Effect of NaCl on Thermal Resistance, Antibiotic Resistance, and Human Epithelial Cell Invasion of *Listeria monocytogenes*

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Abstract

This study evaluated the effects of NaCl on heat resistance and Caco-2 cell invasion of *Listeria monocytogenes* in broth media and sausage. A 10-strain mixture of *L. monocytogenes* was inoculated in tryptic soy broth containing 0.6% yeast extract (TSBYE), and sausage formulated with 0, 2, 4, and 6% NaCl. The medium was stored at 7, 15, 20, and 25°C for 3–16 d, and medium samples were withdrawn at the appropriate time and challenged to 55, 60, and 63°C to evaluate the thermal resistance of the pathogen. Sausage samples were stored at 7 and 25°C, and they were exposed to 63°C to evaluate thermal resistance. NaCl-habituated *L. monocytogenes* strains NCCP10811 and NCCP10943 were examined for 12 antibiotics and Caco-2 cell invasion assay (only *L. monocytogenes* NCCP10943). Bacterial populations of *L. monocytogenes* generally increased ($p < 0.05$) during the heat challenge as NaCl concentrations increased in both TSBYE and sausage samples. The antibiotic resistance of *L. monocytogenes* was not observed ($p \geq 0.05$) when it was exposed to a single concentration of NaCl in TSBYE, but the pathogen obtained resistance to some antibiotics when exposed to a sequential increase of NaCl concentration. Invasion efficiency of *L. monocytogenes* NCCP10943 was not increased ($p \geq 0.05$) with NaCl concentration increase. These results indicate that NaCl may increase the resistance of *L. monocytogenes* to heat and to some antibiotics, but may not increase Caco-2 cell invasion of *L. monocytogenes*.

Key words: *Listeria monocytogenes*, NaCl, heat resistance, antibiotics, Caco-2 cell line

Introduction

Listeria monocytogenes is gram-positive bacteria and widely distributed in environments (Doyle *et al.*, 2001; Schwartzman *et al.*, 2011). The pathogen is able to grow at low oxygen condition and refrigeration temperatures, and to survive for long periods in food and food processing environments (Codex, 2009). *L. monocytogenes* has been isolated from milk, chicken, fresh meat, processed meat products and various ready-to-eat foods, and it could also contaminate food and food-contact surfaces (Raybourne, 2002; Schwartzman *et al.*, 2011). The pathogen infects patients experiencing immunosuppression and chronic disease, neonates, and the elderly, resulting listeriosis with the fatality rate of 20 to 30% (Codex, 2009; Schwartzman *et al.*, 2011).

In processed meat products, sodium is an important ingredient in the food industry due to its positive effects

on water binding, texture, viscosity, flavor and food safety (Breslin and Beauchamp, 1997; Olesen *et al.*, 2010). Sodium is used in various foods such as chicken broth, meat gravy, salami, bacon, cured meat and cheese, and NaCl is usually used as a sodium source. Many studies showed that NaCl increased heat resistance and virulence of foodborne pathogens (Cataldo *et al.*, 2007; Koutsoumanis *et al.*, 2003; Manas *et al.*, 2001).

Exposure of *L. monocytogenes* to sublethal environmental stress conditions can enhance the survival upon subsequent exposure to lethal conditions and virulence gene expressions (Ferreira *et al.*, 2003; Kazmierczak *et al.*, 2003). Recently, Olesen *et al.* (2009) also showed that exposure of *L. monocytogenes* to 4.5% NaCl resulted in increased transcription of genes relevant for the invasion. Taken together, it can be hypothesized that exposure of *L. monocytogenes* to NaCl could influence *L. monocytogenes* virulence characteristics.

L. monocytogenes has been known to be susceptible to most antibiotics, but antibiotic resistant *L. monocytogenes* has recently been isolated from food samples (Wang *et al.*, 2012; Wiczorek *et al.*, 2012). Although antibiotic resistant *L. monocytogenes* were isolated from foods, the

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correlation between food ingredients and antibiotic resistance has not been studied. In addition, since *L. monocytogenes* invades human epithelial cell, using internalin proteins (Lecuit, 2005), it is also necessary to evaluate if NaCl increases *L. monocytogenes* invasion.

Therefore, the objective of this study was to evaluate the effects of NaCl on thermal resistance, antibiotic resistance, and epithelial cell invasion of *L. monocytogenes*.

