

Mathematical Model for Predicting the Growth Probability of *Staphylococcus aureus* in Combinations of NaCl and NaNO₂ under Aerobic or Evacuated Storage Conditions

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Abstract

The objective of this study was to describe the growth patterns of *Staphylococcus aureus* in combinations of NaCl and NaNO₂, using a probabilistic model. A mixture of *S. aureus* strains (NCCP10826, ATCC13565, ATCC14458, ATCC23235, and ATCC27664) was inoculated into nutrient broth plus NaCl (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.75%) and NaNO₂ (0, 15, 30, 45, 60, 75, 90, 105, and 120 ppm). The samples were then incubated at 4, 7, 10, 12 and 15°C for up to 60 d under aerobic or vacuum conditions. Growth responses [growth (1) or no growth (0)] were then determined every 24 h by turbidity, and analyzed to select significant parameters ($p < 0.05$) by a stepwise selection method, resulting in a probabilistic model. The developed models were then validated with observed growth responses. *S. aureus* growth was observed only under aerobic storage at 10-15°C. At 10-15°C, NaCl and NaNO₂ did not inhibit *S. aureus* growth at less than 1.25% NaCl. Concentration dependency was observed for NaCl at more than 1.25%, but not for NaNO₂. The concordance percentage between observed and predicted growth data was approximately 93.86%. This result indicates that *S. aureus* growth can be inhibited in vacuum packaging and even aerobic storage below 10°C. Furthermore, NaNO₂ does not effectively inhibit *S. aureus* growth.

Keywords: predictive model, *S. aureus*, NaCl, NaNO₂, processed meat products

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Introduction

Consumers consider many factors when purchasing certain foods, and their awareness of food safety is becoming more important because volumes of imported and processed foods are increasing (Choe *et al.*, 2005; Kim and Kim, 2003). Some foods contain additives, which are included to improve the quality of the product. Such additives include NaCl and NaNO₂, which play a role in preservation and food safety, especially in processed meat products (Pereira *et al.*, 2015; Shapiro *et al.*, 2016). However, recently, consumers have started to express a preference for processed meat products formulated with low

concentrations of NaCl and NaNO₂, because of the health issues involved (Bedale *et al.*, 2016; Guàrdia *et al.*, 2006; Kim *et al.*, 2012).

The processed meat industry has tried to use substitutes for additives, especially NaNO₂, and consumers are satisfied with the appearance of the products (Lee *et al.*, 2015a). In processed meats, NaNO₂ plays a role in color fixing and inhibiting pathogenic bacteria, such as *Listeria monocytogenes*, *Clostridium botulinum*, and *Staphylococcus aureus* (Hospital *et al.*, 2016; Karina *et al.*, 2011; Latham *et al.*, 2016; Tompkin *et al.*, 1973). Although NaNO₂ substitutes may fix the color in processed meat products, most have no antimicrobial activity. Thus, using a NaNO₂ substitute or a low concentration of NaNO₂ may result in greater pathogenic bacterial growth than in conventional meat products.

S. aureus is a gram-positive enterotoxigenic bacterium (CDC, 2014). Twenty to thirty percent of people are car-

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riers of *S. aureus* (Normanno *et al.*, 2007), and it can contaminate food during processing; the pathogen may produce enterotoxins at 10^5 - 10^6 CFU/g of *S. aureus* (Chiefari *et al.*, 2015; Park *et al.*, 1992). Ham can be contaminated with *S. aureus* during slaughter, processing, or handling (Borch *et al.*, 1996; Ingham *et al.*, 2004). Park *et al.* (2012) reported that they had isolated *S. aureus* from 0.6% of the ham samples they examined, and Atanassova *et al.* (2001) isolated the pathogen from 35.6% of smoked ham.

Therefore, we developed mathematical models to predict the growth probability of *S. aureus* in a combination of NaCl and low-concentration NaNO₂ under both aerobic and vacuum storage conditions.

Materials and Methods

Inoculum preparation

Five *S. aureus* strains (NCCP10826, ATCC13565, ATCC14458, ATCC23235 and ATCC27664) were cultured in 10 mL nutrient broth (NB; Becton, Dickinson and Company, USA) at 35°C for 24 h. The one-tenth milliliter aliquots of the cultures were subcultured in 10 mL fresh NB at 35°C for 24 h. The subcultures were then centrifuged at 1,912 g for 15 min at 4°C, and washed twice with phosphate-buffered saline (PBS, pH 7.4; 0.2 g of KH₂PO₄, 1.5 g of Na₂HPO₄·7H₂O, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water). Each cell suspension of the *S. aureus* strains was mixed, and the mixture was serially diluted with PBS to obtain 4 Log CFU/mL.

