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Evaluation of *Salmonella* Growth at Low Concentrations of $NaNO_2$ and NaCl in Processed Meat Products Using Probabilistic Model

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ABSTRACT: This study developed probabilistic models to predict *Salmonella* growth in processed meat products formulated with varying concentrations of NaCl and $NaNO_2$. A five-strain mixture of *Salmonella* was inoculated in nutrient broth supplemented with NaCl (0%, 0.25%, 0.5%, 0.75%, 0.5%, 1.0%, 1.25%, and 1.75%) and $NaNO_2$ (0, 15, 30, 45, 60, 75, 90, 105, and 120 ppm). The inoculated samples were then incubated under aerobic and anaerobic conditions at 4°C, 7°C, 10°C, 12°C, and 15°C for up to 60 days. Growth (assigned the value of 1) or no growth (assigned the value of 0) for each combination was evaluated by turbidity. These growth response data were analyzed with a logistic regression to evaluate the effect of NaCl and $NaNO_2$ on *Salmonella* growth. The results from the developed model were compared to the observed data obtained from the frankfurters to evaluate the performance of the model. Results from the developed model showed that a single application of $NaNO_2$ at low concentrations did not inhibit *Salmonella* growth, whereas NaCl significantly ($p<0.05$) inhibited *Salmonella* growth at 10°C, 12°C, and 15°C, regardless of the presence of oxygen. At 4°C and 7°C, *Salmonella* growth was not observed in either aerobic or anaerobic conditions. When $NaNO_2$ was combined with NaCl, the probability of *Salmonella* growth decreased. The validation value confirmed that the performance of the developed model was appropriate. This study indicates that the developed probabilistic models should be useful for describing the combinational effect of $NaNO_2$ and NaCl on inhibiting *Salmonella* growth in processed meat products. (**Key Words:** *Salmonella*, $NaNO_2$, NaCl, Probabilistic Model)

INTRODUCTION

Salmonella are facultative and intracellular pathogens, and they cause salmonellosis with the consumption of contaminated food (Jantsch et al., 2011). In the EU, salmonellosis was the second highest zoonotic disease in 2011, following campylobacteriosis (EFSA and ECDC, 2013). Nontyphoidal *Salmonella* infections are estimated to affect 1.4 million people annually in the United States, resulting in 15,000 hospitalizations and 400 deaths (Voetsch et al., 2004). Salmonellosis is mostly caused byanimal foods such as poultry, eggs, pork, beef, and processed meat products (Meyer et al., 2010; EFSA and ECDC, 2013). *Salmonella* contamination of meat products occurs frequently at processing plants through cross contamination (Beery et al., 1988; Izat et al., 1989). According to a study by Busani et al. (2005), *Salmonella* were isolated from 9.9% of raw poultry, 4.9% of raw pork, 0.1% of ice cream, and 5.3% of processed meats product.

$NaNO_2$ is commonly used to fix a red color, to improve flavor, and as an antioxidant in processed meat, increasing the shelf-life of meat products (Jira, 2004). NaCl is also used to enhance flavor and as a protein extractant in processed meat (Aguilera and Karel, 1997; Munasinghe et al., 2004; Kostick, 2010). However, high $NaNO_2$ and NaCl intake is related to cancer and cardiovascular diseases (Maekawa et al., 1982; Perry and Beevers, 1992; Frolich, 1999). Because of the associated health issues, consumers prefer low concentrations of $NaNO_2$ and NaCl in meat products, and thus, these products may allow the growth of foodborne pathogens, especially *Salmonella*, which is commonly found in fresh meat. Therefore, the combinational effects of lower $NaNO_2$ and NaCl concentrations on *Salmonella* growth need to be evaluated and predictive models need to be developed to evaluate the effects.

A probabilistic model can be used to predict the interface

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between the growth and no growth of bacteria or pathogens as a function of various factors (Ratkowsky and Ross, 1995; Schaffner and Labuza, 1997; Lee et al., 2013). For instance, Yoon et al. (2009) suggested a combination of minimum level of lactic acid, dipping time, and storage temperatures to inhibit *Listeria monocytogenes* growth in frankfurters using probabilistic model, and the model could be used to select conditions in meat products. Therefore, the objective of this study was to evaluate the combinational effect of low NaCl and $NaNO_2$ concentration on inhibiting *Salmonella* growth in processed meat products with probabilistic models.

