Journal of Food Protection, Vol. 64, No. 1, 2001, Pages 51–57
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Modeling the Growth Boundary of *Staphylococcus aureus* for Risk Assessment Purposes

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MS 00-83: Received 9 March 2000/Accepted 2 August 2000

ABSTRACT

Knowing the precise boundary for growth of *Staphylococcus aureus* is critical for food safety risk assessment, especially in the formulation of safe, shelf-stable foods with intermediate relative humidity (RH) values. To date, most studies and resulting models have led to the presumption that *S. aureus* is osmotolerant. However, most studies and resulting models have focused on growth kinetics using NaCl as the humectant. In this study, glycerol was used to investigate the effects of a glass-forming nonionic humectant to avoid specific metabolic aspects of membrane ion transport. The experiments were designed to produce a growth boundary model as a tool for risk assessment. The statistical effects and interactions of RH (84 to 95% adjusted by glycerol), initial pH (4.5 to 7.0 adjusted by HCl), and potassium sorbate (0, 500, or 1,000 ppm) or calcium propionate (0, 500, or 1,000 ppm) on the aerobic growth of a five-strain *S. aureus* cocktail in brain heart infusion broth were explored. Inoculated broths were distributed into microtiter plates and incubated at 37°C over appropriate saturated salt slurries to maintain RH. Growth was monitored by turbidity during a 24-week period. Toxin production was explored by enterotoxin assay. The 1,280 generated data points were analyzed by SAS LIFEREG procedures, which showed all studied parameters significantly affected the growth responses of *S. aureus* with interactions between RH and pH. The resulting growth/no growth boundary is presented.

Staphylococcus aureus is one of the leading causes of foodborne illness and is ranked as one of the most prevalent causes of gastroenteritis worldwide *(12),* even though the occurrences of this foodborne disease are grossly underreported. *S. aureus* was determined to be the etiological agent in 367 (19.6%) of 1,869 documented bacterial foodborne outbreaks in the United States *(1)*. Approximately 25 major outbreaks of *Staphylococcus* food poisoning occur annually in the United States *(10). S. aureus* has been estimated by the Centers for Disease Control and Prevention to cause 185,060 illnesses, 1,753 hospitalizations, and 2 deaths per year in the United States, all of which are via consumption of contaminated foods *(18).* Because of its prevalence as a food poisoning organism, *S. aureus* has been extensively studied to determine the physical and chemical parameters that affect its growth and toxin formation *(3, 4, 11, 12).*

S. aureus is ubiquitous to the mucous membranes and skin of warm-blooded animals. It is a poor competitor with other bacteria and is easily destroyed by cooking temperatures; however, its toxins can survive heat processing equivalent to that given to low-acid canned foods *(11).* Staphylococcal food poisoning frequently occurs when food is contaminated after cooking by a person carrying the organism, and subsequently the food is temperature abused for several hours, which allows for toxin production before consumption. It is the ingestion of the toxin that causes the foodborne disease.

S. aureus is highly salt tolerant and has been reported to grow at relative humidities (RHs) as low as 85% in NaCl concentrations up to 25% (wt/wt) *(11).* The RHs limiting growth are typically higher when humectants other than NaCl are used to control RH *(11).* Notermans and Heuvelman *(19)* reported that at a water activity of 0.98 to 0.93, sucrose was more favorable for growth than NaCl, but at a water activity of 0.87, NaCl was more favorable. Marshall et al. *(17)* reported that glycerol inhibition of the growth rate of *S. aureus* was about 10% greater than that caused by NaCl at water activity levels between 0.96 and 0.90.

