ARTICLE

Probabilistic Models to Predict *Listeria monocytogenes* Growth at Low Concentrations of NaNO$_2$ and NaCl in Frankfurters

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Abstract

This study developed probabilistic models to describe *Listeria monocytogenes* growth responses in meat products with low concentrations of NaNO$_2$ and NaCl. A five-strain mixture of *L. monocytogenes* was inoculated in NBYE (nutrient broth plus 0.6% yeast extract) supplemented with NaNO$_2$ (0-141 ppm) and NaCl (0-1.75%). The inoculated samples were then stored under aerobic and anaerobic conditions at 4, 7, 10, 12, and 15°C for up to 60 d. Growth response data [growth (1) or no growth (0)] for each combination were determined by turbidity. The growth response data were analyzed using logistic regression to predict the growth probability of *L. monocytogenes* as a function of NaNO$_2$ and NaCl. The model performance was validated with the observed growth responses. The effect of an obvious NaNO$_2$ and NaCl combination was not observed under aerobic storage condition, but the antimicrobial effect of NaNO$_2$ on the inhibition of *L. monocytogenes* growth generally increased as NaCl concentration increased under anaerobic condition, especially at 7-10°C. A single application of NaNO$_2$ or NaCl significantly ($p<0.05$) inhibited *L. monocytogenes* growth at 4-15°C, but the combination of NaNO$_2$ or NaCl more effectively ($p<0.05$) inhibited *L. monocytogenes* growth than single application of either compound under anaerobic condition. Validation results showed 92% agreement between predicted and observed growth response data. These results indicate that the developed model is useful in predicting *L. monocytogenes* growth response at low concentrations of NaNO$_2$ and NaCl, and the antilisterial effect of NaNO$_2$ increased by NaCl under anaerobic condition.

Keywords: NaNO$_2$, probabilistic model, *L. monocytogenes*, frankfurters

Introduction

L. monocytogenes are gram-positive, facultative anaerobic bacteria with optimum temperatures of 30-35°C (Faber and Peterkin, 1991; Hong *et al.*, 2015). The pathogen is widely found in nature, and they can grow under stressful conditions such as refrigerated temperatures (5°C) and high concentrations of NaCl (10%) (Shin, 2009). In addition, the pathogens are found in many foods, especially in meat and processed meat products (Myersa, 2013; Pan *et al.*, 2009). NaCl is used to improve the flavor and water holding capacity of processed meat products (Rhee and Zipirin, 2001), and to control bacterial growth in meat products (Aguilera and Karel, 1997). NaCl concentrations in meat products range from about 1.5% to 2.3% in South Korea (Kim *et al.*, 2004).

NaNO$_2$ is been known to paly a major role in coloring, controlling fat rancidity and controlling bacteria in meat products (Horsch *et al.*, 2014). However, NaNO$_2$ has chemical precursors that can be transformed into an *N*-nitroso compound under the low pH conditons of the stomach (Sugimura, 2000). This *N*-nitroso compound is reported to be toxic to the human body (Kemp and Dodds, 2002). Therefore, consumers preferentially purchase processed meat products with low NaNO$_2$ concentrations as well as low NaCl concentrations, but microbial safety issues for these products have been raised (Sindelar *et al.*, 2012).

Predictive models have been used to assess microbial behavior under various conditions. In particular, use of probabilistic models is appropriate for determining ideal

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concentration combinations of $NaNO_2$ and NaCl for inhibiting the growth of foodborne pathogens in processed foods. A probabilistic model is developed with logistic regression analysis to produce interfaces between growth and no growth of bacteria under different environmental conditions (Jo *et al.,* 2014; Koseki *et al.,* 2009; Yoon *et al.,* 2012). Therefore, the objective of this study was to describe probabilistic models to predict *L. monocytogenes* growth responses in meat products formulated with low concentrations of $NaNO_2$ and NaCl.

