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Effect of Storage Temperature on the Outgrowth and Toxin Production of *Staphylococcus aureus* in Freeze-Thawed Precooked Tuna Meat

AI KATAOKA,[1]* ELENA ENACHE,[1] CARLA NAPIER,[1] MELINDA HAYMAN,[1] AND LISA WEDDIG[2]

[1]*Grocery Manufacturers Association, 1350 I Street N.W., Suite 300, Washington, D.C. 20005; and* [2]*National Fisheries Institute, 7918 Jones Branch Drive, Suite 700, McLean, Virginia 22102, USA*

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ABSTRACT

The aim of this study was to determine the time for a 3-log CFU/g outgrowth of *Staphylococcus aureus* and its toxin production in previously frozen precooked tuna meat (albacore [*Thunnus alalunga*] prepared as loin, chunk, and flake or skipjack [*Katsuwonus pelamis*] prepared as chunk and flake) held either at 21 or 27°C. A five-strain cocktail of enterotoxin-producing *S. aureus* was surface inoculated with $\sim 10^3$ CFU/g onto tuna samples. The experimental time–temperature conditions were designed to mimic common industry holding conditions. After a 3-h incubation at 37°C, inoculated samples were individually vacuum sealed and stored at −20°C for 4 weeks. Following frozen storage, samples were thawed to the target temperature (21 or 27°C) and then incubated aerobically. Growth of *S. aureus* in tuna was then monitored using Baird Parker agar; simultaneously, aerobic plate counts, enterotoxin production, and sensory profile (color and odor) were determined. The results showed that the time for a 3-log CFU/g increase was >20 h at 21°C and 8 to 12 h at 27°C for albacore, with toxin production observed at 14 to 16 h at 21°C and at 8 h at 27°C. A 3-log CFU/g increase for skipjack occurred at 22 to 24 h at 21°C and at 10 to 14 h at 27°C. The toxin production in skipjack started at 20 to 22 h at 21°C and at 8 to 10 h at 27°C. Toxin production was observed before a 3-log increase was achieved in albacore samples at 21°C. Under all conditions, toxins were detected when the cell density of *S. aureus* was 6 log CFU/g. Overall, significantly faster *S. aureus* growth was observed in albacore compared with skipjack ($P < 0.05$), possibly owing to differences in sample composition (e.g., pH and salt content). The data developed from this study can be used by the tuna industry to model the growth and enterotoxin production of *S. aureus* and to design manufacturing controls that ensure food safety.

Key words: Enterotoxin; Seafood safety; *Staphylococcus aureus*; Tuna

Canned tuna is commonly produced from precooked tuna mixed with edible oils, brine, water, or sauces in cans. Typically, eviscerated whole tuna are precooked to facilitate cleaning, that is, the manual separation of the meat from the skin and bones *(20)*. There are no known cases of staphylococcal food poisoning associated with canned tuna. However, despite the use of pertinent sanitation control procedures during cleaning, there is the potential for contamination and subsequent growth of *Staphylococcus aureus* on the precooked meat before canning owing to the manual cleaning process. Although the canning process will destroy the pathogen, its enterotoxins are thermostable *(13, 17, 30)*. Therefore, to control for this potential hazard, time–temperature control measures should be considered as part of a robust risk management process that also includes effective sanitation and good manufacturing practices.

S. aureus is a foodborne pathogen capable of causing illness through the ingestion of preformed heat-stable staphylococcal enterotoxins (SEs). It is one of the leading causes of foodborne illness in the United States; the Centers for Disease Control and Prevention estimates that ~240,000 illnesses occur annually *(25)*. *S. aureus* is gram positive, ubiquitous in the environment, and capable of aerobic and anaerobic growth from 7 to 47.8°C *(30)*. It is also tolerant of high salt and sugar and can grow at relatively low water activity (a_w), with growth observed at a_w values ranging from 0.83 to 0.99 *(30)*. Humans can be carriers of *S. aureus,* and contamination of foods can occur through handling or via environmental or animal contact. This microorganism grows well in most prepared food, including meats, vegetables, fruits, pastries, and milk products *(1)*. Staphylococcal poisoning is generally associated with foods undergoing extensive manual handling, improper time–temperature control, or both. Staphylococcal poisoning occurs after ingestion of foods containing extracellular SEs; ~25% of *S. aureus* strains isolated from foods produce enterotoxins *(3, 4, 22)*. The intoxication dose of SEs is still unclear and seems to be affected by individual susceptibility; however, it is suggested to be less than 1.0 μg, and generally this toxin level is reached when *S. aureus* populations exceed 5 log CFU/g *(30)*.

* Author for correspondence. Tel: 202-639-5973; Fax: 202-639-5991; E-mail: akataoka@gmaonline.org.

Although several types of SEs have been recognized (A through J), 95% of staphylococcal poisoning outbreaks are caused by SEA through SEE *(7)*. A screen of 125 enterotoxic strains of *S. aureus* isolated from meat and milk products showed that most of the isolates produced SED (33.6%), SEA (18.4%), SEC (15.2%), and SEB (6.4%) *(21)*. A study by Asao et al. *(2)* on an extensive outbreak (13,420 illnesses) of staphylococcal food poisoning due to low-fat milk in Japan reported that SEA was responsible for the outbreak. The detected levels in low-fat milk and the powdered skim milk were low at $\leq$0.38 μg/ml and ~3.7 ng/g, respectively, resulting in an estimated total intake of 20 to 100 ng of SEA on average. SEs are highly heat resistant, especially in food matrices, and they cannot be inactivated by cooking or retorting *(16, 29)*. One reported case of foodborne outbreak associated with *S. aureus* in the United States was caused by commercially sterile canned mushrooms *(17)*. SEs are also resistant to most proteolytic enzymes (i.e. pepsin, trypsin); thus, their structure and activity are not affected by gastric juices during or after digestion *(16)*. Therefore, food safety programs should focus on prevention of enterotoxin formation when the hazard is reasonably likely to occur as a result of the presence of *S. aureus* under conditions where growth is possible.