Empirical microbiological models can be designed to predict how microorganisms relate to the environment. Predictive models are typically broadly applied for the screening of products and/or processes before challenge or shelf-life studies. However, it must be remembered that models are only tools to be used to help design products with food safety concerns addressed at the beginning of the development process and that there must be validation of the system through challenge or shelf-life studies with the real product. Both the U.S. Department of Agriculture Pathogen Modeling Program (PMP) and the Food MicroModel (FMM) developed in the United Kingdom through the Ministry of Agriculture, Fisheries and Food include *S. aureus* growth kinetic models. The PMP models the effects of temperature, initial pH, NaCl concentration, and sodium nitrite

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concentration in a broth system on aerobic and anaerobic growth kinetics of the organism *(7)*. The FMM models the growth responses as affected by NaCl concentration, pH, and storage temperature in a model broth system *(29)*. The PMP also includes a model for the survival of *S. aureus* in nongrowth conditions, which was developed from a model broth system that includes the effects and interactions of pH controlled by lactate buffer, lactic acid concentration, NaCl concentration, sodium nitrite concentration, and varying temperatures *(31)*. For all of the above-mentioned models, NaCl was the humectant chosen to control the RH in the system, and the resulting models are kinetic in nature and, therefore, do not allow the boundary for growth/no growth to be estimated. In contrast to kinetic modeling, probability modeling focuses on determining if the microorganism of concern will or will not grow, in other words, determining the growth/no growth interface *(21)*. This becomes increasingly important when pathogens are of concern, because the rate of growth may be less important than the fact that the organism is present and may be able to grow to an infectious dose or produce toxins *(21)*. In this study, the statistical effects and interactions of RH controlled by glycerol, initial pH, and potassium sorbate or calcium propionate on the boundary for *S. aureus* growth were modeled.

MATERIALS AND METHODS

Organisms and media preparation. The five *S. aureus* strains used to create the bacterial cocktail used in this study were *S. aureus* ATCC 13565 (American Type Culture Collection, Rockville, Md.), which produces staphylococcal enterotoxin A (SEA), ATCC 14458 (SEB), and ATCC 27664 (SEE); D-2 (SED) obtained from Toxin Technology, Inc. (Sarasota, Fla.); and A-100 (SEA) obtained from U.S. Army Natick Labs (Natick, Mass.). All strains showed typical growth on Baird-Parker agar plates and were coagulase positive. Their identification as *S. aureus* was confirmed by use of a Riboprinter (Qualicon, Wilmington, Del.). Stock cultures were grown overnight at 37°C in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.), suspended in a 2:1 broth-glycerol solution, and stored at −80°C until needed.

The BHI broth was reconstituted with the appropriate ratio of water to glycerol to achieve the desired RHs for the study (84, 88, 92, or 95% RH). Appropriate amounts of 10% stock solutions of potassium sorbate (Hoechst, Frankfurt, Germany) or calcium propionate (Aldrich Chemical Co., Milwaukee, Wis.) were added to achieve final concentrations of 500 or 1,000 ppm. The pH was adjusted using 0.1 or 1.0 N HCl (J. T. Baker, Inc., Phillipsburg, N.J.; pH 4.5, 5.0, 6.0, or 7.0). Final RH was determined using the Aqualab CX-2 water activity meter (Decagon Devices Inc., Pullman, Wash.). It was determined that the RH of the media did not change more than ±0.003% when measured at ambient temperature versus 37°C. Each of the 80 broths was filter sterilized and stored in screw-capped tubes at 4°C until needed.

Bioscreen microtiter plate preparation, incubation, and measurements. The five stock cultures were removed from −80°C storage, and one loopful was transferred into 9 ml of BHI broth, separately. The inoculated broths were incubated for 18 h at 37°C. The five cultures were individually adjusted to an optical density (OD) at 530 nm of 0.750 to 0.780 (Perkin-Elmer 35 spectrophotometer) with 0.1% sterile peptone to achieve a concentration of approximately 10^8 CFU/ml. Two milliliters of each diluted culture was transferred into one sterile test tube and vortexed to create the *S. aureus* cocktail used in the studies. Three 1:10 dilutions with sterile 0.1% peptone water were used to make a final working cocktail with a concentration of approximately 10^5 CFU/ml. This working cocktail was kept on ice until used to inoculate the various media used in each experiment. A 1-ml sample of the working cocktail was taken, serially diluted, spread plated onto Baird-Parker agar plates, incubated at 37°C for 48 h, and counted to determine initial concentrations of cells.

