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Effect of the Local Microenvironment on Survival and Thermal Inactivation of *Salmonella* in Low- and Intermediate-Moisture Multi-Ingredient Foods

HAIPING LI,[1]* XIAOWEN FU,[2] YIGE BIMA,[2] JOHN KOONTZ,[1] CHRISTINA MEGALIS,[1] FEI YANG,[2] GREGORY FLEISCHMAN,[1] AND MARY LOU TORTORELLO[1]

[1]*U.S. Food and Drug Administration, Division of Food Processing Science and Technology, 6502 South Archer Road, Bedford Park, Illinois 60501; and* [2]*Institute for Food Safety and Health, Illinois Institute of Technology, 6502 South Archer Road, Bedford Park, Illinois 60501, USA*

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ABSTRACT

Multi-ingredient foods having low- or intermediate-moisture characteristics may pose a special challenge to process design and validation. Ingredients of these foods can create local microenvironments that may have a distinct impact on pathogen survival and processing requirements. In this study, two model systems, each consisting of 80% commercial peanut butter (P) and 20% nonfat dry milk powder (M), were formulated to be identical in composition, but different in the source of the *Salmonella* contamination as originating in either the ingredient P or M. Immediately after inoculation, *Salmonella* showed a 2.0-log reduction when M was the contaminated ingredient compared with a 0.6-log reduction when P was the contaminated ingredient. This pattern of survival was consistent with the single-ingredient control containing only M (2.5-log reduction) or only P (0.7-log reduction), suggesting that the immediate proximity of cells is determined by the contaminated ingredient in the model system. After 5 weeks of storage, the survival rates of *Salmonella* in the two systems remained different, i.e. a 4- and 2-log reduction resulted in the system with M or P as the contaminated ingredient, respectively. Furthermore, thermal inactivation efficacies also differed significantly between the two systems. Fourier transform infrared spectroscopy demonstrated the nonhomogeneous distribution of water, lipid, and protein, indicating that varied local microenvironments were present and likely affected the behavior of the pathogen. The impact of the microenvironment on inactivation and survival of *Salmonella* was further confirmed in a butter cookie formulation in which *Salmonella* was inoculated via four different ingredients. This study shows that the local microenvironment in low- and intermediate-moisture foods affects *Salmonella* survival and thermal inactivation. The ingredient source of the contamination should be taken into account for process design and validation to ensure the safety of the product.

Risk characterization of foodborne pathogen contamination in multi-ingredient foods of low and intermediate water activity (a_w of <0.6 and 0.6 to 0.85, respectively) *(17)* is complex. Ingredients may present different levels of risk to the final product due to their varied likelihoods of being contaminated by specific pathogens. Low- and intermediate-moisture ingredients, such as peanut butter *(5),* chocolate *(6, 7, 19),* and flour *(15),* have been associated with salmonellosis outbreaks, indicating greater risks of *Salmonella* contamination than those that have never been outbreak associated. Moreover, a pathogen surviving in a specific ingredient may be more resistant to processing treatments than in other ingredients. *Salmonella* in flour is desiccated and likely to be more resistant to heat than comparable hydrated cells *(1),* such as *Salmonella* in eggs. It remains unknown how the thermal resistance of *Salmonella* associated with each ingredient affects the inactivation efficacy and survival of *Salmonella* in the final multi-ingredient food formulation.

* Author for correspondence. Tel: 708-728-4110; Fax: 708-728-4176; E-mail: haiping.li@fda.hhs.gov.

Components in multi-ingredient foods having dissimilar physicochemical properties may form different local microenvironments in each food matrix. For example, mixing an ingredient with high lipid content and an ingredient with high water content may form a product appearing homogeneous as a single-phase bulk material on the macroscale, but a two-phase system of water and lipid may exist on the microscale. Hills et al. *(8, 9)* studied the impact of microscopic water distribution and local microstructure on bacterial survival by packing porous beds with glass, silica, and Sephadex beads at the same overall a_w. It was shown that bacteria survived differently in the packed beds having the same a_w, suggesting that the microstructure and the microscopic water distribution in each particulate matrix were different and critically affected bacterial survival *(8, 9).*

Most thermal inactivation and shelf life studies have been carried out by inoculating cells of the target pathogen directly into the final formulation, without considering the local microenvironment of the bacterial cells. In multi-ingredient foods, pathogens associated with different ingredients may reside in these different local microenvironments

within the food matrix. The local microenvironment may affect the inactivation process effectiveness and pathogen survival rates. Although it has been hypothesized that the microenvironment may affect the survival and thermal behavior of the pathogen in multi-ingredient foods *(16),* experimental support for this concept has not been reported in the scientific literature.

