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ARTICLE

Gastric Fluid and Heat Stress Response of *Listeria monocytogenes* Inoculated on Frankfurters Formulated with 10%, 20%, and 30% Fat Content

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Abstract

This study evaluated the effects of frankfurter fat content on *Listeria monocytogenes* resistance to heat stress and gastric fluid, and the Caco-2 cell invasion efficiency of the pathogen. A 10-strain mixture of *L. monocytogenes* was inoculated on frankfurters formulated with 10%, 20%, and 30% fat content (10%: F10, 20%: F20, 30%: F30) and stored at 10°C for 30 d. The samples were analyzed for *L. monocytogenes* resistance to heat stress and a simulated gastric fluid challenge. The total bacteria and *L. monocytogenes* survival rates were measured on tryptic soy agar plus 0.6% yeast extract and Palcam agar, respectively. *L. monocytogenes* colonies inoculated on F10, F20, and F30 samples were used for a Caco-2 cell invasion assay. In general, no obvious differences were observed between the survival rates of total bacteria and *L. monocytogenes* grown on different fat contents under heat stress and gastric fluid challenge. However, *L. monocytogenes* obtained from the F30 samples had a significantly higher Caco-2 cell invasion efficiency than those in the F10 and F20 samples ($p<0.05$). These results indicate that although high fat content in food may not be related to *L. monocytogenes* resistance to heat stress and gastric fluid, it may increase the Caco-2 cell invasion efficiency of the pathogen.

Key words: *Listeria monocytogenes*, fat, heat resistance, gastric fluid, caco-2 cell

Introduction

Listeria monocytogenes causes listeriosis, which can result in miscarriages, severe septicemia in newborns, and can immunocompromise individuals (Bogdan, 2012; Schvartzman *et al*., 2011; Smith *et al*., 2009). Because of its high mortality rate, up to 30%, Listeriosis has become a serious public health problem (Vazquez-Bolland *et al.,* 2001).

Since outbreaks of listeriosis have involved the consumption of ready-to-eat (RTE) meat and poultry products, especially frankfurters formulated with a fat content of approximately 30%, *L. monocytogenes* is considered a dangerous meat-borne pathogen (Grigelmo-Miguel *et al*., 1999). Moreover, RTE meat and poultry products are usually consumed with no additional treatments or minimal heat treatment. Thus, if *L. monocytogenes* is present in RTE meat and poultry products, the pathogen may not be sufficiently eliminated prior to consumption. *L. monocytogenes* is then confronted by the body's primary defense, the low pH of gastric secretions (Waterman and Small, 1998). Extracellular *L. monocytogenes* cells rapidly invade human epithelial cells by secreting invasion-related proteins, and are eventually engulfed in a phagocytic vacuole (Bogdan, 2012; Lecuit, 2005).

Although food-borne pathogens are generally exposed to various ingredients, the effects of these ingredients on the resistance and pathogenicity of bacteria have not been fully studied. Manas *et al*. (2001) studied the effect of ingredients such as sodium chloride on the thermal resistance of *Salmonella* Typhimurium and found that sodium levels in food increased the thermal resistance of *S*. Typhimurium. Previous studies have shown that the virulence and resistance of *L. monocytogenes* are correlated with high osmotic pressure and other stresses (Cataldo *et al*., 2007; Koutsoumanis *et al*., 2003). *L. monocytogenes* is known to grow on foods with high fat content, but the

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effect of fat content on the resistance of *L. monocytogenes* to various stresses and its potential for human epithelial cell invasion has not been fully studied.

The objective of this study was to evaluate the effect of the fat content in frankfurters, which served as a model system, on the resistance of *L. monocytogenes* to heat and gastric fluid, as well as the invasion efficiency of Caco-2 cells.

