Korean J. Food Sci. An. Vol. 34, No. 6, pp. 736~741 (2014)
© 2014 Korean Society for Food Science of Animal Recources
DOI http://dx.doi.org/10.5851/kosfa.2014.34.6.736
ISSN 1225-8563 eISSN 2234-246X

ARTICLE

Probabilistic Models to Predict the Growth Initiation Time for *Pseudomonas* spp. in Processed Meats Formulated with NaCl and $NaNO_2$

Hyunji Jo, Beomyoung Park[1], Mihwa Oh[1], Eunji Gwak, Heeyoung Lee, Soomin Lee, and Yohan Yoon*
Department of Food and Nutrition, Sookmyung Women's University, Seoul 140-742, Korea
[1]National Institute of Animal Science, RDA, Suwon 441-706, Korea

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 time. 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 prolyitc and lipolyic enzymes (Champagne *et al*., 1994; Sorhaug and Stepaniak, 1997). Although *Pseudomonas* spp. causes 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 botulinum* germination in anaerobic condition (Pegg and Shahidi, 2006). However, $NaNO_2$ has the potential to produce *N*-nitroso compounds under acidic conditions in stomach (Sugimura, 2000). *N*-nitroso compounds have been found to cause carcinogenic activity in many animal models (Cassens, 1995). Hence, consumers have low acceptance level for processed meat products. Even though processed meat products formulated with low concentrations of $NaNO_2$ have been developed to avoid potential side effects, but the concern for microbial safety due to lowered concentrations of $NaNO_2$ has now increased (Sinedlar *et al*., 2007).

NaCl has been used to improve water-holding capacity, fat binding properties, flavor and the inhibition of microbial growth in processed meat products (Guàrdia *et al*., 2006; Rhee and Zipirin, 2001). However, the high level of NaCl intake is related to hypertension (Tobian *et al*., 1979), cardiac failure (Frolich, 1999), and stroke (Perry and Beevers, 1992). Thus, consumers are willing to have the low concentration of NaCl in processed meat products, but this low NaCl concentration may not inhibit bacterial growth. Therefore, the minimum concentrations of

*Corresponding author: Yohan Yoon, Department of Food and Nutrition, Sookmyung Women's University, Seoul 140-742, Korea. Tel: +82-2-2077-7585, Fax: +82-2-710-9479, E-mail: yyoon@sookmyung.ac.kr

$NaNO_2$ 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 $NaNO_2$ have focused on the antimicrobial effect of $NaNO_2$ on *Pseudomonas* spp. (Henry and Bessieres, 1984; Nicke *et al*., 2013), but the combination effect of $NaNO_2$ 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 $NaNO_2$ 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 KACC 10323) in Cetrimide agar (Becton Dickinson and Company, USA) were cultured in a nutrient broth (NB; Becton Dickinson and Company) at 35°C for 24 h. One hundred microliter fractions of the cultures were transferred into 10 mL NB for subculture at 35°C for 24 h. After incubation, five strains were mixed and centrifuged at 1,912 g and 4°C for 15 min, and cell pellet was 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 of NaCl, and 0.2 g of KCl in 1 L of distilled water). The cell suspension was diluted with PBS to 5 Log CFU/mL.

Growth/no growth response

The combination of 8 levels (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.75%) of NaCl (Samchun pure chemical Co. Ltd., Korea) and 9 levels (0, 15, 30, 45, 60, 75, 90, 105, and 120 ppm) of $NaNO_2$ (Duksan pure chemicals Co. Ltd., Korea) were prepared in NB. Two hundred and twenty five microliters of the medium were placed in wells of 96-well microtiter plates, and 25 μL of inoculum was inoculated into each well. All microtiter plate wells were then 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. The combinations that became turbid, were considered growth, while the unturbid combination was considered no growth. The growth response was regarded as '1' and no growth response was assigned as '0' (Koutsoumanis *et al*., 2004).