Salmonella presents a great risk for many flour-based bakery foods, chocolate, confectionery, pasta, and noodles, all of which are multi-ingredient foods that undergo a thermal process. Based on estimates by the European Food Safety Authority, *Salmonella* caused about 85% of the 279 outbreaks associated with this food category from 2004 to 2009 *(3)*. As in many other outbreaks, the exact routes of contamination in those outbreaks were often not clear, and there was often insufficient information to distinguish whether the outbreak was caused by a failed thermal process or postprocessing contamination. Nonetheless, the high incidences of *Salmonella* outbreaks that have been associated with thermally processed multi-ingredient foods (bakery, pasta, and chocolate/confectionery) are consistent with the high survival rate for *Salmonella* in this food category *(1)*. A 12-month survival of *Salmonella enterica* serovar Infantis and *Salmonella* Typhimurium was observed when cells were inoculated into pasta containing 12% moisture *(2)*. A study of *Salmonella* survival in different types of chocolate showed that *Salmonella* Eastbourne was detectable in milk chocolate after a 9-month storage and in bitter chocolate after 41 days of storage *(18)*. Burnett et al. *(4)* demonstrated that a cocktail of *Salmonella* strains were able to survive 24 weeks in peanut butter and peanut butter spread. This project aims to understand whether *Salmonella* contamination associated with different ingredients present different survival rates and thermal inactivation efficacy in the final products for this food category, especially those products containing low or intermediate amounts of moisture.

In this study, we created a model multi-ingredient food system to study the impact of the local microenvironment on *Salmonella* behavior, in an effort to provide critical information for industry to consider when designing effective process controls. As a representative bakery food, a simple butter cookie formulation was used to illustrate the impact of each individual ingredient, if contaminated, on process design and validation.

MATERIALS AND METHODS

Strains. *S. enterica* serovar Tennessee strain K4643, a clinical isolate from the 2006 peanut butter–associated outbreak in the United States, was used in the survival and thermal inactivation studies. The nonpathogenic strain *Escherichia coli* K-12 was used as a surrogate in the Fourier transform infrared spectroscopy (FT-IR) study (due to the location of the equipment in a biosafety level 1 laboratory). All strains were grown in tryptic soy broth at 37°C, with orbital shaking for 24 h.

Model food systems. A model food system was formed with 5 g of nonfat dry milk powder (M) (0% fat, 41% protein, 59% carbohydrate; water activity [a_w], 0.29) and 20 g of creamy peanut butter (P) (50% fat, 22% protein, 22% carbohydrate, 0.5% sodium; a_w, 0.33). The a_w of each ingredient was measured at ambient temperature by the AquaLab Water Activity Meter 4TE (Decagon Inc., Pullman, VA).

TABLE 1. *Composition and source of* Salmonella *contamination in model food systems*[a]

Model	Inoculated ingredient	Uninoculated ingredient
MP	5 g of M	20 g of P
PM	20 g of P	5 g of M
Control-M	25 g of M	None
Control-P	25 g of P	None

[a] M, nonfat dry milk powder; P, peanut butter.

Salmonella cells were inoculated into either M or P to create a contaminated ingredient that was subsequently combined with the other ingredient, resulting in two systems. The two systems were of identical composition, with the only difference being that *Salmonella* cells were introduced into the system via a different route of ingredient, either P or M. Briefly, 0.5 ml of a phosphate-buffered solution (PBS, pH 7.0) containing $\sim 10^{10}$ CFU of *Salmonella* cells per ml was added to 5 g of M or 20 g of P; the mixtures were homogenized in a stomacher blender (Masticator Silver Panoramic, Neutec Lab Supplies, Farmingdale, NY) for 2 min, mixed into 20 g of P or 5 g of M, respectively, and then homogenized for another 2 min to form system MP and system PM, respectively (Table 1). Cells were also inoculated into 25 g of pure P or M, respectively, as single-ingredient control systems. Viable cells were enumerated by total plate counting as described below within 1 h of inoculation (immediately after establishing the model system) and after 5 weeks of storage in sealed double stomacher bags within a hermetically sealed container at approximately 25°C. The storage period allowed the moisture to migrate and equilibrate within the model food system.

Bacterial enumeration and plate counts. The cell viability count for each condition was carried out by transferring 1 g of each sample into a stomacher bag containing 5 ml of 0.1% peptone water, and after vortexing for 1 min, the suspension was serially diluted in 0.1% peptone water for enumeration. One hundred microliters of each dilution was plated onto tryptic soy agar plates in duplicate and incubated at 37°C for 18 to 24 h. The *Salmonella* latex test (Remel Inc., Lenexa, KS) was used to confirm the identity of colonies at the beginning of the study and when atypical colonies appeared after thermal treatment. In a preliminary experiment, the uninoculated matrix was plated at the same dilution to verify that the background microflora, if present, did not interfere with the *Salmonella* viable counts.