Sample preparation and inoculation

NB was formulated with NaCl (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.75%) and NaNO₂ (0, 15, 30, 45, 60, 75, 90, 105, and 120 ppm). Two hundred five microliter of the samples were placed into each well of a 96-well microtiter plate (SPL Life Sciences Co., Ltd., Korea), and 25-μL portions of *S. aureus* inoculum were inoculated into the samples. The microtiter plates were sealed with paraffin film (Parafilm M®; Bemis Company Inc., USA) for aerobic storage, or placed in airtight containers with AnaeroGen packs (Oxoid Ltd., UK) for vacuum storage. The AnaeroGen packs were replaced every 24 h. The microtiter plates were stored at 4-15°C for up to 60 d, depending on storage temperature, under aerobic or vacuum conditions. We used plain NB and NB plus *S. aureus* cells for the negative and positive controls, respectively.

Probabilistic model development

During storage, the growth responses for each combi-

nation (n=4) were determined by turbidity every 24 h. If a combination was turbid, it was designated as “growth (score=1)”, otherwise it was designated as “no growth (score=0)”. The growth response data were analyzed by logistic regression as follows:

$$\ln\left[\frac{P}{(1-P)}\right] = a_0 + a_1 \cdot NaCl + a_2 \cdot (NaNO_2/10) + a_3 \cdot \text{Log}(\text{Time}) + a_4 \cdot \text{Temp} + a_5 \cdot NaCl^2 + a_6 \cdot (NaNO_2/10)^2 + a_7 \cdot \text{Log}(\text{Time})^2 + a_8 \cdot \text{Temp}^2 + a_9 \cdot NaCl \cdot (NaNO_2/10) + a_{10} \cdot NaCl \cdot \text{Log}(\text{Time}) + a_{11} \cdot (NaNO_2/10) \cdot \text{Log}(\text{Time}) + a_{12} \cdot \text{Temp} \cdot NaCl + a_{13} \cdot \text{Temp} \cdot (NaNO_2/10) + a_{14} \cdot \text{Temp} \cdot \text{Log}(\text{Time})$$

where P is the probability of growth, a_i are estimates, $NaCl$ is the NaCl concentration, $NaNO_2$ is the NaNO₂ concentration, $Time$ is the storage time and $Temp$ is the storage temperature. Among the parameters, $NaNO_2$ and storage $Time$ were transformed for proper application to the model. In the equation, the significance parameters ($p < 0.05$) were selected by a stepwise selection method using SAS® (Version 9.3; SAS Institute Inc., USA). In addition, the estimates of selected parameters were used to produce growth/no growth interfaces at 0.1, 0.5 and 0.9 probability.

Minimum bactericidal concentration (MBC) test

To determine the MBC of NaCl and NaNO₂ for *S. aureus*, the aqueous portions of the microtiter plate wells that were clear were streaked on mannitol sorbitol agar (MSA; Becton, Dickinson and Company), and the plates were incubated at 37°C for 24 h to observe *S. aureus* survival by colony.

Validation of model performance with emulsion type sausage

To evaluate the performance of the developed probabilistic model, the predicted growth response from the model was compared with the observed growth response from real food. To prepare observed growth response data, emulsion-type sausages were manufactured according to the formulation given in Table 1. Each batch of the formula was mixed for 6 min using a cutter (MSK 760-II; Mado, Germany), and stored at 4°C for 1 h. The mixtures were then filled into collagen casings (30 g per casing) using a Konti A50 automatic sausage can filler (Frey, Germany). The resulting sausages were then smoked at 75°C