Materials and Methods

Inoculum preparation

L. monocytogenes strains NCCP10805, NCCP10806, NCCP10807, NCCP10808, NCCP10809, NCCP10810, NCCP10811, NCCP10920, NCCP10943, and KACC10 764 were cultured in tryptic soy broth (Difco, Becton Dickinson, USA) plus 0.6% yeast extract (Acumedia, USA) (TSBYE) at 30°C for 24 h, and 0.1 mL of the culture was then subcultured in TSBYE at 30°C for 24 h. *L. monocytogenes* strains were then mixed in a centrifuge tube and centrifuged at 1,912×*g* at 4°C for 15 min. The resulting pellet was washed twice and diluted 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).

Sample preparation and inoculation

Frankfurters (5 g) with three different fat contents (10%: F10, 20%: F20, and 30%: F30) were prepared by combining lean pork, vitamin C, phosphate, isolated soy protein, spice, cold water, NaCl, and pork back fat according to a formulation by Kim *et al*. (2010). Then 0.1 mL of the inoculum was inoculated on the surface of the frankfurters to obtain 4-5 Log CFU/g. Two inoculated frankfurter links were placed in a vacuum bag (Food Saver®, Rollpack, Korea), vacuum packaged (Food Guard®, Rollpack, Korea), and stored for 30 d at 10°C, which is the standard temperature for retail refrigerators in Korea (KFDA, 2007). The samples were then analyzed on day 10 and day 30 under conditions of heat stress and gastric fluid.

Heat stress

To assess the effect of fat content on the thermal resistance of *L. monocytogenes*, the vacuum-packed frankfurters were placed in a water bath at 63°C. The vacuum packages were removed and analyzed at 0, 20, 40, and 60 min. Then 40 mL of buffered peptone water (BPW, Difco) was added to the vacuum bag, and we shook each bag 25 times to detach the bacteria from the surface of the frankfurters (Barmpalia *et al*., 2004). For the quantification of bacterial populations, the rinsates were serially diluted with BPW and 0.1 mL portions of the diluents were plated on tryptic soy agar (Difco) plus 0.6% yeast extract (TSAYE) to determine the number of total bacteria, and Palcam agar (Difco) to determine the number of *L. monocytogenes* colonies. The plates were incubated at 30°C for 48 h, and colonies were counted manually.

Gastric fluid experiment

To evaluate the effect of fat on *L. monocytogenes* resistance to gastric fluid, the simulated gastric fluid (Czuprynski *et al*., 2002) was prepared by combining 8.30 g proteose-peptone (Sigma-Aldrich, USA), 3.50 g D-glucose (Samchun Pure Chemical Co. Ltd., Korea), 2.05 g sodium chloride (Duksan Pure Chemicals, Korea), 0.60 g potassium phosphate (Duksan Pure Chemicals), 0.11 g calcium chloride (Samchun Pure Chemical), 0.37 g potassium chloride (Duksan Pure Chemicals), 0.10 g lysozyme (Wako Pure Chemical Industries Ltd., Japan), 50 mg bile salt (Sigma-Aldrich), and 13.30 mg pepsin (Yakuri Pure Chemical Co. Ltd., Japan) per liter of distilled water. The simulated gastric fluid was adjusted to pH 2.0, using 1 N HCl. The frankfurters were transferred from vacuum packages to a filter bag (BagFilter®, Interscience, France) containing 50 mL of simulated gastric fluid, and the samples were homogenized with a pummeler (BagMixer®, Interscience, France) for 30 s. The homogenates were then placed in a water bath at 37°C, and samples were analyzed at 0, 30, 60, 90, and 120 min. The homogenates were diluted with BPW, and 0.1-mL portions of the diluents were then plated on TSAYE and Palcam agar to determine survivals of total bacteria and *L. monocytogenes*, respectively. The plates were incubated at 30°C for 48 h, and colonies were manually counted.