Determination of homogeneous contamination of model food. Homogeneous contamination of *Salmonella* into the model food system was tested according to ISO 13528:2005 (''Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparisons''; ISO, Geneva, Switzerland) *(11)*. Although this statistical method was originally used to assess the uniformity of materials prepared for proficiency testing of laboratory personnel, the statistical calculation is also appropriate to determine homogenous distribution of bacteria within foods. *Salmonella* inoculation at both high (~8 log CFU/g) and low (~5 log CFU/g) levels was carried out in a total of 25 g for each model system (MP and PM). Ten 1-g samples were collected from each model system immediately after mixing. About one-half of the volume of each model system was sampled to enumerate viable cells to test the homogeneity of each inoculation. The sampling variance and

critical value were calculated for each system based on a one-way analysis of variance (ANOVA) calculation, in which the sampling variance equaled $(MS_{between} - MS_{within})/2$ and technical variance, $S_{analytical}^2$, equaled MS_{within}. The target standard deviation (δ) was equal to the mean of all duplicated results (χ) times a coefficient value of 0.15. The critical value was calculated by the formula $[F_1 \times (0.3\delta)^2] + [F_2 \times S_{analytical}^2]$, where F_1 and F_2 are table values. If the sample variances were smaller than the critical value, then the samples passed the homogeneity test. The *z*-score for each sample was calculated by the ratio of its difference from the mean value to the target standard deviation (*z*-score = $(\chi - \text{mean})/\delta$). The *z*-score of the sample with the largest absolute value under each condition was considered its maximum *z*-score. When the absolute value of the *z*-score was ≤ 2, the homogeneity test met the satisfactory standard; when the *z*-score was between 2 and 3, the test was questionable; and when the *z*-score was >3, the homogeneity test was not acceptable.

ATR–FT-IR microscopic imaging. Attenuated total reflectance (ATR)–FT-IR was performed with a Spotlight 400 FT-IR microscope and a Frontier near/mid-IR spectrophotometer (Perkin Elmer Inc, Waltham, MA). The FT-IR microscope was operated in ATR imaging mode with an ATR imaging accessory consisting of a high-refractive-index germanium ATR crystal. An ATR background was collected prior to imaging samples to correct for any spectral effects introduced as the light passed through the instrument and the center of the ATR crystal. Images were scanned, with areas (100 by 100 μm) at 1.56-μm pixel size to study the chemical functional groups of local microenvironments within the model system in the mid-IR region from 4,000 to 750 cm^{-1}. Images and individual spectra were processed with atmospheric and ATR correction by Spectrum Image 1.7 and Spectrum 10.03 software (Perkin Elmer, Inc.), respectively. The distribution of water, lipid, and protein in the food model system was analyzed by measuring the absorption of O-H (3,353 cm^{-1}), ester carbonyl (1,743 cm^{-1}), and amide (1,654 cm^{-1}) groups *(12, 20)*. *E. coli* K-12 (200 μl) was inoculated into 20 g of food matrix consisting of a 4:1 weight ratio of M and P to the same final composition of the model systems MP and PM and mixed in a stomacher blender for 4 min. (*E. coli* K-12 was used as a surrogate for *Salmonella* in these experiments due to the location of the imaging equipment in a biosafety level 1 laboratory.) Several ATR images of the multi-ingredient food system were collected across the area of the bulk material to obtain a representative surface image.

Thermal inactivation of *Salmonella* in a model food system. A device for delivering a thermal treatment to a thin layer of food developed by Keller et al. *(14)* was used with modification. Briefly, immediately after preparation of the model system, 1 g of each sample in duplicate was transferred into a plastic stomacher bag, compressed to a thin layer, and then placed between two metal plates held together by a metal clip. A thermocouple (Fluke Corporation, Everett, WA) was inserted into the sample to monitor the temperature. The plates were immersed in a high-precision oil bath (PolyScience Inc., Niles, IL) heated at 90°C for 10 min. The come-up time was 99.5 ± 3.5 s. After the heat treatment, the plates were transferred into an ice bath and quickly cooled before assaying for total plate counts. Controls consisted of samples handled in the same manner, but without heat treatment. The difference in log reduction between the treated and nontreated samples was calculated to determine the thermal inactivation of the pathogen for each test condition. Three independent experiments were conducted.

***Salmonella* challenge study in baked butter cookies.** Butter cookie dough was formed by mixing 30 g of beaten egg (approximately one-half egg; a_w, 0.995; total fat, 1.6%), 75 g of melted unsalted butter (approximately three-quarters of a stick; a_w, 0.64; total fat, 98.6%), 150 g of all-purpose flour (~1.25 cups; a_w, 0.3; total fat, 1.6%), and 100 g of sugar (~0.5 cup; a_w, 0.47). Approximately 10 log CFU *Salmonella* cells in 200 μl of PBS was inoculated into one of the individual ingredients, followed by mixing into the remaining three uninoculated ingredients according to the above sequence in a bread machine (Breadman Inc, Madison, WI) to prepare dough mixtures with four unique sources of contamination, separately. Dough samples (20 g) were pressed into flat cookie shapes and placed into paper muffin cup liners in an aluminum tray. Three independent experiments were carried out, and each dough mixture was sampled twice. Plate counts were performed immediately after dough preparation. A low baking temperature of 135°C (275°F) was used to mimic the temperature of cold-spot conditions in conventional ovens. Cookies were baked at 135°C (275°F) for 10 min in a convection oven (Environtronics, Grand Rapids, MI). After baking, each cookie was transferred into a stomacher bag followed by the addition of 20 ml of 0.1% peptone water. One gram of each homogenized cookie sample in duplicate was serially diluted for performance of standard plate counts.