MATERIALS AND METHODS

Inoculum preparation

Salmonella Typhimurium NCCP10812, *Salmonella* Agona NCCP12231, *Salmonella* Enteritidis NCCP12243, *Salmonella* Enterica KACC11595, *Salmonella* Montevideo NCCP10141 were cultured in 10 mL nutrient broth (NB; Becton, Dickinson and Company, Sparks, MD, USA) at 35°C for 24 h. Subsequently, 0.1-mL aliquots of the cultures were subcultured in 10 mL NB at 35°C for 24 h. The subcultures of five strains were mixed, and the mixed subcultures were centrifuged (1,912×*g*, 15 min, 4°C). The pellets were then washed twice with phosphate buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 L distilled water). The suspension was serially diluted with PBS to obtain 4 log CFU/mL of inoculum.

Media preparation and inoculation

Twenty five microliter aliquot of the inoculum was inoculated into 225 μL NB supplemented with combinations of NaCl (0%, 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, and 1.75%) and $NaNO_2$ (0, 15, 30, 45, 60, 75, 90, 105, and 120 ppm) in a 96-well microtiter plate. Two hundred fifty microliter aliquot of NB was used as a negative control, and 225 μL of NB inoculated with 25 μL of the cell suspension was used as a positive control. The microtiter plates were then incubated at 4°C, 7°C, 10°C, 12°C, and 15°C for up to 1,440 h, 1,440 h, 1,152 h, 384 h, and 168 h, respectively. For the aerobic conditions, the microtiter plates were sealed with plastic paraffin film (Parafilm M; Bemis Company Inc., Neenah, WI, USA). For the anaerobic conditions, the microtiter plates sealed with paraffin film were placed into tightly sealed plastic containers with Anaerogen (Oxoid Ltd., Basingstoke, Hampshire, UK). The Anaerogens were replaced every 24 h.

Model development

In this study, the combinations of NaCl (0%, 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, and 1.75%), $NaNO_2$ (0, 15, 30, 45, 60, 75, 90, 105, and 120 ppm), storage temperature (10°C, 12°C, and 15°C), and storage time (up to 1,440 h) were prepared. Each combination had eight observations (n = 8), and growth response was evaluated every 24 h during storage. If a well became turbid, it was assigned 1 (growth), otherwise it was assigned 0 (no growth). The growth response data were then analyzed by logistic regression using SAS (Version 9.3; SAS Institute Inc., Cary, NC, USA). The following equation was derived by the logistic regression and a stepwise selection method was used to select significant parameters ($p<0.05$). The selected parameters in the equation were used to describe the combinational effect of NaCl and $NaNO_2$ on *Salmonella* growth by producing growth/no growth interfaces for *Salmonella* at probabilities of 0.1, 0.5, and 0.9.

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

where P is the probability of growth, a_i are estimates, $NaCl$ is the concentration of NaCl, $NaNO_2$ is the $NaNO_2$ concentration, and $Time$ is the storage time.

Validation

To evaluatethe performance of the developed model, the predicted growth probabilities were compared to the results obtained from the frankfurters used as model processed meat products. To prepare the frankfurters, the formulation described by Kim et al. (2010) was used as follows: fat (20%) and lean meat (60%) were well-mixed with ice (20%), isolated soy protein (1.0%), phosphate (0.3%), spice (0.5%), potassium sorbate (0.2%), sugar (0.5%), $NaNO_2$ (0 or 10 ppm), and NaCl (1.0%, 1.25%, or 1.5%). The mixtures were stored at 4°C for 1 h, followed by stuffing them into collagen casings (ca., 30 g/each) using an automatic sausage can filler (Konti A50; Frey, Herbrechtingen, Germany). The frankfurters were subsequently cooked at 75°C for 40 min in a smokehouse (MAXI 3501; Kerres, Backnang, Germany). After cooling, the sausages were vacuum-packaged, and then heated for a second time at 80°C for 15 min, followed by dipping into ice water for 10 min and storage at 4°C overnight. The frankfurters were cut into 25-g samples. Thirty frankfurters were immersed in 500 mL inoculum (3 log CFU/mL) in a sterilized plastic container for 2 min. The samples were air-dried under a laminar flow cabinet for 15 min to allow *Salmonella* attachment then transferred into vacuum bags (Gwak et al., 2015). The bags were sealed for aerobic storage or vacuum packaged for anaerobic storage using a vacuum packager (HFV-500; Fujee Inc., Hwaseong,

