Probabilistic Models to Predict the Growth Initiation Time for Pseudomonas spp. in Processed Meats Formulated with NaCl and NaNO$_2$

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Abstract

This study developed probabilistic models to determine the initiation time of growth of Pseudomonas spp. in combinations with NaNO$_2$ and NaCl concentrations during storage at different temperatures. The combination of 8 NaCl concentrations (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.75%) and 9 NaNO$_2$ concentrations (0, 15, 30, 45, 60, 75, 90, 105, and 120 ppm) were prepared in a nutrient broth. The medium was placed in the wells of 96-well microtiter plates, followed by inoculation of a five-strain mixture of Pseudomonas in each well. All microtiter plates were incubated at 4, 7, 10, 12, and 15°C for 528, 504, 504, 360 and 144 h, respectively. Growth (growth initiation; GI) or no growth was then determined by turbidity every 24 h. These growth response data were analyzed by a logistic regression to produce growth/no growth interface of Pseudomonas spp. and to calculate GI times. NaCl and NaNO$_2$ were significantly effective ($p<0.05$) on inhibiting Pseudomonas spp. growth when stored at 4-12°C. The developed model showed that at lower NaCl concentration, higher NaNO$_2$ level was required to inhibit Pseudomonas growth at 4-12°C. However, at 15°C, there was no significant effect of NaCl and NaNO$_2$. The model overestimated GI times by 58.2±17.5 to 79.4±11%. These results indicate that the probabilistic models developed in this study should be useful in calculating the GI times of Pseudomonas spp. in combination with NaCl and NaNO$_2$ concentrations, considering the over-prediction percentage.

Keywords: Pseudomonas aeruginosa, Pseudomonas fluorescens, NaCl, NaNO$_2$, processed meats

Introduction

Pseudomonas spp. are psychrotrophic bacteria, and they are the main cause for milk spoilage (Reddy et al., 1969), chicken (Pittard et al., 1982), fish (Miller et al., 1973), and meat especially at chill temperatures (Nychas et al., 2008). In food, they produce special fluorescent green, yellow or bluish compounds (Brown et al., 1958). Moreover, they generate off-odors in the meats by producing prolytic and lipolytic enzymes (Champagne et al., 1994; Sor-haug and Stepniak, 1997). Although Pseudomonas spp. cause physicochemical changes, microbiological criteria for the bacteria are not established because they are not pathogenic bacteria.

In processed meat products, NaNO$_2$ plays an important role in developing of cured meat color and flavor, retarding lipid autoxidation, and preventing Clostridium botuli-
NaNO₂ and NaCl need to be determined to inhibit bacterial growth and also to meet consumers’ requirement. Thus, the interactive responses for these two ingredients should be considered in order to determine the minimum concentrations. The probabilistic model should be appropriate to achieve this goal. Probabilistic model using logistic regression can estimate the probabilities of bacterial growth and interface between growth and no growth of bacteria under various conditions (López-Malo et al., 2000; Tienungoon et al., 2000). This mathematical technique can be applied to estimate the GI time of food-related bacteria.

Most studies on the relationship between *Pseudomonas* spp. and NaNO₂ have focused on the antimicrobial effect of NaNO₂ on *Pseudomonas* spp. (Henry and Bessieres, 1984; Nicke et al., 2013), but the combination effect of NaNO₂ and NaCl on *Pseudomonas* spp. in processed meats has not been fully studied yet.

Therefore, the objective of this study was to develop probabilistic models to determine the GI time of *Pseudomonas* spp. in combinations with NaNO₂ and NaCl concentrations.

### Materials and Methods

**Inoculum preparation**

The isolated colonies of *Pseudomonas aeruginosa* strains (NCCP10338, NCCP10250, and NCCP11229) and *Pseudomonas fluorescens* strains (KACC10326 and KACC10323) in Cetrimide agar (Becton Dickinson and Company, USA) were cultured in nutrient broth (NB; Becton Dickinson and Company) in Cetrimide agar (Becton Dickinson and Company). The isolated colonies of *Pseudomonas fluorescens* (NCCP10338, NCCP10250, and NCCP11229) and related bacteria.