Statistical analysis. Three or more independent experiments were carried out with duplicate or triplicate samples in each. Data were analyzed by one-way ANOVA and box-and-whisker plots in Excel (Microsoft Inc., Seattle, WA). In the ANOVA test, when the *F* value was greater than F_{crit} and the *P* value was smaller than 0.05, the difference was considered significant, and pairwise comparisons were then performed with Tukey's honestly significant difference (HSD) test.

RESULTS

Homogeneity testing of model food systems. The two model systems developed in this study were of identical composition, with the only difference being that *Salmonella* cells were introduced into the system via a different route of ingredient, either nonfat dry milk powder (M) or peanut butter (P). Our hypothesis was that the specific contaminating ingredient, i.e., the source of contamination, establishes a local microenvironment for the pathogen in a multi-ingredient food product, which influences pathogen behavior. Thus, it was crucial to ensure the homogeneity of the systems on the macroscale to make the argument that the effect of the local microenvironment, if proven to exist, was not simply due to insufficient mixing of the contaminated ingredient during food processing.

To evaluate the homogeneity of the system, the distribution of *Salmonella* cells in each mixture at a high or low inoculum level was tested in 10 samples at each level. The sampling variances at both high and low inoculum levels were far less than their respective critical values. At the high level, the sample critical values of MP-high (0.148) and PM-high (0.206) were higher than their respective sample variances of 0.004 and 0.015. At the low inoculum level, critical values for MP-low (0.098) and PM-low (0.082) were greater than their respective variances of 0.002 and 0.009. These determinations show that the mixtures passed the homogeneity test (Table 2). Moreover,

TABLE 2. *Homogeneity test of* Salmonella *inoculations in food models*[a]

Matrix	Mean *Salmonella* population (log CFU/g)	SD	Cochran test result	Sampling variance	Critical value	Max *z*-score	Result
MP-low	4.31	0.16	0.40	0.002	0.098	−0.4	Pass
PM-low	4.58	0.10	0.41	0.009	0.082	0.28	Pass
MP-high	6.13	0.09	0.40	0.004	0.148	0.16	Pass
PM-high	7.31	0.13	0.41	0.015	0.206	0.26	Pass

[a] MP, inoculation into nonfat dry milk powder, followed by mixing into peanut butter; PM, inoculation into peanut butter, followed by mixing into nonfat dry milk powder; high, high inoculum; low, low inoculum.

the absolute values of the maximum *z*-scores for all samples were between 0.16 and 0.4 (i.e., <2); thus, the homogeneity of *Salmonella* distribution was determined to be satisfactory (Table 2) *(11)*.

Effect of contamination source on *Salmonella* survival in model food systems. The a_w of the model system was 0.55. Immediately after inoculation, *Salmonella* exhibited different viability levels in systems MP and PM; a 2-log reduction was shown in system MP, and a 0.6-log reduction was the result in system PM (Fig. 1). The log reduction in MP was about fourfold greater ($P < 0.05$) than in PM. This pattern of survival was consistent when *Salmonella* was inoculated individually into M (2.5-log reduction; Control-M) and P (0.67 log reduction; Control-P) in a single-ingredient system ($P < 0.05$), suggesting that the immediate spatial location of the cells within the local microenvironment, as determined by the contaminated ingredient, influenced pathogen survival.

To study whether the impact of the microenvironment remained after a certain level of equilibration, the remaining mixtures were stored in sealed plastic bags at room temperature for 5 weeks. After 5 weeks of storage at 25°C, a twofold-greater log reduction ($P < 0.05$) was observed in the system with contaminated M (4-log reduction; MP and Control-M) than in the one in which P was the contaminating ingredient (2-log reduction; PM and Control-P) (Fig. 1).

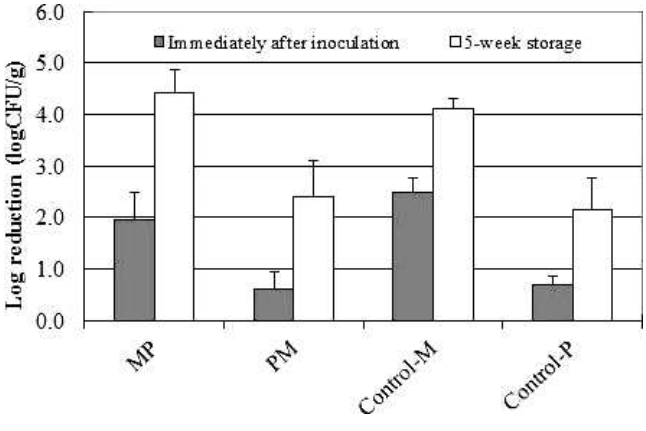

FIGURE 1. Salmonella *viability in model food systems both immediately after inoculation and after 5 weeks of storage. PM and MP have identical compositions but different sources of contamination. PM,* Salmonella*-contaminated peanut butter mixed with nonfat dry milk powder; MP,* Salmonella*-contaminated nonfat dry milk powder, mixed with peanut butter.*

A one-way ANOVA of the impact of sources of contamination on *Salmonella* survival showed a significant difference among four test conditions ($F_{[3,68]} = 153.4961$, $P = 3.3 \times 10^{-30}$). Tukey's HSD test showed a significant difference between the two test conditions MP and PM in which *Salmonella* was introduced into the system via M and P, respectively.

