

# Survival of *Staphylococcus aureus* ATCC 13565 in Intermediate Moisture Foods is Highly Variable

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*Staphylococcus aureus* is a gram-positive, enterotoxin-producing coccus. It is a hardy organism and known to survive over a wide range of water activities, pH values, and temperatures. The objective of this study was to model the survival or gradual inactivation of *S. aureus* ATCC 13565 in intermediate moisture foods (IMFs). Various initial concentrations ( $\sim 10^1$ ,  $10^2$ ,  $10^3$ , and  $10^4$  CFU/g) were used to inoculate three different IMFs (beefsteak, bread, and chicken pockets). Viable counts were determined up to 60 days using tryptic soy agar. Inoculum size did not influence the survival or gradual inactivation of *S. aureus* in these foods. The rate of change (increase or decrease) in log CFU/day was calculated for every consecutive pair of data points and by linear regression for each inactivation curve. Both consecutive pair and linear regression rates of change were fit to logistic distributions (with parameters  $\alpha$  and  $\beta$ ) for each food. Based on the distribution parameters, survival or gradual inactivation of *S. aureus* was predicted by computer simulation. The simulations indicated an overall decline in *S. aureus* population over time, although a small fraction of samples in the consecutive pair simulation showed a slight population increase even after 60 days, consistent with the observed data. Simulation results were compared to predictions from other computer models. The models of Stewart *et al.*<sup>(1)</sup> were fail-safe, predicting the possibility of significant growth only after >3,000 days. The USDA pathogen modeling program predictions were found to be fail-dangerous, predicting declines at least four times faster than observed.

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**KEY WORDS:** *Staphylococcus aureus* ATCC 13565; intermediate moisture foods; probability distribution functions; modeling; simulation

## 1. INTRODUCTION

Intermediate moisture foods (IMFs) are semi-moist foods that have some of their water bound by glycerol, sorbitol, salt, or certain organic acids, thus preventing the growth of many microorganisms.<sup>(2)</sup>

IMFs do not require refrigeration to prevent microbial deterioration because of these low water activities. Preservation occurs partially from the high osmotic pressure associated with the high-solute concentration; an additional preservative effect is contributed by salt, acid, and other specific solutes in some foods.

*Staphylococcus aureus* is an important food-borne pathogen, and staphylococcal food poisoning ranks as one of the most prevalent causes of gastroenteritis worldwide.<sup>(3)</sup> The CDC reports that staphylococcal food poisoning accounts for an estimated 185,060 illnesses, 1,753 hospitalizations, and two deaths in the United States each year.<sup>(4)</sup> *S. aureus* is extremely tolerant of low water activities ( $a_w$ ),

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and its growth has been reported at  $a_w$  values as low as 0.83.<sup>(5)</sup> It can also grow over broad pH (4.8–9.3) and temperature (7–43°C) ranges.<sup>(6)</sup> Acott *et al.*<sup>(7)</sup> showed that a related organism, *S. epidermidis*, grew in dog food with an  $a_w$  level as low as 0.82. Labuza *et al.*<sup>(8)</sup> challenged IMFs (prepared either by freeze drying or direct mixing and then allowing absorption of moisture to desired level) with various microorganisms. *S. aureus* was found to grow in the direct mix food at 25°C at  $a_w$  0.84, while cell death was observed at the same  $a_w$  in the freeze-dried food.

Many predictive models do not include the effect of inoculum size as a factor because the inoculum size often does not appear to affect growth kinetics,<sup>(9–12)</sup> but both Augustin *et al.*<sup>(13)</sup> and Robinson *et al.*<sup>(14)</sup> have reported an inoculum size effect on the lag phase duration of *Listeria monocytogenes* with cells that are severely stressed. These authors suggest that when bacteria are forced to grow at suboptimal conditions, their survival is more likely to be affected by inoculum size than is their growth in an optimal environment. A similar effect, therefore, might be expected in survival or gradual inactivation of *S. aureus* in intermediate moisture foods, which have suboptimal water activity levels. Our study is the first to look at the effect of inoculum size on the survival or gradual inactivation of *S. aureus* under adverse conditions in intermediate moisture foods.

