Open Access
Asian-Australas J Anim Sci
Vol. 30, No. 3:432-438 March 2017
https://doi.org/10.5713/ajas.16.0391
pISSN 1011-2367 eISSN 1976-5517

AJAS
Asian-Australasian Journal of Animal Sciences

Effects of low $NaNO_2$ and NaCl concentrations on *Listeria monocytogenes* growth in emulsion-type sausage

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Submitted May 16, 2016; Revised Aug 18, 2016; Accepted Oct 3, 2016

Objective: The objective of this study was to evaluate the effect of combinations of $NaNO_2$ and NaCl concentrations on *Listeria monocytogenes* (*L. monocytogenes*) growth in emulsion-type sausage.
Methods: Emulsion-type sausages formulated with different combinations of $NaNO_2$ (0 and 10 ppm) and NaCl (1.00%, 1.25%, and 1.50%) were inoculated with a five-strain *L. monocytogenes* mixture, and stored at 4°C, 10°C, and 15°C, under aerobic or vacuum conditions. *L. monocytogenes* cell counts were measured at appropriate intervals, and kinetic parameters such as growth rate and lag phase duration (LPD) were calculated using the modified Gompertz model.
Results: Growth rates increased (0.004 to 0.079 Log colony-forming unit [CFU]/g/h) as storage temperature increased, but LPD decreased (445.11 to 8.35 h) as storage temperature and NaCl concentration increased. The effect of combinations of NaCl and low-$NaNO_2$ on *L. monocytogenes* growth was not observed at 4°C and 10°C, but it was observed at 15°C, regardless of atmospheric conditions.
Conclusion: These results indicate that low concentrations of $NaNO_2$ and NaCl in emulsion-type sausage may not be sufficient to prevent *L. monocytogenes* growth, regardless of whether they are vacuum-packaged and stored at low temperatures. Therefore, additional techniques are necessary for *L. monocytogenes* control in the product.

Keywords: *Listeria monocytogenes*; $NaNO_2$; NaCl; Growth Pattern; Emulsion-type Sausage

INTRODUCTION

Listeria monocytogenes (*L. monocytogenes*) is a foodborne bacterium found in processed meat products; it can contaminate the products during post-processing steps such as slicing, packaging and handling [1,2]. The pathogen causes foodborne illness following intake of meat products, including those that are formulated with $NaNO_2$ and NaCl [3]. NaCl is added to processed meat products to improve flavor and water-holding capacity and preservation [4]. $NaNO_2$ is used as a color-fixing agent in processed meat products, and also has an anti-microbacterial effect, particularly for inhibiting germination of *Clostridium botulinum* [5,6]. Combinations of NaCl (2.7% w/v) and $NaNO_2$ (180 mg/L) had an inhibitory activity of bacteria growth [7]. Xi et al [8] showed that growth of *L. monocytogenes* was inhibited by high $NaNO_2$ (200 ppm), and other studies have also reported that $NaNO_2$ affects *L. monocytogenes* growth by decreasing the growth rate and increasing lag time [9,10].

In general, food additives improve the safety and taste of foods. However, some people suggested that unfavorable chemicals could be produced during curing [11]. A study by Ministry of Food and Drug Safety (MFDS) [12] presented that 54.6% of consumers believe that food additives have negative effects on human health. In addition, Bedale et al [13] presented that consumers

had fear against chemical food additives and antimicrobials. Specifically, consumers believe that $NaNO_2$ forms *N*-nitroso compounds, which are human carcinogens, in an acidic environment [14,15]. Thus, in response to the fact that consumers prefer not to have the food additive in their foods, many companies have recently started to produce meat products with $NaNO_2$ replacements and low $NaNO_2$ concentration, and celery powder, Swiss chard powder and rosemary have been used as $NaNO_2$ replacements [16,17]. However, these replacements lowered quality for flavor and meat color [8,14,18].

Therefore, the objective of this study was to determine the kinetic behavior of *L. monocytogenes* in emulsion-type sausage, which was formulated with either low concentrations of $NaNO_2$ and NaCl or no $NaNO_2$ and NaCl, instead of using $NaNO_2$ replacement.

