment, 1 g of peanut butter was loaded into the bags and spread evenly to a thickness of approximately 1 mm.

The time constant, tau, was also measured for the loaded plate and bag by a thin thermocouple. Tau is defined as the time required for the cold point (i.e., center point) temperature, T, to reach a value such that $[(T_f - T)/(T_f - T_i)] = 0.63$, where T_f is the bath temperature and T_i is the initial temperature of the plate or bag *(13)*.

Thermal treatment of *Salmonella* was conducted in a Thermo silicon oil bath (Neslab RTE-211 circulator, Fisher Scientific, Pittsburgh, PA). Following thermal treatment, individual plates were removed at predetermined time intervals and immediately cooled in an ice bath for approximately 1 min. Following cooling, plates were weighed and then opened; 1 g of peanut butter was aseptically removed, diluted appropriately, and then plated to determine surviving *Salmonella* populations.

Statistical analysis. Separate analyses of covariance were performed for cells tested at 25 and 85°C. The analyses were performed using SAS v9.2 Proc Glm (SAS Institute Inc., Cary, NC). The growth condition of the cells, sessile or planktonic, was the categorical factor, and time and time^2 are continuous factors. The full analyses of covariance model contained the factors Growth, Time, Time × Growth, Time^2, and Time^2 × Growth, with run date as a blocking factor.

RESULTS AND DISCUSSION

Thin-plate versus Whirl-Pak bag performance. Previous studies examined the thermal resistance of *Salmonella* in peanut butter in Whirl-Pak bags *(19)*. Whirl-Pak bags were filled with 1 g of inoculated peanut butter, the bags were loaded on a rack, and the rack was placed in a circulating water bath. This method incompletely submerged the bags to roughly three-fourths of the bag height. In our use of the bags, we encountered limitations. First, a uniform thin layer of a food matrix is important to obtain consistent and accurate results with planar type devices. It was difficult to achieve a uniform layer in the bags, as shown in Figure 2A by the mottled appearance of the peanut butter in the bag. Figure 2B shows the bags having greater irregularities after processing. The bags also had a temperature limitation at around 80°C, noted by the manufacturer and seen in our testing, above which the bag melted as shown in Figure 2C. Another difficulty was that bags were easily dislodged from the commonly used racks and floated in a circulating oil bath unless weighed down. To address all three limitations, the thin-plate apparatus shown in Figure 1 was developed. Since the plates have stiff sides, this allowed a uniform food matrix layer to be established and maintained during heating studies. Figure 1A and 1B show the before and after treatment photos of the loaded device. Because the plates were made of metal, they were able to endure the high temperatures that melted the bags and they did not float in the oil bath, which allowed for easier handling.

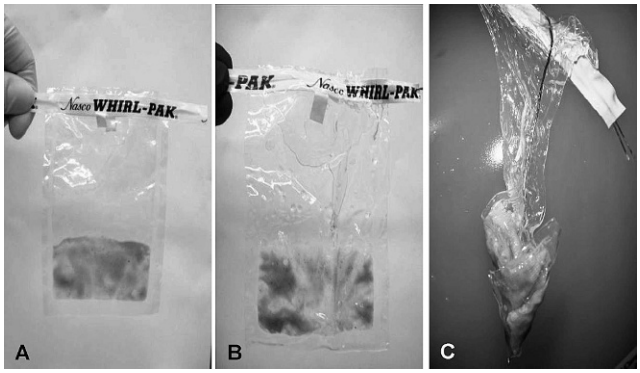

FIGURE 2. *Appearance of the Whirl-Pak plastic bags with peanut butter before and after heat treatment. (A) Prior to heat treatment; (B) heat treated at 90°C for 2 min; (C) heat treated at 121°C for 2 min.*

The only downside to the plates is a longer tau, 32.5 ± 0.9 s, compared with 12.4 ± 1.9 s for the bags. However, the time constants in the bags have a relative variability of about 15% compared with about 3% in the plates. The use of tau as a comparison in performance is preferred over come-up time because the latter is defined as the time to achieve an arbitrary temperature close to the bath temperature and is therefore dependent on the device, its contents, and the bath temperature. Tau, however, is defined as a fixed fraction of the normalized temperature difference between the final (bath) temperature and the initial temperature of the device. It is therefore only a property of the device and its contents and is independent of bath and initial temperatures.