Ten milliliters of each of the 80 broths as described above was aseptically transferred to sterile tubes, and 100 μl of the *S. aureus* cocktail was added to the broth to achieve an initial concentration of approximately 10^3 CFU/ml. The inoculated broth (400 μl) was aseptically transferred into sterile, 100-well microtiter plates in eight replicate wells per medium. The outer wells of the microtiter plates were filled with uninoculated medium to act as a partial moisture loss barrier and as uninoculated controls. Each plate contained media at one RH level only. A piece of sterile Thermaseal film was placed on top of the open wells, and the lid was also in place during incubation. The plates were placed on a rack in a plastic sealable container, which had the bottom filled with appropriate saturated salt slurries to maintain the environmental RH to ensure RH of the media in the plates remained as stable as possible. The saturated salt solutions used were as follows: $ZnSO_4 \cdot H_2O$ (83% RH @ 37°C), KNO (89% RH @ 37°C), KPO_4 (93% RH @ 37°C), and $K_2Cr_2O_7$ (96% RH @ 37°C). The containers were closed and placed into a 37°C incubator. The plates were periodically removed from incubation, and the OD of the 640 individual wells was measured for up to 6 months using the wideband filter on the Bioscreen C system (Labsystems Oy, Helsinki, Finland). The experiments were repeated on separate dates. A total of 1,280 wells were monitored.

Data collection. The *S. aureus* population was considered to have shown growth when the Bioscreen wideband OD (OD_{wb}) measurement increased from an initial reading of 0.200 to 0.220 to 0.350 or higher. Time to growth (TTG) was determined by calculating the geometric mean of the time of the last measurement that showed no growth ($OD_{wb} < 0.350$) and the first time point that showed growth ($OD_{wb} > 0.350$). In instances of no growth, TTG was censored at the final measurement time of 168 days.

OD measurements were made and transferred from the Bioscreen C ASCII file to a DOS text editor and then imported into Statistica software (StatSoft, Tulsa, Okla.) for initial analysis. Data were then transferred to a Microsoft Excel spreadsheet to determine TTG and then to SAS for modeling using LIFEREG (SAS Institute Inc., Cary, N.C.).

Statistical analysis. The TTG data along with a censoring indicator and the RH, pH, and potassium sorbate or calcium propionate levels were input data for construction of two separate models: one based on the potassium sorbate data set and one based on the calcium propionate data set. These two data sets used a common block of data generated from the experimental conditions, which had no preservatives added. The SAS LIFEREG procedure was used to develop predictive models of ln TTG as a function of the factors RH, pH, and preservative level. By default, the procedure fits a model to the log of the dependent variable. The resulting model can easily be transformed to a regular time scale. In growth modeling, there are often conditions where no growth occurs; therefore, TTG is sometimes censored. Under these conditions, ordinary least-squares regression is not applicable, and special procedures are required. The LIFEREG procedure can accommodate such censored data and uses maximum

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likelihood estimation methods to find regression coefficients. When creating models with the LIFEREG procedure, there is a need to have many replicates of conditions equally spaced out about the matrix, with approximately 50% of the conditions allowing and 50% not allowing growth for best-fitting purposes. Therefore, the ranges of conditions studied (RH, pH, and preservative levels) were chosen to satisfy these requirements.

Toxin production. Toxin production for experimental conditions close to the growth or no growth border was explored by enterotoxin assay using the VIDAS Staph Enterotoxin Assay. The VIDAS Staph Enterotoxin Assay is a qualitative enzyme-linked fluorescent immunoassay performed in the automated mini-VIDAS instrument (bioMerieux, Inc., Hazelwood, Mo.). After the final OD_{wb} measurements were completed (24 weeks), the media from the microtiter plates were removed for assay. The broth from each of the eight replicate wells was removed via pipet and combined into two microcentrifuge tubes. The samples were spun down for 10 min, and the supernatant from the two tubes was combined. The pH of the supernatant was adjusted with 1 N NaOH to a pH of 6.0 to 8.0. A half a milliliter of the sample was placed into the appropriate well in a test strip, the strips were placed into the mini-VIDAS, and the assays were run. A positive and negative control were run with each set of test strips. The test is sensitive to 1 ng of toxin per ml of sample.