The current study was designed to reflect common commercial practices using times and temperatures typical for processing canned tuna from frozen loins. The generalized processing flow includes cleaning tuna meat, frozen storage, and thawing, followed by staging and can filling, which may be carried out at a processing facility at ambient temperature *(8)*. The objective of this study was to measure the growth of enterotoxin-producing *S. aureus* in prefrozen cooked tuna meat inoculated with *S. aureus* and held at 21 or 27°C, which reflects the processing facility's ambient temperature. The study measured the time for a 3-log CFU/g increase of *S. aureus,* while monitoring for toxin production, and concurrent increases in aerobic plate counts (APCs). The determination on time to reach a 3-log increase of *S. aureus* for the growth study is recommended in the challenge study guidelines of the U.S. Food and Drug Administration (FDA) *(31)*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Five strains of *S. aureus* capable of producing enterotoxins were used: N-5014, N-5018, ATCC 13566, ATCC 13565, and N-5017. With the exception of N-5017, these strains were selected because they were used in studies by Wu and Su *(32, 33)* that evaluated *S. aureus* growth and enterotoxin production in tuna meat under different conditions. However, one strain (ATCC 25923) used in their studies did not produce enterotoxins in culture broth or inoculated fish (as detected by VIDAS SET2 test assay [bioMérieux, Inc., Marcy l'Etoile, France] described in "Toxin analysis") in our preliminary analysis; therefore, it was replaced with strain N-5017. Each working culture was made from a –80°C stock culture in tryptic soy broth (TSB; Difco, BD, Sparks, MD) supplemented with 20% glycerol and maintained on tryptic soy agar (TSA; Difco, BD) slants stored at 4 ± 1°C. The working cultures were transferred monthly for up to 3 months. A loopful of each isolate was transferred into an individual test tube (10 ml of TSB) and incubated at 35 ± 1°C for 24 h followed by a transfer (10 μl) of enriched culture into 10 ml of fresh TSB in a test tube and incubation at 35 ± 1°C for 16 h (stationary-phase cells). Each culture was harvested by centrifugation at 3,000 × *g* at 5°C for 20 min (SORVALL RC-5B Plus centrifuge, Sorvall, Newtown, CT). The supernatant was discarded and pelleted cells were resuspended and pooled in 50 ml of sterile phosphate-buffered saline to produce a multistrain cocktail suspension. The cell concentration in the cocktail suspension was analyzed at least twice through enumeration onto TSA, and it was ~9 log CFU/ml. The cocktail suspension was further diluted in Butterfield phosphate buffer to achieve an inoculum level of 10^3 CFU/g in a sample. The ability of each strain to produce enterotoxin in broth culture or in fish was verified with VIDAS SET2 assay as described in "Toxin analysis."

Sample preparation and sample evaluation. All precooked and frozen tuna samples were prepared in existing commercial operations by using customary procedures to ensure an effective cook and thorough freezing to ensure a solid core. Two tuna species, albacore (*Thunnus alalunga*) and skipjack (*Katsuwonus pelamis*), were received in different preparation forms (albacore as loin, flake, or chunk; skipjack as chunk or flake). The samples were shipped in coolers overnight and were frozen solid when received. The samples were stored at –20°C upon delivery until cut. Tuna samples of 50 to 55 g were generated by cutting the still-frozen tuna with a table saw (Ryobi Sliding Miter Saw with Laser, model TSS102L, Ryobi Ltd., Anderson, SC). The cut samples (approximate dimensions of 2 by 2 by 1 in. [5.08 by 5.08 by 2.54 cm]) were weighed to ensure correct sample weight and tightly wrapped in bulk with aluminum foil and stored at –20°C until further experiments. Uninoculated tuna samples were analyzed for pH, a_w, moisture (AOAC 950.46; AOAC International, 1950), fat (AOAC 960.39A; 1960), and salt (AOAC 971.27; 1976). The pH was determined using a pH meter (Accumet Research AR 20, Fisher Scientific, Pittsburgh, PA), and the a_w was measured with a water activity meter (AquaLab 4TEV, Decagon Devices, Inc., Pullman, WA). The uninoculated samples also were evaluated for APC and *S. aureus* (see "Microbial analysis").

Sample inoculation and holding conditions. The cut samples (50 to 55 g) were removed from the freezer and were laid in a single layer on a plastic tray in a refrigerator (5 to 7°C) overnight to thaw, and then the samples were brought to 35°C in an incubator at 38 ± 1°C. As soon as the internal temperature of a fish sample had reached 35°C (3 to 3.5 h), the samples were unwrapped and laid in single layer on a sterile plastic tray with a lid (Plastic Instrument Box [17 1/2 by 7 3/4 by 2 3/8 in.], Medi-Dose, Inc./EPS, Inc., Ivyland, PA) and surface inoculated with *S. aureus* by spotting 100 μl of the culture cocktail as five spots (~20 μl per spot) onto the top surface of each sample to create a contamination level of ~10^3 CFU/g. APC and *S. aureus* counts were conducted immediately after inoculation by using duplicate samples as stated below. All inoculated samples were held in a biological safety cabinet for ~5 min to allow the inoculum to be absorbed onto samples. Then, the samples were loosely covered with a lid and held at 37 ± 1°C for 3 h to mimic the exposure of the tuna to elevated temperatures during the cleaning step after precooking. After the incubation of the inoculated samples at 37 ± 1°C, they were packed individually in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) and then vacuum packaged with a vacuum sealer (VacMaster commercial vacuum sealer VP-321, VacMaster, Pleasant Hill Grain, NE). Two samples were also enumerated at this point, and then the remaining samples were held at –20°C for 4 weeks until growth experiments were initiated.

