Journal of Food Protection, Vol. 79, No. 1, 2016, Pages 148–152
doi:10.4315/0362-028X.JFP-15-161

Research Note

Modeling the Effect of Water Activity, pH, and Temperature on the Probability of Enterotoxin A Production by *Staphylococcus aureus*

TIAN DING,[1,2]* YAN-YAN YU,[1] CHENG-AN HWANG,[3] QING-LI DONG,[4] SHI-GUO CHEN,[1] XING-QIAN YE,[1,5] AND DONG-HONG LIU[1,2,5]*

[1]*Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-Food Processing, and* [5]*Fuli Institute of Food Science, Zhejiang University, Hangzhou, Zhejiang 310058, People's Republic of China;* [2]*Synergetic Innovation Center of Food Safety and Nutrition, Northeast Agricultural University, Harbin 150030, People's Republic of China;* [3]*U.S. Department of Agriculture, Agricultural Research Service, Residue Chemistry and Predictive Microbiology Research Unit, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA; and* [4]*Institute of Food Science and Engineering, University of Shanghai for Science and Technology, 516 Jungong Road, Shanghai 200093, People's Republic of China*

MS 15-161: Received 8 April 2015/Accepted 13 August 2015

ABSTRACT

The objectives of this study were to develop a probability model of *Staphylococcus aureus* enterotoxin A (SEA) production as affected by water activity (a_w), pH, and temperature in broth and assess its applicability for milk. The probability of SEA production was assessed in tryptic soy broth using 24 combinations of a_w (0.86 to 0.99), pH (5.0 to 7.0), and storage temperature (10 to 30°C). The observed probabilities were fitted with a logistic regression to develop a probability model. The model had a concordant value of 97.5% and concordant index of 0.98, indicating that the model satisfactorily describes the probability of SEA production. The model showed that a_w, pH, and temperature were significant factors affecting the probability of toxin production. The model predictions were in good agreement with the observed values obtained from milk. The model may help manufacturers in selecting product pH and a_w and storage temperatures to prevent SEA production.

Staphylococcus aureus is a foodborne pathogen that frequently contaminates food products during preparation and processing *(12)*. Human staphylococcal food poisoning (SFP) is caused by the ingestion of heat-stable staphylococcal enterotoxins (SE) produced by *S. aureus (16)*. Symptoms of SFP include nausea, vomiting, and stomachache, which can occur within 2 to 8 h after toxin ingestion *(1)*. Patients with this disease often need hospitalization, especially immunocompromised individuals.

Numerous cases of SFP have been reported. In Japan, there were 13,420 SFP cases caused by contaminated dairy products in 2000 *(2)*. In China, a survey that examined the contamination levels of *S. aureus* in raw milk in Heilongjiang Province showed that the prevalence of *S. aureus* was 83.5% in 400 raw milk samples *(27)*. According to data from the National Foodborne Diseases Surveillance Network, there were 94 outbreaks and 1,186 hospitalizations of 2,223 confirmed SFP cases from 2003 to 2007 in China *(14)*. In 2008, 119 students in Shenzheng, China, a metropolis near the east coast of China, were sickened by SFP after drinking a contaminated milk product *(10)*. More recently, an outbreak of SFP occurred in Deyang, China, with 70 individuals infected from consuming staphylococcal toxin-contaminated milk *(28)*. Due to the typical quick recovery from SFP, unreported minor outbreaks, or misdiagnosis, the actual number of SFP cases is likely to be much higher *(9)*. Since *S. aureus* can cause serious health issues, controlling this pathogen at all stages of the food chain is of public health, economic, and social importance.

Previous studies have indicated that SE would likely be produced when the population of *S. aureus* reaches $>10^5$ CFU/ml *(11, 18, 24)*. However, there were other studies showing that *S. aureus* at $>10^5$ CFU/ml was not necessarily an indication of SE production. For example, Rajkovic et al. *(17)* found that SE was produced in sandwich components when the *S. aureus* counts were below 10^3 CFU/g. Several studies have indicated that inoculum size, temperature, pH, and water activity (a_w) affect the SE production in food products *(6, 7, 13, 19, 23)*. For example, Schelin et al. *(20)* reported that SE production occurred at pH 5.0 to 9.6, with the optimal pH being 7 to 8, and at a_w above 0.86, with the optimal a_w being 0.99.