Materials and Methods

Inoculum preparation

L. monocytogenes NCCP (National Culture Collection for Pathogens) 10805 (poultry isolate), NCCP 10808 (human isolate), NCCP10809 (ruminant brain isolate), NCCP 10810 (human isolate) and NCCP10943 (rabbit isolate) were cultured in 10 mL nutrient broth with 0.6% yeast extract (NBYE; Becton, Dickinson and Company, USA) at 30°C for 24 h. The 0.1 mL aliquots of the cultures were then subcultured in fresh 10 mL NBYE at 30°C for 24 h. The subcultures were centrifuged (1,912 *g*, 15 min, 4°C) and washed twice with phosphate buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4\cdot7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 L distilled water). Suspensions of the five *L. monocytogenes* strains were mixed, and the mixture was serially diluted with PBS to obtain a density of 4 Log CFU/mL for inoculum.

Sample preparation and inoculation

The 225 μL of NBYE supplemented with combinations of $NaNO_2$ (0, 15, 30, 45, 60, 75, 90, 105, 120, and 141 ppm) and NaCl (0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75%) were placed into 96-well microtiter plates containing 25 μL aliquots of the prepared cell suspensions. Sterilized fresh NBYE and 225 μL fresh NBYE inoculated with 25 μL inoculum were used as negative control and positive control, respectively. The microtiter plates were stored under aerobic and anaerobic conditions at 4, 7, 10, 12, and 15°C up to 60 d, depending on storage temperatures. For aerobic condition, the microtiter plates were sealed with Parafilm M® (Bemis Company Inc., USA), while the microtiterplates for anaerobic storage were sealed with Parafilm M® and placed into tightly closed plastic containers with Anaerogen pack (Oxoid Ltd., UK). The Anaerogen packs were replaced every day to maintain anaerobic condition.

Growth response and probabilistic model development

Growth or no growth for the combinations ($NaNO_2\times$ NaCl×storage temperature×storage time; n=8) in aerobic and anaerobic conditions was determined every 24 h by turbidity of the samples. Turbid combinations were scored as 1 (growth) or 0 (no growth). The growth response data were analyzed by logistic regression using SAS® (Version 9.3; SAS Institute Inc., USA) with the following equation to select significant parameters ($p<0.05$) with a stepwise selection method (Koutsoumanis *et al.,* 2004; Lee *et al.,* 2012). The selected parameters were then used to produce growth/no growth interfaces of *L. monocytogenes* at 0.1, 0.5 and 0.9.

$$Logit\ (P) = a_0 + a_1\cdot NaCl + a_2(NaNO_2/10) + a_3\cdot Log\ (Time) + a_4\cdot Temp + a_5\cdot NaCl^2 + a_6\cdot(NaNO_2/10)^2 + a_7\cdot Log\ (Time)^2 + a_8\cdot Temp^2 + a_9\cdot NaCl(NaNO_2/10) + a_{10}\cdot NaCl\cdot Log\ (Time) + a_{11}(NaNO_2/10)\cdot Log(Time) + a_{12}\cdot Temp\cdot NaCl + a_{12}\cdot Temp\cdot(NaNO_2/10) + a_{12}\cdot Temp\cdot Log(Time)$$

Where logit (P) is an abbreviation for $ln[P/(1-P)]$, ln is the natural logarithm, P is the probability of growth, a_i are estimates, *NaCl* is the concentration of NaCl, *$NaNO_2$* is the $NaNO_2$ concentration, *Temp* is the storage temperature, and *Time* is incubation time.

Validation

The performance of the developed probabilistic model was assessed by comparing the predicted growth data to the observed data obtained from frankfuters. To prepare frankfuters, pork meat (60%) and pork fat (20%) were mixed with ice water (20%), phosphate (0.3%), isolated soy protein (1.0%), mixed spice (0.5%), sugar (0.5%), potassium sorbate (0.2%), $NaNO_2$ (0 and 10 ppm), and NaCl (1.0%, 1.25%, and 1.5%). The mixed pastes were then stored at 4°C for 1 h and stuffed into 25-mm diameter collagen casing (#260, NIPPI Inc., Japan; approximate 25 mm diameter) using an automatic sausage can filler (Konti A50, Frey, Germany). The sausages were heated at 75°C for 40 min in a smokehouse (MAXI 3501; Kerres, Germany) and then cooled at room temperature. The emulsion type sausage were then vacuum-packaged with polyethylene and heated at 80°C for 15 min with subsequent cooling in ice water for 10 min. Frankfurters were stored at 4°C until use (Choi *et al.,* 2014). To inoculate *L. monocytogenes* on frankfurters, the vacuum-packages were opened, and 25 g portions of the samples were immersed into 500 mL *L. monocytogenes* inoculum (3 Log

