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Effect of Fat Contents on Thermal Resistance, Antibiotic Sensitivity, and Caco−2 Cell Invasion of *Listeria monocytogenes*

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Abstract

This study evaluates the effects of fat contents on the thermal resistance, antibiotic sensitivity, and Caco-2 cell invasion of *Listeria monocytogenes*. Ten strain mixture of *L. monocytogenes* in milk (0, 1, and 4% fat) and pork sausage patties (10, 20, and 30% fat) were exposed to 63°C. To evaluate effects of fat on the antibiotic sensitivity of *L. monocytogenes*, the *L. monocytogenes* strains NCCP10811 (most antibiotic resistant to streptomycin) and NCCP10943 (most antibiotic sensitive to streptomycin) were exposed to different fat contents in milk and pork sausage patties, and *L. monocytogenes* from the foods were used for antibiotic sensitivity assays. The most invasive *L. monocytogenes* strains (NCCP10943) was exposed to different fat contents in milk or pork sausage patties, and *L. monocytogenes* from the foods were used for the Caco-2 cell invasion assays. The reductions of *L. monocytogenes* populations were not generally influenced by fat contents. The *L. monocytogenes* subjected to milk fat had increased sensitivities ($p<0.05$) due to some antibiotics. In addition, Caco-2 cell invasion efficiency of *L. monocytogenes* NCCP10943 increased ($p<0.05$) as fat contents increased. These results indicated that higher fat contents may be related to *L. monocytogenes* invasions and heat resistances in pork sausage patties, but the relationship between fat and antibiotic sensitivity varied according to antibiotics, strains, and fat contents.

Key words: *Listeria monocytogenes*, fat content, heat, antibiotics, Caco-2 cell

Introduction

Listeria monocytogenes causes listeriosis mostly for neonates, pregnant women, the elderly, and immune compromised patients (Burall *et al*., 2012). The pathogen can survive in foods prior to ingestion and withstand hostile environments such as high salt concentration and low temperature (Sleator *et al*., 2003). *L. monocytogenes* has been isolated from ready-to-eat (RTE) foods such as soft cheese, deli meat and frankfurters, which contain high fat contents up to approximately 30% (Burall *et al*., 2012). In addition, high-fat foods such as frankfurters have been frequently implicated in *L. monocytogenes* outbreaks (Buchanan *et al*., 2000).

L. monocytogenes was usually known to be susceptible to most antibiotics (Wieczorek *et al*., 2012), but *L. monocytogenes* isolates have recently showed antibiotic resistance. Of 4,816 *L. monocytogenes* clinical isolates in France, 1.27% were antibiotic resistant, especially against tetracycline and fluoroquinolones (Morvan *et al*., 2010). *L. monocytogenes* isolates from carcasses and bovine hides also showed antibiotic resistances to oxacillin (72.2%) and clindamycin (37%) (Wieczorek *et al*., 2012). In Taipei, 400 samples of meat products, dairy products, fresh vegetables, seafood, and RTE foods were analyzed for antibiotic resistance, and the isolated *Listeria* spp., were resistant to penicillin (7.58%), chloramphenicol (3.7%), and tetracycline (1.96%) (Wang *et al*., 2012).

Food formulation may affect the bacterial resistances to various lethal stresses. A study by He *et al*. (2011) showed that survival rates of *Salmonella enterica* and *Escherichia coli* O157:H7 were higher ($p<0.05$) in high carbohydrate contents than in low contents. Kuda *et al*. (2012) examined the effects of tryptone and phytone peptoneon human epithelial cell invasion of *L. monocytogenes*, and they found that tryptone increased the cell invasion of the bacteria. Prior experience of *L. monocytogenes* to mild heat stress also increased Caco-2 cell invasion (Bradley *et al*., 2012).

Therefore, this study examined the effect of fat contents of milk and pork sausage patty on thermal resistance, antibiotic sensitivity, and Caco-2 cell invasion of *L. monocytogenes*.

