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Growth delay analysis of heat-injured *Salmonella* Enteritidis in ground beef by real-time PCR

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ABSTRACT

We aimed to estimate the bacterial injury level from ground beef samples that underwent various heat exposure treatments. The growth delay time (GDT) in recovery medium was estimated by real-time PCR monitoring assay. Samples of *Salmonella* Enteritidis in PBS and ground beef were exposed to heat stress in water bath at 52.5–62.5 °C for 0–60 min. Heat-treated samples were transferred to fresh trypticase soy broth, and *S.* Enteritidis growth recovery was monitored by real-time PCR. Sampling was conducted every 2 h, and total DNA was extracted. The *S.* Enteritidis cell number was estimated by real-time PCR, and growth recovery curve was constructed from the DNA copy number of the *Salmonella invA* gene. Growth recovery curve was used for kinetic analysis of GDT. Injured bacteria level in ground beef samples after heat exposure shown differences compared to PBS, where ground beef samples had lower variations in GDT than did PBS samples. Relationship between GDT and heat exposure time was observed where the slope of GDT increased as heat exposure time extended. Recovery of heat-treated *S.* Enteritidis in near sub-lethal conditions in PBS and ground beef samples, which could not be evaluated by traditional culture methods, was successfully monitored by real-time PCR.

1. Introduction

Heat treatments, such as pasteurization and cooking, are widely used in the food industry to reduce the total number of microorganisms in food (Chambliss, Narang, Juneja, & Harrison, 2006). Exposure to extreme temperatures leads to major physiological alterations, such as protein or membrane degradation, which ultimately result in cell inactivation and death (Rowan & Anderson, 1998; Wesche, Gurtler, Marks, & Ryser, 2009). Although common heating and processing methods will kill many bacteria that may spoil food or cause foodborne diseases, some bacteria only become injured and can survive by activating certain survival mechanisms, such as developing cross-protection under environmental stresses (Besse, 2002; Chambliss et al., 2006; Chen & Jiang., 2017; Hurst, 1977; Smith & Archer, 1988). Surviving pathogens that recovered after injury conditions can potentially cause foodborne diseases (Chambliss et al., 2006; Miller, Brandão, Teixeira, & Silva, 2006). Therefore, the study of recovery of injured bacteria after heat stress condition is important to food manufacturers to set an effective thermal process to control the risk of foodborne bacterial hazards, especially in cooked meat products. Broth or model food substrates are commonly used to evaluate bacterial growth conditions, but it is important to use pathogens in real food for practical purposes (Juneja et al., 2007).

To estimate the risk of injured bacteria cell from samples, a conventional culture method is generally used to measure the difference in counts between non-selective and selective media (Hara-Kudo et al., 2000). However, selective medium is not useful to enumerate injured cells because ingredients in the selective medium can cause cells to suffer additional stress and fail to repair the initial damage (Clark & Ordal, 1969; Smith & Archer, 1988). Complicated cases occurred when food materials were already contaminated with naturally occurring background flora, as the determination of the injured cell number is difficult (Silliker, Deibel, & Fagan, 1964). A LIVE/DEAD BacLight™ bacterial viability kit (Molecular Probes) was also used to assess the cell viability (Berney, Hammes, Bosshard, Weilenmann, & Egli, 2007). However, this kit requires optimization of fluorescent dye concentrations as food materials inhibit bacterial cell staining. Moreover, this kit procedure is not suitable to investigate the viability of the pathogens in foods since it is not a species specific technique when the natural endogenous microbiota are present in foods (Elizaquível, Sánchez, & Aznar, 2012). EMA (ethidium bromide monoazide) and PMA (propidium monoazide) are dyes that have been used for differentiation of

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viable and dead cells, and their use has been suggested to reduce PCR signals from DNA originating from dead cells (Barbau-Piednoir et al., 2014; Soejima, Schlitt-Dittrich, & Yoshida, 2011; Wang & Mustapha, 2010). This method also requires optimization of fluorescent dye concentration and cross-linking conditions; it is also not suitable to analyze injured bacteria in food materials.

To assess the viability of bacterial cells exposed to an environment stress, a "growth delay method" was developed (Tsuchido, Koike, & Takano, 1989). The purpose of this method is to cultivate an appropriate liquid assay medium that suspends bacterial cells that have been exposed to environmental stress. The delay in the growth of impaired cells can be assessed from the observation of their growth curves compared to the growth of uninjured cells. The growth delay can also be caused by the addition of inhibitory substances into a cell suspension. For the growth delay determination, growth monitoring for each cultivation time is necessary. In general, growth monitoring is performed using the optical density (O.D.) measurement method. However, the O.D. method is not suitable to measure bacterial cell density accurately, especially when the sample includes food materials containing non-targeted bacteria. The O.D. method requires a high concentration of bacteria within a low quantification range, and turbidity measurements using O.D. method do not measure cell concentrations or the colony-forming units (cfu), as light scattering is most closely related to the dry weight of the cells (D'Arrigo et al., 2006; Koch, 2007; Reichert-Schwillinsky, Pin, Dzieciol, Wagner, & Hein, 2009; Udekwu, Parrish, Ankomah, Baquero, & Levin, 2009). The lag phase prediction monitored by the O.D. method was generally slightly shorter than observed, where the data did not showing goodness fitting with the validation model (Aguirre, González, Özçelik, Rodríguez, & García de Fernando, 2013). This problem occurred because the O.D. method required high detection limit for the measurement, therefore it cannot accurately reflect the actual bacterial growth conditions which also somehow resulting in lower growth rate estimation by this method (Reichert-Schwillinsky et al., 2009). Thus, the development of a sensitive and accurate method to estimate the level of injured bacterial cells exposed to stress in foods is necessary.

