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REVIEW

Predicting the Growth Behavior of Foodborne Pathogenic Bacteria by Real-time Polymerase Chain Reaction Method

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Abstract

This review describes the different applications of real-time polymerase chain reaction (PCR) quantification for monitoring, evaluating, and predicting the growth behavior of foodborne pathogenic bacteria in food materials. Real-time PCR has been successfully used to quantify the cell numbers of target pathogenic bacteria from food materials containing high levels of naturally occurring microbial flora and to monitor their growth under a variety of conditions over time. Real-time PCR has higher accuracy than conventional plate count method, which tends to underestimate bacterial viable cell numbers, particularly after high-stress treatments. Owing to its high accuracy, speed, and throughput, real-time PCR is highly recommended as an alternative tool for obtaining microbial growth data for model construction in predictive microbiology. Furthermore, this technique is particularly useful for evaluating levels of bacterial injury following stress exposure as well as other available estimation methods. Real-time PCR quantification has considerable potential for a wide range of applications on a large scale to ensure microbial safety from farm to table, such as supporting the development of the Hazard Analysis and Critical Control Point system.

Discipline: Food
Additional key words: bacterial growth quantification, bacterial injury analysis, mathematical models, predictive microbiology, real-time PCR

Introduction

Bacterial contamination of food can occur at any stage during production, processing, distribution, or preparation, and it can significantly accelerate the deterioration of food. Although some bacterial contaminants are not harmful to humans, consumption of food contaminated with pathogenic bacteria is associated with foodborne disease outbreaks, causing illnesses ranging from minor abdominal pain to potential fatalities. Consequently, it is important to understanding the responses of pathogenic bacteria under various conditions to elucidate their characteristics. Objective information regarding bacterial growth and survival under different environmental conditions must, therefore, be generated and compiled to enable the establishment of adequate responses to control microbial contamination and spoilage.

Numerous growth monitoring studies have been conducted to analyze bacterial growth in culture media under various environmental conditions (Baev et al. 2006, Bannenberg et al. 2021). However, analyses of growth in culture media typically fail to replicate real-life situations because they are not necessarily valid to reflect the environments that the bacteria encounter in food products. Thus, it is necessary to conduct comprehensive analyses of bacterial growth in different foods to assess the influence of the different constituents, properties of food (e.g., pH, water activity, salts, organic acids, and preservatives), and storage conditions (e.g., temperature, relative humidity, and atmosphere), as well as various combinations of these factors.

Despite being generally used for the enumeration

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of bacterial population to monitor their growth kinetic, the conventional plate count method presents several difficulties, particularly with respect to counting the colonies of target pathogenic bacteria from food materials containing naturally occurring microbial background flora (Kawasaki et al. 2014). The growth of these background microflora typically masks the colonies of target pathogenic bacteria; hence, feasible methods to differentiate between them are not available. Conversely, selective media will tend to underestimate viable numbers of bacterial cells because injured bacteria from high-stress environments cannot form colonies on agar plates (Noviyanti et al. 2021). Moreover, as the conventional plate count method has low specificity, skills and experience are required to distinguish target colonies from background flora that may be present in the samples (Kawasaki et al. 2011).

In recent years, real-time polymerase chain reaction (PCR) analysis has emerged as a novel technique for the rapid, specific, and high-throughput quantification of pathogenic bacteria (Kawasaki et al. 2014; Noviyanti et al. 2018, 2020, 2021). Real-time PCR enables the collection of multiple growth data plots of pathogenic bacteria in various food materials containing background flora. This genome-based assay can be used as an accurate tool for the detection and enumeration of target pathogens because it quantifies their unique genetic sequences. When compared with traditional methods, besides its accuracy, speed, and high throughput, real-time PCR has demonstrated a cost reduction of 38% in terms of labor and materials (Bohaychuk et al. 2007).

This paper reviews the application of the real-time PCR as a rapid and specific quantification method to monitor bacterial growth behavior in samples from diverse food matrices and provide multiple data that are valuable for the construction of predictive microbiological models. Furthermore, the use of real-time PCR quantification to evaluate bacterial injury levels following exposure to stress is discussed in this review.

Bacterial growth monitoring by real-time PCR method

Real-time PCR, which detects target genes rapidly and specifically, has been extensively used for a diverse range of microbial types and samples. Our research group has developed a multiplex real-time PCR method for simultaneous detection and quantification of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in foods (Kawasaki et al. 2005, 2010). The method showed high detection sensitivity and a wide dynamic range, thereby facilitating the quantification of target pathogens. Subsequently, a real-time PCR application that could be employed to characterize the growth of *Salmonella* spp. in pasteurized and unpasteurized milk was developed (Kawasaki et al. 2014), which enabled us to determine the cells number of the target pathogenic bacteria. This method can be used as a substitute for the conventional plate count method.