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Materials and Methods

Preparation of food samples

Three fat contents (0%; nonfat milk, 1%; low fat milk, 4%; whole milk) of commercial milk were purchased, and the samples (10 mL) were transferred into test tubes. They were heated in a water bath at 70°C for 20 min to destroy background microflora, which may interfere the assays in this study. The milk samples were then left at room temperature to cool down.

The pork sausage patties were prepared at 10%, 20%, and 30% of fat contents according to a formulation by Kim *et al.* (2010), followed by homogenizing the formulations with a blender (speed setting of 5; HR1372, Phillips, Netherlands) for 2 min. Ten gram portions of the samples were transferred into a 6-well microtiter plate (well size: 128×85×22 mm), and samples were pressed down by a reagent spoon to remove air, which may impede heat-transfer during heating (Yoon *et al.*, 2009). The microtiter plates were transferred into sterile plastic bags, and they were cooked in a water bath at 80°C for 40 min. The samples were left at room temperature to cool down.

Inoculation and Heat challenge

L. monocytogenes strains NCCP10805, NCCP10806, NCCP10807, NCCP10808, NCCP10809, NCCP10810, NCCP10811, NCCP10920, NCCP10943 and KACC10764 were cultured in 10 mL tryptic soy broth plus 0.6% yeast extract (TSBYE; Difco™, Becton Dickinson and Company, USA) at 30°C for 24 h. The 0.1 mL portions of the cultures were subsequently incubated in 10 mL TSBYE at 30°C for 24 h. The cultures of the 10 strains were mixed, followed by centrifugation (1,912 *g*, 4°C, 15 min). Resulting 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, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water), and diluted in PBS to obtain 4 Log CFU/mL. The 0.1 mL of the inoculum was inoculated into 10 mL milk in a test tube, and the samples were stored at 7 and 25°C for 360 h and 36 h, respectively, to allow the pathogen to adapt to the condition formulated with different fat contents. During storage, milk samples were heat-challenged at 63°C in a water bath every 120 h (7°C) and 12 h (25°C), and heating time period ranged from 30 min to 80 min, depending on storage day and storage temperature.

The 0.1 mL portions of the inoculum were inoculated on surfaces of pork sausage patties in microtiter plates, and spread by a sterile bent glass rod. After a lid was covered, the microtiter plates in sterile plastic bags were stored at 7 and 25°C. For heat challenge, pork sausage patties were withdrawn every 48 h for 192 h (7°C) and 12 h for 36 h (25°C). Pork sausage patties were then transferred into filter bags (BagFilter®, Interscience, France) containing 20 mL of 0.1% buffered peptone water (BPW, Difco™), followed by pummeling (BagMixer® Interscience, France) for 120 s. Resulting homogenates were then subjected to 63°C in a water bath, and total bacterial and *L. monocytogenes* populations were enumerated on tryptic soy agar plus 0.6% yeast extract (TSAYE; Difco™) and *Listeria* selective agar (Oxoid, Thermo Fisher Scientific, UK), respectively. The plates were incubated at 30°C for 48 h, and colonies were manually counted.

Antibiotic sensitivity assay

Because Charpentier and Courvalin (1999) showed that most clinical isolates of *Listeria* spp. were streptomycin resistance, the 10 *L. monocytogenes* strains were screened for streptomycin resistance in a preliminary study. *L. monocytogenes* strains NCCP10811 and NCCP10943 were then selected as the most resistance strain and the most sensitive strain, respectively.