Materials and Methods

Determination of thermal resistance

Inoculum preparation

L. monocytogenes strains were purchased from National Culture Collection for Pathogens (NCCP) and Korean Agriculture Culture Collection (KACC), and they were used in this study. The colonies of *L. monocytogenes* strains NCCP10805, NCCP10806, NCCP10807, NCCP10808, NCCP10809, NCCP10810, NCCP10811, NCCP10920, NCCP10943 and KACC10764 on Palcam agar (Difco™, Becton Dickinson and Company, USA) were inoculated into 10 mL of tryptic soy broth with 0.6% yeast extract (TSBYE; Difco™) and the strains were incubated at 30°C for 24 h. The cultures of each strain were then subcultured in 10 mL of TSBYE at 30°C for 24 h, followed by centrifugation (1,912 g, 4°C, 15 min). Resulting pellet was washed 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), and diluted in PBS to 4-6 Log CFU/mL of inoculum.

Exposure of *L. monocytogenes* to NaCl in broth media

The 0.1 mL portions of inoculum were inoculated into 10 mL of TSBYE plus 0, 2, 4, and 6% of NaCl (w/v) to expose *L. monocytogenes* to single concentrations of NaCl, and the samples were then stored at 7 (16 d), 15 (6 d), 20 (3 d) and 25°C (3 d). To expose *L. monocytogenes* to sequentially increased NaCl concentration, 0.1 mL of the inoculum was incubated in TSBYE (10 mL) at 30°C for 24 h. The 0.1 mL of this culture was sequentially transferred into 10 mL of TSBYE plus 2% NaCl, TSBYE plus 4% NaCl, and then TSBYE plus 6% NaCl every 24 h for incubation at 30°C.

Exposure of *L. monocytogenes* to NaCl in sausage

The formulation of sausage (1,000 g) consisted of pork back fat (780 g), lean (200 g), vitamin C (0.02 g), phos-

phate (0.3 g), isolated soy protein (0.5 g), spice (0.4 g) and cold water (20 mL), and NaCl was also added to have (i) 0%, (ii) 2%, (iii) 4%, and (iv) 6% (w/w) of concentration. These sausage formulations were homogenized with a blender (HR1372, Phillips, China) for 2 min, and the meat batter temperature was maintained at 0-4°C. Ten gram portions of the samples were transferred into a well (128×85×22 mm) of 6-well microtiter plates, and samples were pressed down by a reagent spoon to avoid air pockets (air layer), which may interfere with heat-transfer during heating (Yoon *et al.*, 2009). The microtiter plates were then placed in a sterile bag, and heated at 80°C for 40 min, followed by cooling at room temperature. *L. monocytogenes* inoculum without NaCl treatment was inoculated with sausage samples, and spread by a sterile bent glass rod. The inoculated sausage samples were stored at 7 and 25°C for 8 d and 36 h, respectively.

Heat challenge

L. monocytogenes cells exposed to NaCl in TSBYE media were centrifuged (1,912 g, 4°C, 15 min), and cell pellets were washed with PBS twice. The 1 mL portion of each suspension was inoculated into 9 mL of TSBYE that was preheated at 55, 60, and 63°C in a water bath. *L. monocytogenes* populations were then enumerated on tryptic soy agar plus 0.6% yeast extract (TSAYE; Difco™) at 0, 20, 40 and 60 min, and the plates were incubated at 30°C for 48 h.

To heat-challenge sausage, the samples were withdrawn on day 0, 5, 10 and 15 for 7°C storage, and at 0, 12, 24 and 36 h for 25°C storage. The samples were then transferred into a filter bag (BagFilter®, Interscience, France) containing 20 mL of buffered peptone water (BPW, Difco™), followed by pummeling for 120 s by a pummeler (BagMixer®, Interscience, France). The homogenates were exposed to 63°C in a water bath, and bacterial counts were enumerated on TSAYE for total bacteria and *Listeria* selective agar base (Oxoid, UK) for *L. monocytogenes* at 0, 20, 40, and 60 min. The plates were incubated at 30°C for 48 h, and colonies were manually counted. The survival data of *L. monocytogenes* were then fitted to a simple linear regression to calculate death rates (Log CFU/g/min).

Antibiotic resistance assay

Of 10 *L. monocytogenes* strains, the most sensitive strain (*L. monocytogenes* NCCP10943) and the most resistant strain (*L. monocytogenes* NCCP10811) to streptomycin were selected in a preliminary study. The strains

were cultured and then subcultured in TSBYE at 30°C for 24 h. The cultures were then centrifuged (1,912 g, 4°C, 15 min), and pellets were washed with PBS. The 1 mL portions of suspension were incubated in 10 mL TSBYE supplemented with 0, 2, 4 and 6% of NaCl at 30°C for 24 h. The cultures were plated on TSAYE containing each concentration of NaCl, following incubation at 30°C for 48 h. In addition, *L. monocytogenes* cells subjected to a sequential increase of NaCl concentrations were also prepared up to 6% of NaCl as described above. *L. monocytogenes* strains NCCP10811 and NCCP10943 sequentially exposed up to 6% NaCl were subsequently plated on TSAYE containing 6% NaCl. The plates were then incubated at 30°C for 48 h. The 0.1 mL portions of *L. monocytogenes* cell suspension (6 Log CFU/mL) in PBS were inoculated in the sausage samples formulated with 0, 2 and 4% NaCl, and the samples were stored at 25°C for 36 h. Sausage samples were then pummeled in a filter bag containing 20 mL BPW for 120 s. The homogenates were plated on *Listeria* selective agar base, following incubation at 30°C for 48 h.