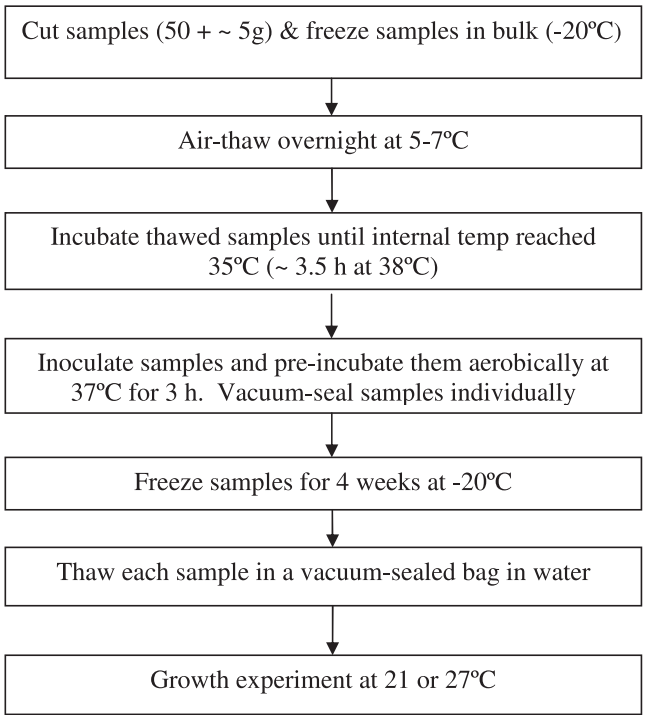

FIGURE 1. *Experimental overview*.

Microbial analysis. Each 50-g sample was hand massaged in a sterile Whirl-Pak bag for ~60 s and then mixed with a sterile spoon for 60 to 120 s to thoroughly break up and mix the sample. Twenty-five grams of the sample was reserved for toxin analysis. Then, the remaining 25-g sample was placed into a filtered Whirl-Pak bag and diluted 1:10 with Butterfield phosphate buffer for microbial analysis. The samples were homogenized for 15 s (Pulsifier, Microgen Bioproducts, Ltd., Camberley, UK). If necessary the samples were serially diluted in Butterfield phosphate buffer. One hundred microliters of the diluted sample was surface plated onto TSA to enumerate APC and onto Baird Parker agar (Oxoid Ltd., Hampshire, UK) to enumerate *S. aureus*. Plates were incubated at 37 ± 1°C for 48 h. TSA plates were counted with a Q-counter (Spiral Biotech, Norwood, MA), and Baird Parker agar plates were counted manually. Typical *S. aureus* colonies formed on the Baird Parker agar plates were confirmed by Fisher HealthCare Sure-Vue SELECT Latex Slide Agglutination test kit and Staph Latex test kit (Fisher HealthCare Inc., Houston, TX) as described in the FDA's *Bacteriological Analytical Manual (27)*.

Toxin analysis. Twenty-five grams of the sample was removed from well-homogenized samples as stated above and tested for toxin by using the VIDAS SET2 assay following the FDA's *Bacteriological Analytical Manual (28)*. This method is able to detect seven forms of enterotoxins (SEA, SEB, $SEC_{1,2,3}$, SED, and SEE), with a detection limit of ≥0.25 ng/ml *(5)*.

Temperature profile study for thawing samples in a water bath. To determine the time needed for the frozen samples to reach 21 or 27°C, a temperature profile study was conducted. Frozen uninoculated samples were thawed in a temperature-controlled water bath (model W45, Thermo Haake, Karlsruhe, Germany) set at either 22.5 or 28.5°C (i.e., samples that were held at 21°C for outgrowth were defrosted at 22.5°C). Each sample bag containing a cut fish sample (50 to 55 g) was hung in the water bath by using a paperclip hung from a metal bar. In this temperature profile study, the internal temperature of a fish sample was monitored using a digital thermometer (Traceable model 15-077-8, Fisher Scientific) positioned at the center of the fish to determine the length of time required to bring the sample from –20°C to the desired incubation temperature. The time to reach to the desired temperatures was within a range of 15 to 60 min. The length varied depending on the number of required samples used for a specific experiment (minimum of 6 bags to maximum of 18 bags) and the desired temperature (e.g. ~25 min was needed to reach 21°C when thawing 16 bags of skipjack samples).

***S. aureus* growth study and detection of enterotoxin in tuna samples.** The experimental design is shown in Figure 1. Inoculated samples were stored at –20°C for 4 weeks to imitate industry practices. Inoculated frozen cut fish samples still individually sealed in Whirl-Pak bags were thawed in a water bath set at either 22.5°C for the 21°C growth study or at 28.5°C for the 27°C growth study as stated above. Uninoculated fish samples were used to monitor an internal temperature of the sample during the thawing process, and all samples were removed from the water bath as soon as the internal temperature of the sample reached the target temperature (21 or 27°C). Each sample bag was dried and surface disinfected and then aseptically cut open and moved to an incubator set at the desired temperature (21 or 27°C). APC and *S. aureus* counts were conducted at time zero, when the fish samples reached the target temperature, and then every 2 h after incubation started using two duplicate samples. The microbiological analysis was continued until SEs were detected at three consecutive sampling points. APC and *S. aureus* counts were performed as described in "Microbial analysis."

Sensory analysis. The color and odor of the samples were assessed at each sampling time on the inoculated samples by using untrained panelists. The samples were graded on a simplified 5-point hedonic scale at each sampling point to monitor spoilage, where 1 = neither like nor dislike, 2 = dislike slightly, 3 = dislike moderately, 4 = dislike very much, and 5 = dislike extremely.

Data analysis. All experiments were conducted in triplicate ($n = 3$), with two duplicate samples per test point. Data were converted to log CFU per gram. The time for a 3-log outgrowth of *S. aureus* was calculated, with the time zero set at the time after water bath defrosting when the sample reached either 21 or 27°C. If the time to toxin production was different among the three replicates, the earliest time among the three replicates was used to denote the time to toxin production. Statistical data analyses were conducted with MINITAB statistical software (Release 8 extended, Minitab, Inc., State College, PA). The response variable in the statistical model was the counts (log CFU per gram). The effects of fish types and preparation were determined using the analysis of variance general linear model. Differences between mean values were considered significant at $P < 0.05$. Time to reach 3-log increase of *S. aureus* in each sample was compared against a calculation on time for 3-log outgrowth of *S. aureus* made from the U.S. Department of Agriculture (USDA) Pathogen Modeling Program (PMP) by using the same pH and NaCl (percentage) concentration for each sample type for skipjack. For albacore samples, the lowest concentration of NaCl (0.5%) with the PMP was used. Other conditions used were aerobic, broth culture, and sodium nitrite (0%).