Korea). The packaged samples were incubated at 4°C, 10°C, and 15°C for up to for 1,440 h. During incubation, *Salmonella* were enumerated on xylose lysine deoxycholate agar (XLD; Beckton, Dickinson, and Company) at the appropriate time interval (6 to 8 times), depending on the incubation temperature. If the *Salmonella* cell counts increased above 1 log CFU/g compared to the cell counts on day 0, it was considered as "growth" and if the cell counts decreased or increased less than 1 log CFU/g, it was considered "no growth" (Koutsoumanis et al., 2004; Yoon et al., 2009).

RESULTS AND DISCUSSION

The estimates of significant parameters are listed in Table 1. These estimates were used to predict the growth and no growth interfaces at probabilities of 0.1, 0.5, and 0.9. For both aerobic and anaerobic conditions, *Salmonella* growth was not observed at 4°C or 7°C (data not shown). *Salmonella* growth was initiated earlier in aerobic conditions than in anaerobic conditions at 10°C to 15°C (Figures 1 to 6). Møller (2012) reported a T_{min} (theoretical minimum temperature for growth) for *Salmonella* in pork at 2.33°C and the T_{min} for *Salmonella* suggested by Pin et al. (2011) was 4.27°C in broth media, which are both lower than the approximate lowest temperature (7°C) for *Salmonella* growth evaluated in this study. Fares (2007) and University of Nebraska Cooperative Extension (2005) also suggested 6°C to 7°C as the minimum temperature for *Salmonella*. Because T_{min} is a theoretical lower temperature limit of growth below which the calculated growth rate is close to zero (Ratkowsky et al., 1991), T_{min} and the minimum observed temperature in food are different. Even though low $NaNO_2$ and NaCl concentrations in processed meat products could be microbiologically unsafe, storage of the samples below 7°C can make the product safe. However, since $NaNO_2$ is very closely related to the inhibition of *Clostridium botulinum* germination (Christiansen et al., 1973), use of low $NaNO_2$ concentration for processed meat products should be considered very carefully.

Table 1. Estimates of the parameters selected from the logistic regression analysis using a stepwise selection method to predict the interfaces between growth and no growth of *Salmonella* at desired probabilities

Storage	Variables	Estimate	SE	p value
Aerobic	Interception	–312.5	5.6395	<0.0001
	Temperature	45.2212	0.8203	<0.0001
	$NaNO_2$/10	1.1584	0.1178	<0.0001
	NaCl	5.5105	0.7921	<0.0001
	Log(Time)	–7.5708	0.6221	<0.0001
	Temperature×$NaNO_2$/10	–0.0533	0.0054	<0.0001
	Temperature×NaCl	–0.1559	0.0362	<0.0001
	$NaNO_2$/10×log(Time)	–0.1207	0.0277	<0.0001
	NaCl×log(Time)	–0.6743	0.1878	0.0003
	Temperature^2	–1.6619	0.0304	<0.0001
	($NaNO_2$/10)^2	–0.0257	0.0020	<0.0001
	NaCl^2	–1.5059	0.0902	<0.0001
	(log(Time))^2	7.3097	0.1890	<0.0001
Anaerobic	Interception	–183.7	2.5665	<0.0001
	Temperature	24.6180	0.3562	<0.0001
	$NaNO_2$/10	0.5442	0.0336	<0.0001
	NaCl	1.1670	0.9812	0.2343
	Log(Time)	–1.4953	0.5295	0.0047
	Temperature*NaCl	–0.1759	0.0469	<0.0001
	$NaNO_2$/10*log(Time)	–0.2190	0.0117	<0.0001
	NaCl *log(Time)	0.5362	0.1988	0.0070
	Temperature^2	–0.8589	0.0132	<0.0001
	($NaNO_2$/10)^2	–0.0165	0.0015	<0.0001
	NaCl^2	–0.7122	0.0685	<0.0001
	(log(Time))^2	3.8334	0.1207	<0.0001