After incubation of TSAYE and *Listeria* selective agar base plates, buffer solution was added over colonies on the plates, and the colonies were collected by scraping with a glass rod. The collected *L. monocytogenes* cells were centrifuged (1,912 g, 4°C, 15 min), and the pellets were washed with PBS twice. The OD_{625 nm} of the suspension was then adjusted to 0.08-0.1 with BPW, and the suspension was plated on a Muller-Hinton agar (Difco™) by spreading with a sterile swab. The Muller-Hinton agar plates were held at room temperature for 10-15 min, and antibiotic discs (Oxoid) were placed on the surface of the plate, using a multi-disc dispenser (Oxoid) (NCCLS, 1999). Tested antibiotics were amoxicillin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), novobiocin (5 µg), oxacillin (1 µg), oxytetracycline (30 µg), streptomycin (10 µg), tigecycline (15 µg), and vancomycin (30 µg). After incubation at 30°C for 48 h, clear zone around each disc was measured.

Human epithelial cell invasion assay

Because *L. monocytogenes* NCCP10943 showed the highest cell invasion efficiency in a preliminary study, the strains were selected for human epithelial cell invasion, using Caco-2 cell line. The cell suspension of *L. monocytogenes* NCCP10943 for Caco-2 cell invasion assay was prepared with the same procedure as used for antibiotic resistance assay. After incubation of TSAYE and *Listeria*

selective agar base at 30°C for 48 h for broth media and sausage samples, respectively, buffer solution was added over colonies on the plates, and the colonies were collected by scraping with a glass rod. The collected *L. monocytogenes* cells were centrifuged (1,912 g, 4°C, 15 min), and the pellets were washed with PBS twice. The Caco-2 cell invasion assay was then performed as described by Garner *et al.* (2006) with minor modifications. Briefly, the *L. monocytogenes* NCCP10943 were diluted to 5×10^5 - 5×10^6 CFU/mL with PBS, and 0.5 mL of this diluents was inoculated into 4.5 mL of MEM medium (Gibco®, New Zealand) supplemented with 20% FBS (Gibco®) to be used as inoculum. The 1 mL of the inoculum was inoculated in a cell monolayer of Caco-2 cells (5.0×10^4 cells/mL) grown in 5% CO₂ at 37°C for 48 h, following incubation in 5% CO₂ at 37°C for 2 h. The upper layer of MEM medium was then discarded, and 1 mL of fresh MEM medium supplemented with 20% FBS and 50 µg/mL of gentamicin were added into the microtiter plate. After incubation of the plate in 5% CO₂ at 37°C for 2 h, the upper layer of the media was discarded and infected Caco-2 cells with *L. monocytogenes* were washed with PBS twice. The 1 mL of 0.5% Triton X-100 was added into each well of the plate on ice and the plate was left for 20 min. Resulting suspension was used for *L. monocytogenes* enumeration on TSAYE. Invasion efficiency was reported as follows; (the number of bacteria recovered from Caco-2 cell lysis over the number of inoculated bacteria) × 100 (Garner *et al.*, 2006).

Statistical analysis

The experiment was repeated twice with two to three samples in each replicate. Bacterial populations were converted to Log CFU/mL or g before statistical analysis. The data values were analyzed by the mixed model procedure and the general linear model procedure of SAS® version 9.2 (SAS Institute Inc., USA). All mean comparisons were performed with pairwise *t*-test at alpha=0.05.

Results and Discussion

Thermal resistance

L. monocytogenes exposed to different NaCl concentrations in TSBYE at 7 and 15°C did not have different thermal resistance ($p \geq 0.05$) among NaCl concentrations, regardless of heating temperature (data not shown in a tabular form). However, NaCl-habituated *L. monocytogenes* in TSBYE at 20 and 25°C showed increased ($p < 0.05$) thermal resistance as NaCl concentration increased, espe-

cially at 60 and 63°C (Tables 1 and 2). Interestingly, when the pathogen experienced sequentially increased NaCl concentration in TSBYE, the thermal resistance was gradually increased ($p<0.05$), regardless of heating temperature (Table 3). In sausage samples at 7°C, death rates (first death phase: 0.08-0.13 Log CFU/g/min, second death phase: 0.01-0.06 Log CFU/g/min) of NaCl-habituated *L. monocytogenes* were not different ($p\geq 0.05$) among NaCl concentrations (data not shown in a tabular form), but the death rates in the sausage samples formulated with 6% NaCl were lower ($p<0.05$) than those in other NaCl concentrations for the sausage samples stored at 25°C (Table 4). Moreover, decrease of total bacterial populations had a similar trend as *L. monocytogenes* (data not

shown in a tabular form). The results indicate that NaCl may increase thermal resistance of *L. monocytogenes*, especially when NaCl concentrations sequentially increase.