## 2. MATERIALS AND METHODS

### 2.1. Rations

All studies were done on three ready-to-eat intermediate moisture rations (beefsteak pH 5.03,  $a_w$  0.732; bread pH 4.88,  $a_w$  0.861; chicken pockets pH 4.97,  $a_w$  0.853) obtained from the U.S. Army Research Development and Engineering Center (Natick, MA). Additional information on product composition is available from this organization. The pH and water activities of the rations were measured by Silliker Laboratories (Garwood, NJ). Association of Official Analytical Chemists (AOAC) reference method 978.18 was used to measure the water activity of all three rations.<sup>(15)</sup> The pH of beefsteak and chicken pockets was measured using the method described in the USDA Microbiology Laboratory Guidebook;<sup>(16)</sup> the pH of bread was measured using reference method American Association of Cereal Chemists method 02-52.<sup>(17)</sup>

### 2.2. Microorganism

*Staphylococcus aureus* ATCC 13565 (also known as FDA 196E) was used for all studies as it is the type

strain for enterotoxin A production<sup>(18)</sup> and has been well studied.<sup>(1,19–21)</sup>

### 2.3. Preparation of Inoculum

The culture was stored on tryptic soy agar (TSA, Difco Laboratories, Detroit, MI) slants at 4°C and transferred monthly to fresh TSA slants. A loopful of culture was transferred to 8 ml of sterile brain heart infusion (BHI) broth (BBL, Cockeysville, MD) and incubated overnight at 37°C at 100 rpm for each experiment. BHI is the preferred medium of choice for growing *S. aureus*.<sup>(20)</sup> After incubation, the culture was washed with 0.05M-phosphate buffer, pH 7.0 (10.84 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 32.72 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 1 l distilled water). To wash the cells, 8 ml of culture from the BHI broth was dispensed into 8 1-ml Eppendorf tubes, centrifuged at 5000–6000 rpm for 5 minutes, the supernatant discarded, and the cell pellets resuspended in 1 ml 0.05 M phosphate buffer. This entire series of steps was repeated twice, and after the second wash, the cells were resuspended in 0.05 M phosphate buffer and pooled. The OD was measured using a Spectronic 501 spectrophotometer (Model UV160; Shimadzu Scientific Instruments, Columbia, MD) at 660 nm. The optical density was adjusted to 0.4–0.6 corresponding to about  $1.0 \times 10^8$  CFU/ml. Serial 10-fold dilutions were made using 0.05 M phosphate buffer to reach the desired final concentration.

### 2.4. Preparation and Inoculation of Food Samples

The rations were divided into 20 g samples. At least 10 samples of 20 g each were inoculated with 0.1 ml of culture to yield a final concentration of  $\sim 10^1$ ,  $10^2$ ,  $10^3$ , or  $10^4$  CFU/g of food. Preliminary experiments showed that inoculum recovery was acceptable and reproducible. For all three rations, replicate experiments were done for each inoculum size. Our analysis showed that the concentrations recovered from the 12 lowest inoculum sizes were always within 0.5 log CFU of the desired concentration, and that replicate samples (for all sampling times) were always within 0.3 log CFU of each other. All samples were incubated at 23°C because it is representative of typical room temperature. The beefsteak and bread samples were surface inoculated while the chicken pockets were inoculated at the meat/bread interface.

### 2.5. Sampling

At time zero and at periodic intervals (3–4 days), 20 ml of 0.05 M phosphate buffer was added to one

sample of each ration. Both bread and chicken pockets were homogenized for 30 seconds in a stomacher lab blender 400 (Cooke Laboratories, Alexandria, VA) and diluted using 0.05 M phosphate buffer. Beefsteak samples were vortexed with 20 ml buffer for 30 seconds before 0.1 ml was spread plated. The pour plate technique was employed for an inoculum size of 10 CFU/g, using 1 ml sample and mixing with 15 ml warm molten TSA. All cultures were incubated at 37°C for 24 hours and then counted manually using backlit illumination. All plating was carried out in duplicate. Colonies were periodically confirmed as *Staphylococcus aureus* by gram staining. Preliminary experiments revealed that uninoculated samples were free of any other microbes. Periodic screening revealed that any infrequently occurring atypical colonies were not *S. aureus*, and these colonies were not counted in the data analysis.

