Heat Tolerance of \textit{Salmonella enterica} Serovars Agona, Enteritidis, and Typhimurium in Peanut Butter

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\textbf{ABSTRACT}

Recent large foodborne outbreaks caused by \textit{Salmonella enterica} serovars have been associated with consumption of foods with high fat content and reduced water activity, even though their ingredients usually undergo pasteurization. The present study was focused on the heat tolerance of \textit{Salmonella enterica} serovars Agona, Enteritidis, and Typhimurium in peanut butter. The \textit{Salmonella} serovars in the peanut butter were resistant to heat, and even at a temperature as high as 90°C only 3.2-log reduction in CFU was observed. The obtained thermal inactivation curves were upwardly concave, indicating rapid death at the beginning (10 min) followed by lower death rates and an asymptotic tail. The curves fitted the nonlinear Weibull model with $\beta$ parameters $<1$, indicating that the remaining cells have a lower probability of dying. $\beta$ at 70°C (0.40 $\pm$ 0.04) was significantly lower than $\beta$ at 80°C (0.73 $\pm$ 0.19) and 90°C (0.69 $\pm$ 0.17). Very little decrease in the viable population (less than 2-log decrease) was noted in cultures that were exposed to a second thermal treatment. Peanut butter is a highly concentrated colloidal suspension of lipid and water in a peanut meal phase. We hypothesized that differences in the local environments of the bacteria, with respect to fat content or water activity, explained the observed distribution and high portion of surviving cells (0.1%, independent of the initial cell number). These results demonstrate that thermal treatments are inadequate to consistently destroy \textit{Salmonella} in highly contaminated peanut butter and that the pasteurization process cannot be improved significantly by longer treatment or higher temperatures.

\textit{Salmonella enterica} is a foodborne pathogen that causes large outbreaks of food poisoning worldwide. The most common and characteristic disease caused by \textit{S. enterica} in humans is self-limited gastroenteritis. Bacteremia and other extraintestinal foci of infection occur usually only in very young, very old, and immunocompromised individuals. In these patients, mortality rates may increase by up to 40% (17). In addition to health aspects, the costs associated with infection can be very high, including expenditures associated with health care, direct costs to patients, and indirect costs due to lost employment. Implications for the food industry include recall of products and lost prestige and income (21).

Most outbreaks of salmonellosis result from consumption of animal products such as poultry, meat, or eggs, but the presence of \textit{Salmonella} on fresh fruits and vegetables has also emerged as a serious concern. Some large outbreaks have been associated with foods that have low water activity ($a_w$). \textit{S. enterica} serovars Napoli, Agona, Mbandaka, and Ealing have been associated with chocolate, snacks, peanut butter, and infant dried milk, respectively (5, 6, 12, 15, 18). Moreover, very low concentrations of \textit{Salmonella} (as low as 3 cells per g) were associated with outbreaks caused by consumption of contaminated high-fat low-$a_w$ foods such as peanut butter (16) or chocolate (5).

An international outbreak of food poisoning due to \textit{Salmonella} Agona was reported during late 1994 and early 1995. More than 2,200 cases were identified in Israel, most of them occurred in children younger than 5 years (18). Twenty-seven cases, 26 of them in children (12), were identified in England and Wales. This outbreak was caused by contamination of a peanut-butter-coated ready-to-eat snack commonly consumed by young children in Israel. The contaminated snacks were manufactured on at least seven separate dates during a 4-month period. A few years later, an outbreak of salmonellosis in Australia caused by \textit{Salmonella} Mbandaka was associated with nine different brands of peanut butter (16).