At 10°C under aerobic conditions, no antimicrobial effects from $NaNO_2$ were observed at all concentrations with 0% NaCl, but 120 ppm $NaNO_2$ showed obvious antimicrobial effects with more than 1% NaCl compared to other $NaNO_2$ concentrations. The antimicrobial effect of $NaNO_2$ became more obvious with 1.75% NaCl (Figure 1). However, at 10°C under anaerobic conditions, the antimicrobial activity of $NaNO_2$ increased as the NaCl concentration increased and became more obvious with 1.75% NaCl (Figure 2). At 12°C, the antimicrobial activity of $NaNO_2$ on *Salmonella* growth was not obvious with 0% NaCl, but NaCl with 0 ppm $NaNO_2$ inhibited *Salmonella* growth ($p<0.05$) under aerobic conditions in a concentration-dependent manner. In addition, the combinational effect of $NaNO_2$ and NaCl was not obvious under aerobic conditions, but the antimicrobial effect of $NaNO_2$ increased as NaCl concentration increased under anaerobic conditions (Figures 3 and 4). At 15°C, the antimicrobial activity of $NaNO_2$ was not observed with 0% NaCl under both aerobic and anaerobic conditions, and the combinational effect of $NaNO_2$ and NaCl was not obvious under aerobic conditions at 12°C, but the antimicrobial effect of $NaNO_2$ increased as NaCl concentration increased under anaerobic conditions (Figures 5 to 6). Jo et al. (2014) also developed probabilistic models to describe *Pseudomonas* growth responses in processed meat products formulated with $NaNO_2$ and NaCl, and found that the antimicrobial effect of $NaNO_2$ on *Pseudomonas* increased as NaCl concentration increased. In addition, Pelroy et al. (1994) reported increased $NaNO_2$ antimicrobial activity against *Listeria monocytogenes* in salmon with high NaCl concentrations. These results suggest that $NaNO_2$ should be combined with NaCl in processed meat products to inhibit *Salmonella* growth, especially in products with low $NaNO_2$ concentrations.