CFU/mL) in sterilized plastic containers for 2 min. The samples were air-dried under a laminar flow cabinet for 15 min, and the sausage were then transferred into sample bags. The samples were sealed for aerobic condition or vacuum-packaged for anaerobic conditon. The samples were incubated at 4°C, 10°C and 15°C for up to 1,416 h, 288 h, and 168 h, respectively. During storage, 40 mL 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, USA) was added into a sample bag and pummeled in a pummeler (BagMixer®, Interscience, France). The homogenates were serially diluted in BPW, and the diluents were spread-plated on PALCAM agar (Becton, Dickinson and Company) to enumerate *L. monocytogenes*. If the *L. monocytogenes* cell counts increased by more than 1 Log CFU/g compared to the initial cell counts, it was scored as "growth." If the cell counts increased by less than 1 Log CFU/g, it was scored as "no growth" (Koutsoumanis *et al.,* 2004). These results were then compared to the predicted growth response data from the developed probabilistic model; if the growth probability was greater than 0.5, it was determined as "growth" (Yoon *et al.,* 2009).

Results and Discussion

The estimates for significant variables selected from the logistic regression analysis are shown in Table 1, and these estimates were used to produce the growth and no growth interfaces at probabilities of 0.1, 0.5, and 0.9. Under aerobic and anaerobic conditions, NaNO₂ and NaCl showed significant effects on the inhibition of *L. monocytogenes* growth (Table 1). The models used the common logarithm of storage time due to its nonlinear effect on the end of the lag time (Koseki *et al.,* 2012). NaNO₂ concentration was divided by 10 to reduce statistical error by minimizing the difference between the observed values.

For aerobic storage condition, an obvious NaNO₂ and

NaCl combination effect was not observed (Fig. 1-3; data for 4°C and 7°C not shown), but the antimicrobial effect of NaNO₂ on the inhibition of *L. monocytogenes* growth generally increased as NaCl concentration increased under anaerobic condition, especially at 7-10°C (Fig. 4-8).

At 4°C and 7°C, *L. monocytogenes* growth was not observed at 75 ppm NaNO₂ or higher for all NaCl concentrations tested in this study under aerobic condition, and the pathogen initiated growth at 720-840 h for 4°C and 480-600 h for 7°C (data not shown). However, *L. monocytogenes* growth was generally observed at 10°C after 192 h storage for all NaNO₂ concentrations under aerobic storage (Fig. 1). Other studies have also reported that refrigeration storage and additives (NaCl and NaNO₂) can cause stress to foodborne pathogens such as *Salmonella* (Laura *et al.,* 2013; Pratt *et al.,* 2012) and *Escherichia coli* (Jones *et al.,* 2008), *Clostridium botulinum* (Derman *et al.,* 2015)*.* Thus, Laura *et al.* (2013) suggested that the effects of stressful conditions on bacteria are influenced by temperature because bacterial stress reponses were influenced by transcriptional changes at variant temperatures (Koutsoumanis *et al.,* 2003). Ribeiro and Destro (2014) also studied that survival of two *L. monocytogenes* serotypes (1/2b and 4b) at high concentrations of NaCl and growth at refrigeration temperatures and found that environmental temperature may affect the virulence of the pathogens, thus temperature should be well controlled during food storage.

Certain countries allow a refrigeration temperature of up to 10°C for retail market, and handmade sausages are usually displayed in aerobic packaging at retail. Our results suggest that NaNO₂ should be present at a concentration of at least 75 ppm in order to inhibit *L. monocytogenes* growth in aerobic storage at 4°C and 7°C, and processed meat product should be sold within 192 h in the event that the products are stored at 10°C.