RESULTS

A threshold OD_{wb} value of 0.350 was used to score wells with *S. aureus* growth ($OD_{wb} \geq 0.350$) versus wells with no growth ($OD_{wb} < 0.350$). This threshold value was determined in two ways. First, OD_{wb} measurements were compared with plate counts, and it was determined that an OD_{wb} reading of 0.350 was approximately the first OD_{wb} value where an increase in turbidity regularly correlated to an increase in plate counts, indicating the approximate OD_{wb} where the instrument was sensitive enough to accurately determine an increase in cell numbers by a change in turbidity (data not shown). Second, the data were analyzed to determine TTG in each well using threshold OD_{wb} values for growth of 0.300, 0.350, and 0.400, and models were created and compared (models using data from OD_{wb} threshold values of 0.300 and 0.400 not shown). The models created with the threshold value set at 0.300 did not make biological sense. We suspect that this was because this OD_{wb} corresponded to a level that is lower than the sensitivity of the instrument to measure an increase in cell numbers. When the threshold OD_{wb} value was raised to 0.400, the models were similar to those created with the TTG data when the threshold OD_{wb} of 0.350 was used; however, the TTG data when the threshold OD_{wb} of 0.350 was used gave a slightly more conservative model. For these reasons, the models were developed with TTG data obtained by making the OD_{wb} of 0.350 or more the lower limit for determining that growth had occurred in a particular well.

The models included all three main effects: RH (rh), pH (ph), and potassium sorbate (sorb) or calcium propionate (cal), their quadratic effects ($RH \cdot RH = rh^2$, $pH \cdot pH = ph^2$, and potassium sorbate·potassium sorbate = $sorb^2$ or calcium propionate·calcium propionate = cal^2), and the three two-factor interactions ($rh \cdot ph$, $rh \cdot sorb$, $ph \cdot sorb$ or $rh \cdot ph$, $rh \cdot cal$, $ph \cdot cal$). These main effects, quadratic effects, and interactions are collectively referred to as the factors. LIFEREG outputs a table of regression coefficient estimates and approximate chi-square distribution *P* values for each factor in the model. The relative importance of each factor can be judged by the *P* value: factors with small *P* values are most influential and predictive of the TTG. LIFEREG also allows the user to specify the error distribution to account for the variation in TTG not explained by the regression model. For model development purposes, Weibull, lognormal, and log-logistic distributions were considered. It was found that all three distributions gave very similar models; the regression coefficients were similar in sign and magnitude. Since the three distributions are not all from the same class of distributions, it is not possible to formally test for goodness of fit using likelihood ratio tests. However, since all models produced similar regression equations, we selected the log-logistic distribution model, because it had the largest sample log likelihood.

The model development process is an iterative process. The first analysis of the data created models that were all inclusive; this allowed for the determination of those factors that had a significant effect on the growth of *S. aureus.* All of the main effects, quadratic effects, and two-factor interactions were significant for each model ($P < 0.005$) except for the interaction between RH and potassium sorbate in the potassium sorbate model and the calcium propionate quadratic term in the calcium propionate model. For this reason, in each model the insignificant factor was dropped and the data were reanalyzed to develop the final models (Tables 1 and 2). The resulting model equation from the calcium propionate data was 2.250 to $3.703 \cdot rh - 1.488 \cdot ph + 0.418 \cdot cal + 1.572 \cdot rh^2 + 0.343 \cdot ph^2 + 0.814 \cdot rh \cdot ph - 0.130 \cdot rh \cdot cal - 0.221 \cdot ph \cdot cal$, and the resulting model equation from the potassium sorbate data was 2.624 to $3.938 \cdot rh - 2.421 \cdot ph + 0.907 \cdot sorb + 1.638 \cdot rh^2 + 0.887 \cdot ph^2 - 0.190 \cdot sorb^2 + 0.903 \cdot rh \cdot ph - 0.756 \cdot ph \cdot sorb$. From the model equations, the TTG contour plots for calcium propionate (Fig. 1) and for potassium sorbate (Fig. 2) were created. For diagnostic purposes, plots of ln predicted time to growth versus ln observed time to growth were examined (Fig. 3). Plots of residuals versus main effects were also examined, and no unusual patterns or anomalies were detected (data not shown).