The growth response data were analyzed with the SAS version 9.2 logistic regression analysis (SAS Institute Inc., USA) to estimate the growth probabilities of *Pseudomonas* spp. Significant parameters were selected through a stepwise selection method (p<0.05).

\[
\text{Logit}(P) = a_0 + a_1 \cdot \text{NaCl} + a_2 \cdot \text{NaNO}_2 + a_3 \cdot \text{Time} + a_4 \cdot \text{NaCl}^2 + a_5 \cdot \text{NaNO}_2^2 + a_6 \cdot \text{Time}^2 + a_7 \cdot \text{NaCl} \cdot \text{NaNO}_2 + a_8 \cdot \text{NaCl} \cdot \text{Time} + a_9 \cdot \text{NaNO}_2 \cdot \text{Time}
\]

Where Logit(P) is an abbreviation of ln[P/(1−P)], P is the probability of growth within the range of 0 to 1, \(a_i\) is the estimates, NaCl is NaCl concentrations, NaNO₂ is NaNO₂ concentrations, and Time is storage time.

**Evaluation of developed model**

Observed data for *Pseudomonas* spp. growth were obtained from commercial frankfurters and bacon. The frankfurters and bacon were cut into 7 g and placed into plastic bags (Food Saver®; Rollpack, Korea). The 0.1 mL portions of the inoculum were inoculated on one side of the sample surface. The inoculated samples were massaged 15 times in order to spread the bacteria and then sealed using a packager (Food Guard®; Rollpack, Korea). The samples were then aerobically stored at 4, 7, 12, and 15°C for 336, 312, 192, and 120 h, respectively. To quantify bacterial populations, the samples were analyzed at appropriate intervals. The 30 mL of 0.1% buffered peptone water (BPW; Becton Dickinson and Company) was added into the sample bag and homogenized using a pummeler (BagMixer®, Interscience, France) for 60 s. The homogenates were serially diluted with 0.1% BPW, and 0.1 mL of the diluents was surface-plated on Cetrimide agar. The plates were incubated at 35°C for 24 h, and the typical colonies were manually counted to determine GI time. A growth greater than 1-log considered growth (Koutsoumanis et al., 2004; Lee et al., 2013). The observed GI times were then compared to the predicted GI times of...
Results and Discussion

The estimates of coefficients selected from the logistic regression analysis, using an automatic variable selection option with a stepwise selection method, are shown in Table 1. The estimates were then used to produce interfaces between growth and no growth of *Pseudomonas* spp. at 0.1, 0.5, and 0.9 of probabilities with the combination for NaNO$_2$ and NaCl level for each storage temperature (Figs. 1-2). This result can also be used to determine the GI time of *Pseudomonas* spp. NaCl, NaNO$_2$ and storage time were generally significant ($p<0.05$) factors for inhibiting *Pseudomonas* spp. growth during storage at 4-12°C (Table 1). However, NaNO$_2$ and NaCl did not have any significant effects on the growth of the bacteria at 15°C. Moreover, a square function for NaCl and NaNO$_2$ was not observed at 12 and 15°C (Table 1).

For 4 and 7°C, the antimicrobial effect of NaNO$_2$ on *Pseudomonas* spp. growth slightly increased to 1% NaCl, but the antimicrobial effect dramatically increased to 1.25% NaCl (Figs. 1 and 2). This result indicates that the obvious antimicrobial effect of NaNO$_2$ to inhibit *Pseudo-*

![Fig. 1. Growth/no-growth interfaces of *Pseudomonas* spp. at 4°C with respect to NaNO$_2$ concentration and storage time as a function of NaCl levels at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.](image)