Probabilistic model development

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 ($p<0.05$).

$$Logit\,(P) = a_0 + a_1 \cdot NaCl + a_2 \cdot NaNO_2 + a_3 \cdot Time + a_4 \cdot NaCl^2 + a_5 \cdot NaNO_2^2 + a_6 \cdot Time^2 + a_7 \cdot NaCl \cdot NaNO_2 + a_8 \cdot NaCl \cdot Time + a_9 \cdot NaNO_2 \cdot Time$$

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, $NaNO_2$ is $NaNO_2$ 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 (Koutsomanis *et al*., 2004; Lee *et al*., 2013). The observed GI times were then compared to the predicted GI times of

Pseudomonas spp. estimated under the developed model.

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-*

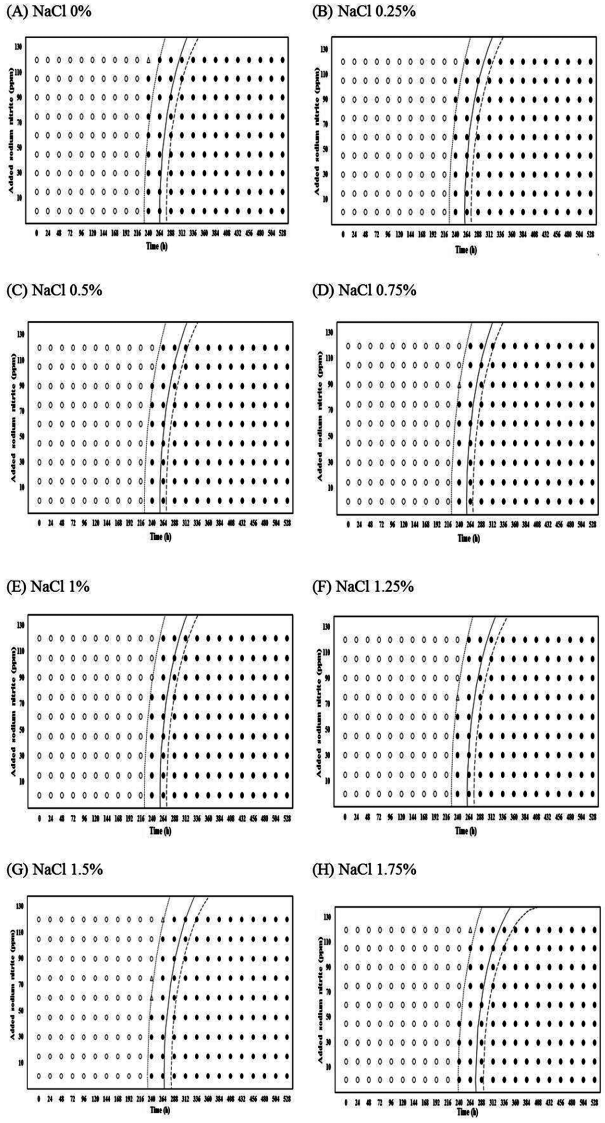

Fig. 1. Growth/no-growth interfaces of *Pseudomonas* spp. at 4°C with respect to $NaNO_2$ concentration and storage time as a function of NaCl levels at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.