TABLE 1. *Characteristics of albacore and skipjack tuna meat*[a]

Fish type	pH	a_w	Moisture (%)	Salt (%)	Fat (%)
Albacore					
Loin	6.3 ± 0.055	0.9870 ± 0.0038	67.65	0.16	1.13
Chunk	6.0 ± 0.032	0.9870 ± 0.0037	66.29	0.15	2.64
Flake	6.6 ± 0.040	0.9883 ± 0.0024	69.25	0.16	3.55
Skipjack					
Chunk	5.8 ± 0.006	0.9774 ± 0.0046	66.69	0.72	0.30
Flake	5.6 ± 0.012	0.9679 ± 0.0018	64.69	1.74	1.52

[a] Samples were tested in triplicate.

RESULTS

Proximate analysis. The tuna meat characteristics (pH, a_w, moisture content, and total salt and fat) are presented in Table 1. The pH of albacore (6.0 to 6.6) was slightly higher than that of skipjack (5.6 to 5.8). The a_w of all samples was high, with slightly higher values in albacore. In general, the moisture of the albacore was slightly higher than that of skipjack. The skipjack had a higher percentage of salt (NaCl) than albacore, with skipjack flake showing the highest salt content (1.74%). The fat content differed between fish type and preparation.

APC and *S. aureus* in uninoculated samples. The average population of background microflora in each tuna sample ranged from 2.6 to 3.8 log CFU/g (Table 2). There was some variation in the population depending on sample type. Generally, flake samples had higher counts. *S. aureus* was not observed in any samples (detection limit 10 CFU/g).

Incubation of inoculated samples. After the tuna was inoculated, it was held at 37°C for 3 h (this time frame is referred to as preincubation). There was a 0.4 to 1.0 log CFU/g increase of *S. aureus* and a 0.3 to 1.1 log CFU/g increase of APC during the 3-h preincubation (Table 3).

Effect of freezing temperature on *S. aureus*. The current study showed that freezing at –20°C for 4 weeks had little effect on *S. aureus* population or APC in tuna. There was no change in *S. aureus* or APC populations after a 4-week frozen storage period (average 0.1 and 0.2 CFU/g increase, respectively; data not shown).

TABLE 2. *Microbiological analysis of uninoculated tuna samples*[a]

Fish type	APC (log CFU/g)	*S. aureus* (log CFU/g)
Albacore		
Loin	3.2 ± 0.42	<0.4
Chunk	2.6 ± 0.09	<0.4
Flake	3.6 ± 0.77	<0.4
Skipjack		
Chunk	2.8 ± 1.26	<0.4
Flake	3.8 ± 0.91	<0.4

[a] Values are reported as mean ± standard deviation ($n \geq 3$).

Growth of *S. aureus*. For the growth study at 21°C, counts of *S. aureus* at time zero (after thawing, as soon as the samples reached an internal temperature of 21°C) ranged from 3.5 to 4.1 log CFU/g in albacore and 3.5 log CFU/g in skipjack (Fig. 2). Growth at 21°C followed typical bacterial growth kinetics; there was a short lag phase followed by exponential growth and then a stationary phase (Fig. 2). A 3-log CFU/g increase did not occur in albacore samples before the three consecutive positive toxin results occurred and growth plateaued at ~6 log CFU/g within 16 to 20 h (Table 4). *S. aureus* reached a 3 log CFU/g increase in skipjack samples at 22 h (chunk) and 24 h (flake) (Table 4).

For the growth study at 27°C, counts of *S. aureus* at time zero (as soon as the samples reached an internal temperature of 27°C) ranged from 3.7 to 4.2 log CFU/g in albacore and 3.6 log CFU/g in skipjack (Fig. 3). Growth at 27°C followed typical bacterial growth kinetics; there was a short lag phase (skipjack samples only) followed by exponential growth and then a stationary phase (Fig. 3). Albacore supported rapid growth of *S. aureus*, and the lag phase was not captured in the current study. A 3-log CFU/g increase occurred within 8 to 12 h in albacore samples and within 10 to 14 h in skipjack samples. Overall, albacore facilitated faster growth for *S. aureus* than skipjack, and the difference was significant at both 21 and 27°C ($P < 0.05$).

SE. At 21°C, toxin production was observed within 14 to 16 h for albacore, a faster time than the 20 to 22 h required in skipjack. In skipjack samples, toxin production occurred ~2 h before observing a 3-log increase. As mentioned, a 3-log CFU/g increase was not observed when albacore was held at 21°C; however, toxin production correlated with the time that the growth transitioned from the exponential phase to the stationary phase. In both albacore and skipjack, toxin production was detected when *S. aureus* cell density was close to or exceeded 6 log CFU/g. At 27°C, toxin production was observed within 8 h in albacore and within 8 to 10 h in skipjack. It is interesting to note the wide gap in time between a 3-log increase (14 h) and toxin production (8 h) in skipjack chunk samples. In both albacore and skipjack, toxin production was detected when *S. aureus* cell density approached or exceeded 6 log CFU/g at 27°C, as observed at 21°C. The time for toxin production was faster in albacore at 21°C, but there was no difference between time for toxin production in albacore or skipjack held at 27°C.

TABLE 3. *Change in total plate count and* S. aureus *during preincubation for 3 h at 37°C*[a]

Plate count	Time (h)	Albacore			Skipjack	
		Loin	Chunk	Flake	Chunk	Flake
APC	0	3.5	3.7	4.2	3.3	3.9
	3	4.6	4.1	5.0	3.7	4.1
		(1.1)[b]	(0.4)	(0.8)	(0.4)	(0.3)
S. aureus	0	2.9	2.9	3.0	3.1	3.0
	3	3.9	3.4	3.8	3.5	3.6
		(1.0)	(0.5)	(0.8)	(0.4)	(0.6)

[a] Values are presented in log CFU per gram. Samples were inoculated with *S. aureus* at $\sim 10^3$ CFU/g and held at 37°C.
[b] Values in parentheses are increase (log CFU per gram).