The processes of bacterial growth monitoring are divided into three steps: inoculum and sample preparation, sample collection, and DNA extraction followed by real-time PCR quantification and data analysis. In contrary to the conventional methods, real-time PCR does not require multiple serial dilutions, as a single dilution is sufficient for sample homogenization, and assay results are available within 2 h. In general, real-time PCR can be used to analyze 96 or 384 templates in a single run using commercially available multiwell PCR plates, thereby enabling the analysis of hundreds of samples in a day. Moreover, real-time PCR has consistently been demonstrated to be superior to traditional agar methods for the quantitative determination of bacterial populations in food samples. Thus, this novel technique is simpler and faster than the conventional plate count method, which requires numerous serial dilutions and days of incubation.

Construction of predictive microbiology models using real-time PCR data plots

Understanding the responses of pathogenic bacteria to different environmental conditions is crucial for implementing measures to control their growth and survival. The information on these responses will contribute to the modification and improvement of food processing technologies. In this regard, predictive microbiology based on mathematical models that correlate intrinsic factors with microbial responses is continuously being developed as an effective tool for estimating bacterial inactivation or growth (Stavropoulou & Bezirtzoglou 2019). In the past two decades, predictive microbiology has established itself as a scientific discipline, which is widely practiced for its use in multiple applications (Schaffner & Labuza 1997). Predictive microbiology models can be used to predict the shifts in microbial numbers due to environmental changes during the processing, distribution, and storage of foods. Furthermore, by utilizing microbial growth rate data, models can be applied to predict the likelihood of certain developments, such as spore germination or toxin formation, occurring within a specified time period (Fujikawa & Morozumi 2006).

Although numerous bacterial growth models have been published (da Silva et al. 2017, Stavropoulou &

Bezirtzoglou 2019), the data was collected solely using agar methods. However, considering the lack of accuracy in determining the actual bacterial viable cell number under different conditions, there are several concerns regarding the construction of predictive models using the data generated from conventional plate counts (Noviyanti et al. 2021). As emphasized previously, accurate estimation of viable cell number is often difficult when samples contain high levels of background microflora. Moreover, large discrepancies between actual viable cell number and the estimated count from agar plates may occur if growth conditions create a high-stress environment for target pathogenic bacteria. Such discrepancies can have a significant impact on the accuracy of models to determine the risk of bacterial contamination in food products.

After successfully applying real-time PCR quantification as a data-generation tool to monitor the growth of foodborne pathogens in food materials (Kimura et al. 2001), the same method was used for the construction of predictive microbiology models. The first study involved modeling the growth behavior of *Salmonella* spp. in pasteurized and unpasteurized milk (Kawasaki et al. 2014). The same method was also applied to modeling *Salmonella* Enteritidis growth in chicken juice as a function of storage time and temperature (Noviyanti et al. 2018).

Furthermore, the growth behavior of *L. monocytogenes* in pasteurized cow milk and ground pork was simulated using time-temperature models (Noviyanti et al. 2020, 2021). In the case study of *L. monocytogenes* growth in ground pork, a series of six growth curves at incubation temperatures of 4°C, 8°C, 12°C, 16°C, 20°C, and 35°C were successfully described (Noviyanti et al. 2021). Figure 1 shows the growth curves of *L. monocytogenes* in ground pork samples at 4°C fitted to a primary Baranyi and Roberts model (Baranyi & Roberts 1994) constructed using the data plots obtained from real-time PCR, direct plate counts on Agar Listeria according to Ottaviani and Agosti (ALOA) medium, and the most probable number (MPN) method (Noviyanti et al. 2021). MPN method (Hitchins et al. 2017, Hurley & Roscoe 1983) was selected for comparison because it can estimate the concentration of viable *L. monocytogenes* using replicate liquid broth in 10-fold dilutions in samples containing background flora that usually affect enumeration by the conventional plate count method (Sutton 2010). The result from this study shows that the concentration of *L. monocytogenes* cells in all data plots obtained using the real-time PCR and MPN methods were comparable, whereas cell concentrations determined from the direct plate counts were somewhat lower. Moreover, according to the analysis using the primary model, growth rate and lag time predicted using the plate count data were correspondingly slower and twice longer than those predicted using real-time PCR data (Noviyanti et al. 2021). Underestimation of bacterial viable cell numbers using selective agar medium results in fail-dangerous predictions (predicting less growth). This phenomenon is particularly prevalent when bacterial cells are suffering from injury following exposure to high-stress conditions (Hosotani et al. 2018). Clais et al (2014) also found that real-time PCR has a better performance compared to conventional plate count and turbidity measurement in terms of replicability, specificity, and speed. Consequently, given its superior accuracy, real-time PCR was established as a better option from the perspective of model construction.