There was a slight difference in the curvature of the contour lines in the plots for the media with no preservatives data for each model (Figs. 1 and 2), despite the fact that the same data for the no preservative condition were used for developing each model. This difference occurs because two distinctly different mathematical models were produced by the analysis, and when different models are used to predict TTG at a zero preservative level, they will produce slightly different predictions. It should be noted that the differences are small and mainly in the low pH areas of the plots. In practice, the target product formulation would be well away from the growth boundary to allow for inherent process variation.

All of the models make good biological sense. As conditions become more and more unfavorable for growth, the

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TABLE 1. *Parameters of the model to predict log_{10} time to growth based on RH (rh), pH (ph), and calcium propionate (cal)*

Variable	Degrees of freedom	Estimate	Standard error	Chi-square	$P >$ chi-square
Intercept	1	2.2496	0.0319	4983.5007	<0.0001
rh	1	−3.7032	0.0562	4338.8734	<0.0001
ph	1	−1.4881	0.0327	2069.5765	<0.0001
cal	1	0.4176	0.0301	192.5908	<0.0001
rh^2	1	1.5717	0.0474	1101.7569	<0.0001
ph^2	1	0.3430	0.0220	243.5212	<0.0001
rh·ph	1	0.8145	0.0300	746.1667	<0.0001
rh·cal	1	−0.1305	0.0302	18.7282	<0.0001
ph·cal	1	−0.2209	0.0208	112.2248	<0.0001

contour lines are closer together, indicating conditions are approaching those that do not allow growth of the organism. As the RH or pH of the system decreases, a corresponding increase in TTG is seen, and the no growth area of the contour plot increases in size. The addition of potassium sorbate at low pH (pH 4.5 to 5.5) dramatically changes the contour plots with steep curvature in the low pH area of the plots. The no growth boundary with respect to RH is increased from approximately 89% to approximately 93% when 1,000 ppm of potassium sorbate is present at pH 4.5. This effect is not as evident when calcium propionate is present; the no growth boundary with respect to RH only increased approximately 1.5% from 88 to 89.5% when 1,000 ppm of calcium propionate is present at pH 4.5. There is little change in the boundary with respect to RH when the system is at pH 7.0 with the addition of either preservative studied.

Various regions on the contour plots generated by the models were explored with enterotoxin assays for all conditions where the final OD_{wb} measurement was borderline. Also, assays were conducted for all samples with treatments at 84% RH, pH 7.0, and 84% RH, pH 4.5, although all OD_{wb} measurements for treatments at 84% RH were less than 0.350. The results are indicated by a plus sign (toxin present) or minus sign (no toxin present) on the contour plots (Figs. 1 and 2). A total of 71 assays were run, and in every case where the data were scored as ‘‘no growth,’’ the toxin assay results were negative for toxin, and in every case where the data were scored as ‘‘growth,’’ the toxin assay results were positive for the presence of toxin. This further supports the choice of an OD_{wb} of 0.350 as the correct cutoff value for time to growth in the individual wells.

DISCUSSION

Comparison of the models developed in this study with other published work is difficult, because most previously published studies have used NaCl to control the RH of the system, whereas this study has used glycerol. The limits of *S. aureus* growth, or the growth of any microorganism, cannot be predicted by the RH of the system alone; instead, both the RH of the system and the physical properties of the humectant used should be considered. For example, NaCl is a non–glass-forming ionic humectant that has specific effects on membrane transport systems. Therefore, both the osmotic and ionic stresses placed on bacterial cells by NaCl could affect the ability of those cells to grow. Glycerol is a nonionic glass former that passively permeates the cell membrane. Therefore, glycerol would not have a direct effect on ion transport systems. Unlike NaCl, glycerol forms an aqueous glass. The glass transition temperature (T_g) is a specific characteristic for each glass-forming compound. The influence of a solute's T_g on molecular mobility directly affects the amount of osmotic stress a specific solute places on cells. For these reasons, it should be expected that the limits of growth based on RH would be different when various humectants are used to control RH, and, therefore, comparing studies based on RH alone could lead to false conclusions *(24)*.