APC. At 21°C, a 3-log CFU/g increase of APC was observed within 16 to 18 h in albacore samples and within 22 to 24 h in skipjack samples (Fig. 4). At 27°C, a 3-log CFU/g increase of APC was observed within 8 to 10 h in albacore and at 12 h in skipjack samples (Fig. 5). APC shows a trend of slower bacterial growth in skipjack than in albacore at 21 or 27°C ($P < 0.05$). Chunk samples provided slower total bacterial growth compared with loin or flake ($P < 0.05$).

Comparison to predictions from PMP. Table 4 includes the predictions on time for 3 log CFU/ml outgrowth of *S. aureus* made from the USDA PMP. For the conditions comparable to albacore samples, the PMP predicted a time for 3 log CFU/ml outgrowth of *S. aureus*, generally faster than the results observed in albacore samples. The differences were >1.2 to 3.2 h at 21°C and within 0.2 to 2.6 h at 27°C, except for albacore flake that had a difference >5 h. For the condition similar to skipjack samples, the PMP predicted a time for 3 log CFU/ml outgrowth of *S. aureus*, generally slower than experimental results for skipjack samples demonstrated in the current study, except chunk samples at 27°C had a 2.8-h faster PMP prediction than the experimental result. For other skipjack samples, the differences were 2.4 to 3.2 h, although flake at 21°C displayed a 7.8-h difference.

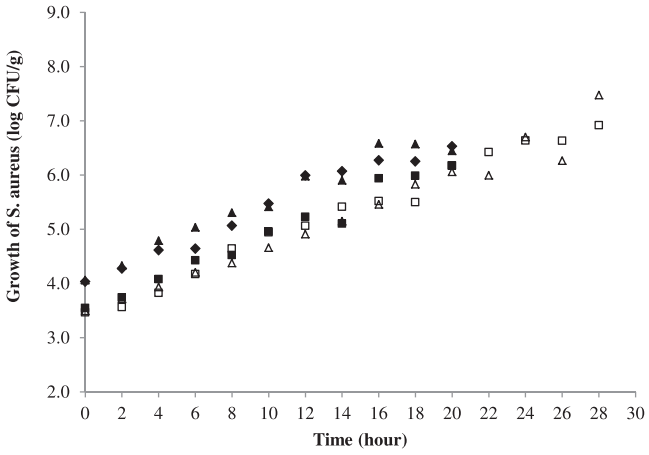

FIGURE 2. *Growth of* S. aureus *in precooked tuna meat samples at 21°C. Samples were initially inoculated at 10^3 CFU/g and then preincubated (held at 37°C for 3 h), after which they were held at –20°C for 4 weeks (◆, albacore loin; ■, albacore chunk; ▲, albacore flake; □, skipjack chunk; △, skipjack flake).*

Sensory analysis. Sensory analysis was conducted by untrained panelists using the 5-point hedonic scale described in "Materials and Methods." For the growth study at 21°C, odor seemed to be a more reliable indicator of spoilage; a deterioration of odor was noted at 8 to 10 h in albacore samples and was most pronounced in the albacore chunk samples. Color did not seem to be a good indicator of spoilage to an untrained panelist in albacore loin or flake, but a noticeable difference was noted within 8 h in albacore chunk samples. These times did not correlate with the 16- to 18-h time frame noted for a 3-log increase of APC. In skipjack, a good correlation between odor and color was noted in flake, but not in chunk. In both types, a difference from the control was noted within 8 to 10 h. Overall, the sensory value did not exceed more than 3 (data not shown), indicating a moderate off-odor or color. At 27°C, a deterioration of odor was noted at 8 h in all samples and was most pronounced in the albacore loin samples. Again, a noticeable difference was observed, especially in the albacore chunk samples, starting at 4 h. Time for a difference in color was noted from 2 h (skipjack chunk) to 10 h (albacore loin). At 27°C, there was more of a correlation between increase of APC and detection of off-odor, particularly for albacore samples.

DISCUSSION

In all the characteristics of fish samples (e.g., pH, moisture, percentages of salt and fat) based on proximate analysis, similar trends were observed to those in Wu and Su *(33)*. Also, the results from APC and *S. aureus* were comparable to the counts of background microflora in precooked tuna meat from a similar study by Wu and Su *(33),* where they observed from 2.7 to 4.8 log CFU/g. Our observation on the freezing effect on *S. aureus* was also similar to the outcome of Wu and Su *(32),* where the effect of frozen storage on *S. aureus* in precooked tuna meat was evaluated. Their results showed less than a 0.6- and 0.3-log CFU/g decrease in *S. aureus* population and APC, respectively, in tuna meat at –20°C after 4 weeks. This was expected since *S. aureus* is known to be resistant to freezing and thawing and survives well in food matrices at freezing temperatures *(11, 13)*. As noted, *S. aureus* grew

TABLE 4. *Comparison of experimental time for microbial growth and SE production in inoculated precooked tuna meat, stored at 21 or 27°C* (n = *3) versus model predictions (in parentheses)*

	Time to reach 3-log CFU/g increase of *S. aureus* (h)		Time for initiation of toxin production (h)		Time to reach 3-log CFU/g increase of APC (h)	
	21°C	27°C	21°C	27°C	21°C	27°C
Albacore						
Loin	22[a] (18.8)[b]	10 (7.4)	14	8	16	10
Chunk	20[a] (21.2)	8 (8.2)	16	8	18	8
Flake	20[a] (17.2)	>12 (7.0)	14	8	18	10
Skipjack						
Chunk	22 (24.4)	14 (11.2)	20	8	22	12
Flake	24 (31.8)	10 (14.2)	22	10	24	12

[a] Sample incubation was discontinued after three consecutive toxin-positive time points. A 3-log increase of *S. aureus* was not observed during this duration. Each log increase observed at the last time point for each fish type was 2.5 (loin), 2.6 (chunk), and 2.4 (flake) log CFU/g.