MATERIALS AND METHODS

Inoculum preparation

L. monocytogenes strains NCCP10805, NCCP10808, NCCP10809, NCCP10810, and NCCP10943, which were isolated from meats and human (Table 1), were cultured in 10 mL nutrient broth plus 0.6% yeast extract (NBYE; Beckton, Dickinson, and Company, Sparks, MD, USA) at 30°C for 24 h. Then, 0.1 mL portions of these cultures were subcultured in 10 mL fresh NBYE at 30°C for 24 h. The subcultures were harvested by centrifugation at 1,912×*g* for 15 min at 4°C, 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 g of NaCl, and 0.2 g of KCl in 1 L distilled water), and resuspended in 10 mL PBS. Each strain was mixed and serially diluted in PBS to obtain 5 to 6 Log colony-forming unit (CFU)/mL of *L. monocytogenes* for use as an inoculum.

Emulsion-type sausage preparation

Fresh pork and pork back fat were purchased from a local butcher shop 24 h after slaughter, and the connective tissue and extra fat layer were removed. Meat and fat were ground using a meat chopper (PM-70, Mainca, Barcelona, Spain) equipped with an 8-mm plate, and emulsified for 6 min using a silent cutter (MSK 760 H II, Mado, Dornhan, Germany). Emulsion-type sausages were then prepared as described in Table 2. Each treatment mixture was stored at 4°C for 1 h to enhance the binding strength and stability of the mixture [19]. Thirty grams of emulsion was filled into a collagen casing (25 mm diameter) using a filling machine (Konti A50, Frey, Herbrechtingen, Germany). For pasteurization, the emulsion-type sausage samples were heated at 75°C for 40 min and then chilled. The emulsion-type sausages were then vacuum-packed and heated again at 80°C for 15 min; subsequently, they were stored at 4°C until use.

Table 1. Origins and serotypes of *Listeria monocytogenes* strains used in this study

Strain	Origin	Serotype
L. monocytogenes NCCP 10805	Poultry	1
L. monocytogenes NCCP 10808	Animal, Tissue (ruminant brain)	4a
L. monocytogenes NCCP 10809	Human	4b
L. monocytogenes NCCP 10810	Chicken	4c
L. monocytogenes NCCP 10943	Rabbit	1/2a

Table 2. Formulation of emulsion-type sausage

Ingredients (%)	0 ppm $NaNO_2$			10 ppm $NaNO_2$		
	1.00% NaCl	1.25% NaCl	1.50% NaCl	1.00% NaCl	1.25% NaCl	1.50% NaCl
Pork meat	60	60	60	60	60	60
Pork fat	20	20	20	20	20	20
Ice	20	20	20	20	20	20
Total	100	100	100	100	100	100
NaCl	1.00	1.25	1.50	1.00	1.25	1.50
$NaNO_2$	-	-	-	0.0029	0.00303	0.00305
Phosphate	0.03	0.03	0.03	0.03	0.03	0.03
Isolated soy protein	1.00	1.00	1.00	1.00	1.00	1.00
Mixed spice	0.50	0.50	0.50	0.50	0.50	0.50
Sugar	0.50	0.50	0.50	0.50	0.50	0.50
Potassium sorbate	0.20	0.20	0.20	0.20	0.20	0.20

Inoculation and microbial analysis

The emulsion-type sausage was cut into 25 g portions. Thirty sausage samples were dipped into a sterile plastic container containing inoculum at 2 to 3 Log CFU/mL which was set up to observe sufficient growth, and stirred gently for 2 min. After dipping, the samples were air-dried under a biosafety cabinet for 15 min to allow the *L. monocytogenes* cells to attach; then, they were transferred to vacuum bags. The bags were either simply sealed (aerobic storage) or vacuum-packaged (vacuum storage). The sealed samples were stored at 4°C for 1,440 h, at 10°C for 528 h and at 15°C for 192 h. During storage, *L. monocytogenes* cell counts were measured on PALCAM medium base agar (Beckton, Dickinson, and Company, USA) at appropriate time intervals (11 to 23 times). Sodium nitrite residues were determined according to Korean Food Standards [20].