The differences in modeling approaches also make comparisons difficult. Both the FMM and PMP have very few kinetic curves generated in which cells would be under

TABLE 2. *Parameters of the model to predict log_{10} time to growth based on RH (rh), pH (ph), and potassium sorbate (sorb)*

Variable	Degrees of freedom	Estimate	Standard error	Chi-square	$P >$ chi-square
Intercept	1	2.6246	0.0646	1649.5082	<0.0001
rh	1	−3.9378	0.0925	1810.6521	<0.0001
ph	1	−2.4207	0.0636	1446.2982	<0.0001
sorb	1	0.9071	0.0381	567.4889	<0.0001
rh^2	1	1.6377	0.0792	428.0078	<0.0001
ph^2	1	0.8870	0.0423	440.1878	<0.0001
$sorb^2$	1	−0.1903	0.0552	11.8978	0.0006
rh·ph	1	0.9027	0.0527	293.0527	<0.0001
ph·sorb	1	−0.7558	0.0363	433.9474	<0.0001

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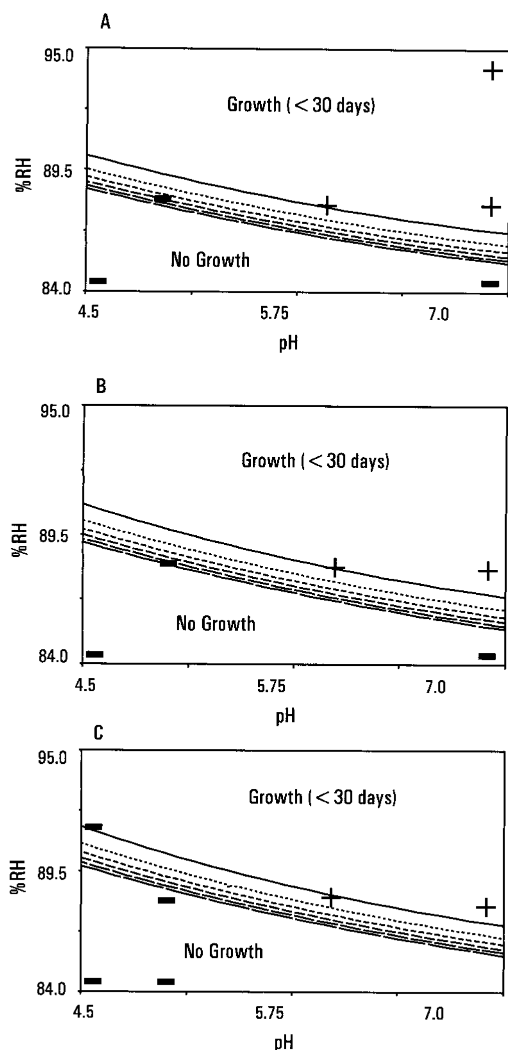

FIGURE 1. *Predicted time to growth of* S. aureus *based on the combined effects of RH (%), pH, and calcium propionate. The contours are scaled in intervals of 30 to 180 days. When moving down from the growth in less than 30-day regions, the first contour encountered is the 30-day line. When moving up from the no growth region, the first contour encountered is the 180-day line. The region above the 180-day contour line is not a plateau but a series of ever-increasing contours or longer TTG. (A) 0 ppm of calcium propionate; (B) 500 ppm of calcium propionate; (C) 1,000 ppm of calcium propionate. +, positive for enterotoxin; –, negative for enterotoxin.*