## 2.6. Analysis of Data

A plot of log CFU/g versus time (in days) was created for each experiment using Excel (Microsoft, Redmond, WA). Replicate samples were averaged, while results from each experiment are presented separately. The rate of change in log CFU/day was calculated between every consecutive pair of data points. Since measurements were taken less frequently than once a day, a single rate was calculated and then converted to change in log CFU/day by assuming log-linear death kinetics.

The values obtained for change in log CFU/day for each ration were fit to a probability distribution using Bestfit (Palisades Corp., Newfield, NY). Another set of three distributions (one for each ration) was created from calculated average rates of decline (as determined by linear regression) for each experiment, also using BestFit. The logistic distribution was always ranked among the top three distributions by chi-squared and Anderson-Darling goodness of fit test statistics.

One limitation of the consecutive pairs method is that the variance of the differences incorporates the covariance of the error terms. Unless the covariance term is zero, any probability distributions based on this method may be confounded by this covariance. Analysis of this particular data set showed that the null hypothesis (correlation between subsequent error terms) could not be rejected ( $r^2 = -0.003$ , the covariance is approximately zero); thus the consecutive pairs method could be used in this case.

## 2.7. Simulation

Two computer simulations were developed using the add-in simulation software @Risk (Palisades Corp., Newfield, NY) for Excel. The simulations were used to predict the decline of *S. aureus* over time in the intermediate moisture foods used in this research. An initial inoculum size of  $1.0 \times 10^2$  CFU/g was chosen to start each simulation. One simulation used the distribution of rates derived from the consecutive pairs of points, while the other used the distribution obtained from linear regression.

The simulations were run at least 1,000 times to predict *S. aureus* concentration from zero to 48 days. The simulation generated a random decline rate for each simulated three-day period based on the logistic distribution parameters for each food.

## 2.8. Comparison with Models

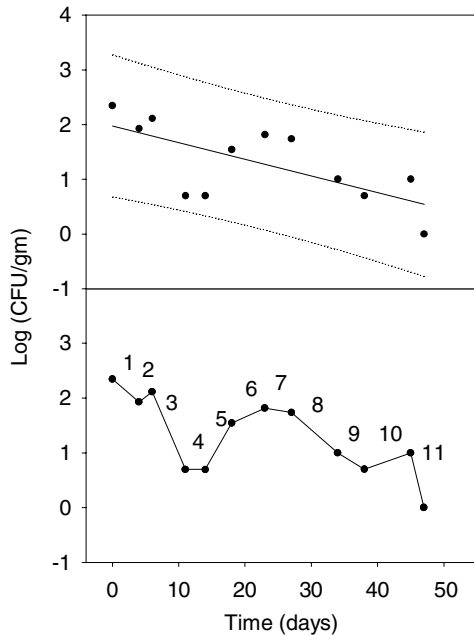
Stewart *et al.*<sup>(1)</sup> created a growth boundary model to study the effect of RH, initial pH, and preservatives on the aerobic growth of a five-strain *S. aureus* cocktail. This model was used to make predictions for *S. aureus* behavior in beefsteak, bread, and chicken pockets. The concentrations of calcium propionate or potassium sorbate in the models were set to 0 ppm, as none of the three rations contained these preservatives. The pathogen-modeling program (PMP) was also used to predict inactivation rates for *S. aureus* for the three rations. The sodium nitrite and lactic acid concentrations in the PMP were set to 0 ppm.

## 3. RESULTS

### 3.1. Effect of Inoculum Size on Mean of Rate of Change

Inspection of each plot of change in log (CFU/g) versus time showed that survival curves were highly variable, but generally declined with time. One representative plot is shown in Fig. 1. The top panel of this figure shows each of the 12 observations made in this experiment, superimposed on the line of best fit, using simple linear regression. Ninety-five percent prediction intervals are also shown as dotted lines. It is important to note that although the  $r^2$  value in this example is low (0.47), the correlation between cell concentration and time is still quite strong ( $p = 0.01$ ), indicating that time has a strong influence on the number of viable cells remaining.