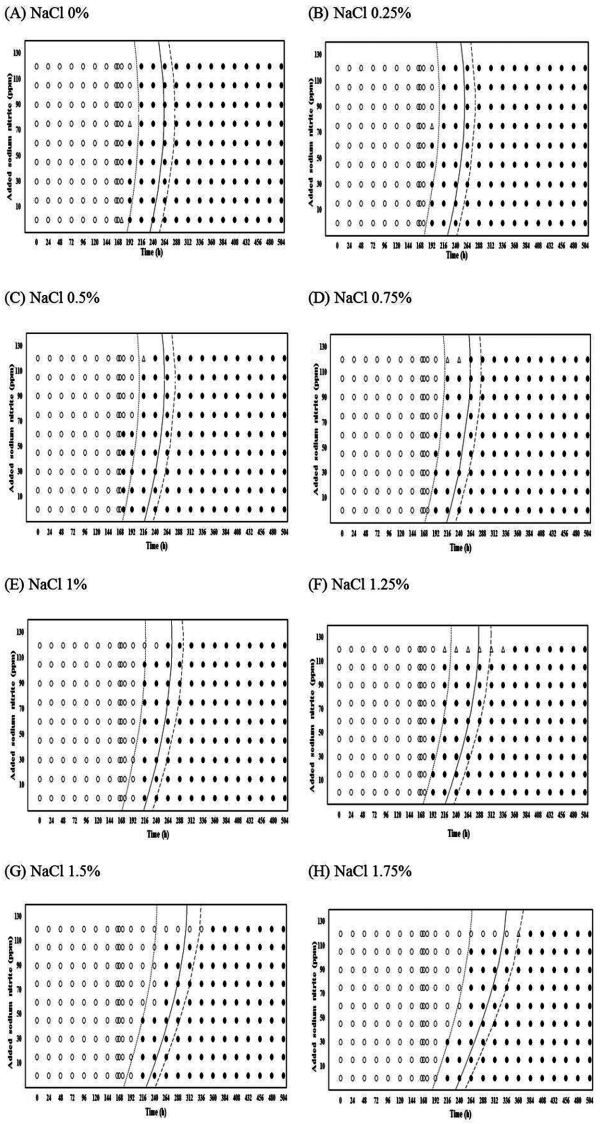

Fig. 2. Growth/no-growth interfaces of *Pseudomonas* spp. at 7°C with respect to $NaNO_2$ concentration and storage time as a function of NaCl levels at growth probabilities of 0.1 (left line), 0.5 (middle line), and 0.9 (right line); no growth: ○, growth: ●, 50% growth: △.

monas spp. growth can be found in high NaCl concentration (>1.25% NaCl). In addition, the combination effect of NaCl and $NaNO_2$ 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 $NaNO_2$ on *Pseudomonas* spp. growth. This is proven by the result from Fig. 3, showing that the difference of growth probability among $NaNO_2$ 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 $NaNO_2$ on *L. monocytogenes* in cold-processed salmon in high NaCl concentrations when stored at 5 and 10°C.

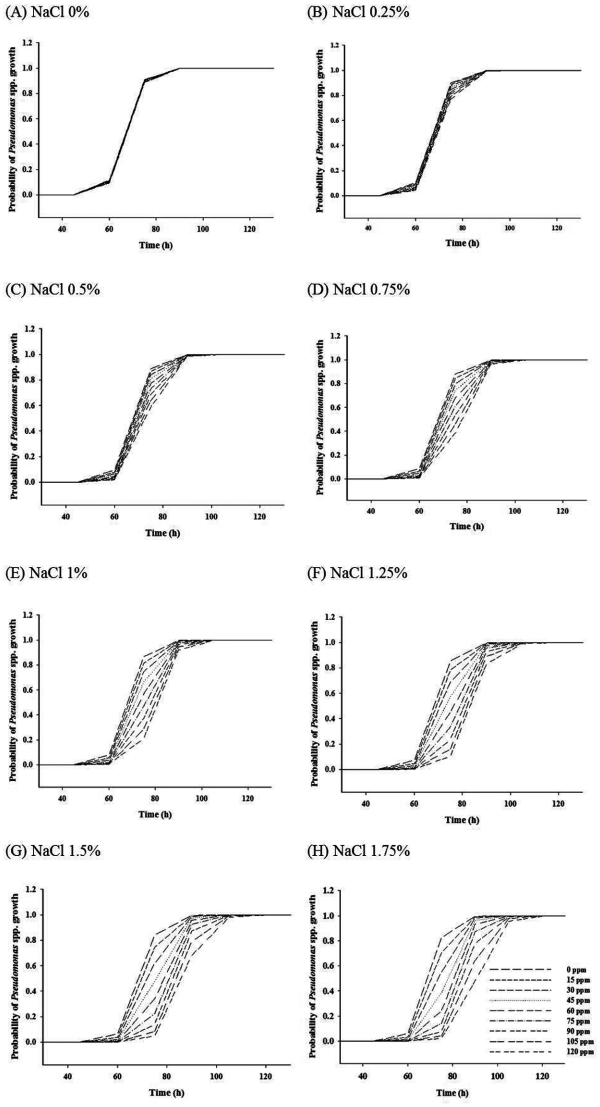

Fig. 3. Probabilities of *Pseudomonas* spp. at different levels of NaCl and $NaNO_2$ when stored at 15°C.