Calculation of kinetic parameters

To calculate kinetic parameters such as growth rate (Log CFU/g/h) and lag phase duration (LPD; h), *L. monocytogenes* growth data for each storage condition were fitted to the modified Gompertz model [21,22] as follows:

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

Where A is the lower asymptotic line of the growth curve as t decreases to zero, B is the growth rate at time M, C is the difference between the upper asymptotic line of the growth curve (N_{max}) minus the lower asymptotic line (N_0), and M is the time at which the growth rate is maximum. According to this equation, growth rate and LPD can be calculated by the equations shown below:

$$\text{Growth rate} = \frac{BC}{e} (e = 2.7182)$$

$$LPD = M - \left(\frac{1}{B}\right)$$

N_{max}=A+C

Statistical analysis

Growth rate (n = 4) and LPD (n = 4) values were analyzed using 3 (4°C, 10°C, and 15°C)×3 (1.00%, 1.25%, and 1.50% NaCl) factorial design for each $NaNO_2$ concentrations (0 ppm and 10 ppm). The data were analyzed by the general linear model procedure using SAS version 9.3 (SAS Institute, Cary, NC, USA) for fixed effects and interactions between fixed effects. Least square (LS) means between the interactions were compared by a pairwise t-test at the significance level of alpha = 0.05.

RESULTS AND DISCUSSION

The growth rates of *L. monocytogenes* in emulsion-type sausage increased under aerobic and vacuum storage as the temperature increased, regardless of $NaNO_2$ and NaCl concentrations (Figures 1 and 2). To determine the kinetic behavior of *L. monocytogenes* in the emulsion-type sausage, kinetic parameters for the pathogen were calculated using the modified Gompertz model. The modified

