Kinetic Behavior of *Salmonella* on Low NaNO₂ 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 (NaNO₂). 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 NaNO₂ (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 bacterial 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) at 10 ppm NaNO₂ concentration than with 0 ppm NaNO₂. The GR values of *Salmonella* decreased (p<0.05) as NaCl concentration increased, especially at 10°C. This result indicates that 10 ppm NaNO₂ may increase *Salmonella* growth at low NaCl concentrations, and that NaCl plays an important role in inhibiting *Salmonella* growth in sausages with low NaNO₂.

**Keywords:** *Salmonella*, kinetic model, NaCl, NaNO₂, sausages

**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).

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 (NaNO₂) (Aguilera and Karel, 1997). NaNO₂ 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 NaNO₂ at anaerobic environmental conditions (Krause et al., 2011). However, NaNO₂ 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 NaNO₂ meat products. However, when NaNO₂ 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 NaN02 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 KH2PO4, 1.5 g of Na2HPO4, 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%), NaNO2 (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 NaNO2 residual (Ham et al., 2004), 10 ppm of NaNO2 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:

\[ N_t = A + C \times \exp(-\exp(-B(1-\frac{t}{M}))) \]

\[ GR = B \times C/e \quad (e \approx 2.7182) \]

\[ LPD = M - (1/B) \]

\[ N_{max} = A + C \]

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 \( a_1 \) 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
NaNO2 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 kinetic behavior of $t$-log. 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, NaNO2 inhibits bacterial growth in sausages (Junttila et al., 1989). However, in this study, $GR$ was higher (p<0.05) in 10 ppm NaNO2 than in 0 ppm NaNO2 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 NaNO2 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 NaNO2 than 0 ppm NaNO2, which was not expected. Recent studies showed that Salmonella produces a nitrite reductase (Gilbert 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 NaNO2 and NaCl concentration at 10°C and 15°C in aerobic conditions

| Storage Temperature (°C) | NaNO2 (ppm) | NaCl (%) | LPD1 (h) | GR† (Log CFU/g/h) | $N^\text{max}_0$ (Log CFU/g) | $N_\text{max}^\text{a}$ (Log CFU/g) | $R^2$ |
|--------------------------|-------------|----------|----------|-----------------|------------------|------------------|------|
| 10                       | 1           | 1.25     | 48.83±22.51<sup>1</sup><sup>BC</sup>D       | 0.042±0.03<sup>IM</sup>D | 3.5±0.5          | 8.0±0.1          | 0.962 |
| 0                        | 1           | 1.5      | 67.17±46.76A                               | 0.023±0.00<sup>IM</sup>D | 3.6±0.5          | 8.2±0.0          | 0.958 |
| 15                       | 1           | 1.25     | 22.22±9.93<sup>1</sup><sup>BC</sup>D       | 0.080±0.05<sup>IM</sup>D | 3.6±0.1          | 8.2±0.3          | 0.962 |
| 10                       | 1.25        | 0.89±1.53<sup>1</sup><sup>BC</sup>D       | 0.064±0.02<sup>BCD</sup>D | 3.4±0.1          | 7.2±0.0          | 0.957 |
| 1.5                      | 0.89±1.53<sup>1</sup><sup>BC</sup>D       | 0.064±0.02<sup>BCD</sup>D | 3.4±0.2          | 7.1±0.1          | 0.956 |
| 15                       | 1.25        | 19.93±12.25<sup>1</sup><sup>BC</sup>D     | 0.037±0.02<sup>BCD</sup>D | 3.4±0.1          | 7.2±0.4          | 0.927 |
| 10                       | 1.25        | 15.89±6.58<sup>1</sup><sup>BCD</sup>D     | 0.091±0.02<sup>AB</sup>D | 3.6±0.2          | 7.8±0.8          | 0.923 |
| 1.5                      | 15.89±6.58<sup>1</sup><sup>BCD</sup>D     | 0.091±0.02<sup>AB</sup>D | 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 NaNO2 and NaCl concentration at 10°C and 15°C in vacuum condition