[b] Values in parentheses are calculated from the USDA Pathogen Modeling Program (PMP) using the same pH and NaCl (percentage) for each sample type for skipjack. For albacore samples, the lowest concentration of NaCl (0.5%) with PMP was used. Other conditions used were aerobic, broth culture, and sodium nitrite (0%).

more rapidly in albacore than in skipjack. This trend was observed when samples were held at either 21 or 27°C. The time for toxin production was faster in albacore at 21°C, but there was no difference between the time for toxin production in albacore or skipjack held at 27°C. The lower pH (5.6 to 5.8) of the skipjack may have contributed to the slower growth of *S. aureus*. Although *S. aureus* can grow over a wide pH range, such as pHs 4 to 10, the optimum pH range is 6 to 7 *(13)*. Metaxopoulos et al. *(18)* also demonstrated that slightly lower pH (pH 4.8 among three pH levels: 4.8, 5.1, and 6.0) by acidification had an inhibitory effect on the growth of *S. aureus* in sausage production with or without starter cultures. In general, skipjack flake showed the slowest time for *S. aureus* growth and toxin production; this fish type had the highest salt, highest fat, lowest pH, lowest moisture, and lowest a_w. Reduced pH, a_w, temperature, or anaerobic environment slows or sometimes inhibits growth of *S. aureus* and production of SEs *(13)*. Therefore, the trend observed in the current study was expected.

Fish preparations (loin, chunk, or flake) demonstrated some influence on the trends for *S. aureus* growth; chunk samples provided the slowest growth of *S. aureus* counts over time and the difference was significant in albacore samples at 21°C and in skipjack samples at 27°C ($P < 0.05$). Loin samples contain a 100% unbroken block of tuna meat and chunk samples consist of loin (~75%) and pieces (~25%); flake samples are made of pieces (100%) *(6)*. It is unclear why loin or flake samples (in albacore or skipjack) supported a greater increase in *S. aureus* population and APC. Based on the results of proximate analysis on samples, there does not seem to be an obvious correlation with this

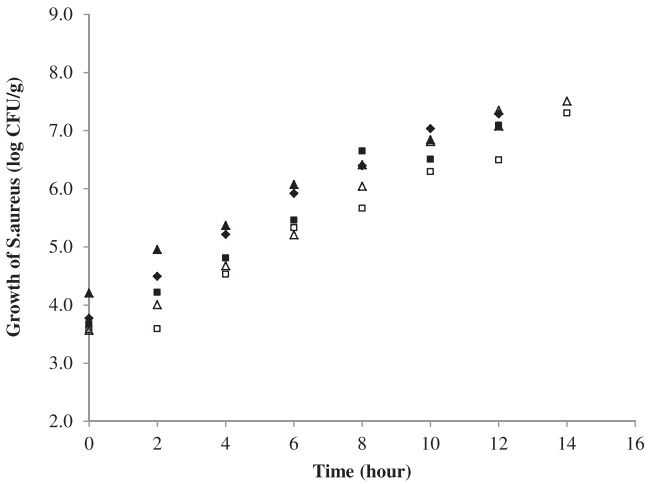

FIGURE 3. *Growth of* S. aureus *in precooked tuna meat samples at 27°C. Samples were initially inoculated at 10^3 log CFU/g and then preincubated (held at 37°C for 3 h), after which they were held at –20°C for 4 weeks (◆, albacore loin; ■, albacore chunk; ▲, albacore flake; □, skipjack chunk; △, skipjack flake).*

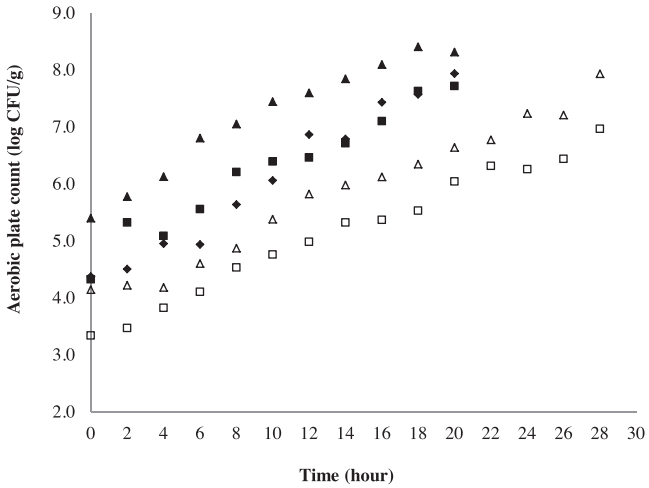

FIGURE 4. *Aerobic plate count (APC) in precooked tuna meat samples at 21°C. APC enumeration was performed on the samples initially inoculated at 10^3 CFU/g and then preincubated (held at 37°C for 3 h), after which they were held at –20°C for 4 weeks (◆, albacore loin; ■, albacore chunk; ▲, albacore flake; □, skipjack chunk; △, skipjack flake).*

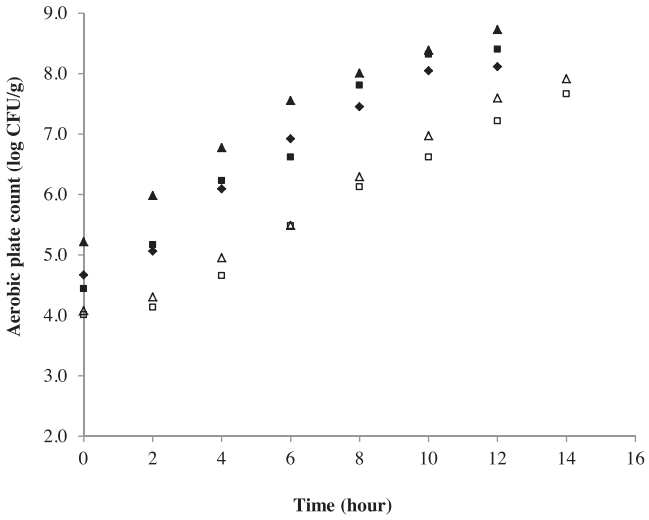

FIGURE 5. *Aerobic plate count (APC) in precooked tuna meat samples at 27°C. APC enumeration was performed on the same samples initially inoculated at 10^3 CFU/g and then preincubated (held at 37°C for 3 h), after which they were held at –20°C for 4 weeks (◆, albacore loin; ■, albacore chunk; ▲, albacore flake; □, skipjack chunk; △, skipjack flake).*

trend. For example, albacore chunk samples had the lowest pH and moisture (percentage) among three sample preparations of albacore; however, the skipjack chunk sample had higher pH and moisture (percentage) than the flake sample.