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

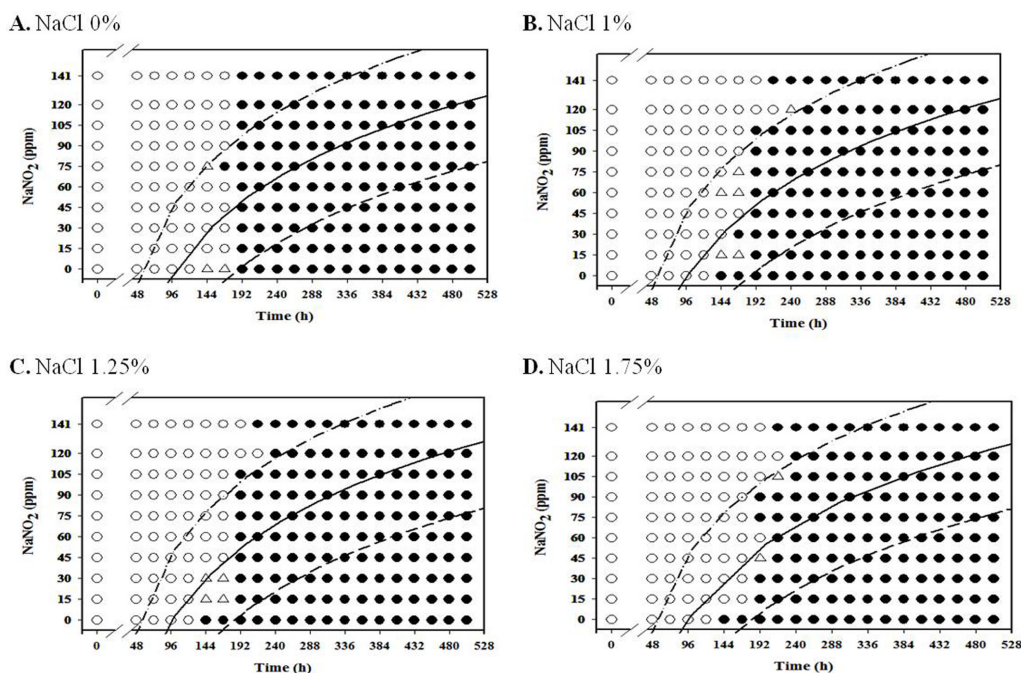| Storage	Variables	Estimate	SE	P value
Aerobic	Interception	-28.9018	0.2029	<0.0001
	Temperature	1.2925	0.0085	<0.0001
	NaNO₂/10	-0.4591	0.0033	<0.0001
	NaCl	0.0704	0.0171	<0.0001
	Log (Time)	8.0034	0.0567	<0.0001
Anaerobic	Interception	-23.1749	0.1456	<0.0001
	Temperature	0.6829	0.0040	<0.0001
	NaNO₂/10	-0.5451	0.0030	<0.0001
	NaCl	0.4213	0.0147	<0.0001
	Log (Time)	7.4567	0.0463	<0.0001

Fig. 1. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 10°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in aerobic condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.
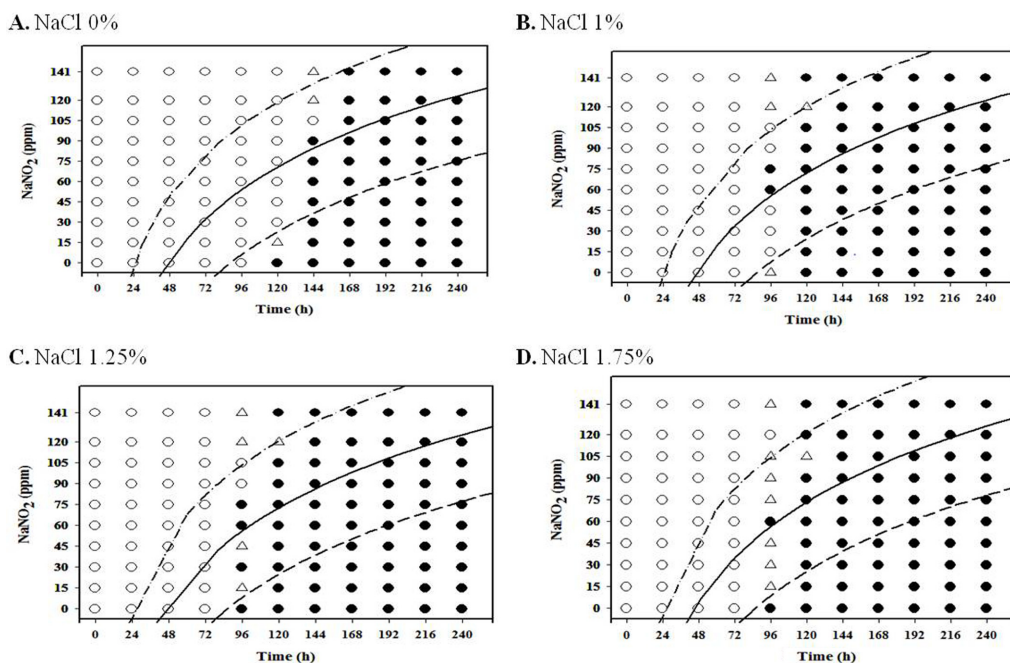

Fig. 2. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 12°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in aerobic condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.