Table 1. Formulation of emulsion-type sausages

Ingredients (%)	No NaNO ₂			10 ppm NaNO ₂		
	1.00% NaCl	1.25% NaCl	1.50% NaCl	1.00% NaCl	1.25% NaCl	1.50% NaCl
Pork meat	60	60	60	60	60	60
Pork fat	20	20	20	20	20	20
Ice	20	20	20	20	20	20
Total	100	100	100	100	100	100
NaCl	1.00	1.25	1.50	1.00	1.25	1.50
NaNO ₂	-	-	-	0.0029	0.00303	0.00305
Phosphate	0.03	0.03	0.03	0.03	0.03	0.03
Isolated soy protein	1.00	1.00	1.00	1.00	1.00	1.00
Mixed spice	0.50	0.50	0.50	0.50	0.50	0.50
Sugar	0.50	0.50	0.50	0.50	0.50	0.50
Potassium sorbate	0.20	0.20	0.20	0.20	0.20	0.20

for 40 min in a smokehouse (MAXI 3501; Kerres, Germany) and chilled. The vacuum-packaged smoked sausages were heated at 80°C for 15 min and stored at 4°C until required. The sausages (25 g) were placed in a sterilized plastic container containing *S. aureus* inoculum at 3 Log CFU/mL, and gently stirred for 2 min to complete inoculation. The samples were air-dried for 15 min to allow *S. aureus* cell attachment, and transferred to sample bags. The bags were sealed for the aerobic or vacuum-packaged experiments and stored at 10°C for 65-70 d and 15°C for 35-43 d, respectively. During storage, *S. aureus* cell counts were enumerated on MSA (Becton, Dickinson and Company). If the *S. aureus* cell count increased by more than 1 Log CFU/g compared with that on day 0, the result was considered to be “growth”, otherwise “no growth” was recorded (Gwak *et al.*, 2015; Koutsoumanis *et al.*, 2004).

Results and Discussion

In vacuum condition, *S. aureus* growth was not observed at any growth temperature up to 60 d, regardless of the NaCl and NaNO₂ concentrations, indicating that *S. aureus* can be inhibited effectively in vacuum packaging, even at low concentrations of NaCl and NaNO₂, and therefore, no probabilistic model was developed (data not shown). Under aerobic conditions, *S. aureus* growth was not observed below 10°C up to 60 d, regardless of the NaCl and NaNO₂ concentrations, but the MBC test showed that *S. aureus* cells were not completely destroyed. Their cell counts were just reduced or retained at all concentrations of NaCl and NaNO₂ examined in this study. This result indicates that NaCl concentrations up to 1.75% and NaNO₂ concentrations up to 120 ppm, and their combinations, may not be sufficient to destroy *S. aureus* at

low temperatures, and *S. aureus* cells that survive below 10°C may grow above 10°C, allowing *S. aureus* to produce enterotoxins. In agreement with this result, Lee *et al.* (2015b) reported that the T_{min} (theoretical minimum growth temperature) value for *S. aureus* was 10.2°C in cheese. However, Lee *et al.* (2013) and Le Marc *et al.* (2009) reported lower T_{min} values for carbonara sauce (5.2°C) and milk (5.8°C). These results indicate that the T_{min} values for *S. aureus* depend on the food matrix. The low temperature adaptation of *S. aureus* is related to the lipamide dehydrogenase gene (*lpd*) in the *bkd* gene cluster, which causes the production of branched-chain fatty acids in phospholipids, resulting in improved membrane fluidity (Singh *et al.*, 2008; Yoon *et al.*, 2015).

S. aureus growth was observed at 10, 12 and 15°C, and the probability model was developed to describe the growth pattern using logistic regression. Significant parameters affecting *S. aureus* growth are presented in Table 2, and the parameters with estimates were used to produce the growth/no growth interfaces at 0.1, 0.5 and 0.9 probabilities in Figs. 1 and 2. The results in Table 2 show that *S. aureus* growth was affected ($p < 0.0001$) by storage temperature, storage time, and the concentrations of NaCl and NaNO₂, but no interaction effects, including for NaCl

Table 2. Estimates of the parameters selected from the logistic regression analysis by a stepwise selection method to produce the interfaces between growth and no growth of *Staphylococcus aureus* at desired probabilities under aerobic conditions

Variables	Estimate	SE	<i>p</i> -value
Interception	-38.2620	0.2078	<0.0001
Temperature	0.7171	0.0038	<0.0001
NaNO ₂ concentration/10	0.0951	0.0021	<0.0001
NaCl concentration	-0.9255	0.0148	<0.0001
Log (Time)	10.2063	0.0599	<0.0001