The inocula of two strains were prepared by the method as described in heat challenge. The 0.1 mL portions of each inoculum were inoculated into 10 mL of 0, 1 and 4% fat milk, and pork sausage patties formulated with 10, 20 and 30% fat, following incubation at 25°C for 36 h. The patty homogenates prepared as described above, and the milk samples were plated on *Listeria* selective agar. After incubation of the plates at 30°C for 48 h, buffer solution was added over *L. monocytogenes* colonies on the plates, and the colonies were scrapped by using a glass rod. The collected colonies were suspended by vortexing, and the turbidity of this *L. monocytogenes* cell suspension was adjusted to 0.1 of OD_{625}. A sterile swab was dampened with the cell suspension, and it was spread on the surface of Muller-Hinton agar (Difco™). The plates were then held at roomtemperature for 10-15 min to allow absorption of free surface liquid. Antibiotic discs (Oxoid) were placed on the surface of Muller-Hinton agar by a multi-disc dispenser (Oxoid); tested antibiotics were amoxicillin (10 μg/disc), ampicillin (10 μg/disc), chloramphenicol (30 μg/disc), ciprofloxacin (5 μg/disc), erythromycin (15 μg/disc), gentamicin (10 μg/disc), novobiocin (5 μg/disc), oxacillin (1 μg/disc), oxytetracycline (30 μg/disc), streptomycin (10 μg/disc), tigecycline (15 μg/disc), and vancomycin (30 μg/disc). After incubation at 30°C for 48 h, the clear zone diameters were measured (CLSI, 2010).

Caco-2 cell invasion assay

Ten *L. monocytogenes* strains were screened for Caco-2 cell invasion in a preliminary study, and the most invasive strain (NCCP10943) of *L. monocytogenes* was selected. The inoculum of *L. monocytogenes* NCCP10943 was prepared by the method as described in heat challenge, and fat-habituated bacterial cell suspension was prepared as described in the antibiotic sensitivity assay. The method described by Garner *et al*. (2006) was used for Caco-2 cell invasion assay with minor modifications. Briefly, a cell monolayer of Caco-2 cell line grown in 24-well tissue culture plates for 72 h was washed twice with PBS. The number (5×10^4 cells/mL) of Caco-2 cells collected from a separate part of the culture plate was determined, using a haemocytometer after trypsin treatment. The fat-habituated *L. monocytogenes* NCCP10943 suspension was diluted to 5×10^5 CFU/mL with PBS, and 0.5 mL of the diluent was inoculated into 4.5 mL MEM medium (Gibco®, New Zealand) supplemented with 20% fetal bovine serum (FBS, Gibco®) to prepare inoculum. One milliliter of this inoculum was inoculated into the cell monolayer of Caco-2 cells, and they were incubated in 5% CO_2 at 37°C for 2 h. The upper layer of MEM medium was then discarded, and 1 mL fresh MEM medium containing 20% FBS and 50 μg/mL gentamicin was added into the microtiter plate. After incubation in 5% CO_2 at 37°C for 2 h, the upper layer of the media was removed and the infected Caco-2 cells with *L. monocytogenes* were washed with PBS twice. The Caco-2 cells were lysed by 1 mL distilled water containing 0.5% Triton X-100 on ice for 20 min. Resulting suspension was plated on TSAYE to enumerate infected *L. monocytogenes* NCCP10943. Caco-2 cell invasion efficiency of *L. monocytogenes* NCCP10943 was expressed 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 replicated entirely twice with three samples in each replicate (n=6). Bacterial populations were converted to Log CFU/mL or /g before statistical analysis. The data were analyzed by the general linear model procedure of SAS® version 9.2 (SAS Institute Inc., Cary, North Carolina, USA), and the LS means among fixed effects were compared with 'pdiff' option to analyze all pairwise comparisons (SAS, 2012).

Results and Discussion

Thermal resistance

To evaluate the protective effect of milk fat on *L. monocytogenes* to heat, the pathogen was exposed to different fat contents (0, 1, and 4%) in milk. When *L. monocytogenes* was then heat-challenged at 63°C, and final reduction (difference between 0 h and end of sampling time) was used to compare the effect of fat contents on *L. monocytogenes* survivals rather than using *L. monocytogenes* cell counts at each sampling time during heat challenge because at 0 h of heat challenge for each storage day, *L. monocytogenes* cell counts were different among fat contents. No differences ($p > 0.05$) in reduction of *L. monocytogenes* cell counts were observed among fat contents, regardless of storage temperature (Table 1). This result indicates that the fat in milk may not have a protective effect on *L. monocytogenes* from heating. After the pork sausage patties were heat-challenge at 63°C, obvious differences in *L. monocytogenes* cell counts were not generally observed ($p > 0.05$) among fat contents, regardless of storage temperature (Table 2). A study by Fain *et al*. (1991) presented that *L. monocytogenes* Scott A had higher *D*-value (5.8 min) in high fat content (30.5%) ground beef than *D*-value (2.6 min) in low fat content (2%) ground beef at 57.2°C. Inc-