Weights of the metal plates taken before and after heating and cooling treatments had a difference less than or equal to ±0.01 g. Nominal weight change was assumed to indicate that there was no transfer of material between the oil bath or cooling water and the plate contents.

Survival in peanut butter at 25°C. Figures 3 and 4 show the survival of *Salmonella* Oranienburg and Tennessee in peanut butter held at 25°C over a 2-week period and

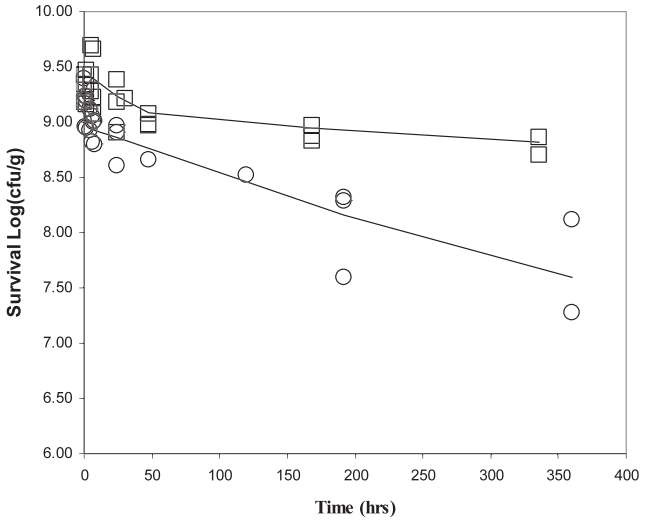

FIGURE 3. *Survival of* Salmonella *Tennessee at 25°C in peanut butter based on type of growth of inoculum. Three trials each are shown for both sessile and planktonic cells. Square symbols (□) represent sessile cells, and circle symbols (○) represent planktonic cells.*

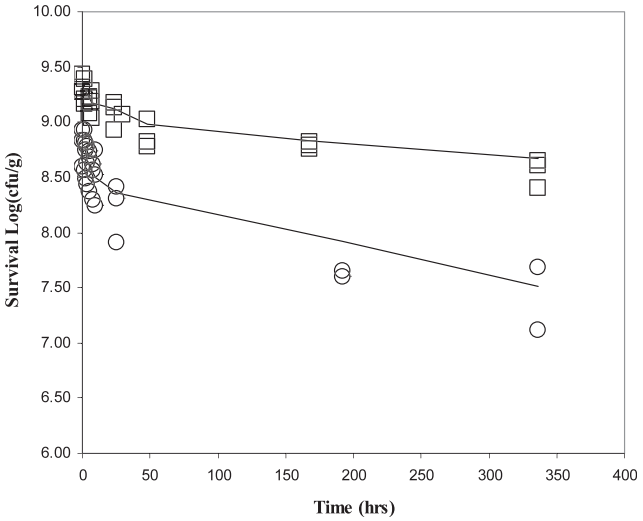

FIGURE 4. *Survival of* Salmonella *Oranienburg at 25°C in peanut butter based on type of growth of inoculum. Three trials each are shown for both sessile and planktonic cells. Square symbols (□) represent sessile cells, and circle symbols (○) represent planktonic cells.*

