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ARTICLE

Kinetic Behavior of *Salmonella* on Low $NaNO_2$ Sausages during Aerobic and Vacuum Storage

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Abstract

This study evaluated the growth kinetics of *Salmonella* spp. in processed meat products formulated with low sodium nitrite ($NaNO_2$). A 5-strain mixture of *Salmonella* spp. was inoculated on 25-g samples of sausages formulated with sodium chloride (NaCl) (1.0%, 1.25%, and 1.5%) and $NaNO_2$ (0 and 10 ppm) followed by aerobic or vacuum storage at 10°C and 15°C for up to 816 h or 408 h, respectively. The bacterial cell counts were enumerated on xylose lysine deoxycholate agar, and the modified Gompertz model was fitted to the *Salmonella* cell counts to calculate the kinetic parameters as a function of NaCl concentration on the growth rate (*GR*; Log CFU/g/h) and lag phase duration (*LPD*; h). A linear equation was then fitted to the parameters to evaluate the effect of NaCl concentration on the kinetic parameters. The *GR* values of *Salmonella* on sausages were higher ($p<0.05$) with 10 ppm $NaNO_2$ concentration than with 0 ppm $NaNO_2$. The *GR* values of *Salmonella* decreased ($p<0.05$) as NaCl concentration increased, especially at 10°C. This result indicates that 10 ppm $NaNO_2$ may increase *Salmonella* growth at low NaCl concentrations, and that NaCl plays an important role in inhibiting *Salmonella* growth in sausages with low $NaNO_2$.

Keywords: *Salmonella,* kinetic model, NaCl, $NaNO_2$, sausages

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Introduction

Salmonella is an invasive and facultative intracellular pathogen, and is mainly transmitted through contaminated food (Wagner *et al*., 2011). Especially, pork can be easily contaminated with *Salmonella* (Davies *et al*., 2000; Uyttendaele *et al*., 1999). Since *Salmonella* is highly resistant to acidic or dry conditions (Bearson *et al*., 1997), the pathogen may survive during sausage manufacturing (Bonnet and Montville, 2005), during which acidic conditions are created by fermentation and dry conditions are created by sodium addition. Therefore, *Salmonella* outbreaks related to sausages have been reported in many countries (Long *et al*., 2002; Nichols and De Louvois, 1995).

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To enhance the flavor and water holding capacity of meat products, sodium chloride (NaCl) is added to processed meat products, usually up to 2.5% (Rhee and Ziprin, 2001). NaCl has also been used as a preservative to control foodborne pathogens in combination with sodium nitrite ($NaNO_2$) (Aguilera and Karel, 1997). $NaNO_2$ in meat products play a major role in coloring agents and fat deterioration (Horsch *et al*., 2014). In particular, the formation of *Clostridium botulinum* spores is controlled and the growth of pathogenic bacteria is inhibited by the addition of $NaNO_2$ at anaerobic environmental conditions (Krause *et al*., 2011). However, $NaNO_2$ is a precursor that changes into *N*-nitroso at the low pH conditions in the stomach (Sugimura, 2000). *N*-nitroso is a compound toxic to the human body, thus consumers prefer to purchase low $NaNO_2$ meat products. However, when $NaNO_2$ concentrations decrease, problems with microbial safety emerge (Sindelar *et al*., 2007).

To describe the kinetic behavior of foodborne pathogens, predictive microbiology has been used with mathematical equations (Whiting and Buchanan, 1997). The results from kinetic models can be used to ensure food safety

in advance by blocking the possible intrinsic and extrinsic factors that can affect food (Yoon, 2010). Primary models are used to calculate kinetic parameters such as growth rate (*GR*; Log CFU/g/h) and lag phase duration (*LPD*; h). Secondary models are used to evaluate the effects of various factors on the kinetic parameters.

Therefore, the objective of this study was to describe the kinetic behavior of *Salmonella* in low $NaNO_2$ sausage formulated with various NaCl concentrations, using mathematical equations.

Materials and Methods

Inoculum preparation

Salmonella Typhimurium NCCP10812, *Salmonella* Agona NCCP12231, *Salmonella* Enteritidis NCCP12243, *Salmonella enterica* KACC11595, and *Salmonella* Montevideo NCCP10141 were cultured in 10 mL nutrient broth (NB; Becton, Dickinson and Company, USA) at 37°C for 24 h. The 0.1 mL portions of each culture were transferred into 10 mL fresh NB for subculture at 37°C for 24 h. The cultures of the five strains were then mixed. The mixture was centrifuged at 1,912 *g* for 15 min at 4°C, and the cell pellets were washed twice with phosphate buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water). Eventually, the cell pellet was diluted with PBS to 6-7 Log CFU/mL for use as inoculum.