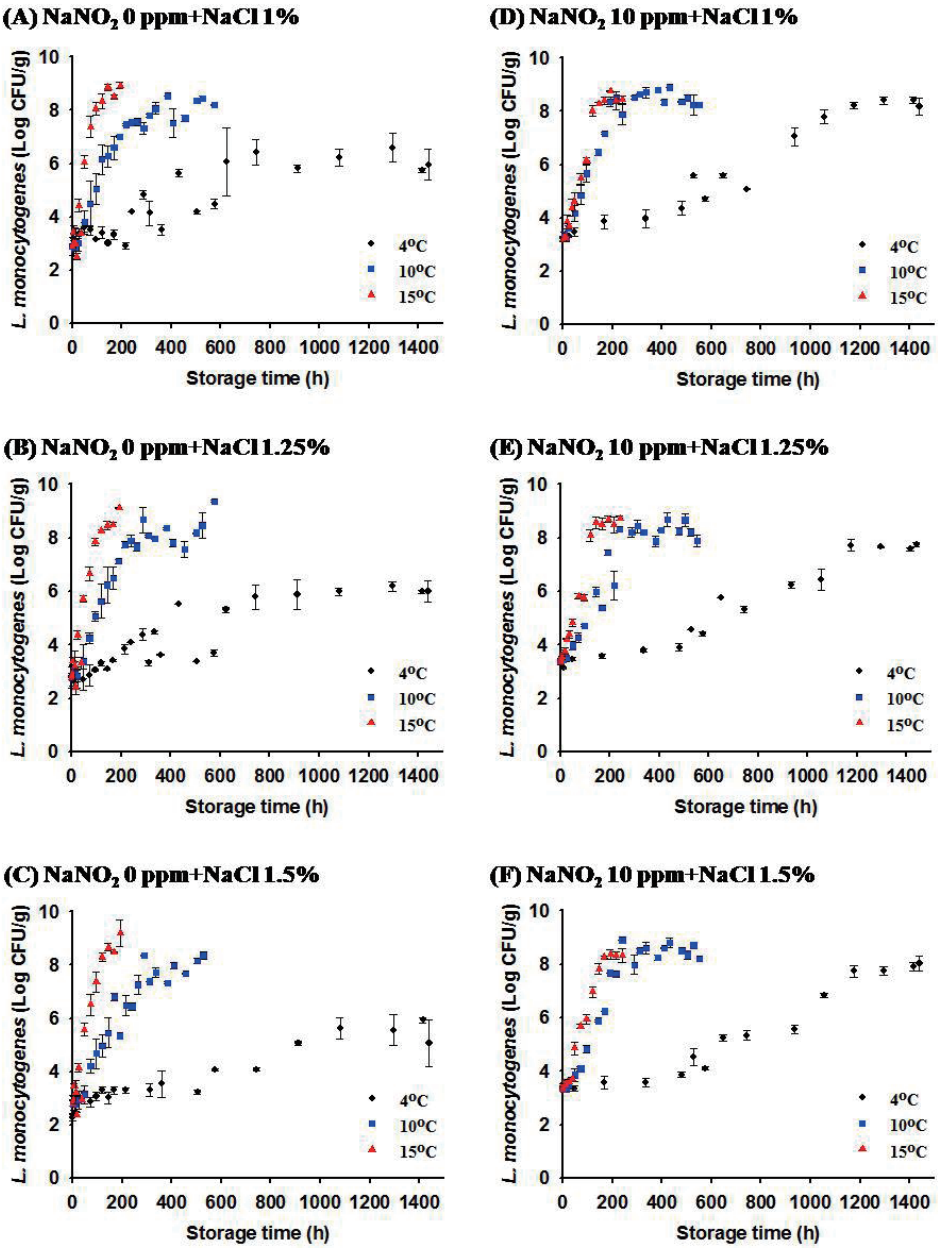

Figure 1. Bacterial populations of *Listeria monocytogenes* cell counts in emulsion-type sausage in aerobic storage.

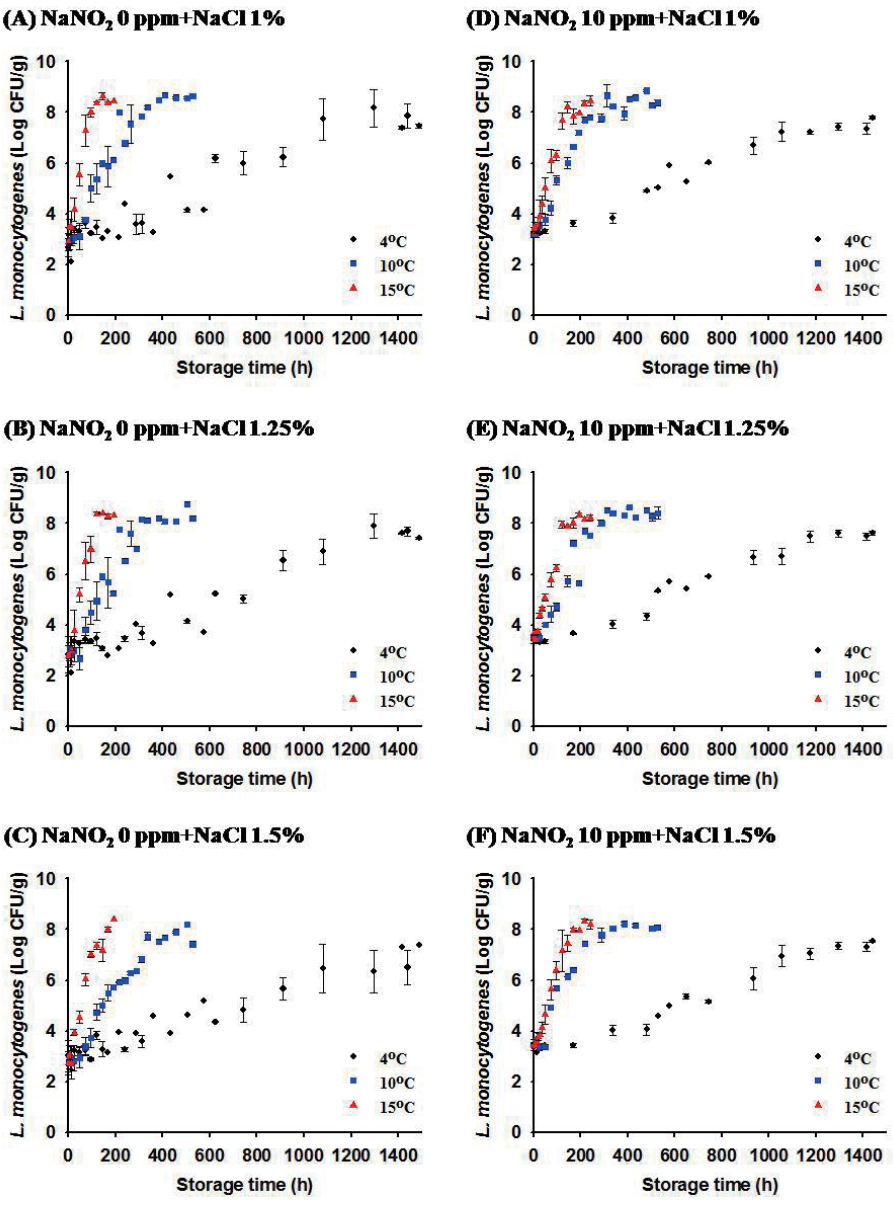

Figure 2. Bacterial populations of *Listeria monocytogenes* cell counts in emulsion-type sausage in vacuum storage.