The bottom panel of this figure shows the same data points, this time with each pair of consecutive



**Fig. 1.** Representative plot for changing population of *Staphylococcus aureus* cells in an intermediate military ration. Top panel shows each of the 12 observations, superimposed on the line of best fit from simple linear regression. Bottom panel shows same data grouped into numbered, consecutive pairs of points.

data points connected by a line, and each line segment numbered, so that point 1 and point 2 are connected by line segment 1, point 2 and point 3 are connected by line segment 2, and so forth. Since each observation is made from a separate sample, each consecutive pair of samples can be thought of as constituting a single observation of the rate of decline (or increase) in the population of organisms. The top panel shows the conventional approach, leading to a single estimate of the rate of decline, while the bottom panel shows an alternative approach leading to nine estimates of the rate of decline.

A summary of the regression analysis for all three food products is shown in Table I. It is clear from the data shown in this table that neither goodness of fit ( $r^2$ ) nor rate of decline is correlated with the initial inoculum concentration. Both analysis of variance and regression analysis (Excel, Microsoft) also showed no statistically significant correlation between inoculum concentration and rate of decline (ANOVA  $F = 1.195$ ,  $r^2 = 0.006$ ) or  $r^2$  (ANOVA  $F = 0.045$ ,  $r^2 = -0.033$ ).

The logistic distribution parameter values are shown in Table II. The mean of the logistic distribution (alpha) is similar no matter which method is used to create the distribution (consecutive pairs or linear re-

**Table I.** Summary of Simple Linear Regression Analysis Results for the Survival of *Staphylococcus aureus* in Three Intermediate Moisture Foods

Product	Initial Concentration (Log CFU/g)	Rate of Decline ( $\Delta$ Log CFU/g/day)	$r^2$	
Beefsteak	1.33	-0.0065	0.1668	
	2.02	-0.0477	0.8665	
	2.30	-0.0143	0.5681	
	2.32	-0.0305	0.4706	
	3.02	-0.0215	0.3865	
	3.04	-0.0354	0.6634	
	3.61	-0.0282	0.7939	
	4.90	-0.0086	0.1437	
	4.75	-0.0027	0.0092	
	Bread	0.48	0.0164	0.3107
		1.00	-0.0215	0.2743
1.96		0.0167	0.3546	
2.70		-0.0355	0.9639	
2.21		-0.0511	0.8273	
2.29		-0.0388	0.5088	
3.05		0.0013	0.0016	
3.12		-0.0194	0.3731	
3.59		-0.0539	0.6685	
3.69		-0.0853	0.8233	
4.50		-0.0349	0.7001	
Chicken pockets	1.10	-0.0160	0.4824	
	1.43	-0.0111	0.7884	
	2.08	0.0029	0.0118	
	2.61	-0.0037	0.0171	
	2.70	-0.0278	0.7004	
	2.88	-0.0046	0.0586	
	2.94	-0.0490	0.7766	
	3.44	-0.0234	0.7543	
	3.96	-0.0058	0.1779	
5.10	-0.0152	0.4450		
5.16	-0.0248	0.7568		

gression), but the beta parameter (which is correlated with the variance) is quite different, generally being about an order of magnitude smaller when linear regression is used to create the distributions. The goodness of fit (as measured by the Anderson-Darling test statistic) is similar between rations for either method. The consecutive pairs method produces higher test statistic values than the linear regression method, but as a different number of observations was used to calculate the test statistic for each method, the numbers cannot be directly compared.