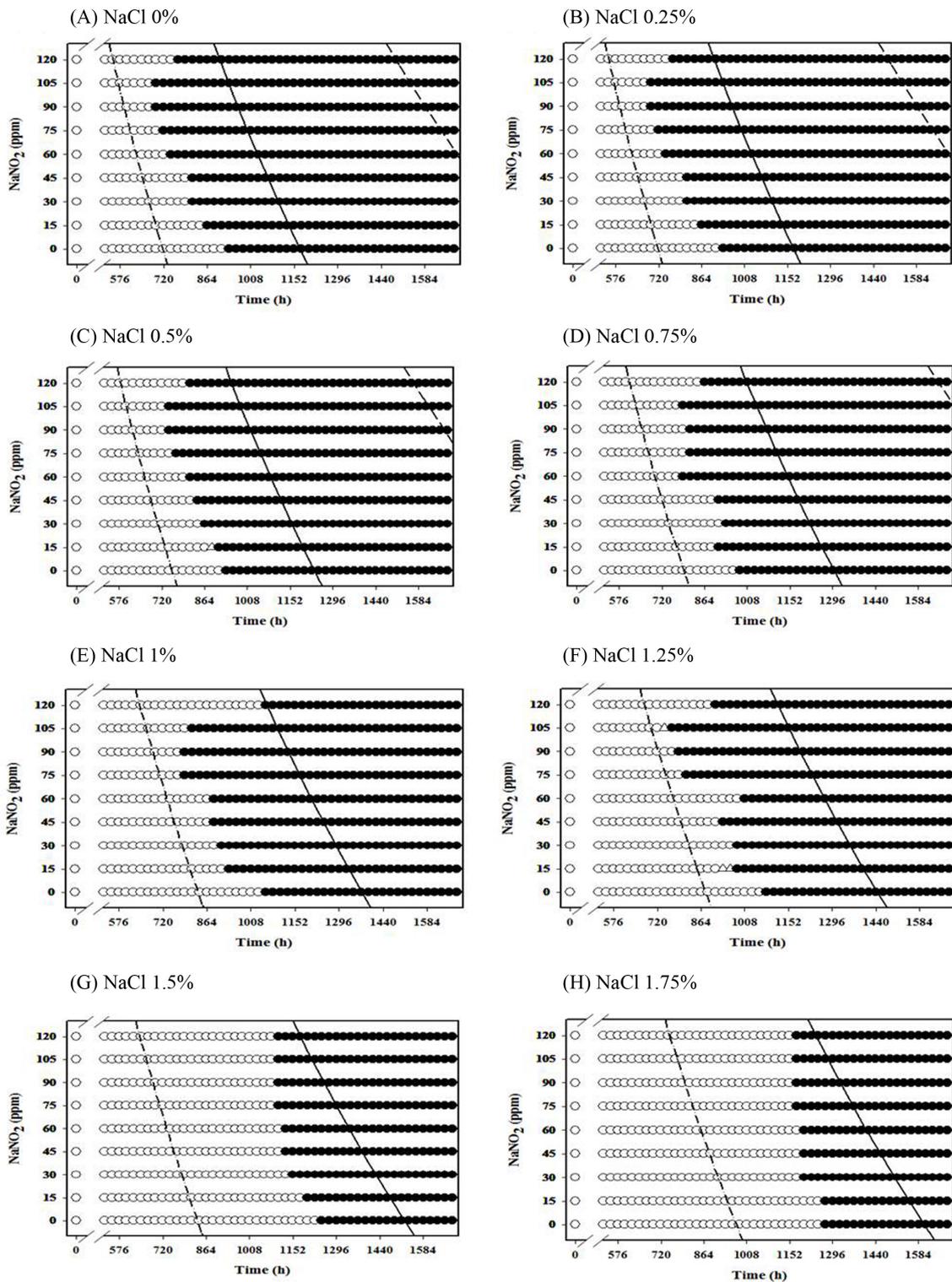


Fig. 1. Growth/no growth interfaces of *Staphylococcus aureus* in nutrient broth at 10°C with respect to NaNO_2 concentration and storage time for various NaCl concentrations under aerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line) and 0.9 (right line); no growth: \circ , growth: \bullet , 50% growth: Δ .

$\times \text{NaNO}_2$, were observed.