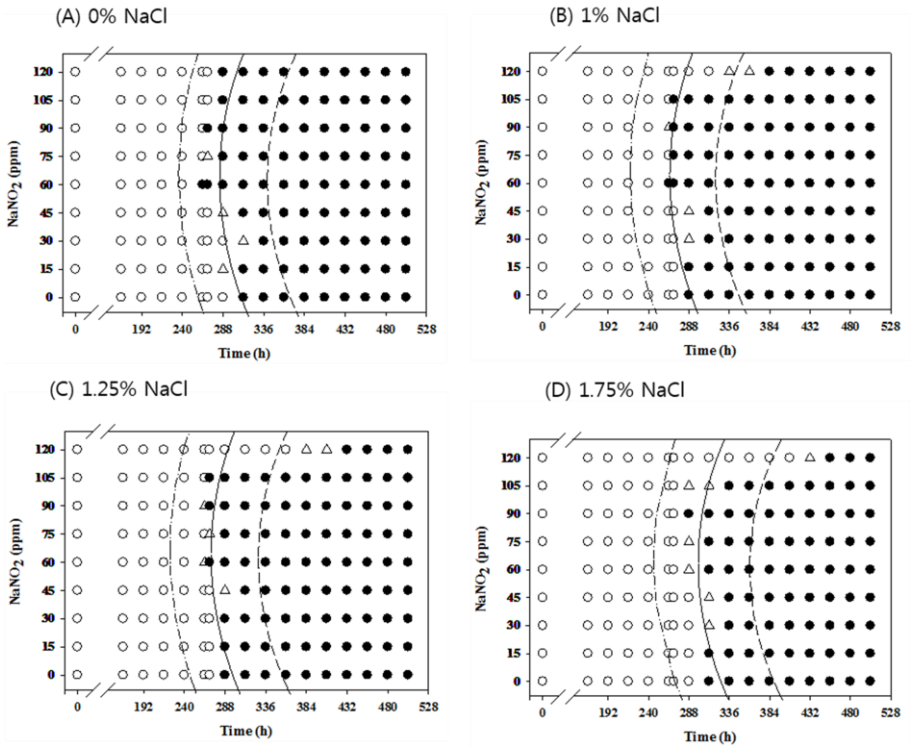

Figure 1. Growth/no-growth interfaces of *Salmonella* in nutrient broth at 10°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in aerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: ∆; only representative data are presented.

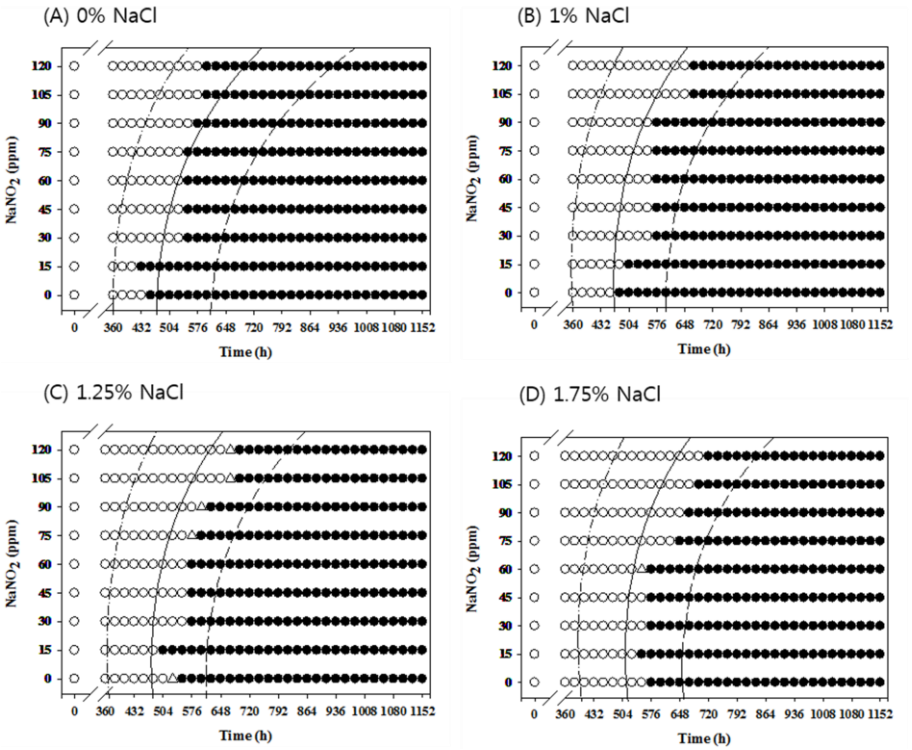

Figure 2. Growth/no-growth interfaces of *Salmonella* in nutrient broth at 10°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in anaerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: ∆; only representative data are presented.