Growth or no-growth models of *S. aureus* in food as affected by environmental factors have been developed *(3, 25)*. To our knowledge, there is no published report regarding the probability of SE production as affected by a_w, pH, and temperature. Therefore, the objectives of this study were to develop a probability model for SE toxin production as a function of a_w, pH, and temperature and evaluate its applicability for ultra-high-temperature-processed milk.

* Authors for correspondence. Tel: +86 57188982169; Fax: +86 57188982154; E-mail: tding@zju.edu.cn (T.D.), dhliu@zju.edu.cn (D.-H.L.).

TABLE 1. *Treatments examined, probabilities of* S. aureus *enterotoxin production in TSB, observed and predicted probabilities for enterotoxin production, and final populations of* S. aureus

a_w	pH	*T* (°C)	Avg no. of positive samples/total no. of samples	Probability of toxin production (%)		*S. aureus* population (log CFU/ml)
				Observed	Predicted	
0.93	6.0	10	0/10	0	0	2.7
0.89	5.4	14	0/10	0	1	2.6
0.89	6.6	14	0/10	0	0	2.8
0.96	5.4	14	0/10	0	0	3.1
0.96	6.6	14	0/10	0	0	3.7
0.99	7.0	14	0/10	0	0	3.9
0.93	6.7	18	2/10	20	8	5.2
0.96	5.2	18	0/10	0	0	3.4
0.96	6.8	18	7/10	70	86	7.0
0.86	6.0	20	0/10	0	0	3.0
0.93	5.0	20	0/10	0	2	3.0
0.93	6.0	20	3/10	30	33	5.8
0.93	7.0	20	6/10	60	53	6.0
0.99	6.0	20	10/10	100	91	8.4
0.89	7.0	25	0/10	0	3	4.4
0.93	5.2	25	6/10	60	50	5.5
0.93	6.8	25	10/10	100	100	8.3
0.98	5.2	25	9/10	90	97	7.7
0.89	5.4	26	0/10	0	1	3.0
0.89	6.6	26	0/10	0	8	3.5
0.96	5.4	26	10/10	100	100	8.7
0.96	6.6	26	10/10	100	100	9.1
0.89	7.0	30	9/10	90	86	8.1
0.93	6.0	30	10/10	100	100	8.3

MATERIALS AND METHODS

Preparation of S. aureus strain. *S. aureus* strain CICC 10786 (*S. aureus* enterotoxin A [SEA] positive) from Shanghai Municipal Center for Disease Control and Prevention was used in this study. The culture was initially grown on Baird-Parker agar (Hangzhou Microbial Reagent Co., Hangzhou, China) at 37°C for 24 h. Cells of several well-grown colonies on the plate were transferred to 50 ml of tryptic soy broth (TSB; Qingdao Hope Bio-Technology Co., Qingdao, China) and incubated at 37°C for 24 h with shaking at 150 rpm to reach the stationary phase. The cell suspension was centrifuged at 5,000 × *g* for 10 min at 4°C and washed twice with sterile 0.85% saline solution. The pellet was diluted with 0.85% saline to approximately 5.0 log CFU/ml for use as inoculum.

Sample preparation. Twenty-four treatments (Table 1) were examined to determine the probabilities of SEA production in TSB of various a_w (0.86 to 0.99) and pH (5.0 to 7.0) values at storage temperatures of 10 to 30°C. The treatments were selected based on the reported growth boundary for *S. aureus* and SEA production *(15, 23, 25)*. The pH of TSB was adjusted with 0.1 M HCl or 0.1 M NaOH, and the a_w was adjusted with sodium chloride (Sinopharm Chemical Reagent Co., Shanghai, China) measured by an Aqualab 4TE a_w meter (Decagon Devices, Pullman, WA). An *S. aureus* inoculum (0.5 ml) was added to 50 ml of sterile pH- and a_w-adjusted TSB. The initial population of *S. aureus* in TSB was approximately 3.0 log CFU/ml, enumerated by plate count agar (Hangwei Co., Hangzhou, China). Samples were stored at the selected temperatures (Table 1) for 7 days. Two trials were conducted. In each trial, 10 samples were prepared for each treatment.