Sausage manufacture and inoculation

To prepare the sausages, pork meat (60%), pork fat (20%), and ice (20%) were mixed. 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%) were added to the mix. Since commercial sausages had approximately 11.5 ppm of $NaNO_2$ residual (Ham *et al*., 2004), 10 ppm of $NaNO_2$ residual was chosen to be examined in this study. All samples were emulsified using a silent cutter (MSK 760 H II, Mado, Germany) for 6 min. The mixed pastes were then stored at 4°C for 1 h, and 30-g portions were stuffed into the collagen casing (#260, NIPPI Inc., Japan; approximate 25 mm diameter) with an automatic sausage filler (Konti A50; Frey, Germany). The sausages were heated at 75°C for 40 min in a smokehouse (MAXI 3501; Kerres, Germany), and the emulsion type sausages were then vacuum-packaged with polyethylene. After the sausages were dipped in ice water for 10 min, the sausages were stored at 4°C until used (Choi *et al*., 2014). To inoculate *Salmonella* on sausages, the vacuum-packages were aseptically opened. Samples were cut into 25 g and inoculated by immersion into 500 mL *Salmonella* inoculum in a sterilized plastic container for 2 min. The sausages were transferred to petri dishes to allow attachment for 15 min, and then transferred into sample bags. The samples were sealed for storage in aerobic condition or vacuum-packaged for the vacuum condition. The samples were incubated at 10°C and 15°C for up to 816 h and 408 h, respectively, and *Salmonella* cell counts were enumerated on xylose lysine deoxycholate agar (XLD; Becton, Dickinson and Company).

Kinetic parameter calculation

The modified Gompertz model (Zwietering *et al*., 1990) were fitted to the *Salmonella* cell counts to calculate kinetic parameters such as *GR* and *LPD* with GraphPad PRISM version 4.0 (GraphPad Software, USA). The model used was as follows;

Modified Gompertz model:

$$Nt = A + C \times \exp(-\exp(-B(t-M)))$$

$$GR = B \times C/e \quad (e = 2.7182)$$

$$LPD = M - (1/B)$$

$$N_{max} = A + C$$

where A is the lower asymptotic line of the growth curve as t decrease to zero, C is the difference between the upper asymptotic line of the growth curve and the lower asymptotic line, B is the relative maximum growth rate at time M, and M is the time at which the growth rate is maximum (Gibson *et al*., 1987).

The secondary model was developed to evaluate the effect of NaCl concentration on the kinetic parameters with the following equation;

$$GR = a_0 + a_1 \times NaCl$$

where a_i is a coefficient, and $NaCl$ is the NaCl concentration.

Statistical analysis

The growth parameter (*GR* and *LPD*) was analyzed using the general linear model procedure in SAS® version 9.3 (SAS Institute, USA). All least squares means comparisons were performed using a pairwise *t*-test at p=0.05.

Results and Discussion

The parameters estimated by the modified Gompertz model for *Salmonella* spp. on sausages formulated with

$NaNO_2$ and NaCl aerobically and anaerobically stored at 10°C and 15°C are shown at Table 1 and Table 2. R^2 values for fitting the modified Gompertz model to the *Salmonella* growth data ranged from 0.913 to 0.979, suggesting that the model was appropriate for describing the kinetic behavior of *Salmonella* in sausage (Table 1, 2).

The *LPD* values were 22.22-67.17 h at 10°C and 6.88-21.51 h at 15°C. The values at 15°C were generally lower than at 10°C (Table 1, 2). In general, $NaNO_2$ inhibits bacterial growth in sausages (Junttila *et al*., 1989). However, in this study, *GR* was higher (p<0.05) in 10 ppm $NaNO_2$ than in 0 ppm $NaNO_2$ at 1% NaCl, when the sausages were stored at 15°C in both aerobic and vacuum conditions (Table 1, 2). In addition, the highest *GR* values were observed with 10 ppm $NaNO_2$ at 1% NaCl for all temperatures in both aerobic and vacuum storage (Table 1, 2). This result indicates that *Salmonella* may have more growth 10 ppm $NaNO_2$ than 0 ppm $NaNO_2$, which was not expected. Recent studies showed that *Salmonella* produces a nitrite reductase (Gilberthorpe and Poole, 2008; Mills *et al*., 2008) and flavohemoglobin (Hmp), which confer tolerance to NO and nitrosoactive stress (Poole and

Table 1. The growth parameters estimated by the modified Gompertz model for *Salmonella* spp. on sausages as a function of $NaNO_2$ and NaCl concentration at 10°C and 15°C in aerobic conditions