| Storage Temperature (°C) | NaNO2 (ppm) | NaCl (%) | LPD1 (h) | GR† (Log CFU/g/h) | $N^\text{max}_0$ (Log CFU/g) | $N_\text{max}^\text{a}$ (Log CFU/g) | $R^2$ |
|--------------------------|-------------|----------|----------|-----------------|------------------|------------------|------|
| 10                       | 1           | 1.25     | 24.86±7.41<sup>1</sup><sup>DE</sup>D       | 0.023±0.00<sup>IM</sup>D | 3.7±0.1          | 7.9±0.2          | 0.949 |
| 0                        | 1           | 1.5      | 33.29±0.33<sup>1</sup><sup>CDE</sup>D     | 0.022±0.01<sup>CD</sup>D | 3.8±0.4          | 8.2±0.0          | 0.979 |
| 10                       | 1           | 1.25     | 28.60±5.54<sup>1</sup><sup>BCD</sup>D     | 0.043±0.03<sup>BC</sup>D | 3.5±0.1          | 7.2±0.0          | 0.950 |
| 1.5                      | 28.60±5.54<sup>1</sup><sup>BCD</sup>D     | 0.043±0.03<sup>BC</sup>D | 3.5±0.1          | 7.2±0.0          | 0.950 |
| 15                       | 1           | 0.915±0.00<sup>1</sup><sup>CD</sup>D     | 0.018±0.00<sup>ID</sup>D | 3.4±0.0          | 6.9±0.0          | 0.975 |
| 1.5                      | 0.915±0.00<sup>1</sup><sup>CD</sup>D     | 0.018±0.00<sup>ID</sup>D | 3.4±0.1          | 6.9±0.2          | 0.962 |
| 10                       | 1           | 16.64±4.23<sup>1</sup><sup>EF</sup>D     | 0.045±0.00<sup>ID</sup>D | 3.7±0.4          | 8.1±0.4          | 0.977 |
| 1.25                     | 16.64±4.23<sup>1</sup><sup>EF</sup>D     | 0.045±0.00<sup>ID</sup>D | 3.7±0.4          | 8.1±0.4          | 0.977 |
| 1.5                      | 16.64±4.23<sup>1</sup><sup>EF</sup>D     | 0.045±0.00<sup>ID</sup>D | 3.7±0.4          | 8.1±0.4          | 0.977 |
| 1.25                     | 20.73±8.77<sup>1</sup><sup>DE</sup>D     | 0.036±0.00<sup>BCD</sup>D | 3.8±0.5          | 8.2±0.8          | 0.978 |
| 1.5                      | 20.73±8.77<sup>1</sup><sup>DE</sup>D     | 0.036±0.00<sup>BCD</sup>D | 3.8±0.5          | 8.2±0.8          | 0.978 |
| 15                       | 1           | 21.51±8.34<sup>1</sup><sup>DE</sup>D     | 0.027±0.01<sup>CD</sup>D | 4.0±0.7          | 8.4±0.6          | 0.948 |
| 1.25                     | 21.51±8.34<sup>1</sup><sup>DE</sup>D     | 0.027±0.01<sup>CD</sup>D | 4.0±0.7          | 8.4±0.6          | 0.948 |
| 1.5                      | 21.51±8.34<sup>1</sup><sup>DE</sup>D     | 0.027±0.01<sup>CD</sup>D | 4.0±0.7          | 8.4±0.6          | 0.948 |

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).
Hughes, 2000). However, Seong et al. (2010) and Birk et al. (2015) showed that 100 ppm NaNO₂ combined with a low concentration (62 g/kg) of salt completely inhibited Salmonella growth. Taken together, it can be suggested that 10 ppm of NaNO₂ was below the threshold needed to destroy Salmonella cells, and thus, Salmonella can resist the low concentration of NaNO₂ because of flavohemoglobin and nitrite reductase, which break down NaNO₂. The nitrogen produced by breaking down NaNO₂ may subsequently be used as a nitrogen source for Salmonella growth (Page and Solberg, 1980), which was higher with 10 ppm NaNO₂ than with no NaNO₂. In addition, although $N_0$ values were 3.4-3.9 Log CFU/g, $N_{\text{max}}$ values were higher (7.8-8.2 Log CFU/g) in 10 ppm NaNO₂ treated samples than in 0 ppm NaNO₂ 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 NaNO₂ on the GR of Salmonella, a linear equation was fitted to the GR values to describe the effect of NaCl and NaNO₂ (Fig. 1). At 10°C, the GR was higher with 10 ppm NaNO₂ than with 0 ppm NaNO₂ at 1% NaCl, but the GR values with 10 ppm NaNO₂ rapidly decreased as NaCl concentration increased. The values became similar to the values with 0 ppm NaNO₂ at 1.5% NaCl, regardless of atmospheric conditions (Table 1, 2, Fig. 1). This result indicates that 10 ppm of NaNO₂ may increase the GR of Salmonella, compared to 0 ppm NaNO₂, but the increase of NaCl in combination with NaNO₂ 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 NaNO₂ and NaCl. At 15°C in aerobic condition, the GR with 10 ppm NaNO₂ was higher ($p<0.05$) than with 0 ppm NaNO₂ at 1% NaCl, but the GR with 10 ppm NaNO₂ 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 NaNO₂ may increase Salmonella growth in processed meat products, and thus, sufficient NaCl must be combined with NaNO₂ to improve food safety, especially for low NaNO₂ products.

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