TABLE 2. *Comparison of predicted and observed probabilities in TSB broth and milk*

Substrate	a_w	pH	*T* (°C)	No. of positive samples/total no. of samples	Probability of toxin production (%)	
					Observed	Predicted
TSB	0.93	6.5	18	2/10	20	8
	0.92	6.5	20	0/10	0	17
	0.93	6.7	20	2/10	20	53
	0.93	5	30	9/10	90	86
Milk	0.98	6.7	14	0/10	0	0
	0.98	6.7	18	10/10	100	97
	0.98	6.7	20	10/10	100	100

Detection of SEA. The presence of SEA in samples at the end of storage was determined by using a Ridascreen SET Total *S. aureus* enterotoxin immunoassay kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. The assay's detection limit for SEA is 0.25 ng/ml. A sample that was positive according to the cut-off absorbance value was recorded as a toxin-contaminated sample. The probability of toxin production for a treatment was the ratio of the number of toxin-contaminated samples to the total number of samples.

Model fitting. The probabilities of SEA production for the 24 treatments were fitted with a linear logistic regression model, using the Logistic procedure of SAS 9.1.2 (SAS Institute, Cary, NC), as follows:

$$\begin{aligned}\text{Logit}(p) = {} & a_0 + a_1 \times b_w + a_2 \times pH + a_3 \times T + a_4 \\ & \times b_w \times pH + a_5 \times b_w \times T + a_6 \times pH \times T + a_7 \\ & \times {b_w}^2 + a_8 \times pH^2 + a_9 \times T^2\end{aligned}$$

where a_0 to a_9 are the coefficients to be estimated, pH is the broth pH, $b_w = \sqrt{1 - a_w}$, which was transformed to stabilize the variance and provide a more suitable model *(8),* and *T* is the incubation temperature (°C). Since Logit(*p*) is equal to $\ln[p/(1 - p)]$, the probability of toxin production (*p*) can be estimated by the equation $p = 1/[1 + e^{-\text{Logit}(p)}]$.

Validation of model performance. Model validation is an essential step in establishing a predictive model for practical uses. The validation includes internal and external validations. In internal validation, goodness-of-fit indices, including concordance, discordance, and the concordance index, *c (26),* were used to assess the agreement between the predicted values and observed values. The external validation step is commonly conducted using independent data for selected conditions within the experimental design range (a_w of 0.86 to 0.99 and pH of 5.0 to 7.0 at 10 to 30°C) not included in model development *(21)*. Therefore, the probability of SEA production in an additional 5 treatments in TSB and 3 treatments in milk (Table 2) was determined. The probability data used to validate the model employed indices that included median relative error (MRE) and mean absolute relative error (MARE) *(4),* which is determined as follows:

TABLE 3. *Parameter estimates of the logistic regression*

Parameter	df	Estimate	SE	Wald chi-square	$P >$ chi-square
Intercept	1	−146.9	74.3081	3.9084	0.0480
b_w	1	575.3	196.0	8.6163	0.0033
pH	1	21.3353	17.2280	1.5337	0.2156
T	1	−0.2442	1.2547	0.0379	0.8456
$b_w \times$ pH	1	−54.7167	23.7801	5.2944	0.0214
$b_w \times T$	1	−9.0328	3.6625	6.0824	0.0137
pH $\times T$	1	0.5187	0.2460	4.4450	0.0350
b_w^2	1	−232.8	106.1	4.8155	0.0282
pH^2	1	−1.2628	1.1605	1.1841	0.2765
T^2	1	0.0126	0.0307	0.1689	0.6811

$$\text{MARE} = (1/n) \times \sum_i \left| \left((X_p - X_o)/X_o \right)_i \right|$$

where X_o is the observed value and X_p is the predicted value.