\textit{Salmonella} can survive in nuts or low-$a_w$ foods for long periods, although optimal growth of \textit{Salmonella} strains occurs at an $a_w$ of 0.99. \textit{Salmonella} inoculated onto pecan halves, for instance, survived for at least 32 weeks after contamination (1), and \textit{Salmonella} in peanut butter survived more than 24 weeks (2, 13). Peanut butter usually undergoes pasteurization before packaging at temperatures around 70 to 75°C. Because several researchers have suggested that \textit{Salmonella} have increased heat resistance in low-$a_w$ foods or in foods with high lipid content (10, 11, 13), we hypothesized that the pasteurization of contaminated peanut butter is inadequate. Data about the survival of \textit{Salmonella} in peanut butter at temperatures above 70°C have not been available. Thus, the focus of this research was to study the inactivation of \textit{Salmonella} serovars in peanut butter after either one-step or two-step applications of high-temperature treatments.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains.} Three serovars of \textit{S. enterica} were used: \textit{Salmonella} Agona (a clinical isolate associated with the peanut-
butter-coated snack outbreak in Israel), *Salmonella Enteritidis* (a clinical isolate from human feces), and *Salmonella Typhimurium* ATCC 14028.

**Preparation of inoculum.** Cultures of each serovar were prepared by inoculation of a single colony into 50 ml of Luria-Bertani (LB) broth and incubation overnight at 37°C with aeration. *Salmonella* cultures were then centrifuged (3,800 × g for 10 min at 4°C) and resuspended in 5 ml of saline (0.85% NaCl) to a final concentration of about 10¹⁰ CFU/ml.

**Inoculation and thermal treatment of peanut butter.** Pure peanut butter, the raw material used to produce the peanut butter-coated ready-to-eat snack that was associated with the outbreak in Israel in 1994 and 1995, was used for the experiment. Peanut butter samples (25 g) were aseptically placed in sterile 400-ml stomacher bags (Steriblend, Bibby Sterilin, Stone, Staffordshire, UK). Bags were sealed with clips and kept for 45 min in a heated water bath (Fried Electric, Haifa, Israel) adjusted to 70, 80, or 90°C. At these temperatures, the peanut butter is turned into a viscous liquid. One bag (which was not used for the experiments) contained thermometers to ensure that the temperature in the bags was homogeneous and reached the experimental temperature. *Salmonella* inoculum (0.25 ml) was added to each sample to final concentrations of about 5 × 10⁴ or 5 × 10⁸ CFU/g and vigorously mixed in the stomacher for 15 s for homogenization. The flattened peanut butter (0.2 mm thickness) was immediately reimmersed in the water bath. The temperature decrease during this step was approximately 1°C and come-up time was less than 1 min. At each predetermined time point (0, 5, 7.5, 10, 15, 20, 30, 40, and 50 min), a bag was removed from the water bath, and the sample was immediately diluted in 100 ml of saline at room temperature (1:5 dilution). This mixture was pummeled in a stomacher at normal speed for 1 min, serially diluted in saline, and pour plated onto LB agar plates in duplicate. The bacterial concentration (CFU per gram) was counted after 24 h of incubation at 37°C. The limit of detection was 5 CFU/g. The control was peanut butter that was not contaminated with *Salmonella*. *Salmonella* was also similarly treated in saline for comparison. The initial natural microflora concentration was determined by dilution of 25 g of peanut butter in 100 ml of saline, homogenization in the stomacher, and plating. Less than 50 CFU/g was obtained, and these microorganisms were killed within 5 min at 70°C. All experiments were conducted at least three times in duplicate.

In these experiments, bacteria were introduced into preheated peanut butter to eliminate the effect of heating time. However, the treated *Salmonella* cells were simultaneously exposed to several stresses, including low a₀, high fat concentration, and high temperatures. To allow adjustment to the peanut butter environment prior to the exposure to the challenge temperatures (as would probably occur in a low-a₀, food ingredient before heat processing), cells were introduced into the peanut butter and the peanut butter was kept at room temperature (~25°C) for 1 and 24 h. These peanut butter cultures in the bags were then flattened to decrease the thickness and to facilitate heat transfer (about 0.2 mm thickness) and incubated in the water baths at 70, 80, or 90°C for 10, 20, 30, and 40 min. Samples reached the final temperatures within less than 4 min.