![Fig. 2. Growth/no-growth interfaces of *Pseudomonas* spp. at 7°C with respect to NaNO$_2$ concentration and storage time as a function of NaCl levels at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.](image)
growth can be found in high NaCl concentration (>1.25% NaCl). In addition, the combination effect of NaCl and NaNO₂ on the inhibition of Pseudomonas spp. growth was also observed at 10, 12, and 15°C (data not shown). According to these results, it is suggested that NaCl concentration of ready-to-eat meat products should be at a certain level to have the obvious antimicrobial effect of NaNO₂ on Pseudomonas spp. growth. This is proven by the result from Fig. 3, showing that the difference of growth probability among NaNO₂ concentrations became more obvious as NaCl concentration increased. Similarly, a study by Pelroy et al. (1994) also showed that the concentration-dependent antimicrobial effect of NaNO₂ on L. monocytogenes in cold-processed salmon in high NaCl concentrations when stored at 5 and 10°C.

Shahamat et al. (1980) and Buchanan et al. (1989) examined the antimicrobial effects of NaNO₂ on L. monocytogenes and suggested that the antilisterial effect is improved with NaCl and other factors such as pH, and temperature. Hence, Allaker et al. (2001) suggested that even though the specific inhibitory modes of nitrite are not well clarified, its antimicrobial effectiveness depends on several factors including salt concentration, pH, reductants, iron content, and others.

The concordance index was used in order to measure the goodness of fit in the developed probabilistic model. The concordance index indicated the degree of agreement between the observations and calculated probabilities. In this study, the concordance index was 94.5-98.1%, while the discordance was 1.9-5.3%, depending on the storage temperature (data not shown).

To evaluate the performance of the developed probabilistic models in this study, the model performance was assessed with the observed data. The predicted GI times calculated by the estimates of the parameters listed in Table 1 at the probability level of 0.5 were then compared to the predicted GI times (Table 2). A growth more than 1-log scale was considered ‘growth’. The developments of the growth/no growth model were compared with the observed growth data. The predicted GI times were generally overestimated when compared to the observed values by 58.2±17.5% to 79.4±11%. This result indicates that Pseudomonas spp. initiated to grow earlier in frankfurter and bacon than in broth media by 58.2-79.4%. Over-prediction percentages were 79.4±11% (4°C), 66.4±14.6% (7°C), 58.2±17.5% (12°C), and 68.2±2.1% (15°C) (Table 2). In our study, the broth media became turbid, when Pseudomonas spp. grew up to approximately 5-6 Log CFU/mL, and at the point, Pseudomonas spp. growth was determined. However, data from ready-to-eat meats were considered as “growth” if a growth greater than 1-log was observed. Because of this reason, there was a difference between the predicted data and the observed data. Therefore, decreased GI time by 58.2 to 79.4 % compared to the predicted GI time from developed probabilistic model should be applied for real processed meat products such as frankfurters and bacon.

In conclusion, the probabilistic models developed in this study can be used to calculate the GI times of Pseudomonas spp. in frankfurters and bacon as a function of NaCl and NaNO₂ concentrations, considering the over-prediction percentage, and thus, the probabilistic models can be useful in controlling bacterial spoilage in the processed meats by Pseudomonas spp.
Table 1. Estimates of the parameters selected from the logistic regression analysis by a stepwise selection method to calculate the growth probability of *Pseudomonas* spp. at 4-15°C