For anaerobic condition, *L. monocytogenes* growth was inhibited at NaNO₂ concentrations above 60 ppm for all

NaCl concentrations under anaerobic condition at 4°C, and the growth of the pathogen was inhibited at NaNO₂
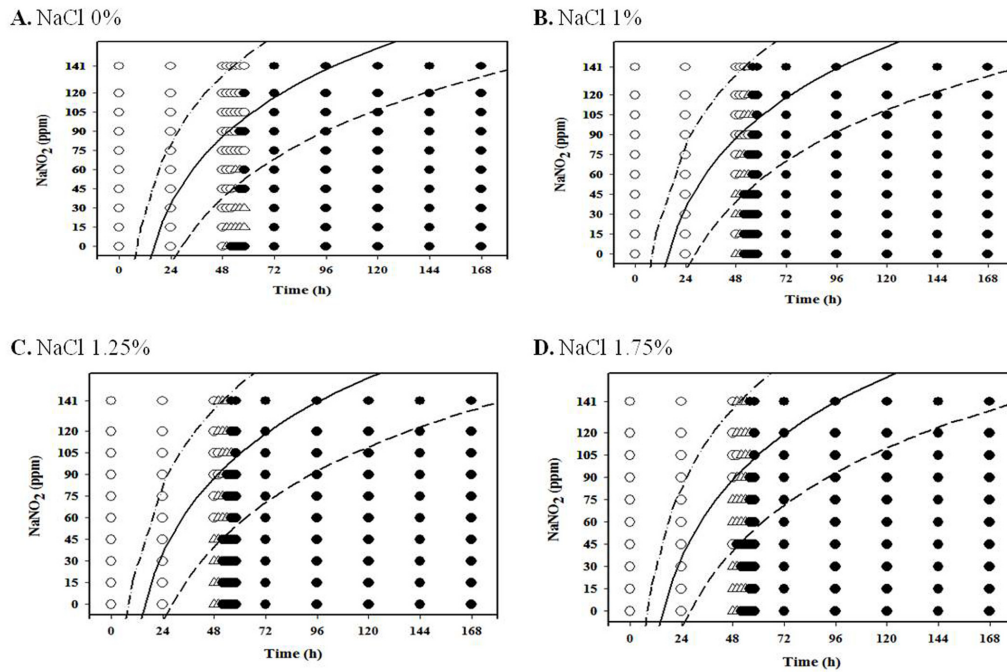
Fig. 3. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 15°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in aerobic condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.
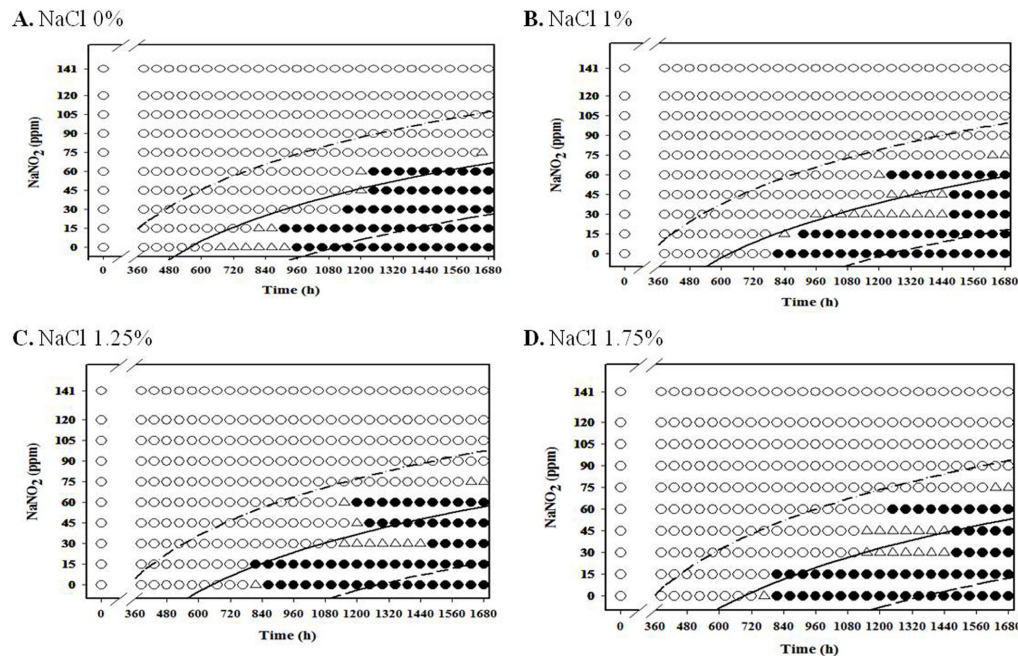