At 10°C, NaCl and NaNO_2 did not inhibit the growth of

S. aureus, as well as combination of NaCl and NaNO_2 at less than 1.25% NaCl. However, interestingly, the initia-

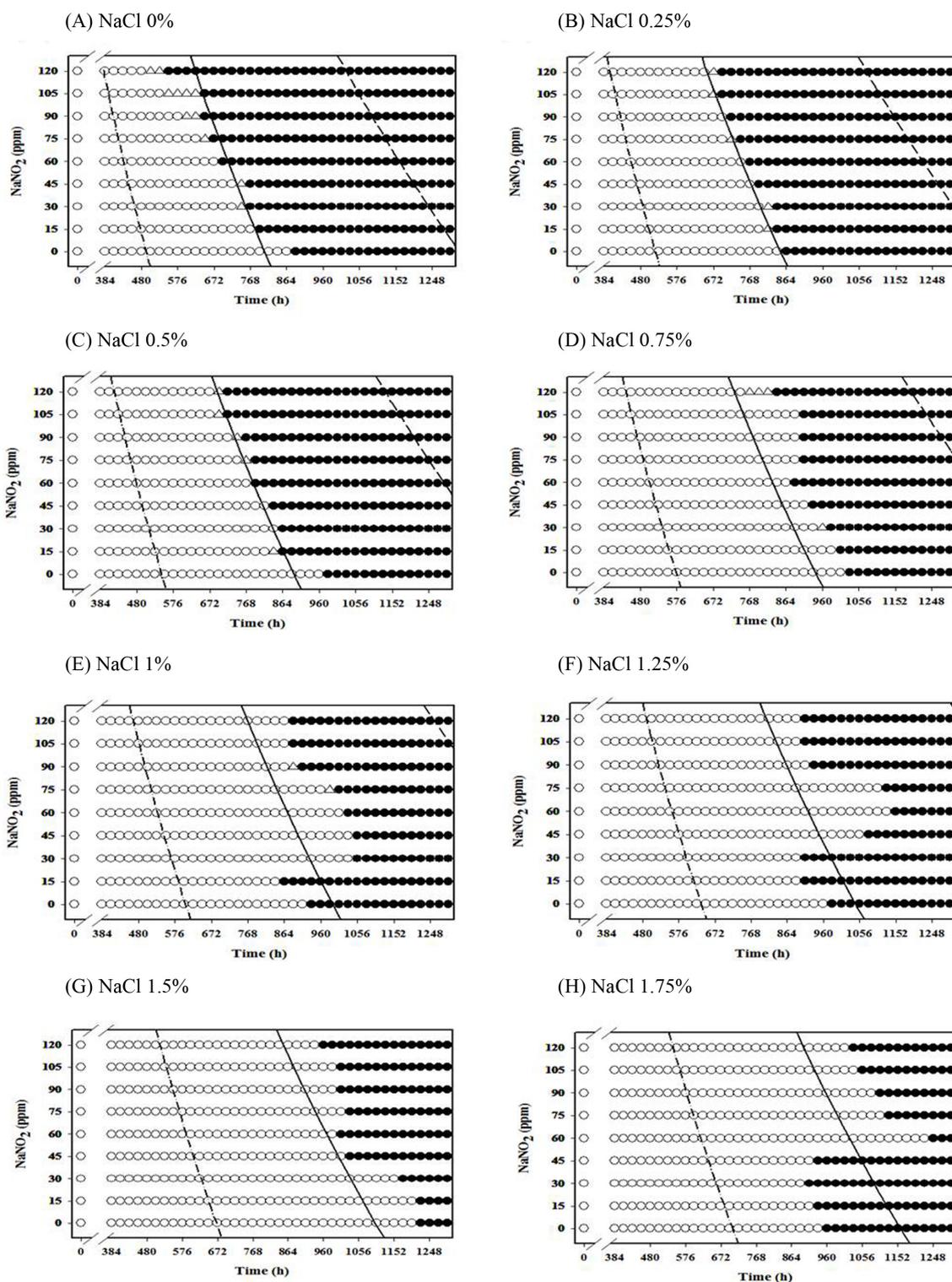


Fig. 2. Growth/no growth interfaces of *Staphylococcus aureus* in nutrient broth at 15°C with respect to NaNO_2 concentration and storage time for various NaCl concentrations under aerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line) and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.

tion time for *S. aureus* growth decreased as NaNO_2 concentration increased at less than 1.25% NaCl (Fig. 1).

Schlag *et al.* (2008) reported that the *nreABC* gene is involved in nitrate reduction. Therefore, the antibacterial

effect of NaNO₂ on *S. aureus* was not detected.