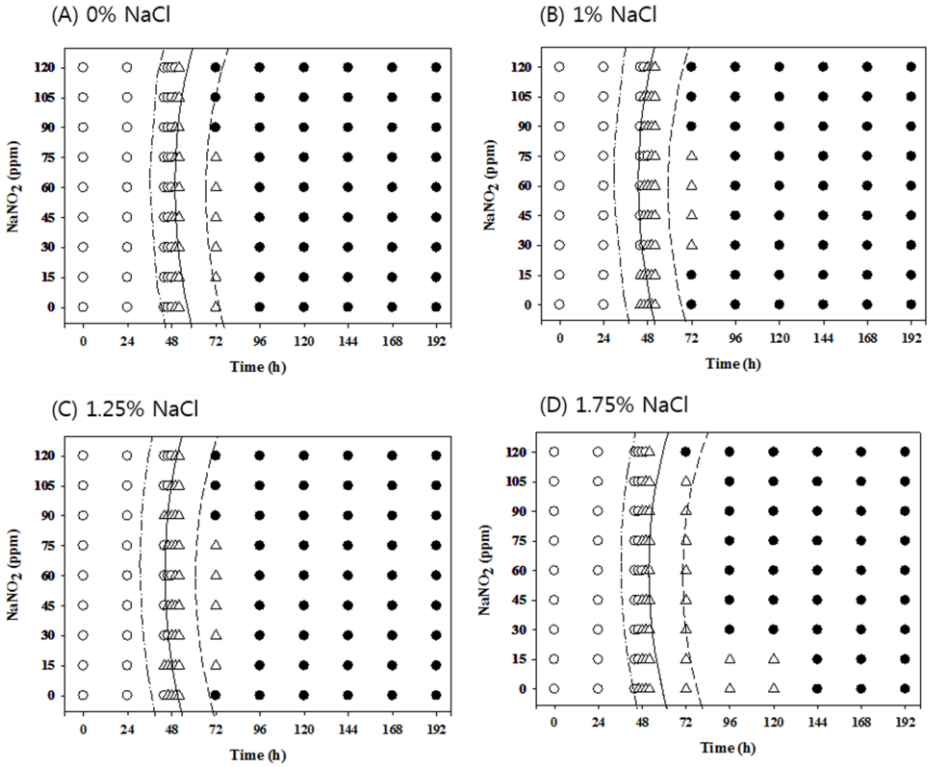

Figure 3. Growth/no-growth interfaces of *Salmonella* in nutrient broth at 12°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in aerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: ∆; only representative data are presented.

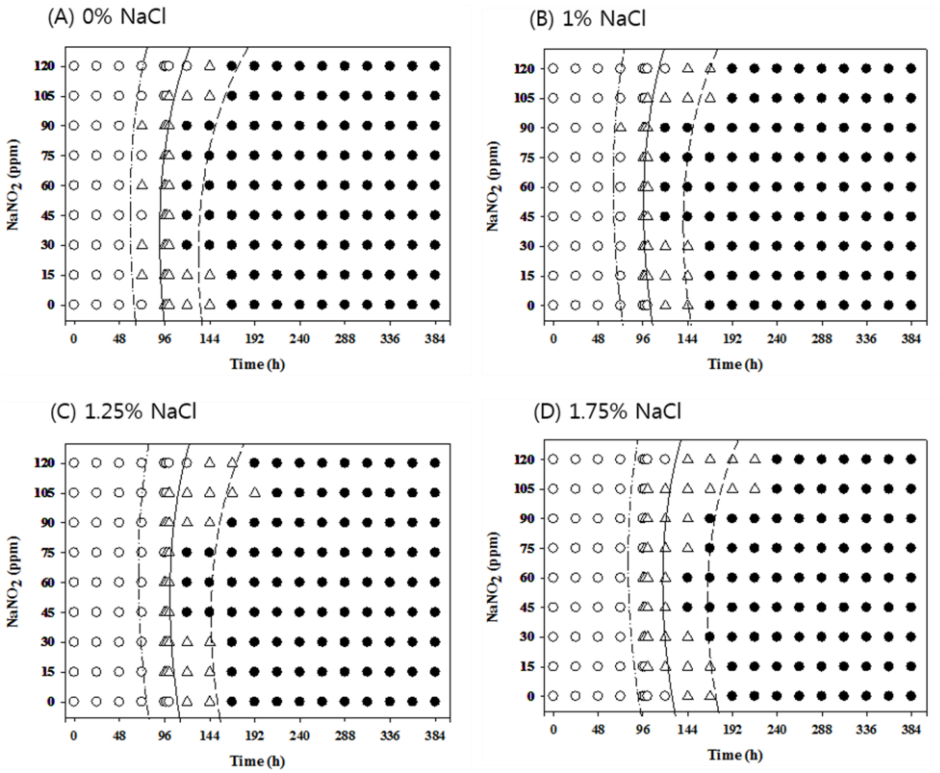

Figure 4. Growth/no-growth interfaces of *Salmonella* in nutrient broth at 12°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in anaerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: ∆; only representative data are presented.