Nonhomogeneous physicochemistry of the model food system. Our initial attempt was to use *E. coli* K-12 in PBS as a surrogate for *Salmonella* Tennessee to demonstrate the possible distribution of the microbial cells within the food matrix. However, it was found that FT-IR spectroscopy was unable to differentiate whether identical chemical functional groups originated from food components or bacterial sources; thus, the location of the *E. coli* K-12 cells could not be directly identified by imaging. Instead, the FT-IR peak absorption of water was viewed as an indication of the spatial distribution of the population of hydrated bacterial cells. More importantly, FT-IR imaging was used to illustrate the phase distribution of water, lipid, and other nutrients. Regardless of the distribution of the pathogen, FT-IR microscopic image analysis quantified the nonhomogeneous physicochemical distribution in the model food system.

Figure 2A displays a single wave number ATR image of an area of 100 by 100 μm, in which the color spectrum represents absorption of the O-H stretching vibrations and intermolecular hydrogen bonding of water at 3,353 cm^{-1}, referenced to the absorption peak of pure water. The color scale represents the absorbance from low (purple) to high (red). Figure 2B and 2C display the lipid (1,743 cm^{-1}) and protein (1,654 cm^{-1}) distributions, respectively, at the same location as in Figure 2A, indicating that the physicochemical composition was nonhomogeneously distributed in the field examined. Applying atmosphere correction resolved the overlap of amide and water peaks. The resolution of the ATR imaging accessory at 3.1 μm is diffraction limited over most of the spectral range, and near-optimum oversampling was provided by utilizing a pixel spacing of 1.56 μm. Considering that those areas having the highest water absorption or lipid content measured greater than about 20 by 15 μm, and with reference to the 1- to 2-μm length of bacterial cells, different local microenvironments that were