Table 1. Final reduction [difference between 0 h and end of sampling time (mean±standard deviation; Log CFU/mL)] of *Listeria monocytogenes* in milk samples formulated with 0, 1, and 4% fat stored at 7°C and 25°C for 360 h and 36 h during heat challenge at 63°C

Storage temperature (°C)	Storage (h)	Fat contents (%)		
		0	1	4
7	0	2.0±0.5[Da]	2.1±0.3[Da]	2.1±0.1[Ea]
	120	4.4±0.5[Ca]	4.6±0.4[Ca]	4.4±0.4[Fa]
	240	6.3±0.2[Aa]	6.3±0.6[Aa]	6.0±0.4[Aa]
	360	6.4±0.5[Aab]	6.8±0.5[Aa]	6.2±0.4[Ab]
25	0	2.3±0.2[Da]	2.3±0.3[Da]	2.3±0.2[Ea]
	12	4.6±0.4[Ca]	4.9±0.1[BCa]	4.8±0.2[DFa]
	24	5.4±0.4[Ba]	5.2±0.5[Ba]	5.3±0.6[CDa]
	36	5.6±0.2[Ba]	5.7±0.7[Ba]	5.6±0.4[BCa]

[A-F]: means within the same column with different superscript letters are different ($p < 0.05$).
[a-b]: means within the same row with different superscript letters are different ($p < 0.05$).

Table 2. Final reduction [difference between 0 h and end of sampling time (mean±standard deviation; Log CFU/g)] of *Listeria monocytogenes* in pork sausage patties formulated with 10, 20, and 30% fat stored at 7°C and 25°C for 192 hand 36 h during heat challenge at 63°C

Storage temperature (°C)	Storage (h)	Fat content (%)		
		10	20	30
7	0	2.9±0.3^{Ca}	3.1±0.1^{Ba}	3.0±0.2^{Ba}
	48	3.0±0.1^{Ca}	2.7±0.4^{Ca}	3.0±0.4^{Ba}
	96	3.1±0.0^{Ca}	3.0±0.1^{BCa}	3.0±0.1^{Ba}
	144	3.0±0.1^{Ca}	3.3±0.2^{Ba}	3.0±0.3^{Ba}
	192	3.3±0.3^{Ca}	3.2±0.2^{Ba}	3.2±0.2^{Ba}
25	0	3.3±0.6^{Ca}	3.1±0.7^{Ba}	3.5±0.6^{Ba}
	12	4.2±0.8^{Ba}	3.8±0.3^{Aab}	3.4±0.3^{Bb}
	24	5.0±0.7^{Aa}	3.9±0.4^{Ab}	4.5±0.3^{Aa}
	36	4.2±0.2^{Bb}	4.4±0.3^{Aab}	4.8±0.4^{Aa}

$^{A-C}$: means with the same column with different superscript letters are different ($p<0.05$).
$^{a-b}$: means with the same row with different superscript letters are different ($p<0.05$).

Table 3. Clear zone diameter (mean±standard deviation; mm) formed by *Listeria monocytogenes* strains NCCP10811 and NCCP 10943 in tryptic soy broth plus 0.6% yeast extract after exposure to different fat contents of milk at 25°C for 36 h