contain data from three experiments each for both planktonic and sessile cells. Each experiment used separate freshly grown inoculum in a separate aliquot (100 g) of peanut butter. Triplicate samples were taken at each time point from three different locations in the bag to ensure uniformity of the distribution of *Salmonella* within each 100-g lot. The variability of the triplicate samples at each time point was ≤0.2 log CFU/g. Both *Salmonella* serovars showed significantly more survival in peanut butter when originally grown on solid media ($P = 0.001$) with a <1-log loss over 2 weeks as opposed to a 1- to 2-log loss when grown in liquid culture. Regardless of growth method, the fastest loss in viability occurred in the first 48 h after inoculation, after which the decline in population was much slower for both serovars. A similar observation was noted by Uesugi et al. *(26),* in whose work surface-grown cultures of *Salmonella* were used to inoculate whole almond kernels. In that study, a greater reduction of *Salmonella* in the first 2 days of drying was observed when cells were grown in liquid culture than when grown on solid agar. However, following the drying period on almond kernels, no further difference in behavior related to solid or liquid medium for growing cells was noted. It was speculated that cells grown on solid agar are more resistant to desiccation than cells grown in a liquid medium. In our study, we noted a similar phenomenon. Although no deliberate drying step has been implemented, the act of inoculating a low-a_w paste such as peanut butter (a_w, approximately 0.3) may result in the loss of water from the cell, hence in drying of the cell. Consequently, the similar phenomenon seen here may also indicate that cells grown on solid agar surfaces may be more resistant to desiccation than are cells grown on liquid.

Thermal resistance in peanut butter. Thermal resistance measurements of *Salmonella* Oranienburg and Tennessee using cells grown in liquid media were conducted both immediately after inoculation into peanut butter and after 1 and 2 weeks of incubation in peanut butter that was held at 25°C (data not shown). Statistical analysis of the rate of thermal inactivation showed no significant dependence of length of incubation time at 25°C on the rate of survival at 85°C ($P = 0.2$ and 0.4 for *Salmonella* Oranienburg and *Salmonella* Tennessee, respectively). Consequently, subsequent thermal inactivation experiments were conducted only with peanut butter samples directly after inoculation.

For both *Salmonella* Oranienburg and *Salmonella* Tennessee, four separate trials were completed with sessile cells, and three separate trials were completed with planktonic cells. All data from all trials are shown in Figures 5 and 6. The two serovars showed similar thermal resistance in peanut butter regardless of growth as sessile or planktonic cells when thermal resistance was calculated as the time required for 4- or 5-log reduction in population (Figs. 5 and 6). However, a subsequent analysis of covariance was performed separately for serovars. Growth condition, sessile or planktonic, was the categorical factor, and time and time squared were continuous factors in the analysis. Experiment date was treated as a blocking factor. A statistically significant ($P < 0.05$) test of the quadratic time^2 × growth estimate indicates a different curvature of the survival plots. At 85°C the planktonic quadratic estimate was significantly greater than the sessile condition for both *Salmonella* Oranienburg and *Salmonella* Tennessee ($P = 0.0198$ and 0.0047, respectively). Consequently, the thermal destruction curves from sessile cultures showed far less curvature than those obtained from planktonic cells. Since the use of sessile cells resulted in more linearity in the corresponding reduction curve, a calculation of the *D*-value using standard first-order kinetics is possible. The *D*-values and standard deviations calculated for *Salmonella* Tennessee and *Salmonella* Oranienburg at 85°C, using data from