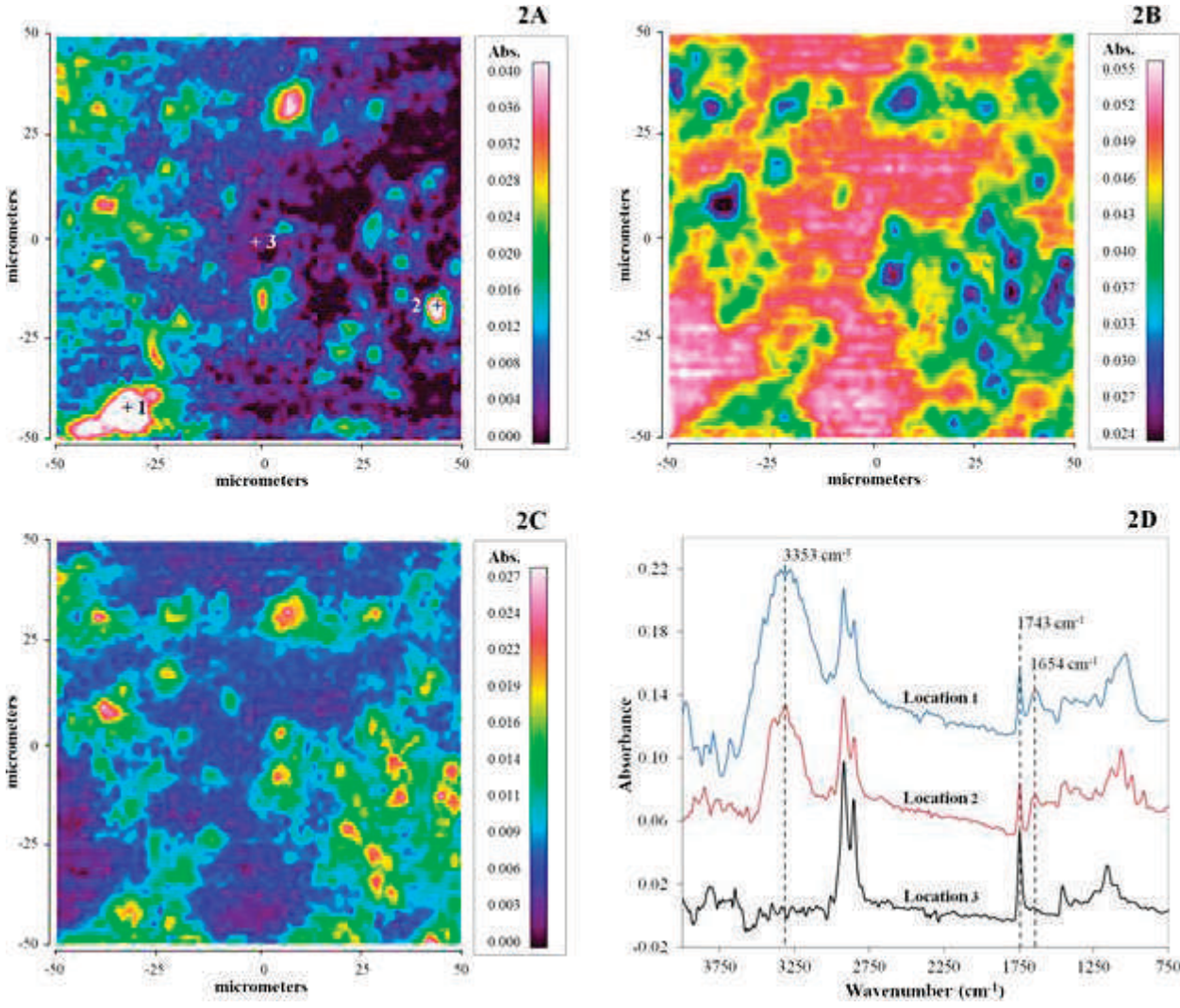

FIGURE 2. *ATR–FT-IR images (100 by 100 μm) of the food model system inoculated with* E. coli *K-12 acquired at single wave number values (A to C). The image color scale represents relative absorbance at the specific wave number values of 3,353 cm^{-1} (A), 1,743 cm^{-1} (B), and 1,654 cm^{-1} (C). The mid-IR spectra presented in panel D compare water absorption peaks at three different locations labeled in panel A. Dashed lines in panel D indicate the absorption maxima of water at 3,353 cm^{-1}, lipid at 1,743 cm^{-1}, and protein at 1,654 cm^{-1}.*

of sufficient size to accommodate a number of cells were identified.

Figure 2D semiquantitatively illustrates individual mid-IR spectra of water absorption peaks at 3,353 cm^{-1} at three spatial locations of interest labeled on the ATR image (Fig. 2A). A notable difference was presented at these three locations in the ATR image at peaks 3,353, 1,743, and 1,654 cm^{-1}, each representing water, fatty acid, and protein *(12, 20)*. The nonhomogeneous distribution of water, lipid, and protein was demonstrated at the micrometer scale by ATR imaging, suggesting that the homogenously distributed cells (Table 2) may actually reside in different microenvironments, which include phase separation and different a_w environments, thereby affecting pathogen survival.

Impact of microenvironment on *Salmonella* thermal inactivation efficacy. The effect of microenvironment on thermal resistance of *Salmonella* was also evaluated by measuring log reductions in both MP and PM after thermal treatment at 90°C for 10 min. Box-whisker plot graphs showed that the median log reduction for MP was 4.9 log and the 25th and 75th percentiles were 4.6 and 5.2 log, respectively, which was significantly different from the PM median log reduction of 3.2 log, with 25th and 75th percentiles of 1.8 and 3.6 log, respectively (Fig. 3). A one-way ANOVA showed the impacts of the specific ingredient in the immediate proximity of cells on the survival of *Salmonella* Tennessee between the two conditions ($F_{[1,12]} = 12.8$, $P = 0.003$). The statistical analysis suggested that the contamination source significantly affected the thermal resistance of the pathogen. In other words, the two ingredients that determined the *Salmonella* Tennessee local microenvironment made significantly different impacts on the pathogen's thermal resistance.

Behavior of *Salmonella* associated with different ingredients in butter cookies. To study the above phenomena in real multi-ingredient foods, a simple butter cookie formulation was used as a representative bakery food product. *Salmonella* was introduced into the cookies by

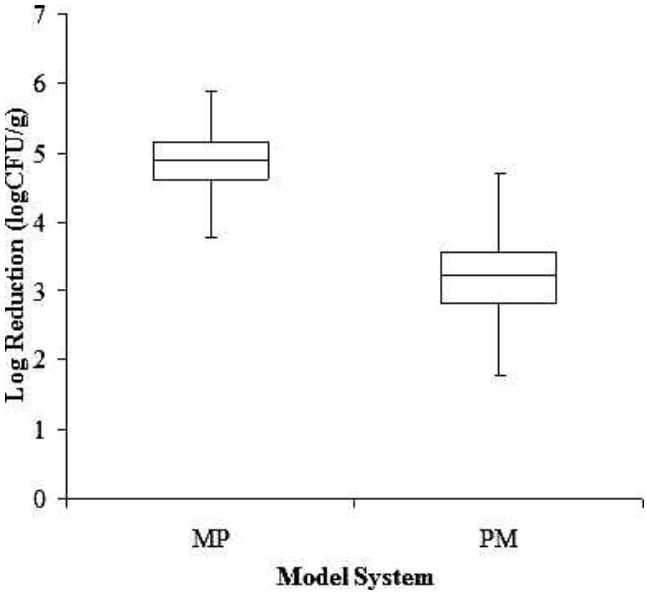

FIGURE 3. *Box-whisker plot of the log reduction of* Salmonella *in two model food systems after heating at 90°C for 10 min. The 75th percentile (top edge of the box), 25th percentile (bottom edge of the box), and median (line inside the box) provide an overview of data distribution. MP,* Salmonella*-contaminated nonfat dry milk powder mixed with peanut butter; PM,* Salmonella*-contaminated peanut butter mixed with nonfat dry milk powder.*