RESULTS AND DISCUSSION

Effect of a_w, pH, and temperature on *S. aureus* growth and SEA production. The SEA production and final populations of *S. aureus* in 24 treatments are shown in Table 1. The results indicated that the populations of *S. aureus* in TSB ranged from 2.6 to 9.1 log CFU/ml after 7 days of incubation. When the final population of *S. aureus* was at approximately 3.0 log CFU/ml, no SEA was detected. When the population was greater than 5.2 log CFU/ml, SEA was detected. Based on the cell population and toxin production, it is reasonable to assume that when the population of *S. aureus* in TSB is greater than 5.0 log CFU/ml, SEA is likely to be produced. It is recognized that SE production is related to the growth of *S. aureus*. Notermans and Heuvelman *(15)* reported that SEA was produced in brain heart infusion broth under nearly all conditions of a_w of 0.87 to 0.99, pH 4.0 to 7.0, and temperature of 8 to 30°C that allowed the growth of *S. aureus,* whereas Fujikawa and Morozumi *(7)* reported that SE was produced in milk after the *S. aureus* population reached 6.5 log CFU/ml. In addition, SE production is also influenced by the *S. aureus* strain and food matrix *(17, 23)*.

Probability of toxin production and model development. The average probability of SEA production for each treatment is listed in Table 1. Among the 24 treatments, 12 treatments had no toxin production ($p = 0\%$), and 5 treatments had toxin production in 10 samples ($p = 100\%$). The probabilities of toxin production were fitted with a logistic model, as follows:

$$\begin{aligned}\text{Logit}(p) = &-146.9 + 575.3 \times b_w - 54.7167 \times b_w \\ &\times \text{pH} - 9.0328 \times b_w \times T + 0.5187 \\ &\times \text{pH} \times T - 232.8 \times {b_w}^2\end{aligned}$$

The coefficients and corresponding standard errors and p values are presented in Table 3. The concordance, discordance, and c values were 97.5%, 1.6%, and 0.98, respectively. The concordance value is the ratio of the number of pairs of observations that are concordant to the total number of pairs of observations, and c ranges from 0.5 to 1, with 1 indicating that the model perfectly predicts the responses. When the concordance is 100%, discordance is 0%, and c is 1, the predicted and observed