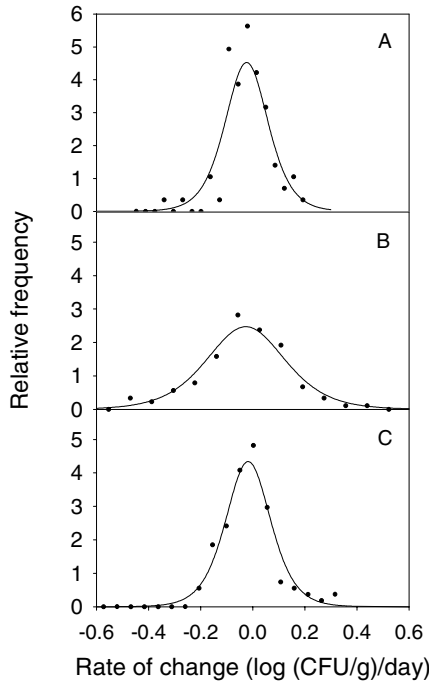
### 3.2. Probability Distributions for *S. aureus* Rate of Change

Fig. 2 shows the probability distributions for the consecutive pairs, pooled rate of change in *S. aureus* population in beefsteak (A), bread (B), and chicken pockets (C) for all inoculum sizes. A logistic

**Table II.** Comparison of Logistic Distribution Parameters for the Survival of *Staphylococcus aureus* in Three Intermediate Moisture Foods Based on Either Consecutive Pairs of Points or Linear Regression

Ration	Consecutive Pairs			Linear Regression		
	Alpha	Beta	A-D*	Alpha	Beta	A-D
Beefsteak	-0.023546	0.055232	0.8276	-0.021186	0.008530	0.2320
Bread	-0.026894	0.101110	0.6570	-0.028122	0.017241	0.2509
Chicken pockets	-0.017602	0.057600	0.6199	-0.014954	0.007746	0.2582

\*Anderson-Darling test statistic.



**Fig. 2.** Probability distribution of the rate of change in *S. aureus* population in beefsteak (A), bread (B), and chicken pockets (C). The solid circles represent the histogram of actual changes in log CFU/day, and the line is the logistic distribution fit to the data from each ration.

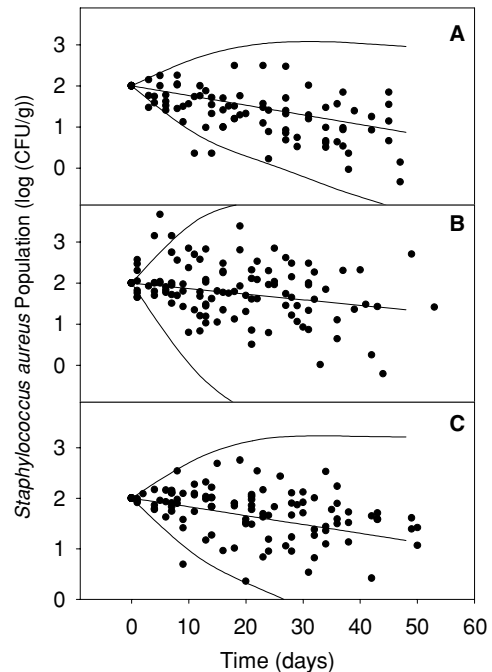
distribution was the best-fitting distribution for all three data sets and has been used previously to model microbial survival.<sup>(22)</sup> A comparison of the distributions (Fig. 2) demonstrates that the median rate of change of log (CFU/g)/day is not very different for the three rations used (although the variability is significantly greater in bread). This may indicate that *S. aureus* survival is not particularly affected by the food composition in the ranges found in these foods.

**3.3. Comparison of Data and Consecutive Pair Simulations**

Actual data and consecutive pair simulation predictions are shown in Fig. 3. The raw data (normalized

to an initial concentration of 10<sup>2</sup> CFU/g) for beefsteak (Fig. 3A) are generally bounded by the highest and lowest values obtained in the simulation, although some of the lowest observed values are below the lowest observed simulation results. This is likely due to the fact that the logistic distribution for decline in beefsteak (Fig. 2A) does not encompass some more extreme decline values. One solution to this problem would be to use a combined logistic-uniform distribution as detailed elsewhere,<sup>(22)</sup> which would increase the probability for occurrence of extreme declines.