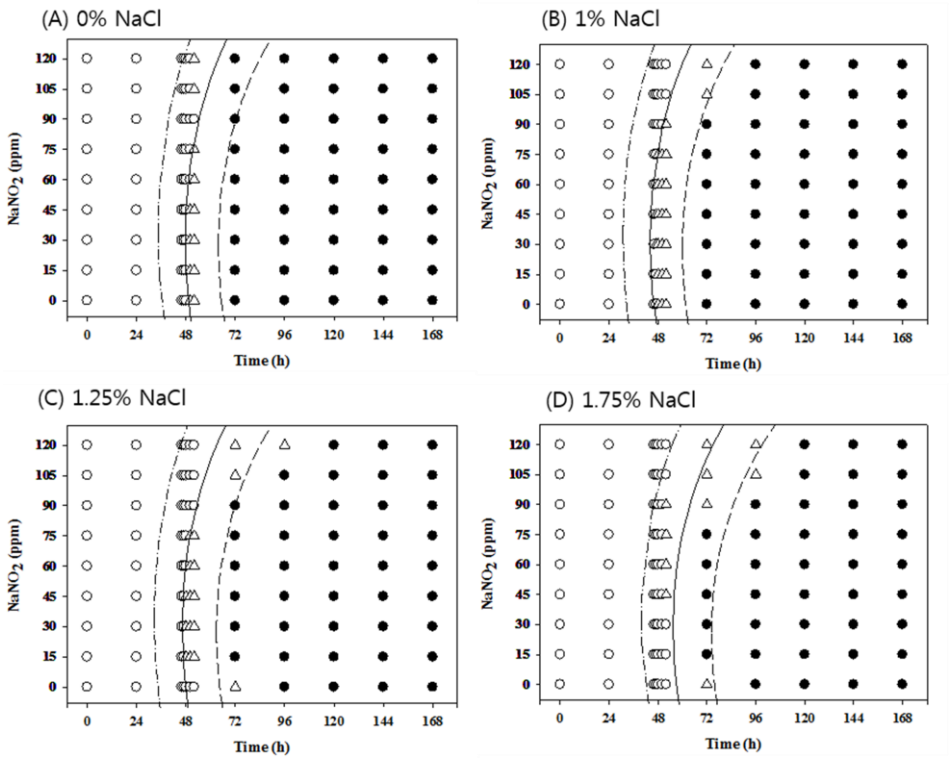

Figure 5. Growth/no-growth interfaces of *Salmonella* in nutrient broth at 15°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in aerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: ∆; only representative data are presented.