Shahamat *et al*. (1980) and Buchanan *et al*. (1989) examined the antimicrobial effects of $NaNO_2$ on *L. monocytogenes* and suggested that the antilisterial effect is improved with NaCl and other factor 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 $NaNO_2$ 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	4°C			7°C			10°C			12°C			15°C		
	Estimate	SE[a]	*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	0.000099	0.00	0.004	0.00024	0.00	<0.001	-	-	-	-	-	-
$NaCl^2$	-0.8441	0.19	<0.001	-0.8103	0.16	<0.001	-	-	-	-	-	-	-	-	-
$Time^2$	-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	-	-	-	0.00004	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[a]	230	168	79.4±11
	Frankfurt B	1.3	0	228	168	
	Bacon	1.6	26	234.6	216	
7	Frankfurt A	1.4	5	186	96	66.4±14.6
	Frankfurt B	1.3	0	180	120	
	Bacon	1.6	26	208	168	
12	Frankfurt A	1.4	5	121	48	58.2±17.5
	Frankfurt B	1.3	0	119	72	
	Bacon	1.6	26	129	96	
15	Frankfurt A	1.4	5	69.6	48	68.2±2.1
	Frankfurt B	1.3	0	68.8	48	
	Bacon	1.6	26	73	48	

[a]added $NaNO_2$ level

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

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.