**Determination of heat tolerance after a second exposure of peanut butter cultures to high temperatures.** To determine whether cells that survived a heat inactivation treatment would have higher tolerance to a second treatment, these cells were exposed to heat a second time. Initially, bagged peanut butter samples contaminated with about 10⁸ CFU/g were incubated at 80°C for 30 min and then removed from the water bath and stored on the benchtop at ambient temperature (~25°C) for 24 h. Samples were then incubated in the water baths at 70, 80, or 90°C. Cells were counted before and after the first heat treatment, after the storage period at room temperature, and at predetermined time points (up to 50 min) during the second heat treatment. All experiments were conducted at least three times in duplicate.

**Data analysis.** The observed survival data were examined graphically to determine the type of curve that needed to be modeled. Experiments usually appeared to form concave curves with an asymptotic tail. Thus, the applicability of the Weibull model to describe thermal inactivation of *Salmonella* in the peanut butter was evaluated (3, 20). Data analysis was performed with Microsoft Excel version 7 (Microsoft, Redmond, Wash.) using the Solver capability. Each survival curve was then fitted according to equation 1 by a linear regression:

\[
\frac{N}{N_0} = \exp\left(-\frac{t}{\alpha}\right)^\beta
\]

Data were analyzed using a one-way analysis of variance followed by the Tukey-Kramer test. Results with *P* values of <0.05 were considered significant.

**RESULTS AND DISCUSSION**

*Salmonella* is a gram-negative mesophilic bacterium that is usually sensitive to temperatures above 55°C. All three *Salmonella* serovars tested here (Agona, Typhimurium, and Enteritidis) were rapidly killed at 70°C in saline, and a greater than 7-log reduction was observed within less than 5 min. However, in the peanut butter environment the tolerance to heat dramatically increased. We did not observe significant differences between the serovars, and the averaged results of all three serovars are given in Figures 1 and 2. When peanut butter containing viable bacteria at approximate 8 log CFU/g was exposed to heat for 5 min,
FIGURE 2. Inactivation of low initial concentrations of Salmonella Agona, Salmonella Enteritidis, and Salmonella Typhimurium in peanut butter. Bacteria (approximately $5 \times 10^4$ CFU/g) were introduced into preheated 25-g samples of peanut butter, and the number of surviving cells was determined from plate counts. Values are the log CFU per gram of sample. Bacteria were treated in peanut butter at 70°C ( ), 80°C ( ), or 90°C ( ). The standard error of the mean for the results from the three serovars is shown.

A 1.4-log reduction was observed at 70°C, a 2.2-log reduction was observed at 80°C, and a 2.5-log reduction was observed at 90°C (Fig. 1). Numbers of cells continued to decline, with lower death rates up to approximately 2.7-log reduction at 70°C and 3.0-log reduction at 80 and 90°C, but concentrations did not change significantly after 20 min of exposure to the challenge temperatures ($P > 0.05$). About 0.1% of the cells remained viable at all three temperatures even after 50 min. The higher temperatures (80 and 90°C) were slightly more effective at killing cells than was 70°C, but the differences were not significant. Similar results were obtained when bacteria were introduced into the peanut butter 1 or 24 h before heat was applied; however, the variability was much higher during the first 10 min, probably because of the heating time (results not shown).

Any food might be contaminated with lower numbers of pathogenic cells. Because death rates were lower when the number of Salmonella cells decreased, we hypothesized that the initial number of cells affected the survival properties. Thus, we repeated the experiments with lower initial inoculum concentrations. We inoculated the peanut butter samples with approximately $5 \times 10^4$ CFU/g, the lowest number of cells that could still be detected under our experimental conditions after heat treatment. Figure 2 presents averaged results of all three serovars. Like the profiles generated with higher numbers of cells, an elevated death rate was observed during the first 5 min at all three temperatures, with reductions of 1.5 (70°C), 1.9 (80°C), and 2.6 (90°C) log CFU/g, followed by much lower death rates. About 0.1% of the cells at 70°C and 0.05% at 80 and 90°C remained viable. These results indicate that the initial cell concentration did not significantly affect the survival percentage.