Storage Temperature (°C)	$NaNO_2$ (ppm)	NaCl (%)	*LPD*[1] (h)	*GR*[2] (Log CFU/g/h)	N_0[3] (Log CFU/g)	N_{max}[4] (Log CFU/g)	R^2
10	0	1	48.83±22.51^{ABCD}	0.042±0.03^{CD}	3.5±0.5	8.0±0.1	0.962
		1.25	58.76±35.53^{ABC}	0.024±0.00^{D}	3.5±0.5	7.9±0.4	0.942
		1.5	67.17±46.76^{A}	0.023±0.00^{D}	3.6±0.5	8.2±0.0	0.958
	10	1	22.22±0.95^{BCD}	0.080±0.05^{ABC}	3.6±0.1	8.2±0.3	0.962
		1.25	24.14±0.69^{BCD}	0.038±0.01^{CD}	3.7±0.0	8.2±0.6	0.964
		1.5	62.90±8.42^{AB}	0.028±0.02^{D}	3.5±0.2	8.1±0.3	0.944
15	0	1	9.22±11.53^{D}	0.064±0.02^{BCD}	3.4±0.1	7.2±0.0	0.957
		1.25	11.76±10.33^{D}	0.063±0.02^{BCD}	3.4±0.2	7.1±0.1	0.956
		1.5	19.93±12.25^{CD}	0.037±0.02^{CD}	3.4±0.1	7.2±0.4	0.927
	10	1	8.93±3.35^{D}	0.117±0.01^{A}	3.9±0.4	8.2±0.4	0.934
		1.25	15.89±6.58^{D}	0.091±0.02^{AB}	3.6±0.2	7.8±0.8	0.923
		1.5	16.65±6.48^{CD}	0.091±0.02^{AB}	3.7±0.7	7.8±1.1	0.973

[1]lag phase duration.
[2]growth rate.
[3]initial cell concentration.
[4]maximum cell concentration.
[A-D]Means with the same column with different superscript letters are significantly different (p<0.05).

Table 2. The growth parameters estimated by the modified Gompertz model for *Salmonella* spp. on sausages as a function of $NaNO_2$ and NaCl concentration at 10°C and 15°C in vacuum condition

Storage Temperature (°C)	$NaNO_2$ (ppm)	NaCl (%)	*LPD*[1] (h)	*GR*[2] (Log CFU/g/h)	N_0[3] (Log CFU/g)	N_{max}[4] (Log CFU/g)	R^2
10	0	1	24.86±7.41^{CDE}	0.023±0.00^{CD}	3.7±0.1	7.9±0.2	0.949
		1.25	26.23±6.33^{CDE}	0.022±0.01^{CD}	3.8±0.4	8.2±0.0	0.979
		1.5	33.29±0.33^{ABC}	0.022±0.00^{CD}	3.7±0.1	8.2±0.3	0.974
	10	1	28.60±5.54^{BCD}	0.043±0.03^{BCD}	3.5±0.1	7.2±0.0	0.950
		1.25	39.15±0.00^{AB}	0.018±0.00^{D}	3.4±0.0	6.9±0.0	0.975
		1.5	43.65±4.45^{A}	0.020±0.01^{CD}	3.4±0.1	6.9±0.2	0.962
15	0	1	16.64±4.23^{EF}	0.045±0.00^{BC}	3.7±0.4	8.1±0.4	0.977
		1.25	20.73±8.77^{DE}	0.036±0.00^{BCD}	3.8±0.5	8.2±0.8	0.978
		1.5	21.51±8.34^{DE}	0.027±0.01^{CD}	4.0±0.7	8.4±0.6	0.948
	10	1	6.88±2.03^{F}	0.098±0.02^{A}	3.8±0.1	7.2±0.2	0.952
		1.25	8.59±0.00^{F}	0.062±0.00^{B}	3.7±0.0	6.7±0.0	0.924
		1.5	16.12±0.00^{EF}	0.049±0.00^{BC}	3.9±0.0	6.8±0.0	0.913

[1]lag phase duration.
[2]growth rate.
[3]initial cell concentration.
[4]maximum cell concentration.
[A-F]Means with the same column with different superscript letters are significantly different (p<0.05).

Hughes, 2000). However, Seong *et al*. (2010) and Birk *et al*. (2015) showed that 100 ppm $NaNO_2$ combined with a low concentration (62 g/kg) of salt completely inhibited *Salmonella* growth. Taken together, it can be suggested that 10 ppm of $NaNO_2$ was below the threshold needed to destroy *Salmonella* cells, and thus, *Salmonella* can resist the low concentration of $NaNO_2$ because of flavohemoglobin and nitrite reductase, which break down $NaNO_2$. The nitrogen produced by breaking down $NaNO_2$ may subsequently be used as a nitrogen source for S*almonella* growth (Page and Solberg, 1980), which was higher with 10 ppm $NaNO_2$ than with no $NaNO_2$. In addition, although N_0 values were 3.4-3.9 Log CFU/g, N_{max} values were higher (7.8-8.2 Log CFU/g) in 10 ppm $NaNO_2$ treated samples than in 0 ppm $NaNO_2$ treated samples at 15°C, but not at 10°C under aerobic storage. This phenomenon, however, was not observed under vacuum storage (Table 1, 2).