References

1. Allaker, R. P., Silva Mendez, L. S., Hardie, J. M., and Benjamin, N. (2001) Antimicrobial effect of acidified nitrite on periodontal bacteria. *Oral Microbiol. Immunol.* **16**, 253-256.
2. Brown, A. D. and Weidemann, J. F. (1958) The taxonomy of the psychrophilic meat-spoilage bacteria: a reassessment. *J. Appl. Bacteriol.* **21**, 11-17.
3. Buchanan, R. L., Stahl, H. G., and Whiting, R. C. (1989) Effects and interactions of temperature, pH, atmosphere, sodium chloride, and sodium nitrite on the growth of *Listeria monocytogenes. J. Food Prot.* **52**, 844-851.
4. Cassens, R. G. (1995) Use of sodium nitrite in cured meats today. *Food Technol.* **49**, 72-80.
5. Champagne, C. P., Laing, R. R., Roy, D., and Mafu, A. A. (1994) Psychrotrophs in dairy products: Their effects and their control. *Crit. Rev. Food Sci. Nutr.* **34**, 1-30.
6. Frolich, E. D. (1999) Risk mechanisms in hypertensive heart disease. *Hypertension*. **34**, 782-789.
7. Guàrdia, M. D., Guerrero, L., Gelabert, J., Gou, P., and Arnau, J. (2006) Consumer attitude towards sodium reduction in meat products and acceptability of fermented sausages with reduced sodium content. *Meat Sci*. **73**, 484-490.
8. Henry, Y. and Bessieres, P. (1984) Denitrification and nitrite reduction: *Pseudomonas aeruginosa* nitrite-reductase. *Biochimie* **66**, 259-289.
9. Koutsoumanis, K. P., Kendall, P. A., and Sofos, J. N. (2004) Modeling the boundaries of growth of *Salmonella* Typhimurium in broth as a function of temperature, water activity, and pH. *J. Food Prot*. **67**, 53-59.
10. Lee, S., Lee, H., Lee, J. Y., Skandamis, P., Park, B. Y., Oh, M. H., and Yoon, Y. (2013) Mathematical models to predict kinetic behavior and growth probabilities of *Listeria monocytogenes* on pork skin at constant and dynamic temperatures. *J. Food Prot*. **76**, 1868-1872.
11. López-Malo, A., Guerrero, S., and Alzamora, S. M. (2000) Probabilistic modeling of *Saccharomyces cerevisiae* inhibition under the effects of water activity, pH, and potassium sorbate concentration. *J. Food Prot*. **63**, 91-95.
12. Miller, A., III, Scanlan, R. A., Lee, J. S., and Libbey, L. M. (1973) Volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas putrefaciens*, *Pseudomonas fluorescens*, and an *Achromobacter* species. *Appl. Microbiol*. **26**, 18-21.
13. Nicke, T., Schitzer, T., Munch, K., Adamczack, J., Hqufschildt, K., Buchmeier, S., Kucklick, M., Felgentrager, U., Jansch, L., Riedel, K., and Layer, G. (2013) Maturation of the cytochrome *cd1* nitrite reductase NirS from *Pseudomonas aeruginosa* requires transient interactions between the three proteins NirS, NirN and NirF. *Biosci. Rep*. **33**, 529-539.
14. Nychas, G. J. E., Skandamis, P. N., Tassou, C. C., and Koutsoumanis, K. P. (2008) Meat spoilage during distribution. *Meat Sci*. **78**, 77-89.
15. Pegg, R. B. and Shahidi, F. (2006) Processing of nitrite-free cured meats. In L. Nollet, M. L., and Todra, F. (Eds.). Advanced Technologies for Meat Processing. Boca Raton, FL: Taylor & Francis Group, CRC Press, LLC.
16. Pelroy, G., Peterson, M., Paranjpye, R., Almond, J., and Eklund, M. (1994) Inhibition of *Listeria monocytogenes* in cold-process (smoked) salmon by sodium nitrite and packaging method. *J. Food Prot*. **57**, 114-119.
17. Perry, I. J. and Beevers, D. G. (1992) Salt intake and stroke: a possible direct effect. *J. Hum. Hypertens*. **6**, 23-25.
18. Pittard, B. T., Freeman, L. R., Later, D. W., and Lee, M. L. (1982) Identification of volatile organic compounds produced by fluorescent pseudomonads on chicken breast muscle. *Appl. Environ. Microb*. **43**, 1504-1506.
19. Reddy, M. C., Bills, D. D., and Lindsay, R. C. (1969) Ester production by *Pseudomonas fragi*. II. Factors influencing ester levels in milk cultures. *Appl. Microbiol*. **17**, 779-782.
20. Rhee, K. S. and Zipirin, Y. A. (2001) Pro-oxidative effects of NaCl in microbial growth-controlled and uncontrolled beef and chicken. *Meat Sci*. **57**, 105-112.
21. Shahamat, M., Seaman, A., and Woodbine, M. (1980) Influence of sodium chloride, pH and temperature on the inhibitory activity of sodium nitrite on *Listeria monocytogenes. Soc. Appl. Bacteriol*. **15**, 227-237.
22. Sindelar, J. J., Cordray, J. C., Olson, D. G., Sebranek, J. G., and Love, J. A. (2007) Investigating quality attributes and consumer acceptance of uncured, no-nitrate/ nitrite-added commercial hams, bacons, and frankfurters. *J. Food Sci*. **72**, 551-559.
23. Sorhaug, T. and Stepaniak, L. (1997) Psychrotrophs and their enzymes in milk and dairy products: quality aspects. *Trends Food Sci. Technol*. **8**, 35-40.
24. Sugimura, T. (2000) Nutrition and dietary carcinogens. *Carcinogenesis*. **21**, 387-395.
25. Tienungoon, S., Ratkowsky, D. A., McMeekin, T. A., and Ross, T. (2000) Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. *Appl. Environ. Microb*. **66**, 4979-4987.
26. Tobian, L., Lange, J., Iwai, J., Hiller, K., Johnson, M. A., and Goossens, P. (1979) Prevention with thiazide of NaCl-induced hypertension in Dahl "S" rats. Evidence for a Na-retaining humoral agent in "S" rats. *J. Am. Heart Assoc*. **1**, 316-323.

(Received 2014.4.14/Revised 2014.9.15/Accepted 2014.9.16)