The results in Figures 1 and 2 with the very high survival rates are consistent with previous observations that only a 1.5-log reduction of Salmonella in peanut butter was obtained after 196 min at 55°C, 48 min at 65°C, and 12 min at 74°C (14). Our main conclusion from this experiment is that Salmonella is likely to survive in peanut butter for the duration of the expected shelf life, as was previously shown (2), even after pasteurization. Moreover, it can survive at temperatures as high as 90°C for 50 min.

Historically, heat inactivation of populations of bacteria has been described using first-order kinetics. However, Figures 1 and 2 illustrate that first-order kinetics do not apply for these data because the obtained curves are upwardly concave, with an initially rapid death rate followed by lower death rates and an asymptotic tail. Because nonlinear models, particularly the Weibull model, have been used to describe the survival of Salmonella in a variety of foods such as ground beef, ground chicken, egg yolk, and milk (20) and chorizo (a kind of sausage) (7) and the survival of other heat-inactivated foodborne pathogens such as Listeria monocytogenes (8), we evaluated the classical test for judging the applicability of the Weibull model by analyzing the double logarithmic plot of $\ln(-\ln N/N_0)$ versus $t$. If the Weibull model applied, a straight line would be obtained (9, 20). Reasonable plots were obtained, with $R^2$ values higher than 0.95 (Table 1), indicating the appropriateness of the Weibull distribution function to model the survival of Salmonella in peanut butter after heating.

The two parameters of the Weibull distribution, $\alpha$ (the scale parameter) and $\beta$ (the shape parameter), were calculated (Table 1). At all three temperatures $\beta$ was $< 1$, which means that the remaining cells have a lower probability of dying. The value of $\beta$ at 70°C was significantly different from $\beta$ at 80 and 90°C, indicating that $\beta$ is dependant on temperature only at lower temperatures. In a broad review, van Boekel (20) concluded that in most cases of bacterial inactivation in different media or foods $\beta$ is greater than 1, suggesting that in the majority of the cases accumulated damage occurs and that the remaining cells have a tendency to become weaker when heating time increases. However, in some examples from foods such as Salmonella Enteritidis in egg yolk, which also has a low $a_w$ value, $\beta$ was less than 1 (0.8). In only a very few cases was $\beta$ dependent on temperature, but in most cases the temperature range was limited and always below 70°C (20).

Why do death profiles of Salmonella in peanut butter...
and egg yolk differ from those in other environments? Peanut butter is a highly concentrated colloidal suspension of lipid and water in a peanut meal phase. It contains about 50% fats (2), and its aw was approximately 0.5 as measured with the AwQuick (Rotronic Instrument Corp., Huntington, N.Y.). Thus, the peanut butter environment is characterized by two factors that can affect the heat tolerance of microorganisms: high fat content and low water activity. Van Asselt and Zwietering (19) extensively studied factors that affect the thermal inactivation of various pathogens. They found that for most pathogens there was no significant effect of aw on inactivation in a variety of products or media. However, low aw markedly affected the survival of Salmonella. Chocolate had a protective effect on Salmonella, and the D-value at 70°C was more than 3-log higher than that in other food products (19). In other studies specific to Salmonella, there was a significant effect of low aw on heat resistance, and this effect depended on the solutes used to decrease aw (4, 13). Mattick et al. (14) demonstrated that although low aw is protective for Salmonella at temperatures above 70°C, it promotes more rapid death at lower temperatures.

Another important characteristic of peanut butter is high fat content, which might protect the cells at high temperatures. Increased heat resistance in oily products was shown for Bacillus cereus (19). The combination of both, high fat and low water in foods such as peanut butter or chocolate might have a synergistic effect. Mattick et al. (14) found that death of Salmonella in low-aw foods such as pecorino cheese, pepperoni sausage, strawberry jam, and dried apricots occurred at the rate predicted by their thermal inactivation models, Salmonella survived in peanut butter for a much longer time.