Gompertz model was fitted to the *L. monocytogenes* cell counts, and R^2 values were 0.944 to 0.987 for aerobic storage, and 0.933 to 0.992 for vacuum storage. These results indicate that the model is appropriate for calculating the kinetic parameters of *L. monocytogenes* in emulsion-type sausage.

For aerobic storage, higher LPD values (245.56 to 445.11 h; $p<0.05$) were obtained at 4°C than 10°C (22.76 to 47.24 h) or 15°C (8.35 to 23.33 h) storage. Growth rate values increased as storage temperature increased. At 4°C, the growth rate (0.004 to 0.008 Log CFU/g/h) of *L. monocytogenes* was very low ($p<0.05$), as compared to those (0.028 to 0.079 Log CFU/g/h) at 10°C and 15°C, regardless of $NaNO_2$ and NaCl concentration (Table 3). Even though the growth rate of *L. monocytogenes* was very low, the pathogen gradually grew at 4°C (Table 3). At 4°C and 10°C, a combination effect of $NaNO_2$ and NaCl on *L. monocytogenes* was not observed, but at 15°C, growth rates (0.043 to 0.050 Log CFU/g/h) were lower ($p<0.05$) in 10-ppm $NaNO_2$ treated samples than in 0-ppm $NaNO_2$ treated samples (0.065 to 0.079 Log CFU/g/h), regardless of NaCl concentration, indicating that under aerobic storage a combiation effect of $NaNO_2$ and NaCl on *L. monocytogenes* is temperature-dependent (Table 3). Because the growth at 4°C and 10°C was too slow to show antimicrobial effect, combinations of $NaNO_2$ and NaCl on *L. monocytogenes* may not be observed at low temperature. The higher temperatures had higher N_{max} values (4°C: 7.0 to 7.6 Log CFU/g, 10°C: 8.0 to 8.6 Log CFU/g, 15°C: 8.6 to 8.9 Log CFU/g) (Table 3). These results suggest that a low $NaNO_2$ concentration may allow *L. monocytogenes* growth even in NaCl combination,

Table 3. The growth parameters estimated by the modified Gompertz model for *Listeria monocytogenes* on emulsion-type sausage as a function of $NaNO_2$ and NaCl concentration at 4°C, 10°C, and 15°C in aerobic storage

Temperature (°C)	$NaNO_2$ (ppm)	NaCl (%)	LPD (h)	Growth rate (Log CFU/g/h)	N_0 [1] (Log CFU/g)	N_{max} [2] (Log CFU/g)	R^2
4	0	1.00	245.56 ± 124.86^{B}	0.008 ± 0.00^{A}	3.6 ± 0.7	7.0 ± 0.0	0.951
		1.25	297.97 ± 197.76^{AB}	0.006 ± 0.00^{F}	0.5 ± 0.3	7.0 ± 0.0	0.944
		1.50	445.11 ± 0.00^{A}	0.004 ± 0.00^{F}	3.3 ± 0.0	7.2 ± 0.0	0.954
	10	1.00	275.07 ± 188.80^{AB}	0.006 ± 0.00^{F}	4.1 ± 0.3	7.1 ± 0.0	0.977
		1.25	346.56 ± 128.17^{AB}	0.006 ± 0.00^{F}	3.9 ± 0.2	7.7 ± 0.0	0.981
		1.50	350.50 ± 125.96^{AB}	0.005 ± 0.00^{F}	4.2 ± 0.5	7.6 ± 0.0	0.985
10	0	1.00	22.76 ± 13.82^{C}	0.036 ± 0.01^{E}	3.7 ± 0.6	8.0 ± 0.3	0.987
		1.25	25.17 ± 10.75^{C}	0.035 ± 0.01^{E}	3.8 ± 0.6	8.0 ± 0.5	0.970
		1.50	39.77 ± 4.27^{C}	0.028 ± 0.00^{E}	3.7 ± 0.5	8.0 ± 0.0	0.961
	10	1.00	26.79 ± 21.85^{C}	0.035 ± 0.00^{E}	4.2 ± 0.1	8.6 ± 0.4	0.980
		1.25	46.74 ± 1.14^{C}	0.028 ± 0.01^{E}	4.2 ± 0.2	8.4 ± 0.2	0.986
		1.50	47.24 ± 1.72^{C}	0.029 ± 0.01^{E}	4.2 ± 0.0	8.6 ± 0.0	0.984
15	0	1.00	8.35 ± 5.40^{C}	0.079 ± 0.01^{B}	4.1 ± 0.8	8.7 ± 0.3	0.979
		1.25	10.07 ± 4.90^{C}	0.073 ± 0.01^{BC}	4.0 ± 0.7	8.7 ± 0.2	0.983
		1.50	13.83 ± 4.84^{C}	0.065 ± 0.00^{C}	4.1 ± 0.6	8.8 ± 0.1	0.973
	10	1.00	16.96 ± 2.66^{C}	0.050 ± 0.00^{D}	4.2 ± 0.1	8.7 ± 0.1	0.968
		1.25	20.04 ± 2.00^{C}	0.048 ± 0.00^{D}	4.5 ± 0.0	8.9 ± 0.2	0.974
		1.50	23.33 ± 5.89^{C}	0.043 ± 0.01^{DE}	4.3 ± 0.2	8.6 ± 0.2	0.983