Many factors such as strain variation, previous growth conditions, exposure to heat shock, acid, and other stresses could be related to the thermal resistance of *L. monocytogenes* (Doyle *et al.*, 2001). Heat-shock response in gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* is regulated by σ^B factor, which regulates various chaperones, redox balance, and oxidative stress protection (Cebrián *et al.*, 2009, Price *et al.*, 2001). Thus, the result showing correlation of NaCl with increased thermal resistance of *L. monocytogenes* could be caused by σ^B factor.

Table 1. Survivals (Mean $\pm$ SD; Log CFU/mL) of NaCl-habituated *Listeria monocytogenes* in tryptic soy broth plus 0.6% yeast extract formulated with 0, 2, 4, and 6% of NaCl at 20°C for 3 d during heat challenge at 55, 60, and 63°C for 60 min

Heating temperature (°C)	Exposure time (min)	NaCl concentration (%)			
		0	2	4	6
55	0	7.2 $\pm$ 0.1 ^{Aa}	7.5 $\pm$ 0.0 ^{Aa}	7.5 $\pm$ 0.0 ^{Aa}	7.4 $\pm$ 0.2 ^{Aa}
	20	5.0 $\pm$ 0.1 ^{Ba}	4.9 $\pm$ 0.1 ^{Ba}	5.1 $\pm$ 0.0 ^{Ba}	5.2 $\pm$ 0.2 ^{Ba}
	40	3.8 $\pm$ 0.4 ^{Ca}	3.7 $\pm$ 0.3 ^{Ca}	4.0 $\pm$ 0.1 ^{Ca}	4.2 $\pm$ 0.2 ^{Ca}
	60	3.1 $\pm$ 0.3 ^{Db}	3.1 $\pm$ 0.3 ^{Db}	3.2 $\pm$ 0.2 ^{Dab}	3.7 $\pm$ 0.2 ^{Da}
60	0	7.2 $\pm$ 0.1 ^{Aa}	7.5 $\pm$ 0.0 ^{Aa}	7.5 $\pm$ 0.0 ^{Aa}	7.4 $\pm$ 0.2 ^{Aa}
	20	3.9 $\pm$ 0.1 ^{Bb}	4.7 $\pm$ 0.2 ^{Ba}	4.9 $\pm$ 0.4 ^{Ba}	4.9 $\pm$ 0.3 ^{Ba}
	40	3.3 $\pm$ 0.4 ^{Cb}	3.5 $\pm$ 0.3 ^{Cb}	3.6 $\pm$ 0.5 ^{Cb}	4.1 $\pm$ 0.1 ^{Ca}
	60	2.3 $\pm$ 0.7 ^{Db}	2.9 $\pm$ 0.1 ^{Da}	2.8 $\pm$ 0.5 ^{Da}	3.3 $\pm$ 0.2 ^{Da}
63	0	7.2 $\pm$ 0.1 ^{Aa}	7.5 $\pm$ 0.0 ^{Aa}	7.5 $\pm$ 0.0 ^{Aa}	7.4 $\pm$ 0.2 ^{Aa}
	20	3.7 $\pm$ 0.3 ^{Bc}	4.3 $\pm$ 0.3 ^{Bb}	5.0 $\pm$ 0.2 ^{Ba}	5.0 $\pm$ 0.0 ^{Ba}
	40	2.7 $\pm$ 0.2 ^{Cc}	3.4 $\pm$ 0.6 ^{Cb}	4.0 $\pm$ 0.3 ^{Ca}	3.9 $\pm$ 0.3 ^{Ca}
	60	2.0 $\pm$ 0.4 ^{Db}	2.7 $\pm$ 0.5 ^{Da}	2.9 $\pm$ 0.2 ^{Da}	3.1 $\pm$ 0.4 ^{Da}

^{a-c}Means within the same row with different superscript letters are different ($p<0.05$).

^{A-D}Means within the same column with different superscript letters are different ($p<0.05$).