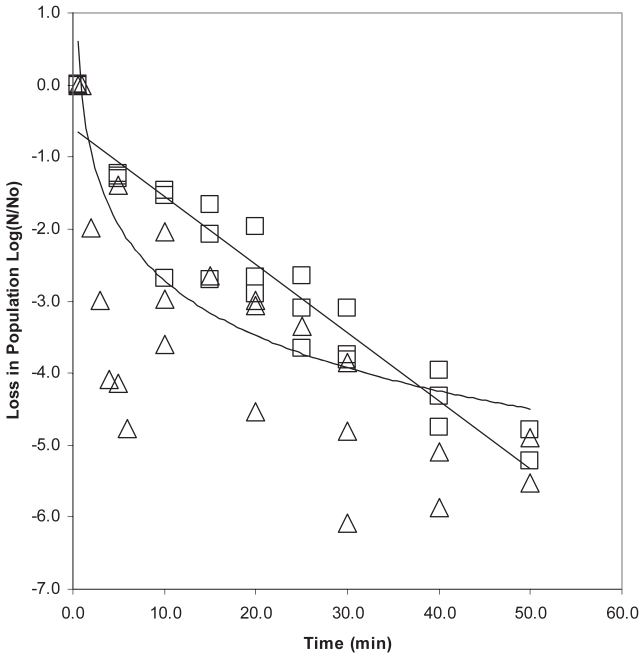

FIGURE 5. *Survival of* Salmonella *Tennessee at 85°C in peanut butter based on type of growth of inoculum. Square symbols (□) represent four separate trials with sessile cells, and triangle symbols (△) represent three separate trials with planktonic cells.*

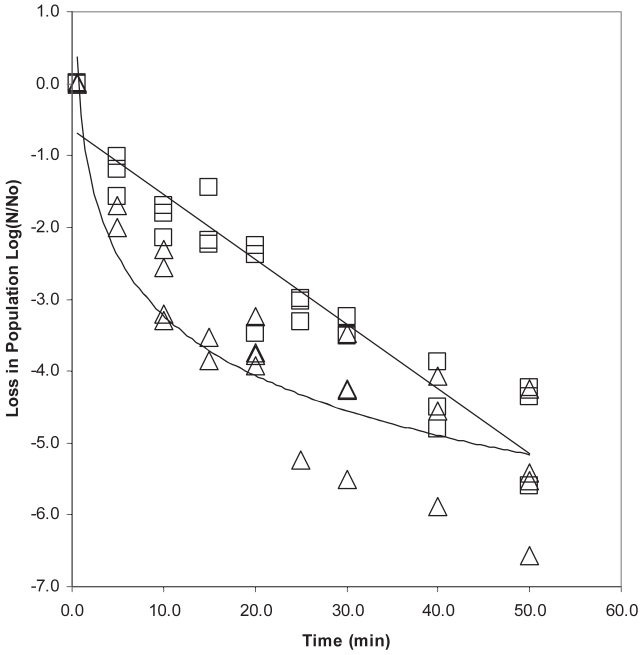

FIGURE 6. *Survival of* Salmonella *Oranienburg at 85°C in peanut butter based on type of growth of inoculum. Square symbols (□) represent four separate trials with sessile cells, and triangle symbols (△) represent three separate trials with planktonic cells.*

sessile cells, were 11.95 $\pm$ 1.55 min and 12.83 $\pm$ 2.35 min, respectively. Data from planktonic cells were not used.

Two different factors may have played a role in the reduced variability seen in thermal destruction curves when using solid-grown sessile cells. Based on the loss of viability at 25°C, it is likely that inactivation of *Salmonella* in peanut butter at 85°C is the result of two different effects, the first being the result of the cell desiccation when inoculated into the peanut butter and the second being thermal death. The lesser variability and hence the more linear trend of the rate of thermal inactivation displayed when cells are grown on solid media may reflect the greater desiccation resistance displayed by *Salmonella* when grown on solid media than on liquid media. The second factor that may play an important role in the reduced variability is the use of a rigid thin-layer metal device that is completely submergible in the heating medium. This method likely reduced errors that can occur with the use of a flexible plastic holder that is not itself heat resistant and is not completely submerged.

In summary, the data presented here indicate that the lack of linearity in thermal destruction curves in previous literature reports must be treated with caution if the cause of nonlinearity is not well understood. Methods of analysis should not impact rates of inactivation of microorganisms. Furthermore, an attempt should be made to understand physiological conditions that may affect the results of such analyses. The use of *Salmonella* species grown on solid media may result in greater resistance to the physiological changes that occur when moving cells from high- to low-a_w environments. As a consequence, results for thermal inactivation may more accurately assess survival in low-a_w environments.

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