LPD, lag phase duration; CFU, colony-forming unit.
[1] Initial cell concentration. [2] Maximum cell concentration.
[A-F] Different letters in the same column mean significantly different $p < 0.05$.

and therefore, alternative or additional techniques are necessary to inhibit *L. monocytogenes* growth in the emulsion-type sausage formulated with low $NaNO_2$ and NaCl concentrations.

For vacuum storage, LPD values were higher ($p<0.05$) at 4°C (114.23 to 442.33 h) than at 10°C (24.48 to 90.25 h) or 15°C (9.26 to 17.40 h). As shown for aerobic storage, growth rates (0.003 to 0.005 Log CFU/g/h) at 4°C were very low ($p<0.05$), compared to those (0.019 to 0.072 Log CFU/g/h) at 10°C and 15°C, regardless of $NaNO_2$ and NaCl concentration (Table 4). In addition, no differences in growth rates were observed for

Table 4. The kinetic parameters estimated by the modified Gompertz model for *Listeria monocytogenes* on emulsion-type sausage as a function of $NaNO_2$ and NaCl concentration at 4°C, 10°C, and 15°C in vacuum storage

Temperature (°C)	$NaNO_2$ (ppm)	NaCl (%)	LPD (h)	Growth rate (Log CFU/g/h)	N_0 [1] (Log CFU/g)	N_{max} [2] (Log CFU/g)	R^2
4	0	1.00	227.92 ± 31.16^{C}	0.005 ± 0.00^{F}	4.1 ± 0.4	7.3 ± 0.2	0.969
		1.25	271.97 ± 9.01^{B}	0.005 ± 0.00^{F}	4.1 ± 0.4	7.3 ± 0.4	0.965
		1.50	442.33 ± 0.00^{A}	0.003 ± 0.00^{F}	3.7 ± 0.0	7.5 ± 0.1	0.933
	10	1.00	114.23 ± 27.49^{E}	0.005 ± 0.00^{F}	4.1 ± 0.2	7.3 ± 0.4	0.985
		1.25	174.63 ± 17.27^{D}	0.005 ± 0.00^{F}	4.2 ± 0.2	7.6 ± 0.2	0.984
		1.50	228.80 ± 43.54^{C}	0.004 ± 0.00^{F}	4.3 ± 0.2	7.7 ± 0.5	0.972
10	0	1.00	24.48 ± 14.95^{F}	0.026 ± 0.00^{E}	4.0 ± 0.4	8.8 ± 0.2	0.985
		1.25	26.19 ± 13.18^{F}	0.025 ± 0.00^{E}	3.9 ± 0.4	8.8 ± 0.8	0.989
		1.50	33.27 ± 12.75^{F}	0.019 ± 0.00^{E}	3.8 ± 0.8	8.0 ± 0.1	0.973
	10	1.00	33.04 ± 23.99^{F}	0.028 ± 0.00^{E}	4.2 ± 0.0	8.4 ± 0.2	0.983
		1.25	44.59 ± 20.73^{F}	0.025 ± 0.00^{E}	4.3 ± 0.0	8.5 ± 0.4	0.991
		1.50	90.25 ± 0.00^{F}	0.023 ± 0.00^{E}	4.2 ± 0.0	9.0 ± 0.0	0.972
15	0	1.00	9.26 ± 8.69^{F}	0.072 ± 0.00^{A}	4.1 ± 0.8	8.7 ± 0.0	0.989
		1.25	12.04 ± 7.80^{F}	0.062 ± 0.00^{AB}	3.9 ± 1.0	8.7 ± 0.1	0.982
		1.50	15.75 ± 2.95^{F}	0.058 ± 0.01^{BC}	3.8 ± 0.5	8.0 ± 0.7	0.971
	10	1.00	10.34 ± 7.93^{F}	0.048 ± 0.01^{CD}	4.4 ± 0.2	8.5 ± 0.1	0.986
		1.25	10.96 ± 8.63^{F}	0.043 ± 0.01^{D}	4.5 ± 0.0	8.5 ± 0.1	0.978
		1.50	17.40 ± 17.48^{F}	0.041 ± 0.01^{D}	4.4 ± 0.0	8.5 ± 0.2	0.992