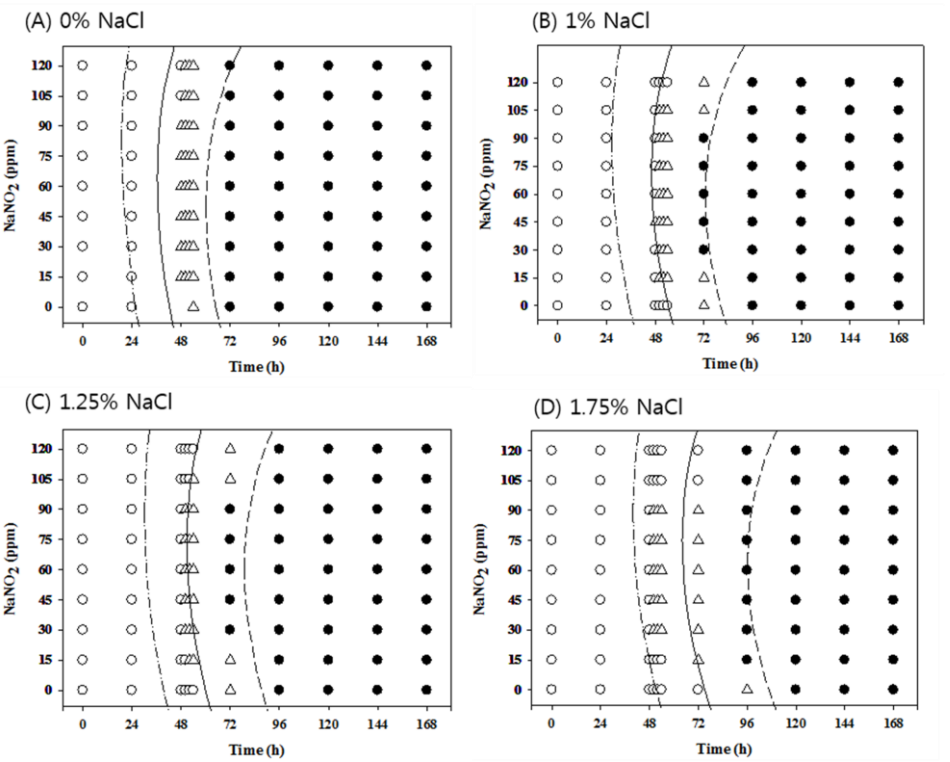

Figure 6. Growth/no-growth interfaces of *Salmonella* in nutrient broth at 15°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in anaerobic conditions at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: ∆; only representative data are presented.

To evaluate the performance of the developed probabilistic model, *Salmonella* growth response data were collected from frankfurters formulated with various concentrations of NaCl and $NaNO_2$, and the growth response data were compared to the results predicted from the probabilistic model under aerobic and anaerobic conditions; growth probability above 0.5 was considered growth (Koutsoumanis et al., 2004). The percentage of agreement between the observed and predicted growth response was approximately 81% (Table 2), indicating that the developed probabilistic model in this study was acceptable for evaluating the effect of low NaCl and $NaNO_2$ concentrations on *Salmonella* growth.

In conclusion, processed meat products containing low concentrations of $NaNO_2$ and NaCl should be stored below 7°C to inhibit *Salmonella* growth, regardless of the atmospheric conditions. However, if the products must be stored above 7°C, low $NaNO_2$ concentrations should be combined with NaCl to inhibit *Salmonella* growth under both aerobic and anaerobic conditions. In addition, the developed probabilistic model is appropriate for predicting the growth probability of *Salmonella* in processed meat products formulated with $NaNO_2$ and NaCl, which should be useful for describing the effect of $NaNO_2$ and NaCl on *Salmonella* growth in processed meat products.

Table 2. Comparison between observed growth responses and the predicted growth responses of *Salmonella* under aerobic condition

Temperature (°C)	$NaNO_2$ (ppm)	NaCl (%)	Time (h)	Observed growth response	Predicted growth responses	Temperature (°C)	$NaNO_2$ (ppm)	NaCl (%)	Time (h)	Observed growth response	Predicted growth responses
10	0	1	12	NG	NG	15	0	1	6	NG	NG
			24	NG	NG				12	NG	NG
			48	NG	NG				24	NG	NG
			192	G	NG				48	G	G
			288	G	NG				96	G	G
			336	G	G				120	G	G
		1.25	12	NG	NG			1.25	6	NG	NG
			24	NG	NG				12	NG	NG
			48	NG	NG				24	NG	NG
			192	G	NG				48	G	G
			288	G	NG				96	G	G
			336	G	G				120	G	G
		1.5	12	NG	NG			1.5	6	NG	NG
			24	NG	NG				12	NG	NG
			48	NG	NG				24	NG	NG
			192	G	NG				48	NG	NG
			288	G	NG				96	G	G
			336	G	G				120	G	G
	10	1	24	NG	NG		10	1	24	G	NG
			96	NG	NG				48	G	G
			144	NG	NG				72	G	G
			192	G	NG				96	G	G
			240	G	G				120	G	G
			384	G	G			1.25	24	NG	NG
		1.25	24	NG	NG				48	G	G
			96	NG	NG				72	G	G
			144	NG	NG				96	G	G
			192	G	NG				120	G	G
			240	G	NG			1.5	24	NG	NG
			384	G	G				48	G	NG
		1.5	24	NG	NG				72	G	G
			96	NG	NG				96	G	G
			144	NG	NG				120	G	G
			192	**G**	NG						
			240	**G**	NG						
			384	**G**	G						

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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