Fig. 4. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 4°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in vacuum condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.

concentrations about 75 ppm at 7°C for all NaCl concentrations (Fig. 4 and 5). Under anaerobic storage at 4°C and

7°C, the antimicrobial effect of NaNO$_2$ on *L. monocytogenes* increased as NaCl concentration increased (Fig. 4
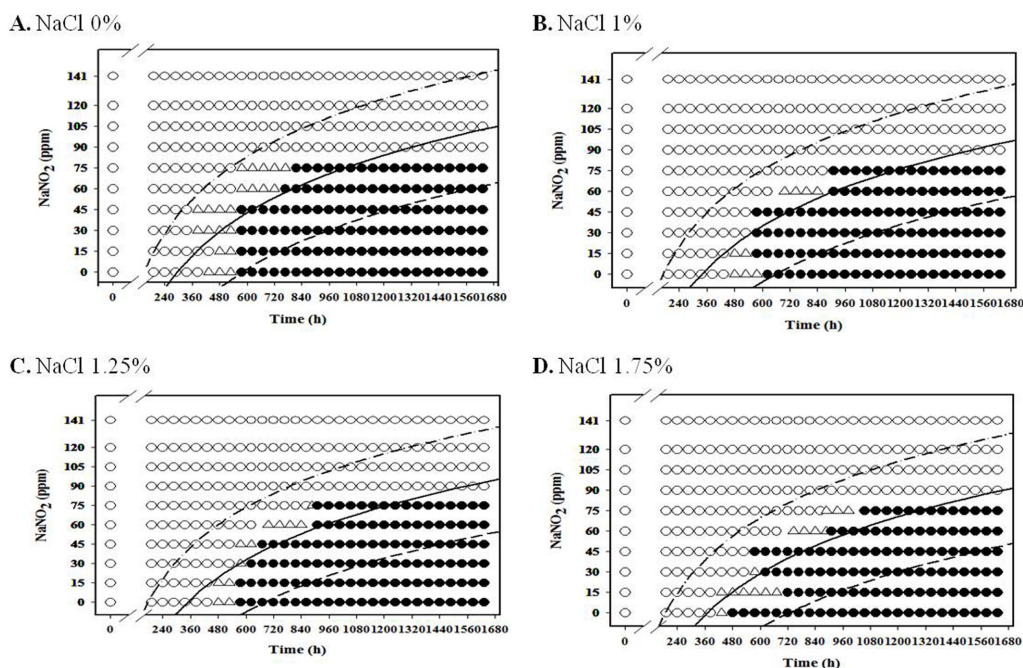
Fig. 5. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 7°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in vacuum condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.
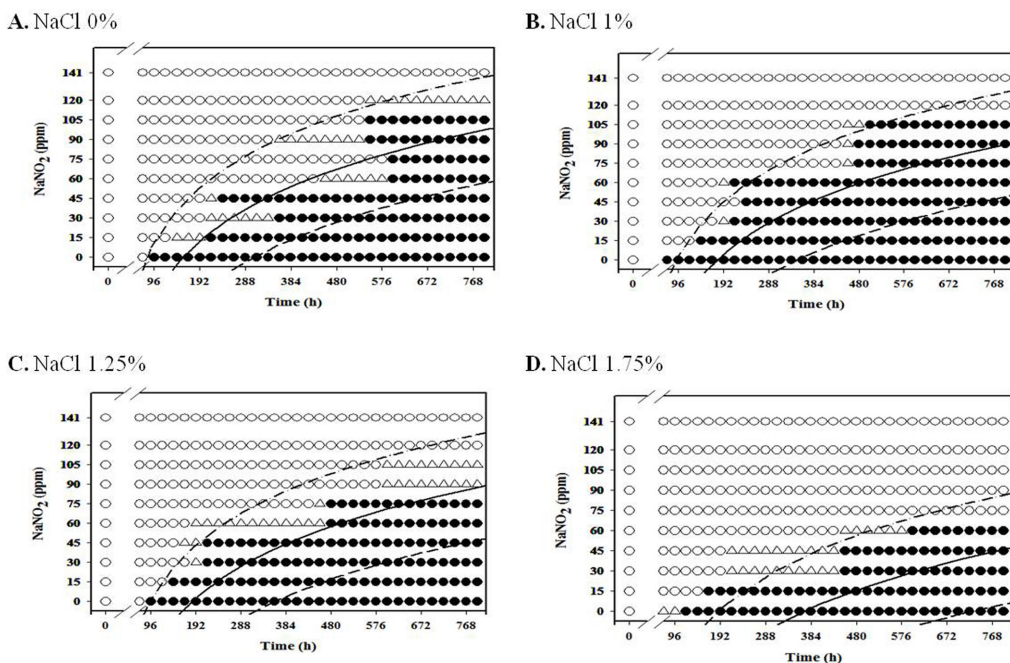