inoculating individual ingredients of egg, sugar, butter, or flour, separately. The a_w of the final cookie dough was approximately 0.8. Prior to baking, a significantly different log reduction of *Salmonella* Tennessee was observed at room temperature in the dough immediately after preparation, depending on the contamination source. Contamination via sugar led to the greatest log reduction (3.0 ± 0.1), followed by a <2-log reduction when *Salmonella* was introduced into the dough via butter, flour, or egg (Fig. 4A). A one-way ANOVA showed a significant impact ($F_{[3,11]} = 24.2$, $P = 3.8 \times 10^{-5}$) of the contamination source (ingredient) on *Salmonella* Tennessee survival in the cookie doughs. Tukey's HSD test showed that the microbial log reduction in the doughs made with contaminated sugar showed a significant difference from the reductions obtained when any of the other ingredients were contaminated.

The cookie doughs prepared under four conditions were then baked at 135°C (275°F), a temperature that is lower than normal baking and represents the temperature at a cold spot condition in baking, for 10 min. Cookies with flour as the contamination source showed higher log reductions (4.1 ± 0.4) than those obtained when other ingredients were contaminated (Fig. 4B). A one-way ANOVA showed an overall significant impact ($F_{[3,12]} = 4.65$, $P = 0.02$) of different contaminated ingredients on *Salmonella* Tennessee thermal resistance in cookies. Tukey's HSD test showed that baking caused a significantly greater microbial log reduction in cookies made with contaminated flour than that obtained with any of the other ingredient sources of contamination. There was no significant difference in microbial log reduction between any of the other test conditions. Clearly, different thermal inactivation efficacies of *Salmonella* Tennessee were associated with the four ingredient sources of contamination under this thermal treatment. These experiments confirmed that in a real food system, the local microenvironment affects the thermal inactivation and survival of *Salmonella*. Nonetheless, the impact of the local microenvironment was shown only for low-temperature baking; when the cookies were baked at 177°C (350°F) for 10 min, *Salmonella* was not detectable in any of the four test formulations.

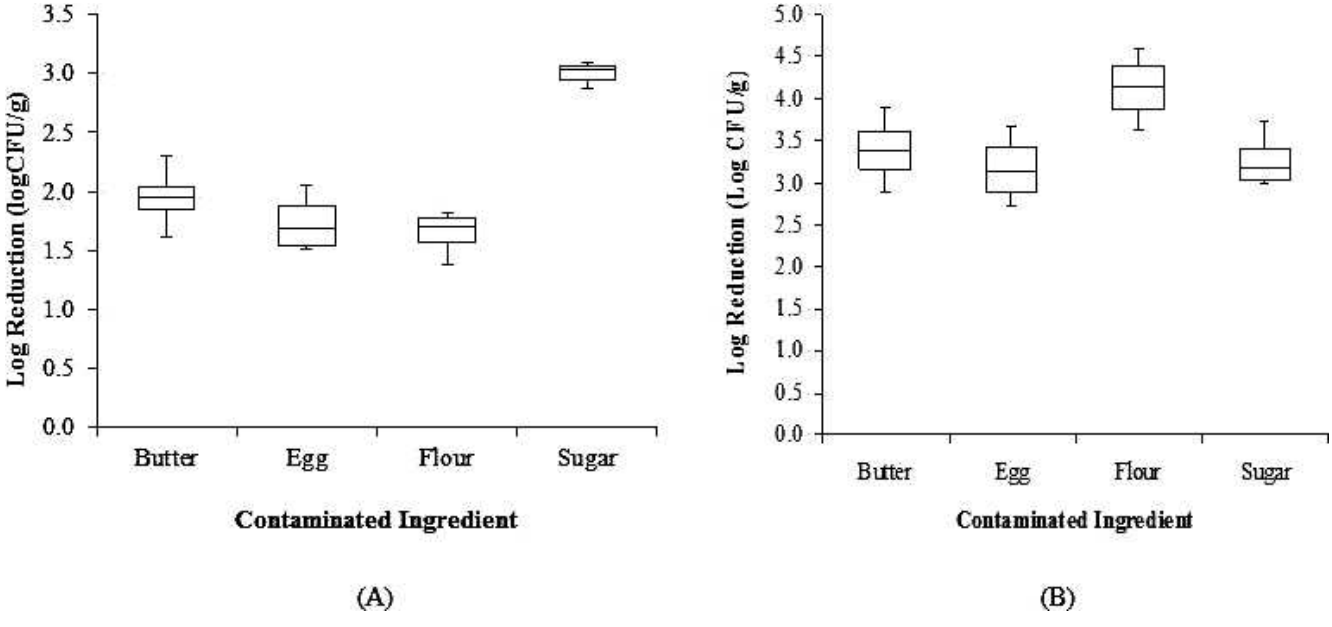

FIGURE 4. *Box-whisker plot of the log reduction of* Salmonella *in cookies containing different contaminated ingredients before (A) and after (B) baking at 135°C (275°F) for 10 min. The log reduction after baking (B) displayed the log difference of viable cells between the treated and nontreated samples. The 75th percentile (top edge of the box), 25th percentile (bottom edge of the box), and median (line inside the box) provide an overview of data distribution.*