Figure 2a and Figure 2b show the changes in lag time (λ, h) and maximum specific growth rate (μ_{max}, 1/h) as a response of *L. monocytogenes* to the increase in incubation temperatures, respectively (Noviyanti et al. 2021). It was revealed that the duration of λ decreased significantly with an increase in incubation temperature; meanwhile, the μ_{max} values increased linearly in response to increasing temperature. The relationship between μ_{max} and temperature was analyzed using the secondary Ratkowsky's square root analysis model to predict the theoretical minimum temperature of growth (T_{min}) of *L. monocytogenes* in ground pork, giving a T_{min} of −2.7°C. The relationship between the square root and incubation temperature was linear, as indicated by a high coefficient of determination (R^2). By obtaining the T_{min} value from a scenario-based study, prediction of the μ_{max} values for *L. monocytogenes* in ground pork at storage temperatures

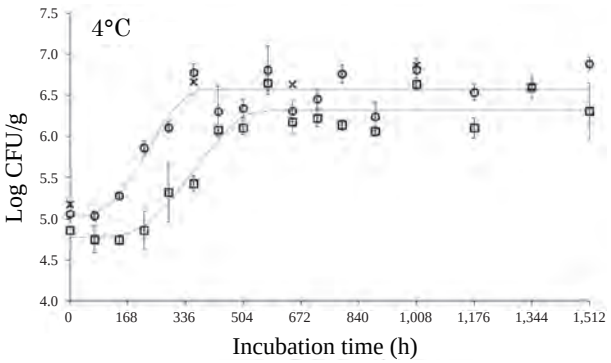

Fig. 1. Measured and predicted numbers of viable *Listeria monocytogenes* in ground pork incubated at 4°C
Quantitative data obtained by real-time polymerase chain reaction (○), direct plate count on selective agar medium (Agar Listeria according to Ottaviani and Agosti) (□), and most probable number (×) methods were fitted to the Baranyi and Roberts model (n = 3).

that were not measured in this study can be performed using Ratkowsky's square root equation, as follows:

$$\sqrt{\mu_{max}} = 0.013\ (T + 2.7) \quad (1)$$

Predicting bacterial growth behavior in dynamic temperature conditions by real-time PCR method

To prevent spoilage during transportation, food products must be maintained at the requisite temperatures specified for specific types of food. In practice, however, the incidence of temperature abuse or unacceptable deviation from the requisite product temperature for a certain period of time often occurs. Such incidences potentially provide conditions facilitating the growth of undesirable bacteria in food products, thereby increasing the deterioration rate of food. Moreover, there is an increase in the potential risk of infection with pathogenic bacteria since the shifts in temperatures enable pathogenic bacteria to attain infectious levels (Noviyanti et al. 2018). In this section, the application of real-time PCR techniques for data collection will be discussed from the perspective of constructing microbiological growth models that can predict bacterial responses to dynamic temperature conditions.

A study was conducted to model the growth of *L. monocytogenes* in pasteurized cow milk under fluctuating temperature conditions (Noviyanti et al. 2020). In this study, three dynamic temperature profiles were designed to represent the chilling temperature of milk (2°C), refrigeration temperature (5°C), slight temperature abuse (8°C), severe temperature abuse (12°C and 15°C), and a control temperature near the optimum condition for *L. monocytogenes* (30°C). The model construction method used for dynamic kinetic analysis of *L. monocytogenes* in this study was similar to that described for the *S.* Enteritidis dynamic modeling study in chicken juice samples (Noviyanti et al. 2018). In this study, however, the conventional plate count method was also applied to collect growth data for *L. monocytogenes* under fluctuating temperatures for comparison with the real-time PCR method. Figure 3 represents one of the scenarios of *L. monocytogenes* growth behavior in pasteurized cow milk under fluctuating temperatures for 16 h at 2°C, 8 h at 8°C, and 8 h at 15°C. The results of this study indicate that the conventional culture method underestimated *L. monocytogenes* cell concentrations when compared with the data obtained using real-time PCR analysis. Moreover, the prediction line constructed from the data obtained using the conventional plate count method was lower than that based on the real-time PCR data. Predictions that underestimate the levels of bacterial contamination may be dangerous with regards to potential industrial applications, where the models would fail to estimate the existing risks. Consequently, considering its higher accuracy, real-time PCR is considered superior to the conventional plate count method in this context. Thus, real-time PCR would be a promising option and a powerful tool for precisely assessing bacterial growth data for constructing predictive microbiology models for microbial safety risk analysis in food industries. Moreover, considering that not all parameters or conditions are encompassed by the existing models in the available