Fig. 6. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 10°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in vacuum condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.

and 5). Interestingly, the observed growth inhibition concentrations (60-75 ppm) of NaNO$_2$ were similar for both

aerobic and anaerobic storage (Fig. 4 and 5; data not shown for aerobtic storage at 4°C and 7°C), indicating that the

A. NaCl 0%

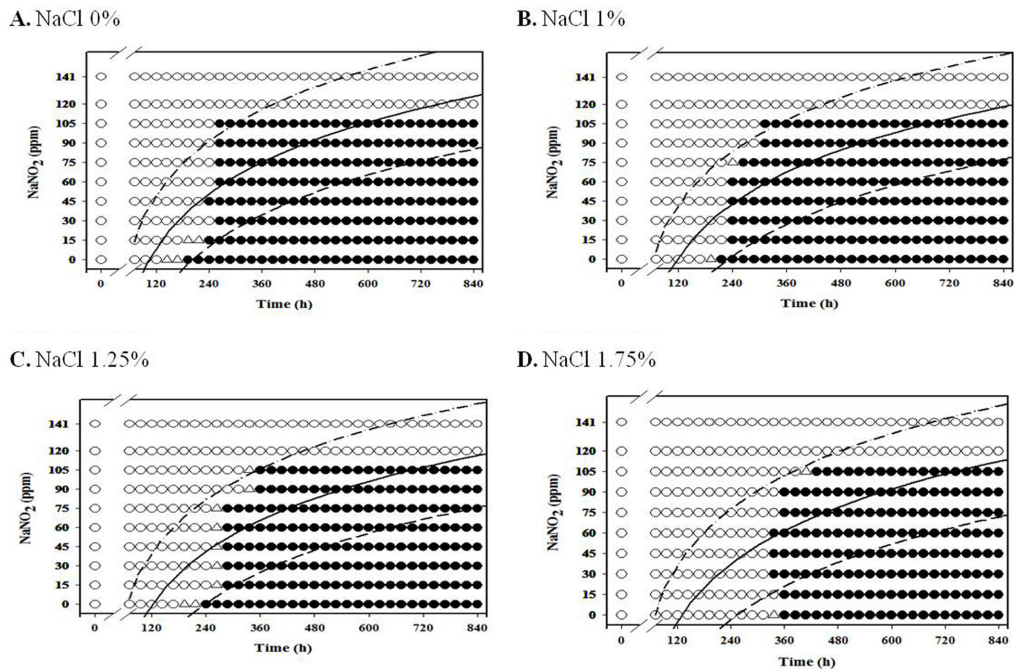**B.** NaCl 1%

C. NaCl 1.25%

D. NaCl 1.75%

Fig. 7. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 12°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in vacuum condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○ , growth: ● , 50% growth: △ .