Our observations of decreased heat sensitivity of bacteria in peanut butter over time, with a tailing effect for survivors, can be explained by the heterogeneous nature of the peanut butter matrix, exposing different cells to different local environments. We hypothesized that Salmonella cells would aggregate into clumps within or near the water phase of the peanut butter (2, 13); thus, cells would be found in different microenvironments. A portion of the bacteria would be located in a relatively wet environment, others in a drier environment but still in a low-fat area, or in a more fatty and hydrophobic environment. Upon exposure to heat, the least protected bacteria would die rapidly, followed more slowly by the other bacterial groups. It is not obvious which local environment is more protective, but we assume that it has lower water activity and higher fat content. This hypothesis is supported by the fact that survival was always about 0.1%, independent of the initial cell concentration. If the peanut butter dries as heat is applied, then inactivation rates are further slowed. Adaptation to the low-aw environment is unlikely because bacteria in the peanut butter were heated within seconds.

The main reason to investigate temperature-dependant thermal inactivation is to allow accurate calculation of the time needed to pasteurize or sterilize foods. With the non-linear model, we were able to calculate the minimum time needed to reach a 7-log reduction. Based on the calculated parameters of the Weibull model, more than 260 min are needed to reduce Salmonella by 7 log units at 70°C, and more than 1 h is needed at 90°C. This estimate is an extrapolation, and the real inactivation time could be even longer because of the asymptotic tail in the model. However, even with these minimum estimations, the thermal treatments currently used to pasteurize peanut butter (usually less than 20 min) are not sufficient for destruction of Salmonella. However, 4 h at 70°C or 1 h at 90°C is not practical and could adversely affect the properties of the products by increasing denaturation or browning.

Because the first 10 min of treatment were when most of the bacteria were killed, we examined the effect of two sequential heat treatments on survival of Salmonella, assuming that each treatment would result in a rapid reduction of about 3 log units. In the first step, Salmonella cells were incubated in the peanut butter at 80°C for 30 min and then stored for 24 h at room temperature. As expected, an approximately 3-log reduction was observed at this stage. The number of cells was increased twofold during 24 h of incubation at room temperature. It is not clear, however, whether cells grew during storage or whether injured cells recovered. During the second treatment, cells were exposed to 70, 80, or 90°C, and the kinetics of death was determined. Bacteria survived much better after the second treatment (Fig. 3). A less than 1-log reduction was observed at the lower temperatures, and a less than 2-log reduction was observed after treatment at 90°C. The death rates were not linear ($R^2 < 0.7$); however, because the reduction was very low modeling of the death curve was not relevant.

Results indicated that the history of the cells had a strong influence on their response to heat; a second heat inactivation treatment, even when administered long after
the first treatment (24 h), was significantly less effective. This finding supports our hypothesis that the surviving cells are protected in specific microenvironments in the peanut butter suspension. This local protection might continue during the second heat treatment. It is less likely but also possible that phenotypic changes in the surviving cells enhance their survival. Further research is needed to determine the spatial location of cells in the peanut butter suspension and any correlation between location and heat tolerance.

The recent international outbreak caused by Salmonella Agona in a peanut butter–coated snack (for which the peanut butter was heated to 75°C before being used for coating) led us to examine the lethal effect of heat treatment on Salmonella serovars in a peanut butter environment. The standard pasteurization process was ineffective for achieving a 7-log reduction in Salmonella, but effectiveness was not significantly approved by longer treatment (up to 50 min) or higher temperatures (up to 90°C). Two sequential treatments also were not effective for achieving a 7-log reduction. The survival of Salmonella in low-aw foods raises specific issues for food safety, particularly when investigations of outbreaks indicate that only a very few cells (3 CFU) may be required to cause disease when consumed in low-aw foods (16). Survival of even 0.1% of the bacterial cells (several thousand bacteria) might be hazardous. A large proportion of low-aw foods are snack foods that are a regular part of the modern diet of young children. Increased consumption of these food types could result in more frequent outbreaks of salmonellosis unless appropriate management steps are taken. Investigation of other inactivation treatments is needed to improve the safety of these kinds of foods.

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REFERENCES