DISCUSSION

This study provided the first evidence that the local microenvironment in low- and intermediate-moisture (a_w of 0.55 and 0.8, respectively) multi-ingredient foods critically affects *Salmonella* survival and thermal inactivation. When a contaminated ingredient is used in a multi-ingredient food, the immediate proximity (microenvironment) of bacterial cells at the microscale is determined by this specific ingredient. The comparable survival of *Salmonella* cells between system MP and its single-ingredient control M supports that the nonfat dry milk (M) formed a local microenvironment in system MP that was a factor in determining the pathogen behavior. This observation is also supported by the comparable viability between system PM and its single-ingredient control P. While it is unknown why a greater viability reduction was observed in *Salmonella* when in contact with nonfat dry milk powder (M) than with peanut butter (P), the different log reductions of *Salmonella* associated with the two ingredients allowed us to differentiate the role of each ingredient on pathogen survival. The different log reductions of *Salmonella* in systems MP and PM, which had identical material compositions but different contaminated ingredient sources, provide unmistakable evidence for the existence of the local microenvironment in multi-ingredient foods and its impact on *Salmonella* survival (Fig. 1).

The local microenvironments in a multi-ingredient food may contain different microbial nutrients and demonstrate water activities and solution dynamics different from the bulk properties of the food. Neighboring niches can be either immiscible or in a dynamic mass transfer to reach a moisture equilibrium. Figure 1 showed that survival rates of *Salmonella* Tennessee between model systems MP and PM remained different even after 5 weeks of storage, suggesting that the miscibility or mass transfer rate between neighboring niches during storage was too low to diminish the impact of the local microenvironment. The phase separation of the two ingredients, peanut butter (50% fat, 22% protein) and nonfat dry milk powder (0% fat, 41% protein), was demonstrated by the reciprocal absorption patterns between the ATR image at 1,743 cm^{-1} (Fig. 2B, lipid) and 1,654 cm^{-1} (Fig. 2C, protein). The ATR image at 1,743 cm^{-1} largely reflects the distribution of the peanut butter, since no absorption at this wave number in the nonfat dry milk powder was observed *(20)*. A similar pattern of phase separation between lipid and water was also supported by the reciprocal absorption patterns between the ATR images at 1,743 cm^{-1} (Fig. 2B) and at 3,353 cm^{-1} (Fig. 2A), respectively. In contrast, the miscibility between water and protein was supported by the relatively similar absorption distribution patterns between peak wave numbers of 3,353 cm^{-1} (Fig. 2A) and 1,654 cm^{-1} (Fig. 2C). The implication of the water and lipid phase separation can be illustrated by water microdroplets within the peanut butter matrix; i.e., cells residing in a higher-a_w location within a water microdroplet will experience different survival rates from those in areas with low a_w and high lipid content, even after a long storage time. Nonetheless, between neighboring spatial locations with highly miscible components such as milk protein and water, moisture migration into areas with a low a_w and high protein content may support the growth of a pathogen in an otherwise overall inhibitory environment. These results suggest the need to consider nonhomogeneous microenvironments for thorough risk assessment in the process design of low- and intermediate-moisture multi-ingredient foods.

Another finding from this study is that the thermal inactivation efficacy of *Salmonella* in the multi-ingredient food systems was also affected by the contamination source. In our model system, when *Salmonella* was inoculated into nonfat dry milk powder first and then mixed with peanut butter (MP) and thermally processed at 90°C for 10 min, an approximately 2-log-greater reduction resulted, compared with the system in which *Salmonella* was inoculated into peanut butter first (PM). This result may be due to a protective effect of the higher lipid content in peanut butter than the nonfat dry milk powder, which forms the local microenvironment in each respective system *(10, 13)*. In the butter cookie challenge study, when *Salmonella* was inoculated into different ingredients to simulate different sources of contamination, the thermal inactivation was significantly different among test conditions, despite the identical composition of the final formulations. This study delivered information that may be useful in process design and validation; i.e., process boundaries should reflect ingredients of highest risk in the formulation of multi-ingredient foods. For example, in butter cookies, baking time-temperature parameters that ensure a sufficient kill of *Salmonella* contaminating the dough via flour may not render a sufficient kill for a contamination originating in the egg or butter; thus, more-rigid process conditions may be required.

In summary, local microenvironments formed by specific ingredients within low- and intermediate-moisture multi-ingredient foods can impact pathogen survival. Therefore, hazard characterization and implementation of preventive controls should be performed with an understanding of the existence of local microenvironments in such foods. The ingredient that is able to form the most favorable local microenvironment for pathogen survival in the final product should be taken into account when determining the process boundary, whether for product development, processing validation, or reconditioning needs.

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