A. NaCl 0%

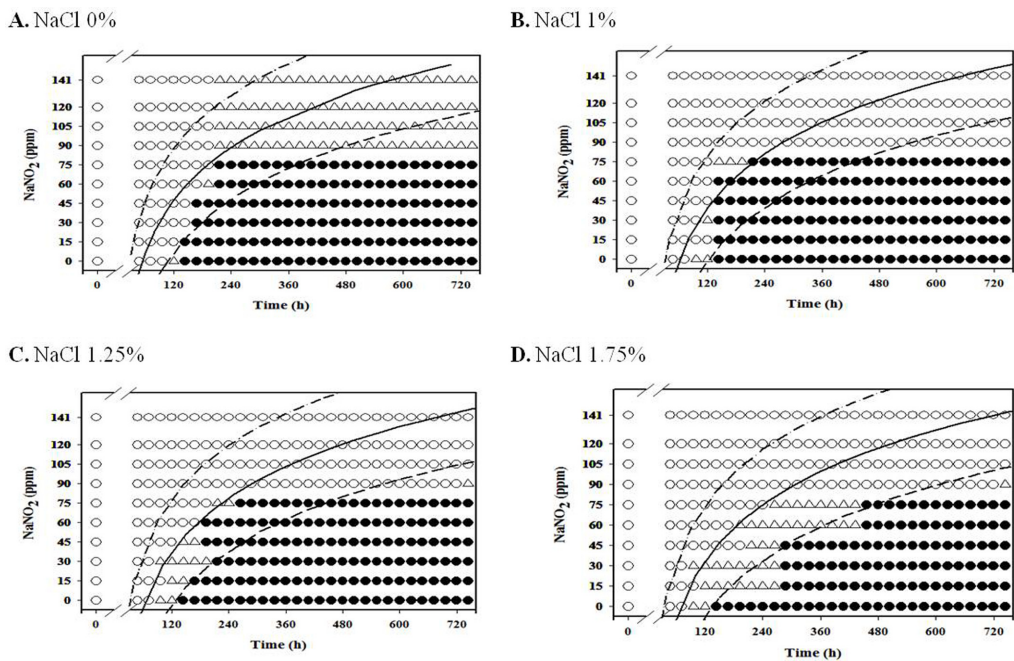**B.** NaCl 1%

C. NaCl 1.25%

D. NaCl 1.75%

Fig. 8. Growth/no-growth interfaces of *L. monocytogenes* in nutrient broth with 0.6% yeast extract at 15°C with respect to sodium nitrite concentration and storage time as a function of NaCl levels in vacuum condition at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○ , growth: ● , 50% growth: △ .

inhibition effect of NaNO$_2$ on *L. monocytogenes* may not be significantly influenced by oxygen concentration at 4°C

and 7°C (Fig. 4 and 5). At 7°C and 10°C, the combined effect of NaNO$_2$ and NaCl on the inhibition of *L. mono-*

cytogenes became very clear under anaerobic storage, indicating that the antilisterial effect of $NaNO_2$ increased as NaCl concentration increased (Fig. 5 and 6). These results indicate that $NaNO_2$ should be combined with NaCl to effectively inhibit the growth of *L. monocytogenes* in processed meat. In addition, the NaCl concentration should be higher than 1% for emulsion type sausage such as frankfurters in order to induce emulsion in sausage.

The performance of the developed model was assessed by a comparison between predicted and observed growth responses from the experiment with frankfurters. The predicted growth responses of the developed model were in agreement with most observed data under aerobic and anaerobic conditions. The concordance percentage between observed and predicted growth responses was about 92% (data not shown).

Conclusion

In conclusion, the antilisterial effect of $NaNO_2$ increased as NaCl concentration increased under anaerobic conditions, and the developed model should be useful in predicting the minimum concentrations of NaCl and $NaNO_2$ combinations for the inhibition of *L. monocytogenes* growth in processed meat products formulated with low concentrations of $NaNO_2$ and NaCl. Also, these results should be useful in quantitative microbial risk assessments.

Acknowledgements

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