Strain	Antibiotic	Fat content (%)		
		0	1	4
L. monocytogenes NCCP10811	Amoxycillin	36.5±2.5^{A}	36.0±3.7^{A}	36.5±1.0^{A}
	Ampicillin	31.5±4.4^{A}	34.0±4.9^{A}	34.0±3.7^{A}
	Chloramphenicol	29.0±2.6^{A}	27.5±2.5^{A}	29.5±1.9^{A}
	Ciprofloxacin	26.0±1.6^{AB}	23.0±2.0^{B}	25.5±1.9^{AB}
	Erythromycin	34.5±3.4^{A}	34.5±2.5^{A}	34.5±1.9^{A}
	Gentamicin	26.0±3.7^{A}	26.5±3.0^{A}	26.0±2.3^{A}
	Novobiocin	23.5±3.0^{A}	22.5±1.9^{A}	24.0±2.8^{A}
	Oxacillin	10.5±1.0^{A}	10.5±1.0^{A}	6.0±6.9^{A}
	Oxytetracycline	31.0±1.2^{AB}	30.5±3.0^{B}	35.5±1.9^{A}
	Streptomycin	17.5±1.0^{A}	17.0±4.8^{A}	20.5±1.0^{A}
	Tigecycline	26.0±2.8^{A}	25.0±2.0^{A}	27.5±1.0^{A}
	Vancomycin	31.5±5.3^{B}	31.0±4.8^{B}	39.5±1.0^{A}
L. monocytogenes NCCP10943	Amoxycillin	36.0±1.6^{A}	36.0±1.6^{A}	35.0±2.6^{A}
	Ampicillin	36.5±7.7^{A}	35.5±3.0^{A}	36.0±3.7^{A}
	Chloramphenicol	25.0±4.8^{A}	25.8±3.1^{A}	28.0±2.8^{A}
	Ciprofloxacin	25.0±2.0^{A}	26.5±2.5^{A}	26.0±2.8^{A}
	Erythromycin	33.5±1.0^{A}	33.5±1.9^{A}	35.0±3.8^{A}
	Gentamicin	24.0±3.7^{B}	30.5±5.5^{A}	30.0±3.7^{AB}
	Novobiocin	24.0±2.3^{A}	22.5±1.0^{A}	25.0±2.6^{A}
	Oxacillin	8.5±2.4^{A}	10.8±1.5^{A}	12.3±4.2^{A}
	Oxytetracycline	29.5±1.0^{A}	34.0±3.7^{A}	31.0±1.2^{A}
	Streptomycin	18.0±2.8^{B}	21.5±1.0^{A}	21.5±1.0^{A}
	Tigecycline	30.0±3.7^{A}	28.0±0.0^{A}	29.5±2.5^{A}
	Vancomycin	30.0±8.2^{A}	27.0±2.6^{A}	26.5±1.9^{A}

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

reasing the fat content in pork sausage patties may induce the protective effect of fat on *L. monocytogenes* because increased fat content resulted in decreased water activity and this may lead to poor heat penetration (Juneja and Eblen, 2000). However, in our study the differences (0-4% for milk; 10-30% for pork sausage patties) of fat contents may be below the threshold to make difference of water activity, the effect of fat on thermal resistance may not be observed.

Antibiotic sensitivity

The antibiotic sensitivity test of 10 *L. monocytogenes* strains to streptomycin was performed, and the most resistant strain (*L. monocytogenes* NCCP10811) and the most sensitive strain (*L. monocytogenes* NCCP10943) were selected. Both strains were then exposed to 12 antibiotics.

For the *L. monocytogenes* strains exposed to milk fat, a clear zone diameter of *L. monocytogenes* NCCP10811 increased ($p<0.05$) only to oxytetracycline and vancomycin ($p<0.05$) as fat contents increased (Table 3). For *L. monocytogenes* NCCP10943, clear zone diameters to gentamicin and streptomysin increased ($p<0.05$) when fat contents increased (Table 3). The reason for such an effect is not clear, and thus a further research is necessary to elucidate the relationship between fat contents and changes in antibiotic sensitivity. The diameters were not different ($p>0.05$) for *L. monocytogenes* strains NCCP10811 and NCCP10943 habituated in pork sausage patties (data not shown in a tabular form). The result indicates that the fat effect on antibiotic sensitivity of *L. monocytogenes* depends on antibiotic, strain, and food type. Sodium also influenced the antibiotic sensitivity of *L. monocytogenes*. A study by Hood *et al.* (2010) demonstrated that *Acinetobacter baumannii* resistance to antibiotic (colistin) increased in response to NaCl concentrations. Lee *et al.* (2012) proved that monosodium glutamate increased acid resistance of *Escherichia coli*. According to these results, further researches to find the relationship between food components and bacterial stress responses are necessary.