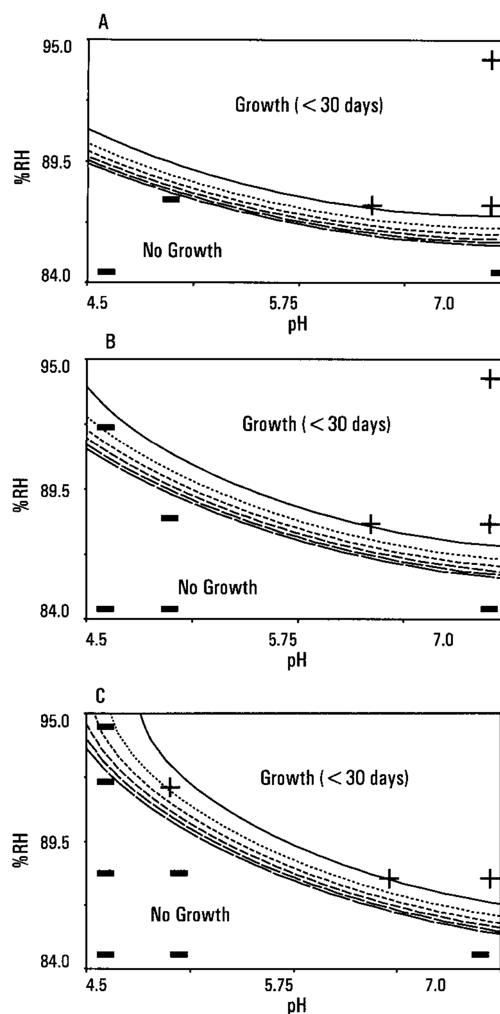

FIGURE 2. *Predicted time to growth of* S. aureus *based on the combined effects of RH (%), pH, and potassium sorbate. The contours are scaled in intervals of 30 to 180 days. When moving down from the growth in less than 30-day regions, the first contour encountered is the 30-day line. When moving up from the no growth region, the first contour encountered is the 180-day line. The region above the 180-day contour line is not a plateau but a series of ever-increasing contours or longer TTG. (A) 0 ppm of potassium sorbate; (B) 500 ppm of potassium sorbate; (C) 1,000 ppm of potassium sorbate. +, positive for enterotoxin; –, negative for enterotoxin.*

stressed conditions, the most difficult conditions in which to obtain kinetic information. Also, there are few true replicates in these areas, with most of the experimental design similar to a central composite design in which most of the replicates are in the middle of the design. Kinetic models also model the mean value, which, due to natural biovariability in the bacterial population, can be meaningless under stressed conditions. The LIFEREG procedure used to create the boundary models handles this by fitting the distribution to the error term, hence allowing probabilities to be calculated. This is useful for risk assessment purposes and allows the model to be easily used in Monte Carlo simulations. One consequence of modeling this way is that there is a need to have many replicates equally spaced about the matrix, with approximately 50% of the conditions allowing and 50% not allowing growth for best-fitting purposes. The log time to growth is used to normalize the variance.

It has generally been accepted that the limits for growth of *S. aureus* are 85% RH when NaCl is used as the humectant and 89% when glycerol is used *(11)*. Our models, based on glycerol, show the limiting RH for growth to be approximately 86% (at pH 7.0), which disagrees with work published by Marshall et al. *(17)*, who found the growth limits were 89 and 86% RH when glycerol and NaCl were used as the humectants, respectively. This disagreement could be due to strain variation or to the fact that only quarter-strength BHI broth was used in the work by Marshall et al. *(17)*, whereas full-strength BHI broth was used in our model system. Shapero et al. *(23)* reported growth