LPD, lag phase duration; CFU, colony-forming unit.
[1] Initial cell concentration. [2] Maximum cell concentration.
[A-F] Different letters in the same column mean significantly different $p < 0.05$.

the different $NaNO_2$ and NaCl concentrations at either 4°C or 10°C (Table 4). However, at 15°C, growth rates (0.041 to 0.048 Log CFU/g/h) were lower ($p<0.05$) in 10 ppm $NaNO_2$ than in 0 ppm $NaNO_2$ (0.058 to 0.072 Log CFU/g/h), and $NaNO_2$ growth rates decreased as NaCl concentration increased in 0 ppm (Table 4), which was an opposite result for aerobic storage at 15°C. N_{max} values were lower ($p<0.05$) at 4°C (7.3 to 7.7 Log CFU/g) than at 10°C (8.0 to 9.0 Log CFU/g) or 15°C (8.5 to 8.7 Log CFU/g) (Table 4). These results indicate that low $NaNO_2$ concentration is not sufficient to inhibit *L. monocyogenes* growth completely.

Growth rates were similar between aerobic and vacuum storage (Tables 3 and 4). Taken together, these results suggest that 10 ppm $NaNO_2$ may not completely inhibit *L. monocytogenes* growth even in combination with NaCl and in vacuum storage and even when samples are stored at 4°C.

In other literature, antimicrobial effects of $NaNO_2$ and NaCl were observed. Doyle and Glass [23] showed that generation time of *L. monocytogens* became longer in broth media supplemented high $NaNO_2$ and NaCl. Jo et al [24] showed that there is a combination effect of low $NaNO_2$ with NaCl on *Pseudomonas* spp. in processed meat products. Also, Lee et al [25] showed that the growth of *Enterococcus* spp. was also inhibited by added $NaNO_2$ and NaCl. Gill and Holley [7] found that pathogenic bacteria such as *Staphylococcus aureus* and *L. monocytogenes* were inhibited by $NaNO_2$ not by NaCl. In addition, single-effect of $NaNO_2$ and NaCl was identified in several Gram-negative bacteria related to meat (*Salmonella* Typhimurium, *Escherichia coli*, *Serratia grimesii*, and *Shewanella putrefaciens*), but multi-effect was observed in only *S.* Typhimurium and *S. putrefaciens*. *Lactobacillus* which cause spoilage in processed meat produtcs [26,27] was inhibitied by a combination of $NaNO_2$ and NaCl in frankfurters [28]. Also, Sallam and Samejima [29] showed that NaCl-treated ground beef supplemented with sodium lactate could delay the growth of lactic acid bacteria and Enterobacteriaceae at refrigerated storage condition. The sporulation of *Clostridium perfringens* and production of *C. perfringens* enterotoxin were also inhibited by nitrate salts as suppression of gene expression [30]. These results indicate that antimicrobial effect of $NaNO_2$ and NaCl was clearly observed, and a combination effect of $NaNO_2$ and NaCl is bacteria dependent.

In conclusion, our studies suggest that emulsion-type sausages that contain low $NaNO_2$ levels, as preferred by consumers, are not safe for *L. monocytogenes* growth even under NaCl combination, regardless of atmospheric storage conditions. Therefore, aleternative or additional techniques are necessary for *L. monocytogenes* control in the product.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

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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