Table 2. Survivals (Mean $\pm$ SD; Log CFU/mL) of NaCl-habituated *Listeria monocytogenes* in tryptic soy broth plus 0.6% yeast extract formulated with 0, 2, 4, and 6% of NaCl at 25°C for 3 d during heat challenge at 55, 60, and 63°C for 60 min

Heating temperature (°C)	Exposure time (min)	NaCl concentration (%)			
		0	2	4	6
55	0	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}
	20	4.7 $\pm$ 0.1 ^{Ba}	4.8 $\pm$ 0.1 ^{Ba}	5.1 $\pm$ 0.0 ^{Ba}	5.1 $\pm$ 0.0 ^{Ba}
	40	3.5 $\pm$ 0.1 ^{Cc}	3.8 $\pm$ 0.2 ^{Cb}	3.9 $\pm$ 0.1 ^{Ca}	4.1 $\pm$ 0.1 ^{Ca}
	60	3.0 $\pm$ 0.3 ^{Db}	3.4 $\pm$ 0.3 ^{Da}	3.6 $\pm$ 0.2 ^{Ca}	3.8 $\pm$ 0.0 ^{Da}
60	0	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}
	20	4.1 $\pm$ 0.2 ^{Bc}	4.5 $\pm$ 0.2 ^{Bb}	4.8 $\pm$ 0.1 ^{Bb}	5.0 $\pm$ 0.1 ^{Ba}
	40	2.6 $\pm$ 0.3 ^{Cc}	3.4 $\pm$ 0.2 ^{Cb}	3.6 $\pm$ 0.2 ^{Cab}	3.9 $\pm$ 0.2 ^{Ca}
	60	1.8 $\pm$ 0.4 ^{Db}	2.9 $\pm$ 0.4 ^{Da}	3.0 $\pm$ 0.2 ^{Da}	3.1 $\pm$ 0.2 ^{Da}
63	0	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}	7.0 $\pm$ 0.2 ^{Aa}
	20	4.0 $\pm$ 0.4 ^{Bb}	4.4 $\pm$ 0.3 ^{Bab}	4.4 $\pm$ 0.1 ^{Bab}	4.8 $\pm$ 0.0 ^{Ba}
	40	3.2 $\pm$ 0.2 ^{Ca}	3.1 $\pm$ 0.4 ^{Ca}	3.5 $\pm$ 0.2 ^{Ca}	3.6 $\pm$ 0.3 ^{Ca}
	60	2.3 $\pm$ 0.2 ^{Db}	2.7 $\pm$ 0.5 ^{Cb}	2.9 $\pm$ 0.3 ^{Db}	3.1 $\pm$ 0.5 ^{Ca}

^{a-c}Means within the same row with different superscript letters are different ($p<0.05$).

^{A-D}Means within the same column with different superscript letters are different ($p<0.05$).

Table 3. Survivals (Mean±SD; Log CFU/mL) of *Listeria monocytogenes* sequentially exposed to tryptic soy broth plus 0.6% yeast extract formulated with 0, 2, 4, and 6% of NaCl at 30°C during heat challenge at 55, 60, and 63°C for 60 min

Heating temperature (°C)	Exposure time (min)	Sequential increase of NaCl concentration (%)					
		0→0	0→2	0→0	2→4	0→0	4→6
55	0	7.0±0.0 ^{Aa}	7.0±0.0 ^{Aa}	6.9±0.4 ^{Aa}	6.9±0.4 ^{Aa}	7.4±0.4 ^{Aa}	7.4±0.4 ^{Aa}
	20	4.7±0.2 ^{Bb}	5.0±0.1 ^{Bab}	4.5±0.4 ^{Bb}	5.1±0.0 ^{Ba}	4.5±0.1 ^{Bb}	5.6±0.1 ^{Ba}
	40	3.7±0.1 ^{Cd}	4.1±0.1 ^{Cc}	3.7±0.3 ^{Cd}	4.3±0.1 ^{Cb}	3.6±0.2 ^{Cd}	4.8±0.1 ^{Ca}
	60	2.8±0.2 ^{Dc}	3.6±0.2 ^{Db}	3.2±0.4 ^{Cc}	4.0±0.4 ^{Cab}	2.9±0.3 ^{Dc}	4.3±0.3 ^{Ca}
60	0	7.0±0.0 ^{Aa}	7.0±0.0 ^{Aa}	6.9±0.4 ^{Aa}	6.9±0.4 ^{Aa}	7.4±0.4 ^{Aa}	7.4±0.4 ^{Aa}
	20	4.1±0.5 ^{Bb}	4.5±0.1 ^{Bab}	4.0±0.2 ^{Bb}	4.9±0.1 ^{Ba}	4.1±0.4 ^{Bb}	5.0±0.2 ^{Ba}
	40	3.2±0.2 ^{Cc}	3.4±0.2 ^{Cbc}	2.9±0.1 ^{Cc}	3.7±0.3 ^{Cbc}	3.1±0.6 ^{Cc}	4.3±0.1 ^{Ca}
	60	2.7±0.3 ^{Dc}	2.8±0.2 ^{Dc}	2.2±0.3 ^{Dc}	3.6±0.4 ^{Db}	2.2±0.5 ^{Dc}	3.8±0.4 ^{Da}
63	0	7.0±0.0 ^{Aa}	7.0±0.0 ^{Aa}	6.9±0.4 ^{Aa}	6.9±0.4 ^{Aa}	7.4±0.4 ^{Aa}	7.4±0.4 ^{Aa}
	20	3.9±0.3 ^{Bc}	4.2±0.1 ^{Bbc}	3.7±0.6 ^{Bc}	4.7±0.3 ^{Ba}	3.8±0.4 ^{Bc}	5.0±0.1 ^{Ba}
	40	2.9±0.2 ^{Cc}	3.3±0.2 ^{Cbc}	2.8±0.4 ^{Cc}	3.5±0.4 ^{Cbc}	3.1±0.5 ^{Cc}	3.9±0.2 ^{Ca}
	60	2.3±0.4 ^{Dc}	2.6±0.2 ^{Dbc}	2.0±0.5 ^{Dc}	3.1±0.6 ^{Dab}	2.0±0.9 ^{Dc}	3.3±0.3 ^{Da}