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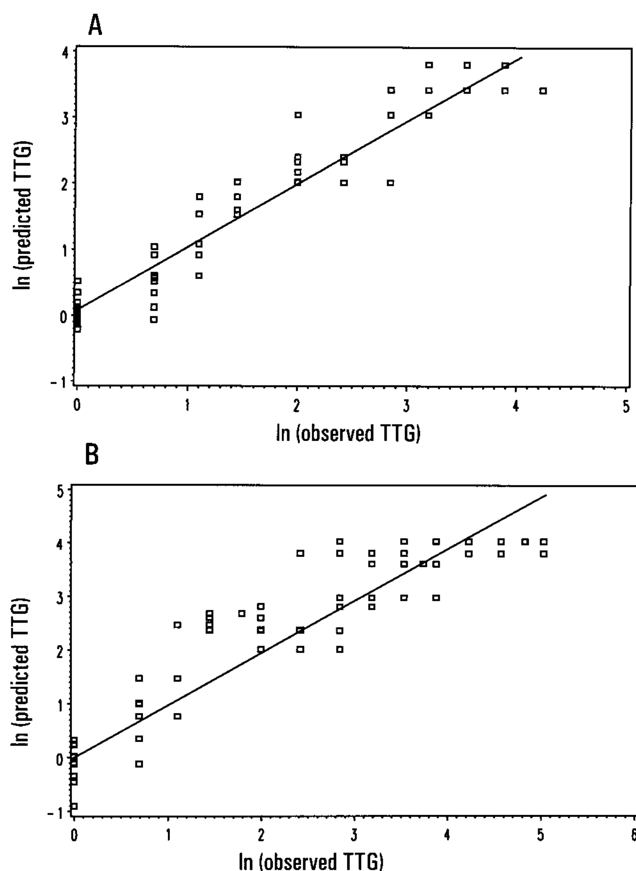

FIGURE 3. *Plots of ln predicted time to growth versus ln observed time to growth for* S. aureus. *(A) Calcium propionate model; (B) potassium sorbate model.*

of *S. aureus* at 88% RH on tryptic soy agar plates with RH adjusted with glycerol but did not conduct experiments with the system at any lower RH values. Studies with sucrose (a glass former that is not cell membrane permeable and has its own T_g) used as the humectant showed growth at 90% RH but not at 87% RH *(19),* and Scott *(22)* reported growth at 88 but not at 86% RH. Broughall et al. *(5, 6)* developed models for growth of *S. aureus* in UHT milk, with RH adjusted by the addition of glucose (a glass former that is not cell membrane permeable and has a different T_g). They found growth at 88% RH but did not explore the RH boundary further, because the additional glucose caused the system to become saturated. There is published literature that describes the ability of *S. aureus* to grow in various food systems, mainly meats and cheeses, but most of these systems used NaCl as the RH depressant *(2, 13, 15, 16, 20, 25, 30)* and, therefore, cannot be used to validate the models created with glycerol. Often in experiments conducted with food systems, the pH of the product is not reported, nor is the list of ingredients published, which also leads to difficulty when trying to compare data from various published studies.

The effectiveness of potassium sorbate to inhibit growth was considerably greater compared with calcium propionate, which was especially noticeable at low pH. Potassium sorbate and calcium propionate have some key similarities that may lead to the belief that they should act similarly as preservatives; potassium sorbate has a pK_a value of 4.74 and a molecular weight of 150.2, whereas calcium propionate has a pK_a value of 4.87 and molecular weight of 186.2. Both have been reported in the literature to act as weak acids; the molecules remain undissociated at low pH, diffuse through the bulk lipids of the cell membrane, then enter and dissociate in the cytoplasm, where the pH is close to neutral. It is the dissociated molecules that decrease the internal pH of the cell, which may prevent growth by perturbing metabolism *(14, 28).* Recent studies have shown that sorbic acid does have inhibitory effects in the undissociated form, suggesting not only that it may act as a weak acid but also that it may have a secondary mode of action associated with membrane-resident sorbic acid that acts as an uncoupler, which may disable active transport *(8, 26, 27).* It has also been reported that for *S. aureus* the minimum inhibitory concentration of propionic acid is much greater than sorbic acid *(8, 9).* This suggests that the levels of calcium propionate used in this study that are in line with the typical use levels in foods may have been too low to observe the inhibitory effect of calcium propionate on *S. aureus* growth.

In summary, two models describing the growth boundaries for *S. aureus* with respect to RH controlled by glycerol, pH, and preservative were developed. The growth boundaries were explored with toxin assays. For the first time, these models describe the growth/no growth boundaries for *S. aureus* with respect to RH controlled by glycerol and pH in addition to the preservatives potassium sorbate and calcium propionate. These models will allow product developers to visualize the "safe space" for the formulation of shelf-stable intermediate moisture foods using these preservation factors, will allow microbiologists to assess risks more effectively over a wide range of products, and will ultimately allow the consumer to have greater assurance of food safety.

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