Human epithelial cell invasion

After storage at 10°C for 30 d, the vacuum packages were opened, and 40 mL BPW was added to the packages. The vacuum packages were shaken 25 times to detach *L. monocytogenes* from the samples as described above, and the rinsates were plated on Palcam agar. After incubation at 30°C for 48 h, 3 mL PBS was added to the *L. monocytogenes* colonies on the plates, and the colonies were scraped using a glass rod. The collected *L. monocytogenes* cells were centrifuged, and the pellets were washed twice with PBS. The *L. monocytogenes* suspension was then diluted to 1.1×10^6 to 1.8×10^6 CFU/mL with PBS, and 0.5 mL of this suspension was inoculated into 4.5 mL

of Eagle's minimum essential medium (MEM medium, Gibco®, Penrose, New Zealand) plus 20% fetal bovine serum (FBS, Gibco®). Next, 1 mL of the inoculum was inoculated in Caco-2 cell monolayer grown at 1×10^5 cells/mL in 5% CO_2, at 37°C for 48 h, and this mixture was incubated in 5% CO_2 at 37°C for 2 h. The upper layer of the culture was then discarded to eliminate detached bacteria, and 1 mL of MEM medium supplemented with 20% FBS and gentamicin (50 μg/mL) was added into each well, following incubation at 37°C for 2 h in 5% CO_2. The medium was then removed and *L. monocytogenes*-infected Caco-2 cells were washed twice with PBS. Subsequently, 0.5% Triton X-100 (1 mL) was added into each well of the plate on ice and left for 20 min. The resulting suspension was plated on TSAYE to enumerate viable *L. monocytogenes* within Caco-2 cells. Invasion efficiency was reported as follows; (Bacterial populations recovered from Caco-2 cell lysis/inoculated bacterial populations) × 100 (Garner *et al*., 2006).

Statistical analyses

All data (n=4) were analyzed using general linear models in SAS® version 9.2 (SAS Institute Inc., USA). LS means among the fixed effects were compared with pairwise *t*-test at alpha=0.05.

Results and Discussion

L. monocytogenes was inoculated in frankfurters formulated with different fat contents. The total bacterial and *L. monocytogenes* populations on the frankfurter samples significantly increased ($p<0.05$) from 4 Log CFU/g up to approximately 8 Log CFU/g during storage at 10°C for 30 d (data not shown). No obvious differences in bacterial populations of *L. monocytogenes* on the frankfurters were observed among samples with different fat contents, suggesting that fat content is not related to bacterial growth (Fig. 1). On being subjected to temperatures of 63°C on F10, F20, and F30 frankfurters the *L. monocytogenes* was, populations significantly decreased ($p<0.05$). The survival rate of *L. monocytogenes* following the heat stress did not differ among the three fat contents (Fig. 1). Moreover, subjecting of *L. monocytogenes* on frankfurter samples to simulated gastric fluid caused a gradual decrease of bacterial populations for 120 min, but no correlation between fat content and *L. monocytogenes* resistance was observed (Fig. 2). In contrast, Barmpalia-Davis *et al*. (2009) showed that the survival of *L. monocytogenes* after the gastric fluid challenge was 1-Log unit higher in high fat (32.5%) beef frankfurters than low fat (4.5%) frankfurters after storage at 7°C, and they suggested that high fat content had a protective effect against gastric fluid for *L. monocytogenes*. The discrepancy between our study and the study by Barmpalia-Davis *et al*. (2009) may be explained by the different fat contents and storage temperatures used in the two studies. The physical state of cell membrane lipids is closely related to temperature, which also changes the lipid composition of cell membrane (Annous *et al*., 1997). Moreover, *L. monocytogenes* is a psychrotrophic bacterium, and thus the pathogen is capable of growth in temperatures lower than 7°C, which was the temperature used in the Barmaplia-Davis *et al*. (2009) study. Hence, the different storage temperatures in the two studies may cause a discrepancy in results due to the different physical states