^{a-c}Means within the same row with different superscript letters are different ($p<0.05$).

^{A-D}Means within the same column with different superscript letters are different ($p<0.05$).

Table 4. Death rates (Mean±SD; Log CFU/g/min) of *Listeria monocytogenes*, which were habituated to 0, 2, 4, and 6% NaCl formulated in sausage samples at 25°C for 36 h, during heat challenge at 63°C for 60 min

Storage time (h)	NaCl concentration (%)	First death phase	Second death phase
0	0	0.10±0.02 ^{BC}	0.02±0.01 ^A
	2	0.12±0.00 ^{AB}	0.02±0.00 ^{AB}
	4	0.11±0.01 ^{AB}	0.01±0.01 ^{AB}
	6	0.10±0.02 ^{BC}	0.02±0.02 ^{AB}
12	0	0.11±0.03 ^{AB}	0.05±0.01 ^{AB}
	2	0.10±0.02 ^{BC}	0.03±0.02 ^{AB}
	4	0.10±0.01 ^{BC}	0.02±0.02 ^{AB}
	6	0.07±0.01 ^{CD}	0.01±0.03 ^{AB}
24	0	0.12±0.01 ^{AB}	0.06±0.05 ^{AB}
	2	0.11±0.04 ^{AB}	0.03±0.04 ^{AB}
	4	0.11±0.04 ^D	0.03±0.01 ^{AB}
	6	0.06±0.02 ^{AB}	0.03±0.00 ^{AB}
36	0	0.15±0.00 ^A	0.03±0.01 ^{AB}
	2	0.14±0.01 ^A	0.03±0.00 ^B
	4	0.13±0.02 ^{AB}	0.02±0.00 ^B
	6	0.07±0.00 ^{CD}	0.01±0.03 ^B

^{A-D}Means within the same column with different superscript letters are different ($p<0.05$).

Antibiotic resistance

To evaluate effects of NaCl on *L. monocytogenes* antibiotic resistance, *L. monocytogenes* strains NCCP10811 and NCCP10943 were exposed to a single concentration of NaCl in TSBYE during storage at 30°C for 24 h, and both strains did not have significantly decreased ($p\geq 0.05$) diameter of clear zone for antibiotics (data not shown in a tabular form). However, exposure of *L. monocytogenes* to

Table 5. Clear zone diameters (Mean±SD; mm) by *Listeria monocytogenes* strains NCCP10811 and NCCP10943 to 12 antibiotics after sequential exposure up to 6% of NaCl supplemented in tryptic soy broth plus 0.6% yeast extract

Strain	Antibiotic	NaCl concentration (%)	
		0	6
<i>L. monocytogenes</i> NCCP10811	Amoxycillin	31.0±5.8 ^A	35.0±2.0 ^A
	Ampicillin	31.5±3.0 ^A	34.5±1.9 ^A
	Chloramphenicol	26.5±3.4 ^B	29.5±3.0 ^{AB}
	Ciprofloxacin	24.5±1.9 ^A	26.0±1.6 ^A
	Erythromycin	29.5±1.9 ^A	29.5±2.5 ^A
	Gentamycin	22.5±1.9 ^A	21.5±2.5 ^A
	Novobiocin	22.0±0.0 ^A	19.5±2.5 ^A
	Oxacillin	9.3±1.5 ^A	11.5±2.5 ^A
	Oxytetracycline	28.5±3.4 ^A	24.5±1.9 ^A
	Streptomycin	17.0±1.2 ^A	14.5±1.0 ^B
	Tigecycline	26.0±1.6 ^A	26.5±1.9 ^A
	Vancomycin	29.5±6.0 ^A	35.5±2.5 ^A
<i>L. monocytogenes</i> NCCP10943	Amoxycillin	33.0±1.2 ^A	35.0±1.2 ^A
	Ampicillin	33.5±1.0 ^A	35.0±1.2 ^A
	Chloramphenicol	24.5±1.9 ^A	25.5±1.0 ^A
	Ciprofloxacin	26.0±1.6 ^A	25.0±2.6 ^A
	Erythromycin	35.0±1.2 ^A	33.0±2.0 ^A
	Gentamycin	27.0±1.2 ^A	24.5±1.9 ^A
	Novobiocin	20.0±2.8 ^A	22.0±1.6 ^A
	Oxacillin	10.0±1.6 ^A	10.5±1.9 ^A
	Oxytetracycline	31.0±2.6 ^A	28.5±2.5 ^A
	Streptomycin	20.0±0.0 ^A	20.0±1.6 ^A
	Tigecycline	30.0±1.6 ^A	26.5±3.4 ^A
	Vancomycin	25.0±1.2 ^A	22.0±0.0 ^B