S. aureus did not reach a 3 log CFU/g increase in the albacore samples held at 21°C, with growth plateauing at ~6 log CFU/g. Foodborne illness is typically associated with foods that may have a low background flora or characteristics such as low a_w and high salt that favor *S. aureus* growth while slowing the growth of competing microorganisms. *S. aureus* is considered to be a poor competitor in foods that allow a broad spectrum of organisms to grow, such as the high-moisture, high-a_w, low-salt fish products in the current study *(12, 19)*. This may have contributed to the effect observed here. In particular, at the lower temperature (21°C), *S. aureus* may not compete against background microflora, whereas the higher incubation temperature (27°C), which is more favorable to *S. aureus,* might allow *S. aureus* to better compete against background microflora. This phenomenon was observed in other studies where a greater inhibitory effect on growth of *S. aureus* compared with competing microorganisms was demonstrated at 15°C compared with 30°C *(9, 14)*.

The current study was similar to the study conducted by Wu and Su *(33)*; however, their study did not include growth at 21°C, incorporate preincubation right after inoculation, or freezing at –20°C for 4 weeks before the growth study at 27°C. When comparing the data to determine a 3-log increase of *S. aureus,* the studies showed similar results, with data from the current study indicating slightly faster time (2 h less) than that of Wu and Su *(33)* for most of the fish types. However, there was a large difference in the time for toxin production data; data from the current study showed toxin detection at 8 to 10 h (for at least one positive replicate; all fish types), whereas data from Wu and Su showed that it took 24 h (all fish types) at 27°C *(33)* and 24 h at 37°C *(32)* for toxin to be detected. Given that toxin production is reported to correspond with a cell density of 5 to 6 log CFU/g, data from the current study are not unexpected. The difference in the times (8 versus 24 h) could be related to the detection method used; the current study used the VIDAS assay described in the FDA's *Bacteriological Analytical Manual* (a limit of detection ≥0.25 ng/ml in extraction and ≥0.75 ng/g in food when a 1:2 dilution is used) *(5, 28),* whereas Wu and Su *(32, 33)* used a SET-RPLA kit that relies on visual interpretation of agglutination to detect toxin, with a limit of detection of 0.5 ng/ml in extraction and 1 ng/g when a 1:1 dilution is used *(23)*. Both methods use immunological reactions. Park and Szabo *(24)* evaluated the SET-RPLA kit and demonstrated a minimum detection limit of 0.75 ng/g in food samples such as salami, ham, noodles, and turkey. In their study, the lack of absolute verification of agglutination reaction in the kit was also discussed as a difficulty for result determination. Other variations in experimental methods and techniques may also contribute to the difference.

The results from a comparison to PMP calculations underline that a mathematical model established with laboratory conditions (i.e. broth culture) may display different predictions from outcomes in real food having a complex matrix with competing microflora. Therefore, care needs to be taken when using general predictive models such as the PMP, and it is best to establish a predictive model to describe the target microorganism behavior in the actual food of interest *(26)*.

There seems to be few mathematical models developed for prediction of SEs. Several researchers developed a model for SEA production in milk medium *(10)*, and there is a model based on data in milk product used for quantitative risk assessment for pork-based food at food service *(15)*. These models present some difficulties such as a short temperature range or prediction only for a single enterotoxin in a particular food matrix (milk), considering *S. aureus* grow and produce SEs in a wide temperature range in variety of food matrices and several SEs exist. Therefore, data from the current study may contribute to establishing predictive models for growth of *S. aureus* and production of SEs in tuna meat.

In summary, growth temperature significantly affected the time to 3-log outgrowth of *S. aureus,* enterotoxin production, and APC; all were more rapid at 27°C compared with 21°C. Albacore samples supported faster growth of *S. aureus* and faster toxin production than the skipjack samples; this was more pronounced in the 27°C data. Toxin production was detected much earlier than in Wu and Su *(33)* when comparing results from 27°C growth experiments. This may be due to the detection method, the different strain, or other variations in methods or techniques. Toxin production was detected earlier than the time to reach a 3-log increase of *S. aureus* at 21°C. In the albacore samples, three (consecutive) toxin positives were observed at 21°C before the samples showed a 3-log increase in *S. aureus*. SEs were detected when the counts of *S. aureus* in samples approached or exceeded 6 log CFU/g at 21 or 27°C, which is aligned with the literature. Reducing the incubation temperature from 27 to 21°C seems to delay onset of toxin

production by 6 to 12 h in tuna samples. The data developed from this study can be used by the tuna industry to model the growth and enterotoxin production of *S. aureus* and to design manufacturing controls that ensure food safety.