The situation for bread (Fig. 3B) is the reverse, where most raw data points are encompassed by the lowest observed simulation results, while a number

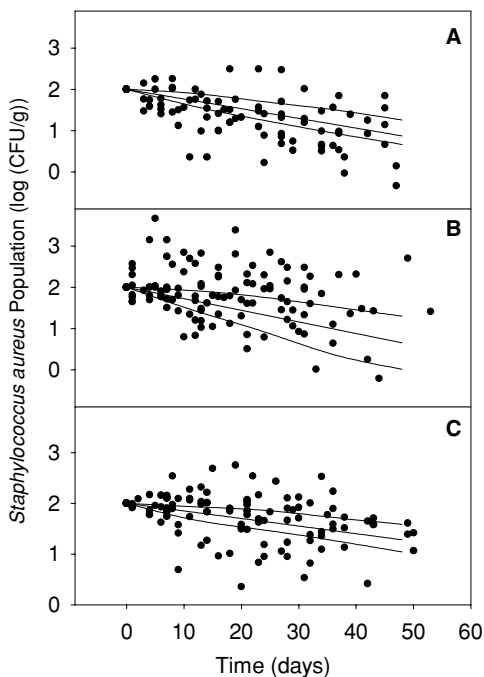


**Fig. 3.** Comparison of raw data (normalized to 10<sup>2</sup> CFU/g initial concentration) for *Staphylococcus aureus* in beefsteak (A), bread (B), and chicken pockets (C) with consecutive pair simulation predictions where lines represent lowest, average, and highest simulation results.

of data points between 0 and 20 hours are above the highest results observed in the simulation. Nevertheless, most of the observed data are within the bounds of the simulation. Most of the chicken pockets (Fig. 3C) raw data lie within the bounds of the simulations, although one point does fall below the lines indicating the simulation bounds.

### 3.4. Comparison of Data and Linear Regression Simulations

The data and linear regression simulation predictions shown in Fig. 4 are markedly different from those seen for the consecutive pair simulations. Although the central tendency lines are very similar to those seen in the consecutive pair simulations, the upper and lower bounding lines are considerably closer than in Fig. 3, and are able to encompass only a small fraction of the actual data points. Fig. 4 provides a compelling endorsement for using the consecutive pairs approach for determining microbial inactivation variability instead of linear regression.



**Fig. 4.** Comparison of raw data (normalized to  $10^2$  CFU/g initial concentration) for *Staphylococcus aureus* in beefsteak (A), bread (B), and chicken pockets (C) with linear regression simulation predictions where lines represent lowest, average, and highest simulation results.

### 3.5. Comparison to Published Models

Predicted values for time-to-growth (TTG) in days derived from the two models developed by Stewart *et al.*<sup>(1)</sup> were extremely large (thousands of days or more) for all three rations. An increase in TTG is expected as conditions become less favorable for growth. The largest TTG value was observed for beefsteak, which had the lowest water activity (0.732) of the three rations used. Table III displays the predicted number of days needed to achieve a one log decline (D value) in *S. aureus* according to the pathogen-modeling program (PMP). The fastest decline is seen at the lowest water activity level allowed in the PMP *S. aureus* model (0.834). The fastest decline in our study was seen in beefsteak, which had the lowest water activity (0.732). The predicted D values from the @Risk simulations were always longer than the values predicted by the PMP.

## 4. DISCUSSION

Research involving inoculum size generally seems to indicate that there is no inoculum size effect on the lag phase duration (LPD), maximum population density (MPD), and exponential growth rate (EGR) of vegetative cells studied in both broth and food systems.<sup>(12,23–25)</sup>

Augustin *et al.*<sup>(13)</sup> reported that for *Listeria monocytogenes* growing at low nutrient concentrations and suboptimal pH, the lag time was extended when inoculum size was very small. Stephens *et al.*<sup>(26)</sup> have also reported longer lag periods with considerable variability in lag times for heat-injured *Salmonella* at very low inoculum levels. This variability was reduced significantly at inoculum levels greater than 100 cells/ml. These results also indicate that inoculum size affects the LPD during recovery from stress. Jackson and Woodbine<sup>(27)</sup> pointed out that if staphylococci were subjected to sublethal heat treatment, they showed a delayed lag phase, accompanied by a fall in viable count before growth was observed. One logical interpretation would be that if cells are under stress, then their behavior might be affected by a change in inoculum size. The inoculum size effect could be explained by an increase in the variation of individual cells' lag times when cells are stressed.<sup>(28)</sup> Baranyi used a stochastic approach to describe how the lag distribution of cells relates to the deterministic population lag.<sup>(29)</sup> He based his formula on physiological state of cells rather than the traditional definition of the population lag of bacterial growth curves.