^{A,B}Means with the same row with different superscript letters are different ($p<0.05$).

sequentially increased NaCl concentrations up to 6% decreased ($p<0.05$) clear zone diameters to vancomycin

(*L. monocytogenes* NCCP10943) and streptomycin (*L. monocytogenes* NCCP10811) (Table 5). For *L. monocytogenes* exposed to NaCl in sausage samples, higher concentration of NaCl caused small ($p<0.05$) diameters of clear zones for gentamycin and oxacillin (*L. monocytogenes* NCCP10943) and for ciprofloxacin, tigecycline, and vancomycin (*L. monocytogenes* NCCP10811) (Table 6). Furthermore, more antibiotics showed resistance to *L. monocytogenes* when the pathogen was exposed to sausage compared to NaCl in TSBYE (Tables 5-6). These results indicate that the effect of NaCl on antibiotic resistance may depend on type of foods, antibiotics, and *L. monocytogenes* strain. In agreement, Doyle *et al.* (2001) and Lianou *et al.* (2006) also suggested that stress resistance of bacteria depends on growth condition and strain. Although a study by Hood *et al.* (2010) was not conducted in food-related condition, the study also showed that *Acinetobacter baumannii* used a global regulatory cascade to physiological NaCl concentrations, resulting in broad-spectrum tolerance to antibiotics. In fact, many scientists have considered that antibiotic resistances are caused mostly by inappropriate uses of antibiotics (WHO,

2012). However, NaCl in food should be also considered as one of the causes in response to the development of antibiotic resistance according to the results of our study and a study by Hood *et al.* (2010), which can be also supported by the fact that NaCl is ubiquitous within the hospital environment, drug formulations, wound dressings, intravenous fluids and body fluids, where pathogenic bacteria are exposed to (Hood *et al.*, 2010).

Human epithelial cell invasion

Of 10 *L. monocytogenes* strains, the most invasive strain (*L. monocytogenes* NCCP10943) on Caco-2 cell was selected for the further cell invasion assay. After *L. monocytogenes* NCCP10943 was exposed to TSBYE media supplemented with single concentrations of NaCl, the invasion of the pathogen on Caco-2 cell line were not increased ($p\geq 0.05$), and a sequential increase of NaCl in TSBYE did not also increase ($p\geq 0.05$) the Caco-2 cell invasion of *L. monocytogenes* (Fig. 1). However, *L. monocytogenes* NCCP10943 exposed to the sausage samples formulated with 2 and 4% of NaCl had lower ($p<0.05$) Caco-2 cell invasion, compared to the sausage sample

Table 6. Clear zone diameters (Mean $\pm$ SD; mm) by *Listeria monocytogenes* strains NCCP10811 and NCCP10943 to 12 antibiotics after NaCl-habitation at 0, 2, and 4% in sausage samples for 36 h at 25°C