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REFERENCES

1. Ahanotu, E., D. Alvelo-Ceron, T. Ravita, and E. Gaunt. 2006. Staphylococcal enterotoxin B as a biological weapon: recognition, management, and surveillance of staphylococcal enterotoxin. *Appl. Biosaf.* 11:120–126.
2. Asao, T., Y. Kumeda, T. Kawai, T. Shibata, H. Oda, K. Haruki, H. Nakazawa, and S. Kozaki. 2003. An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiol. Infect.* 130:33–40.
3. Belay, N., and A. Rasooly. 2002. *Staphylococcus aureus* growth and enterotoxin A production in an anaerobic environment. *J. Food Prot.* 65:199–204.
4. Bergdoll, M. S. 1989. *Staphylococcus aureus,* p. 463–523. *In* M. P. Doyle (ed.), Food bacterial pathogens. Marcel Dekker, New York.
5. bioMérieux, Inc. VIDAS® Staph enterotoxin II (SET2) test assay instruction brochure. bioMérieux, Inc., Marcy l'Etoile, France.
6. Colley, J. (jcolley@cosintl.com). 14 April 2014. Personal communication.
7. Cremonesi, P., M. Luzzana, M. Brasca, S. Morandi, R. Lodi, C. Vimercati, D. Agnellini, G. Caramenti, P. Moroni, and B. Castiglioni. 2005. Development of a multiplex assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. *Mol. Cell. Probes* 19:299–305.
8. De Beer, J. (jdebeer@cosintl.com). 10 April 2014. Personal communication.
9. DiGiacinto, J. V., and W. C. Frazier. 1966. Effect of coliform and *Proteus* bacteria on growth of *Staphylococcus aureus*. *Appl. Microbiol.* 14:124–129.
10. Fujikawa, H., and S. Morozumi. 2006. Modeling *Staphylococcus aureus* growth and enterotoxin production in milk. *Food Microbiol.* 23:260–267. doi:10.1016/j.fm.2005.04.005.
11. Haines. R. B. 1938. The effect of freezing on bacteria. *Proc. R. Soc.* 124:451–463.
12. Haines, W. C., and L. G. Harmon. 1973. Effect of variations in conditions of incubation upon inhibition of *Staphylococcus aureus* by *Pediococcus cerevisiae* and *Streptococcus lactis*. *Appl. Microbiol.* 25:169–172.
13. International Commission on Microbiological Specifications for Foods. 1996. *Staphylococcus aureus,* p. 299–333. *In* Microorganisms in food 5: characteristics of microbial pathogens. Blackie Academic & Professional, London.
14. Kao, C. T., and W. C. Frazier. 1966. Effect of lactic acid bacteria on growth of *Staphylococcus aureus*. *Appl. Microbiol.* 14:251–255.
15. Kim, H. J., M. W. Griffiths, A. M. Fazil, and A. M. Lammerding. 2009. Probabilistic risk model for staphylococcal intoxication from pork-based food dishes prepared in food service establishments in Korea. *J. Food Prot.* 72:1897–1908.
16. Le Loir, Y., F. Baron, and M. Gautier. 2003. *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res.* 2:63–76.
17. Levine, W. C., R. W. Bennett, Y. Choi, K. J. Henning, J. R. Rager, K. A. Hendricks, D. P. Hopkins, R. A. Gunn, and P. M. Griffin. 1996. Staphylococcal food poisoning caused by imported canned mushrooms. *J. Infect. Dis.* 173:1263–1267.
18. Metaxopoulos, J., C. Genigeorgis, M. J. Fanelli, C. Franti, and E. Cosma. 1981. Production of Italian dry salami: effect of starter culture and chemical acidulation on staphylococcal growth in salami under commercial conditions. *Appl. Environ. Microbiol.* 42:863–871.
19. Noleto, A. L., L. M. Malburg Júnior, and M. S. Bergdoll. 1987. Production of staphylococcal enterotoxin in mixed cultures. *Appl. Environ. Microbiol.* 53:2271–2274.
20. Nolte, F., D. G. Black, J. DeBeer, and E. Enache. 2014. Use of end point internal product temperature to control histamine formation in tuna at pre-cooking step. *Food Prot. Trends* 34:94–100.
21. Normanno, G., G. La Salandra, A. Dambrosio, N. C. Quaglia, M. Corrente, A. Parisi, G. Santagada, A. Firinu, E. Crisetti, and G. V. Celano. 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *Int. J. Food Microbiol.* 115:290–296.
22. Notermans, S., and R. L. M. van Otterdijk. 1985. Production of enterotoxin A by *Staphylococcus aureus* in food. *Int. J. Food Microbiol.* 2:145–149.
23. Oxoid Ltd. SET-RPLA (TD0900) test assay instruction brochure. Oxoid Ltd., Hampshire, UK. Available at: http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=TD0900&org=153&c=uk&lang=en. Accessed 10 April 2015.
24. Park, C. E., and R. Szabo. 1986. Evaluation of the reversed passive latex agglutination (RPLA) test kits for detection of staphylococcal enterotoxins A, B, C, and D in foods. *Can. J. Microbiol.* 32:723–727.
25. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7–15.
26. Schelin, J., N. Wallin-Carlquist, M. Thorup Cohn, R. Lindqvist, G. C. Barker, and P. Rådström. 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence* 2:580–592. doi:10.4161/viru.2.6.18122.
27. U.S. Food and Drug Administration. 2001. *Staphylococcus aureus,* chap. 12. *In* Bacteriological analytical manual. Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071429.htm. Accessed 20 February 2015.
28. U.S. Food and Drug Administration. 2001. Staphylococcal enterotoxins: micro-slide double diffusion and ELISA-based methods, chap. 13A. *In* Bacteriological analytical manual. Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm073674.htm. Accessed 20 February 2015.
29. U.S. Food and Drug Administration. 2011. Fish and fishery products hazard and controls guidance, 4th ed. Available at: http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM251970.pdf. Accessed 20 February 2015.
30. U.S. Food and Drug Administration. 2012. *Staphylococcus aureus*. *In* Bad bug book: foodborne pathogenic microorganisms and natural toxins, 2nd ed. Available at: http://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf. Accessed 20 February 2015.
31. U.S. Food and Drug Administration. 2013. Evaluation and definition of potentially hazardous foods - Chapter 6. Microbiological challenge testing. Available at: http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm094154.htm. Accessed 15 June 2015.
32. Wu, X., and Y.-C. Su. 2014. Effects of frozen storage on survival of *Staphylococcus aureus* and enterotoxin production in precooked tuna meat. *J. Food Sci.* 79:1554–1559.
33. Wu, X., and Y.-C. Su. 2014. Growth of *Staphylococcus aureus* and enterotoxin production in pre-cooked tuna meat. *Food Control* 42:63–70.