A NaCl concentration of more than 1.25% inhibited *S. aureus* growth (Fig. 1). In addition, no difference in initiation time for *S. aureus* growth was observed among the various NaNO₂ concentrations, and the initiation times were longer than those at less than 1.25% NaCl. Even at more than 1.25% NaCl, the combination effect was not observed (Fig. 1), as shown in Table 2, and the *S. aureus* growth response at 0 ppm NaNO₂ was similar to that at 120 ppm (Fig. 1). This result indicates that a NaCl concentration of more than 1.25% is needed to inhibit *S. aureus* growth, but NaNO₂ is not effective in inhibiting *S. aureus* growth. However, Lee *et al.* (2015c) reported a NaCl and NaNO₂ combination effect on *Lactobacillus* in frankfurters, and Jo *et al.* (2014) reported a combination effect on *Pseudomonas* spp. in processed meats. These results suggest that the NaCl and NaNO₂ combination effect depends on the type of foodborne bacteria. *S. aureus* grew better at 12°C than at 10°C, and demonstrated a NaCl concentration-dependent growth response (Fig. 2). In addition, no obvious effect of NaNO₂ on the inhibition *S. aureus* growth was observed (Fig. 2), which was similar to the result at 15°C (data not shown). In agreement with these observations, a study by Bang *et al.* (2008) also showed that nitrite had no effect on inhibiting *S. aureus* growth.

To evaluate the performance of the developed probabi-

listic models in this study, observed growth responses were collected from real food (emulsion-type sausages) in an additional study, and the observed growth responses from the study were compared with the predicted growth responses from developed probabilistic models. Because the predictions from the developed probabilistic models were expressed as numbers, growth was determined at more than 0.5 of growth probability (Yoon *et al.*, 2012). In addition, growth responses (growth or no growth) from the sausages were determined at 1 Log CFU/g of *S. aureus* growth (Gwak *et al.*, 2015; Koutsoumanis *et al.*, 2004). Comparisons between predicted and observed growth response are presented in Table 3; the observed growth responses mostly agreed with the predicted growth responses. The accordance percentage between the predicted and observed growth responses was 93.86%, indicating that the developed probabilistic model was capable of predicting the growth responses of *S. aureus* in emulsion-type sausages, formulated with various concentrations of NaCl and NaNO₂.

In conclusion, the probabilistic models were appropriate for describing the growth responses of *S. aureus* at different concentrations of NaCl and NaNO₂. Vacuum storage can inhibit *S. aureus* growth in emulsion-type sausages, and storage below 10°C can inhibit *S. aureus* growth under aerobic storage conditions, even at low concentrations of NaCl and NaNO₂. In storage above 10°C, a NaCl

Table 3. Comparisons between observed and predicted growth responses of *Staphylococcus aureus* in emulsion-type sausage under aerobic conditions

Temperature (°C)	NaNO ₂ (ppm)	NaCl (%)	Time (h)	Observed growth response	Predicted growth response
10	0	1.00	0-1,320 ¹⁾	NG	NG
			1,440	NG	G
			1,560	G	G
		1.25	0-1,320	NG	NG
			1,440	NG	NG
			1,560	G	G
	1.50	0-1,320	NG	NG	
		1,440	NG	NG	
		1,560	G	G	
	10	1.00	0-1,320	NG	NG
			1,440	NG	G
			1,560	G	G
1.25		0-1,320	NG	NG	
		1,440	NG	G	
		1,560	G	G	
1.50	0-1,320	NG	NG		
	1,440	NG	NG		
	1,560	G	G		
			1,680	G	G

Table 3. Comparisons between observed and predicted growth responses of *Staphylococcus aureus* in emulsion-type sausage under aerobic conditions (Continued)

Temperature (°C)	NaNO ₂ (ppm)	NaCl (%)	Time (h)	Observed growth response	Predicted growth response	
15	0	1.00	0-528 ²⁾	NG	NG	
			696	NG	G	
			864	G	G	
			1,032	G	G	
		1.25	0-528	NG	NG	
			696	NG	G	
			864	G	G	
			1,032	G	G	
		1.50	0-528	NG	NG	
			696	NG	G	
			864	G	G	
			1,032	G	G	
	10	1.00	0-480 ³⁾	600	NG	NG
				720	G	G
				840	G	G
				840	G	G
		1.25	0-480	600	NG	NG
				720	G	G
				840	G	G
				840	G	G
		1.50	0-480	600	NG	NG
				720	G	G
				840	G	G
				840	G	G

¹⁾Time interval (h): 0, 120, 240, 360, 528, 696, 864, 1,080, 1,320. ²⁾Time interval (h): 0, 120, 240, 360, 528. ³⁾Time interval (h): 0, 120, 240, 360, 480.

concentration of more than 1.25% is necessary to inhibit *S. aureus* growth effectively, but NaNO₂ may not effectively inhibit *S. aureus* growth.

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