Real-time PCR is known as a specific and sensitive method for quantification of bacterial populations from food materials (Botteldoorn et al., 2008; Hong et al., 2007; Josefsen et al., 2010; Kawasaki, Shimizu, Koseki, & Inatsu, 2014; Kimura, Kawasaki, Nakano, & Fujii, 2001). In a previous study, Kawasaki et al. (2014) described the estimation of *Salmonella* growth using real-time PCR in pasteurized and non-pasteurized milk. To validate the precision of the previous study, the data was compared with the microbial response viewer (MRV) database (Koseki, 2009). The results showed that *Salmonella* cells counted by a traditional culture method and real-time PCR were similar, and these results reflect other reference growth data. Therefore, their results clearly indicated that the real-time PCR method can be considered as a fast and reliable method to quantify bacterial growth in food materials.

The aim of this study was to estimate the bacterial injury level from ground beef samples that underwent various heat exposure treatments. Real-time PCR was used to monitor *S.* Enteritidis growth in recovery medium, growth delay time (GDT) was determined using growth fitting software. Moreover, the injured bacteria level was compared in phosphate-buffered saline (PBS) and ground beef samples after heat exposure.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Salmonella enterica serovar Enteritidis IFO3313 (the Institute for Fermentation Osaka, Japan) was used in this study. The strain was grown overnight at 35 °C in trypticase soy broth (TSB, BBL, Becton Dickinson and Company). The optical density (Abs 600 nm) of the *S.* Enteritidis culture was monitored until it reached an O.D. of 0.80, as determined using an automated O.D. instrument (BioPlotter, Toyo Sokki Co. Ltd., Japan). Enriched *S.* Enteritidis cultures were diluted in 9 mL of PBS with a 10-fold dilution series as follows: 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 cfu/mL. To measure the concentration of the prepared bacterial solution, a 10^4 cfu/mL diluted bacterial solution was spread onto trypticase soy agar (TSA, Difco, Becton, Dickinson and Company) using a spiral plater (Eddy Jet 2, IUL, S.A., Spain) and incubated at 35 °C for 24 h before enumeration. The number of colonies was counted to determine the number of initial inoculated bacteria.

2.2. Preparation of S. Enteritidis inoculate samples

The pre-cultivation culture was diluted with PBS, and the cell density was adjusted to 10^4 cfu/mL. For preparation of heat-injured *S.* Enteritidis cells, 2 mL of cell suspension aliquots were transferred to test tubes (hard glass, id 7 mm). Thereafter, 25-g ground beef samples were inoculated with diluted pre-cultivation culture in PBS, and the final cell density was adjusted to 10^4 cfu/g. Inoculated ground beef samples were mixed and vacuum packed in gas-impenetrable laminate film bags. These samples were stored in ice water for 1 h and then used for the heat treatment study.

2.3. Heat treatment

The inoculated samples of PBS and ground beef were exposed to heat treatment in a water bath. The temperature was monitored using a temperature data logger (Thermo recorder tr-52, T&D Corporation). A temperature probe was inserted into the center of the PBS sample inside the glass tube or ground beef sample, and the exposure time was counted when the temperature of the sample was within ± 1 °C of the target temperature. The come up time to reach target temperature was 48 s in PBS and approximately 4 min in ground beef samples. The tubes and ground beef samples were placed in the water bath at 52.5–62.5 °C for 0–60 min and then immediately placed into ice water.

Heat-treated PBS samples (50 μL) were spread onto non-selective and selective media including TSA and deoxycholate hydrogen sulfide lactose agar (DHL, Eiken Chemical, Japan) plate using a spiral plater instrument (Eddy Jet 2, IUL, Spain) and incubated at 35 °C for 24 h before enumeration. The colonies were counted to determine *S.* Enteritidis quantities, and the rate of injured *S.* Enteritidis was estimated by subtracting the number of cfu on DHL from the number of cfu on TSA (Hara-Kudo et al., 2000).

2.4. Inoculation to recovery liquid medium and recovery monitoring

The 50-μL heat-treated samples in test tube were transferred into 9.95 mL of TSB. A total of 225 mL of TSB was added to 25-g heat-treated ground beef samples, which were then pummeled for 1 min by a stomacher in sterile stomacher bags. After stomaching, 500 μL of the slurries was transferred into 9.5 mL of TSB. These samples were incubated at 35 °C, and 25 μL of the sample was transferred every 2 h for a total of 30 h. Each sample was immediately frozen at −20 °C until DNA extraction to estimate the *S.* Enteritidis cell number by real-time PCR.

2.5. DNA extraction

The 25-μL recovery medium samples and diluted *S.* Enteritidis cultures (10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 cfu/mL) were processed with a QIAGEN DNeasy Blood and Tissue Kit (QIAGEN GmbH, Germany). The DNA extraction procedure was performed by following the Qiagen DNeasy Blood and Tissue protocol to obtain total DNA from *S.* Enteritidis in chicken juice samples. A total of 200 μL of flow-through extracted solutions obtained from this step was used as a DNA template for the real-time PCR.

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