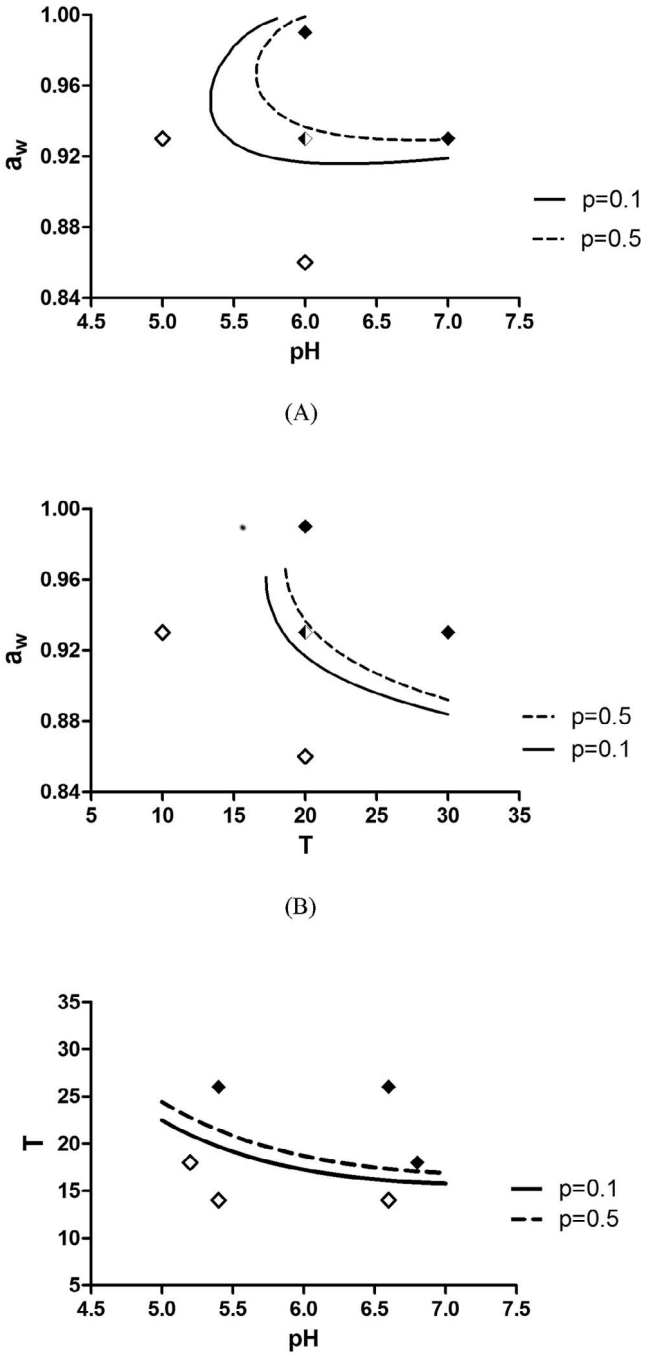

FIGURE 1. *Boundaries for predicted "no SEA production" (*$P \leq 0.1$*) and "SEA production" (*$P \geq 0.5$*) at* T *of 20°C (A), pH of 6.0 (B), and* a_w *of 0.96 (C). Probabilities obtained from treatments with the same* T*, pH, or* a_w *(Table 1) were superimposed to compare the predicted and observed probabilities (◇, no SEA production; ◆, likely SEA production; ◆, SEA production).*

values are completely in agreement *(5)*. The results indicated that the probability model had a good predictive ability. The b_w, $b_w \times$ pH, $b_w \times T$, and pH $\times T$ and the quadratic term of b_w significantly affect ($P < 0.05$) the probability of toxin production. Comparing the predicted and observed probabilities in Table 1, the predicted values are in agreement with the observed values for the majority of the treatments. The prediction overestimated the probability for the treatment with a_w of 0.96 and pH of 6.8 at 18°C and underestimated the probabilities for treatments with a_w of 0.93 and pH of 6.7 at 18°C and a_w of 0.93 and pH of 5.2 at 25°C.

Assuming that $P \leq 0.1$ means a "no SEA production" region, $P > 0.5$ means an "SEA production" region, and $0.1 < P \leq 0.5$ means an "SEA likely to be produced" region, which are similar to the growth, likelihood of growth, and no-growth boundaries of foodborne pathogens *(22, 25),* the predicted SEA production and no-production boundaries at T of 20°C, pH of 6.0, or a_w of 0.96 are shown in Figure 1. The SEA production and no-production predictions indicated that the conditions for SEA production were pH of >5.0 and a_w of >0.86 at temperatures of >15°C. Similar results were reported by Schelin et al. *(20),* who found that the enterotoxin production in ultra-high-temperature-processed milk was restricted at a pH of <5.0 and a_w of <0.86 at temperatures of ≤14°C.

Model validation. In China, milk consumption is becoming popular. Milk serves as a suitable growth medium for microorganisms, and *S. aureus* is a pathogen of particular concern for milk safety. Cases of SFP are frequently linked to the consumption of milk and milk products *(15)*. Therefore, a model validation experiment was conducted using TSB and ultra-high-temperature-processed milk with treatments not included in the model development (Table 2). The predictive ability of the model for validation treatments was assessed by MRE and MARE. The MRE of the model predictions was used as a measure of bias by estimating the mean difference between the observed and predicted values. MARE was used to measure the model's predictive accuracy, which assesses how close the predicted values are to the observed values. The results showed that the predicted probabilities were close to the measured values, and the predictions were acceptable (MRE = 0.030334 and MARE = 0.332909). Especially for milk, the model predictions were in agreement with the observed probabilities, indicating a good applicability of the probability model to milk.

In conclusion, the probability model for SEA production developed in this study provided reasonable predictions for the probabilities of SEA production at the ranges of a_w, pH, and temperature tested and identified the a_w, pH, and temperature limits for SEA production. The model may be used to select a_w, pH, and storage temperatures for milk that prevent the production of SEA by *S. aureus*. Further studies are needed to evaluate the applicability of this model to other types of food products.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Funds of China (grant no. 31401608 and 31271896) and Chinese National Key Technology R & D Program (grant no. 2012BAD29B07).

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