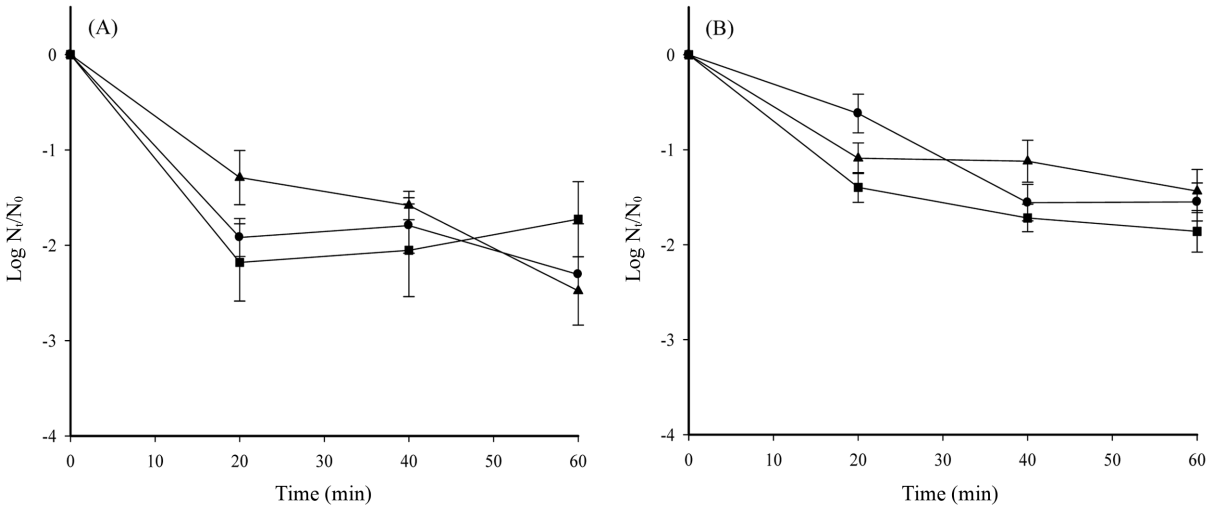

Fig. 1. Thermal resistance of *Listeria monocytogenes* at 63°C for 60 min after habituation to different fat contents (10%: F10 (●), 20%: F20 (▲), 30%: F30 (■)) at 10°C for 0 (A) and 30 d (B).

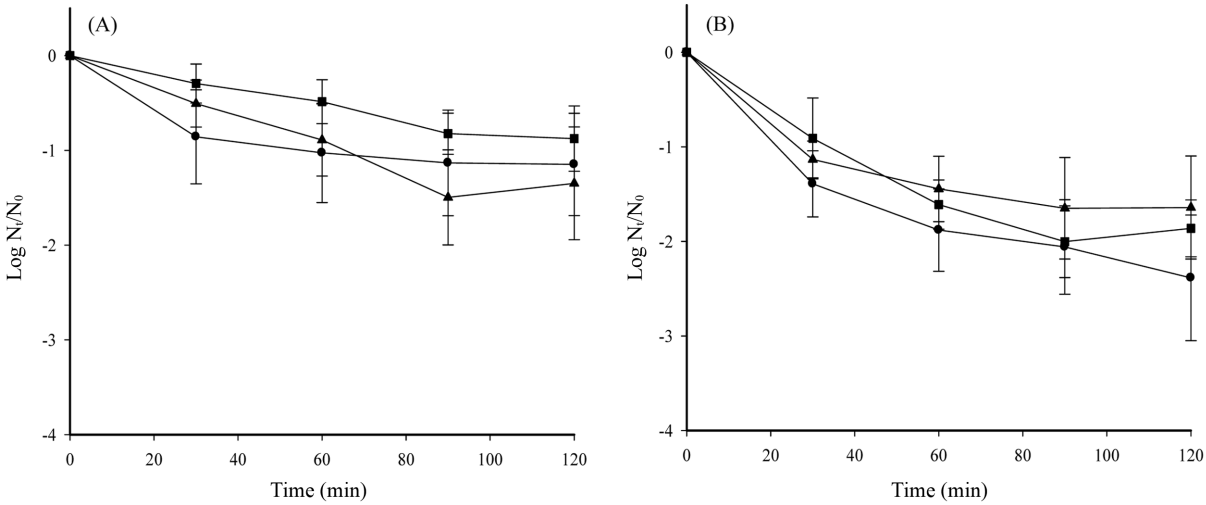

Fig. 2. Resistance of *Listeria monocytogenes* to simulated gastric fluid at 37°C for 120 min after habituation to different fat contents (10%: F10 (●), 20%: F20 (▲), 30%: F30 (■)) at 10°C for 0 (A) and 30 d (B).

of the cell membranes. In addition, there are differences in between pork and beef frankfurters, and different *L. monocytogenes* strains were examined in the two studies may have also contributed to the discrepancy in results (Lianou *et al*., 2006).