Caco-2 cell invasion

Of the 10 strains tested in this study, *L. monocytogenes* NCCP10943 was selected as the most invasive strain to Caco-2 cell. This strain was then used to evaluate effects of fat contents on *L. monocytogenes* invasion efficiency. Caco-2 cell invasion efficiency of *L. monocytogenes* NCCP10943 increased ($p<0.05$) when the strain was exposed to milk fat (Fig. 1A). In addition, *L. monocytogenes* NCCP10943 exposed to 30% fat in pork sausage patties also had a higher ($p<0.05$) invasion efficiency compared to 10 and 20% fat contents (Fig. 1B).

According to the result from this study, fat in the foods may increase *L. monocytogenes* invasion efficiency to Caco-2 cell line, but genomic and proteomic studies need to be conducted to identify the related genes and proteins for increased invasion. The other studies found that food components such as sodium chloride and organic acids increased virulence of foodborne pathogens (Beckingsale *et al.*, 2011; Gancz *et al.*, 2008; Garner *et al.*, 2006; Jensen *et al.*, 2007). Therefore, further researches are necessary to elucidate the effect of fat on regulatory cascades of *L. monocytogenes* virulence and resistance.

In conclusion, high fat contents examined in this study may not be related to the thermal resistance of *L. monocytogenes*, and effect of fat on antibiotic sensitivity of the
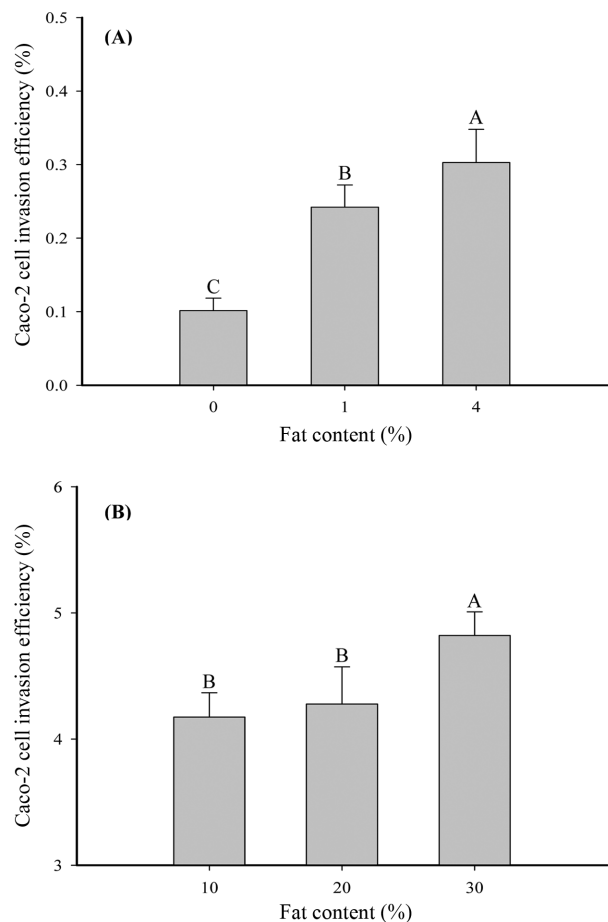
Fig. 1. Caco-2 cell invasion efficiency of *Listeria monocytogenes* subjected to different fat contents in milk (A) and pork sausage patties (B) at 25°C for 36 h. [A-C]means with different letters are significantly different ($p<0.05$).

pathogen depends on antibiotic, strain, and fat content in food. In addition, fat in foods may increase Caco-2 cell invasion efficiency of *L. monocytogenes.*

Acknowledgements

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