To evaluate the effects of NaCl and $NaNO_2$ on the *GR* of *Salmonella*, a linear equation was fitted to the *GR* values to describe the effect of NaCl and $NaNO_2$ (Fig. 1). At 10°C, the *GR* was higher with 10 ppm $NaNO_2$ than with 0 ppm $NaNO_2$ at 1% NaCl, but the *GR* values with 10 ppm $NaNO_2$ rapidly decreased as NaCl concentration increased. The values became similar to the values with 0 ppm $NaNO_2$ at 1.5% NaCl, regardless of atmospheric conditions (Table 1, 2, Fig. 1). This result indicates that 10 ppm of $NaNO_2$ may increase the *GR* of *Salmonella*, compared to 0 ppm $NaNO_2$, but the increase of NaCl in combination with $NaNO_2$ can decrease the *GR* (Fig. 1). Jo *et al*. (2014) also showed that *Pseudomonas* spp. growth in processed meat was inhibited by a combination of $NaNO_2$ and NaCl. At 15°C in aerobic condition, the *GR* with 10 ppm $NaNO_2$ was higher ($p<0.05$) than with 0 ppm $NaNO_2$ at 1% NaCl, but the *GR* with 10 ppm $NaNO_2$ did not become the same as the *GR* with 0 ppm as NaCl concentration increased, which were different from the results at 10°C (Fig. 1).

In conclusion, 10 ppm $NaNO_2$ may increase *Salmonella* growth in processed meat products, and thus, sufficient NaCl must be combined with $NaNO_2$ to improve food safety, especially for low $NaNO_2$ products.

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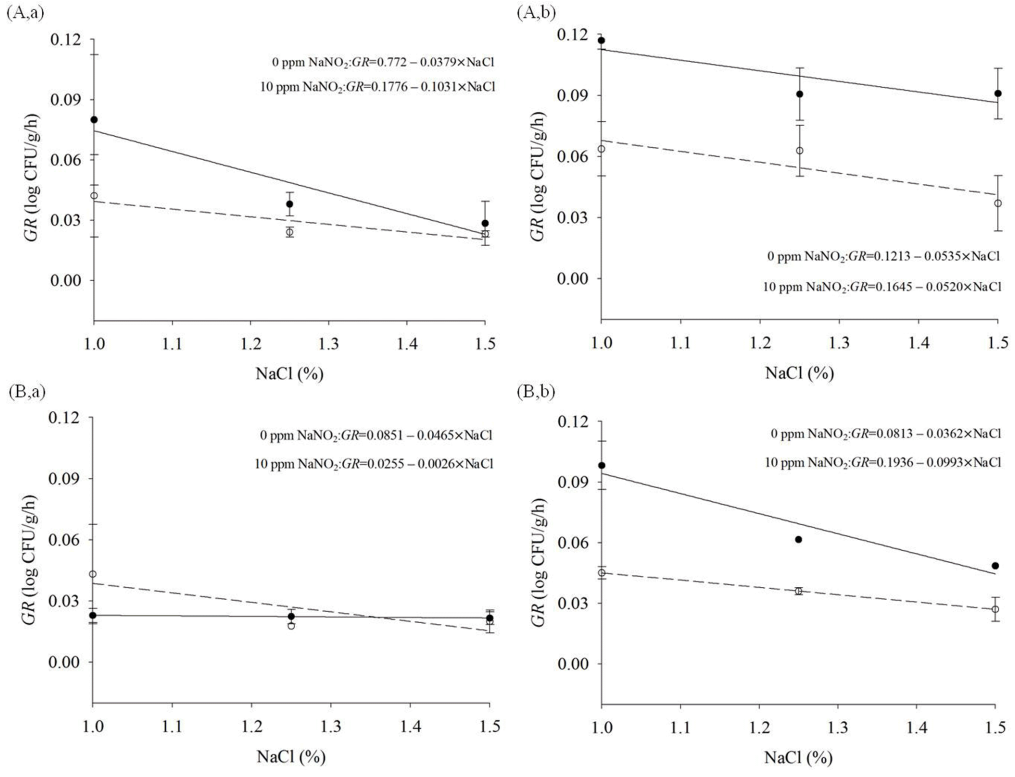

Fig. 1. Effect of NaCl on growth rate (*GR*) according to the amount of NaCl under (A) aerobic conditions, (B) vacuum storage at (a) 10°C and (b) 15°C. 0 ppm: -----, 10 ppm: ——.

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