In recent times, the necessity for elucidating the correlation between food components and the resistance of food-borne bacteria to heat and antimicrobial agents has increased, and the correlation between the two factors has been suggested in previous studies (Bacon *et al*., 2003; Juneja *et al*., 2001; Yoon *et al*., 2013a). Pflug and Holcomb (1991) and Yoon *et al*. (2013b) suggested that environmental factors such as carbohydrate availability, water activity, salt concentration, pH, and the presence of organic or inorganic compounds increases the heat resistance of food-borne pathogens. Thus, more potent antimicrobials have been examined to control resistant bacteria (Yoon *et al*., 2013a). Although Ding *et al*. (2010) showed that fat in food plays an important role in bacterial pathogenicity; the thermal resistance of *L. monocytogenes* was not influenced by fat during the heat experiment in our study, which simulated the minimal heat treatment that these meat products generally receive before consumption.

To examine the effect of fat content on *L. monocytogenes* invasion into Caco-2 cells, *L. monocytogenes* cells were collected after growth on different fat contents for 30 d. Caco-2 cell invasion efficiency was higher ($p<0.05$) in F30 samples (0.916%) than F10 (0.731%) and F20 samples (0.809%); MOI (multiplicity of infection) ranged from 17.20 to 19.95 (Fig. 3). This result indicated that as *L. monocytogenes* is exposed to meat with a high fat content, the invasion efficiency of *L. monocytogenes* into Caco-2 cells might increase. The entry of *L. monocytogenes* into Caco-2 cells is triggered by at least two surface proteins, InlA and InlB, which are the virulence factors for *L. monocytogenes* invasion, and this protein family is characterized by leucine-rich repeats (Cabanes *et al*., 2002; Cossart *et al*., 2003). The pathogen uses the proteins to enter host cells like *Yersinia*, using a so-called "zipper" mechanism (Bou Ghanem *et al*., 2012; Isberg and Tran Van Nhieu, 1994; Mengaud *et al*., 1996). Thus, it could be suggested that exposure of *L. monocytogenes* to fat in frankfurters may up-regulate *inlA* and *inlB* expression, resulting in an increase in Caco-2 cell invasion of *L. monocytogenes*, but more research is still required to prove this hypothesis.

In conclusion, fat in frankfurters may not have protective effects on *L. monocytogenes* resistance to thermal

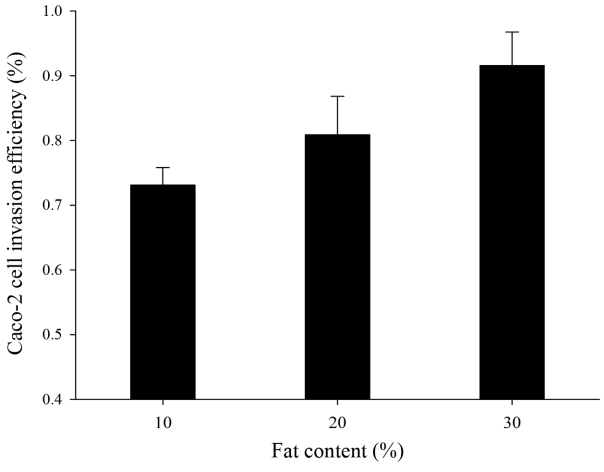

Fig. 3. Caco-2 cell invasion efficiency of *Listeria monocytogenes* habituated to different fat contents at 10°C for 30 d.

and gastric fluids, both of which the pathogen is usually exposed to. However, human Caco-2 cell invasion efficiency of the pathogen can be increased by high fat content in frankfurters.

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