Strain	Antibiotics	NaCl concentration (%)		
		0	2	4
<i>L. monocytogenes</i> NCCP10811	Amoxycillin	29.0 $\pm$ 10.4 ^A	27.0 $\pm$ 8.2 ^A	29.5 $\pm$ 9.8 ^A
	Ampicillin	30.5 $\pm$ 12.8 ^A	27.3 $\pm$ 10.1 ^A	34.5 $\pm$ 1.9 ^A
	Chloramphenicol	18.5 $\pm$ 11.1 ^A	22.8 $\pm$ 4.1 ^A	26.0 $\pm$ 1.6 ^A
	Ciprofloxacin	35.0 $\pm$ 5.8 ^A	29.5 $\pm$ 7.9 ^{AC}	24.0 $\pm$ 4.9 ^C
	Erythromycin	26.0 $\pm$ 2.8 ^A	30.0 $\pm$ 2.8 ^A	28.5 $\pm$ 2.5 ^A
	Gentamycin	26.8 $\pm$ 5.6 ^A	30.3 $\pm$ 8.7 ^A	22.3 $\pm$ 2.6 ^A
	Novobiocin	20.5 $\pm$ 3.0 ^A	20.5 $\pm$ 1.0 ^A	19.0 $\pm$ 2.6 ^A
	Oxacillin	13.5 $\pm$ 2.5 ^A	12.5 $\pm$ 2.9 ^A	11.5 $\pm$ 1.9 ^A
	Oxytetracycline	22.0 $\pm$ 8.1 ^A	31.5 $\pm$ 6.4 ^A	25.5 $\pm$ 1.9 ^A
	Streptomycin	16.3 $\pm$ 1.3 ^A	18.0 $\pm$ 3.7 ^A	18.0 $\pm$ 3.3 ^A
	Tigecycline	28.0 $\pm$ 1.6 ^A	28.5 $\pm$ 3.0 ^A	23.5 $\pm$ 3.0 ^B
	Vancomycin	28.5 $\pm$ 3.8 ^A	22.3 $\pm$ 1.7 ^C	23.0 $\pm$ 2.6 ^{BC}
<i>L. monocytogenes</i> NCCP10943	Amoxycillin	26.0 $\pm$ 3.7 ^B	32.5 $\pm$ 4.1 ^A	35.5 $\pm$ 1.0 ^A
	Ampicillin	27.5 $\pm$ 5.7 ^A	29.0 $\pm$ 5.8 ^A	32.5 $\pm$ 1.9 ^A
	Chloramphenicol	21.0 $\pm$ 3.8 ^B	26.5 $\pm$ 4.1 ^{AB}	27.5 $\pm$ 4.1 ^A
	Ciprofloxacin	33.5 $\pm$ 7.9 ^A	26.5 $\pm$ 3.4 ^A	25.5 $\pm$ 1.9 ^A
	Erythromycin	26.0 $\pm$ 0.0 ^B	30.5 $\pm$ 3.4 ^A	30.0 $\pm$ 1.6 ^A
	Gentamycin	31.3 $\pm$ 4.4 ^A	23.0 $\pm$ 1.2 ^{BC}	25.0 $\pm$ 1.2 ^C
	Novobiocin	15.8 $\pm$ 3.9 ^B	17.0 $\pm$ 1.2 ^B	21.0 $\pm$ 1.2 ^A
	Oxacillin	19.3 $\pm$ 2.8 ^A	9.0 $\pm$ 1.2 ^B	11.0 $\pm$ 1.2 ^B
	Oxytetracycline	31.3 $\pm$ 1.0 ^A	25.8 $\pm$ 2.6 ^B	29.0 $\pm$ 1.2 ^A
	Streptomycin	19.5 $\pm$ 2.4 ^A	17.0 $\pm$ 6.6 ^A	19.5 $\pm$ 1.0 ^A
	Tigecycline	27.5 $\pm$ 2.5 ^A	26.5 $\pm$ 1.9 ^A	27.5 $\pm$ 1.0 ^A
	Vancomycin	24.0 $\pm$ 2.2 ^A	26.3 $\pm$ 0.5 ^A	23.5 $\pm$ 1.0 ^A

^{A-C} Means with the same row with different superscript letters are different ($p<0.05$).

Fig. 1. Invasion efficiency of *Listeria monocytogenes* NCCP10943 to Caco-2 cell line after exposure to single concentrations (0, 2, 4, and 6%) of NaCl (A) and sequential exposure up to 6% (B) of NaCl supplemented in tryptic soy broth plus 0.6% yeast extract.

Fig. 2. Invasion efficiency of *Listeria monocytogenes* NCCP10943, which was habituated to 0, 2 and 4% of NaCl in sausage samples for 36 h at 25°C, to Caco-2 cell line. ^{A-B}Means with different superscript letters are different ($p < 0.05$).

formulated with 0% NaCl (Fig. 2). This result indicates that NaCl in food may not increase cell invasion of *L. monocytogenes* into Caco-2 cell line. Moreover, NaCl in

sausage may decrease *L. monocytogenes* cell invasion capability. In contrast, Olesen *et al.* (2009) showed that invasion gene transcriptions of *L. monocytogenes* increased after they were subjected to 4.5% NaCl. However, a study by Olesen *et al.* (2009) included only genotype data other than phenotype data. Since there are the qualitative differences between genotype and phenotype data (Olsen and Woese, 1993), the discrepancy for Caco-2 cell invasion efficiency could be induced.

In conclusion, NaCl in food formulation may increase the thermal resistance and the resistance of *L. monocytogenes* to some antibiotics, but NaCl may not increase Caco-2 cell invasion efficiency of *L. monocytogenes*.

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