Probabilistic Models to Predict Listeria monocytogenes Growth at Low Concentrations of NaNO₂ 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 NaNO₂ and NaCl. A five-strain mixture of L. monocytogenes was inoculated in NBYE (nutrient broth plus 0.6% yeast extract) supplemented with NaNO₂ (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 NaNO₂ and NaCl. The model performance was validated with the observed growth responses. The effect of an obvious NaNO₂ and NaCl combination was not observed under aerobic storage condition, 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. A single application of NaNO₂ or NaCl significantly (p<0.05) inhibited L. monocytogenes growth at 4-15°C, but the combination of NaNO₂ 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 NaNO₂ and NaCl, and the antilisterial effect of NaNO₂ increased by NaCl under anaerobic condition.

Keywords: NaNO₂, 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).

NaNO₂ has been known to play a major role in coloring, controlling fat rancidity and controlling bacteria in meat products (Horsch et al., 2014). However, NaNO₂ has chemical precursors that can be transformed into an N-nitroso compound under the low pH conditions 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 NaNO₂ 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
concentration combinations of NaNO₂ 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 NaNO₂ and NaCl.

Materials and Methods

Inoculum preparation

L. monocytogenes NCCP (National Culture Collection for Pathogens) 10805 (poultry isolate), NCCP 10808 (human isolate), NCCP 10809 (ruminant brain isolate), NCCP 10810 (human isolate) and NCCP 10943 (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 0.1 mL aliquots of the cultures were centrifuged (1,912 g, 15 min, 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 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 NaNO₂ (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 (NaNO₂×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.

\[
\text{Logit}(P) = a_0 + a_1 \cdot \text{NaCl} + a_2 \cdot (\text{NaNO}_2/10) + a_3 \cdot \log(T) + a_4 \cdot \text{Temp} + a_5 \cdot \text{NaCl}^2 + a_6 \cdot (\text{NaNO}_2/10)^2 + a_7 \cdot \log(T)^2 + a_8 \cdot \text{Temp}^2 + a_9 \cdot \text{NaCl}(\text{NaNO}_2/10) + a_{10} \cdot \text{NaCl} \cdot \log(T) + a_{11} \cdot (\text{NaNO}_2/10) \cdot \log(T) + a_{12} \cdot \text{Temp} \cdot \text{NaCl} + a_{13} \cdot \text{Temp} \cdot (\text{NaNO}_2/10) + a_{14} \cdot \text{Temp} \cdot \log(T)
\]

Where logit(P) is an abbreviation for ln[P/(1-P)], ln is the natural logarithm, P is the probability of growth, aᵢ are estimates, NaCl is the concentration of NaCl, NaNO₂ is the NaNO₂ 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 frankfurters. To prepare frankfurters, 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%), NaNO₂ (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 condition. 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 \textit{L. monocytogenes}. If the \textit{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, \textit{NaNO}_\textsubscript{2} and \textit{NaCl} showed significant effects on the inhibition of \textit{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). \textit{NaNO}_\textsubscript{2} concentration was divided by 10 to reduce statistical error by minimizing the difference between the observed values.

For aerobic storage condition, an obvious \textit{NaNO}_\textsubscript{2} and \textit{NaCl} combination effect was not observed (Fig. 1-3; data for 4°C and 7°C not shown), but the antimicrobial effect of \textit{NaNO}_\textsubscript{2} on the inhibition of \textit{L. monocytogenes} growth generally increased as \textit{NaCl} concentration increased under anaerobic condition, especially at 7-10°C (Fig. 4-8).

At 4°C and 7°C, \textit{L. monocytogenes} growth was not observed at 75 ppm \textit{NaNO}_\textsubscript{2}, or higher for all \textit{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, \textit{L. monocytogenes} growth was generally observed at 10°C after 192 h storage for all \textit{NaNO}_\textsubscript{2} concentrations under aerobic storage (Fig. 1). Other studies have also reported that refrigeration storage and additives (\textit{NaCl} and \textit{NaNO}_\textsubscript{2}) can cause stress to foodborne pathogens such as \textit{Salmonella} (Laura et al., 2013; Pratt et al., 2012) and \textit{Escherichia coli} (Jones et al., 2008), \textit{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 \textit{L. monocytogenes} serotypes (1/2b and 4b) at high concentrations of \textit{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 \textit{NaNO}_\textsubscript{2} should be present at a concentration of at least 75 ppm in order to inhibit \textit{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 \textit{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 |
|         | \textit{NaNO}_\textsubscript{2}/10 | -0.4591 | 0.0033 | <0.0001 |
|         | \textit{NaCl} | 0.0704 | 0.0171 | <0.0001 |
|         | Log (Time) | 8.0034 | 0.0567 | <0.0001 |
| Anaerobic | Interception | -23.1749 | 0.1436 | <0.0001 |
|          | Temperature | 0.6829 | 0.0040 | <0.0001 |
|          | \textit{NaNO}_\textsubscript{2}/10 | -0.5451 | 0.0030 | <0.0001 |
|          | \textit{NaCl} | 0.4213 | 0.0147 | <0.0001 |
|          | Log (Time) | 7.4567 | 0.0463 | <0.0001 |
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 concentrations above 60 ppm for all NaCl concentrations under anaerobic condition at 4°C.
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 NaNO₂ on *L. monocytogenes* increased as NaCl concentration increased (Fig. 4 and 5).
and 5). Interestingly, the observed growth inhibition concentrations (60-75 ppm) of NaNO₂ were similar for both aerobic and anaerobic storage (Fig. 4 and 5; data not shown for aerobic storage at 4°C and 7°C), indicating that the

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: △.
L. monocytogenes at Low Levels of NaNO₂ and NaCl

The inhibition effect of NaNO₂ 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 NaNO₂ and NaCl on the inhibition of L. mono-
cytogenes became very clear under anaerobic storage, indicating that the antilisterial effect of NaNO$_3$ increased as NaCl concentration increased (Fig. 5 and 6). These results indicate that NaNO$_3$ 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$_3$ increased as NaCl concentration increased under anaerobic conditions, and the developed model should be useful in predicting the minimum concentrations of NaCl and NaNO$_3$ combinations for the inhibition of L. monocytogenes growth in processed meat products formulated with low concentrations of NaNO$_3$ and NaCl. Also, these results should be useful in quantitative microbial risk assessments.

**Acknowledgements**

This work was carried out with the support of “Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009237)” Rural Development Administration, Republic of Korea.

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