| Variable | Estimate | SE \(^a\) | \(p\) | Estimate | SE | \(p\) | Estimate | SE | \(p\) | Estimate | SE | \(p\) | Estimate | SE | \(p\) | Estimate | SE | \(p\) |
|----------|----------|----------|------|----------|------|------|----------|------|------|----------|------|------|----------|------|------|----------|------|------|
| Intercept | -19.4190 | 0.77 | <0.001 | -9.1128 | 0.43 | <0.001 | -7.0291 | 0.34 | <0.001 | 10.5739 | 0.45 | <0.001 | 25.3165 | 2.24 | <0.001 |
| NaNO\(_2\) | 0.00320 | 0.01 | 0.623 | -0.0165 | 0.00 | 0.001 | 0.0180 | 0.01 | 0.001 | 0.00645 | 0.00 | 0.018 | 0.00212 | 0.00 | 0.573 |
| NaCl | 1.4815 | 0.36 | <0.001 | 1.3652 | 0.32 | <0.001 | -0.0798 | 0.15 | 0.587 | -0.9429 | 0.26 | 0.001 | -0.4328 | 0.25 | 0.083 |
| Time | 0.1149 | 0.01 | <0.001 | 0.0592 | 0.00 | <0.001 | 0.0517 | 0.00 | <0.001 | 0.1223 | 0.00 | <0.001 | 0.4673 | 0.04 | <0.001 |
| NaNO\(_2\)^2 | -0.00011 | 0.00 | 0.005 | 0.000999 | 0.00 | 0.004 | 0.00024 | 0.00 | <0.001 | - | - | - | - | - | - |
| NaCl | 1.4815 | 0.36 | <0.001 | 1.3652 | 0.32 | <0.001 | -0.0798 | 0.15 | 0.587 | -0.9429 | 0.26 | 0.001 | -0.4328 | 0.25 | 0.083 |
| Time^2 | -0.00014 | 0.00 | <0.001 | -0.0006 | 0.00 | <0.001 | 0.00006 | 0.00 | <0.001 | 0.00023 | 0.00 | <0.001 | 0.00132 | 0.00 | <0.001 |
| NaNO\(_2\)×NaCl | -0.00689 | 0.00 | 0.003 | -0.00783 | 0.00 | 0.001 | -0.00535 | 0.00 | 0.009 | 0.00902 | 0.00 | 0.001 | -0.0244 | 0.00 | <0.001 |
| NaNO\(_2\)×Time | -0.00004 | 0.00 | 0.001 | -0.0003 | 0.00 | 0.003 | 0.0004 | 0.00 | 0.003 | - | - | - | - | - | - |
| NaCl×Time | - | - | - | - | - | - | - | - | - | 0.00333 | 0.00 | 0.017 | - | - | - |

\(^a\) Standard error

Table 2. Comparison of observed and predicted growth initiation (GI) time of *Pseudomonas* spp. at various concentrations of NaCl and NaNO\(_2\)

| Temperature (°C) | Product | Conditions | Predicted GI time (h) | Observed GI time (h) | Over-prediction percentage (%)\(^b\) |
|-----------------|---------|------------|------------------------|----------------------|-------------------------------|
|                 | NaCl (%) | NaNO\(_2\) (ppm) |                        |                      |                               |
| 4               | Frankfurt A | 1.4 | 5\(^c\) | 230 | 168 | 79.4±11 |
|                 | Frankfurt B | 1.3 | 0 | 228 | 168 |                               |
|                 |           | 1.6 | 26 | 234.6 | 216 |                               |
|                 | Frankfurt A | 1.4 | 5 | 186 | 96 |                               |
| 7               | Frankfurt B | 1.3 | 0 | 180 | 120 | 66.4±14.6 |
|                 |           | 1.6 | 26 | 208 | 168 |                               |
|                 | Frankfurt A | 1.4 | 5 | 121 | 48 |                               |
| 12              | Frankfurt B | 1.3 | 0 | 119 | 72 | 58.2±17.5 |
|                 |           | 1.6 | 26 | 129 | 96 |                               |
|                 | Frankfurt A | 1.4 | 5 | 69.6 | 48 |                               |
| 15              | Frankfurt B | 1.3 | 0 | 68.8 | 48 | 68.2±2.1 |
|                 |           | 1.6 | 26 | 73 | 48 |                               |

\(^c\) added NaNO\(_2\) level

\(^b\) Percentage (%) = (the observed data/the predicted data)×100; values are means±standard errors.
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