**Table III.** D Values (Time for One-Log CFU/gm Decline) for *Staphylococcus aureus* Predicted by the Pathogen-Modeling Program and Simulations

Food	pH		$a_w$		D Value (Days)	
	Measured in Food	Used for PMP Prediction	Measured in Food	Used for PMP Prediction	Based on	
					Paired Points Simulation	Based on PMP Prediction
Beefsteak	5.0	5.0 <sup>a</sup>	0.732	0.834 <sup>aw</sup>	51.0	11.6
Bread	4.9	4.9	0.861	0.861	73.0	14.1
Chicken pockets	5.0	5.0	0.853	0.853	115.0	14.5

<sup>a</sup>This is the lowest  $a_w$  allowed in the PMP.

According to this approach, when cells are stressed, there would be a greater variation in their individual physiological states and this would consequently affect the population lag. Our results show no concomitant effect in stressed *S. aureus* cells undergoing slow inactivation due to environmental stress.

For all three rations, the range of expected *S. aureus* populations widened as storage time increased (Fig. 3). The data show the presence of elevated counts (above the initial concentration), in some cases after even 50 days (Fig. 3 or 4). The simulations predict elevated counts up to and beyond 50 days for all rations. These results are similar to the observations made by Duffy and Schaffner<sup>(22)</sup> regarding simulation of *E. coli* O157:H7 during storage of cider. They also observed that the distribution of results in the simulation became wider and shifted lower over time.

When the simulation results were compared to the predictions made by the PMP, the D-value predictions of the PMP were always lower than the predictions made by our simulation. This indicates that the PMP predictions may be fail-dangerous because they predict the pathogen will become inactivated faster than actually observed in these foods. Our simulations were based on data obtained from challenge studies performed in the laboratory using real food systems, whereas the PMP is based on experiments carried out in aqueous laboratory medium. This difference may help to explain why the inactivation rate based on the simulations was much slower.

It was not surprising that the time-to-growth (TTG) values predicted by models from Stewart *et al.*<sup>(1)</sup> were extremely large (on the order of thousands of days). The TTG values predicted by the models indicate that it would take a very long time (if ever) for *S. aureus* to grow in the food used in our study. These predictions support our results, since the organism did not grow in our experiments. These results, in conjunction with the simulation results (which

showed a decline in *S. aureus* population over time), strongly suggest that these foods do not support the growth of *S. aureus* ATCC 13565 (even at high inoculum sizes) and are therefore a good choice to be used as military rations. Our results also suggest that even if these foods become contaminated with high ( $\sim 10^4$  CFU/g) levels of *S. aureus*, the population is likely to decline with time, not grow to population densities where it could produce toxin, and thus poses a negligible public health risk.

This study is the first of its kind to look at the effect of inoculum size on the survival rate of *Staphylococcus aureus* ATCC 13565 in intermediate moisture foods. We found that inoculum size does not affect survival rate of *S. aureus* in three representative intermediate moisture foods. Our results indicate that these products may not be adversely affected by contamination with even large numbers of *S. aureus*.

The fact that an apparent short-term increase in cell numbers occurs at low inoculum sizes (even under conditions where most cells are inactivated) is of interest. It is important to study this response and especially the variability surrounding it. Such studies would be useful in assessing the risk posed by these products. Survival modeling is an important but understudied part of predictive food microbiology. More research is needed to validate the simulation results presented in this study and to develop standardized survival modeling protocols.

## 5. CONCLUSIONS

Simulated behavior of *S. aureus* at room temperature in intermediate moisture foods supported our findings that *S. aureus* does not grow in three such low moisture foods. Although the distributions showed a general decline in *S. aureus* numbers over a period of two months, occasionally occurring actual and simulated elevated counts are a tribute to the hardiness of *S. aureus* and its ability to survive in these

low moisture foods. Our results show the value in using probability distributions derived from consecutive pairs of points, rather than linear regression, to model survival of pathogens in foods.

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