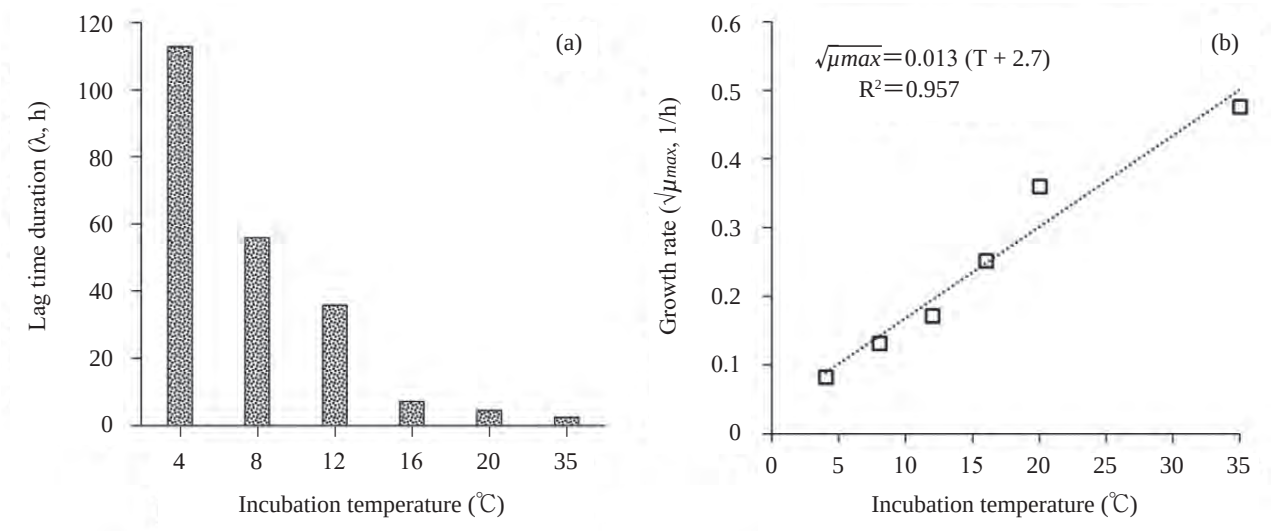

Fig. 2. Temperature-dependent growth behavior of *Listeria monocytogenes* in ground pork
Change in lag time duration (λ, h) from a primary Baranyi and Roberts model (a) and maximum specific growth rate (μ_{max}, 1/h) from a secondary Ratkowsky's square root model (b).

databases or online modeling programs, it is particularly important to construct customized prediction models on specific food types based on food company conditions.

Application of real-time PCR for evaluating bacterial injury levels as a response to stress exposure using the growth delay time (GDT) method

A diverse range of treatments is applied in the food industry to eliminate microbial contaminants in food products. However, despite such measures, some pathogenic bacteria have been reported to survive in an attenuated form by activating certain survival mechanisms, such as developing cross-protection under environmental stresses and facilitating a subsequent resumption of growth (Chambliss et al. 2006). Considering the potential risk posed by injured bacteria, Kawasaki et al. (2018) developed a rapid analysis method to evaluate the level of bacterial injury based on the GDT approach using real-time PCR. Principally, the GDT analysis can be used to estimate the levels of bacterial injury based on the differences in repair time observed as lag time, which follows exposure to different physical stresses of varying intensity (Fig. 4).

The injury conditions of *S.* Enteritidis in ground beef samples were created by heat exposure at between 52.5°C and 62.5°C for 0-60 min. Then, the growth recovery of injured *S.* Enteritidis cells was monitored using the real-time PCR assay. Growth recovery curves constructed using the real-time PCR data were then used for GDT analysis of *S.* Enteritidis, which revealed that the slope of the GDT increased with a prolonged duration of heat exposure. In addition, the real-time PCR analysis method was found to enable an evaluation of *S.* Enteritidis recovery in near sublethal conditions in both phosphate-buffered saline (PBS) and ground beef. However, the recovery was not observed by the conventional plate count method. Figure 5 shows a comparison of the GDT of *S.* Enteritidis in PBS and ground beef after heat exposure at 55°C (Kawasaki et al. 2018). Compared with PBS, ground beef samples were found to exhibit lower GDT values, despite the similarity in central temperature. These results highlight the fact that food matrices can have a significant influence on the heat stress imposed on target pathogenic bacteria. It is possible that the constituents of different food products, such as proteins and lipids, provide varying degrees of protection against heat inactivation. Furthermore, the difference in GDTs between PBS and ground beef serves to emphasize that bacterial stress responses in laboratory culture media are likely to differ from those in actual food materials. Therefore, to obtain precise estimation for industrial applications, it is essential to analyze bacterial responses using specific food matrices.

Hosotani et al. (2018) studied the applicability of real-time PCR in evaluating stress-related bacterial injury in simultaneous comparisons with the conventional culture-based methods, a bacterial membrane destruction assay, an NADPH content assay, and an intercellular ATP content assay. A high degree of correlation among the different approaches indicated that real-time PCR

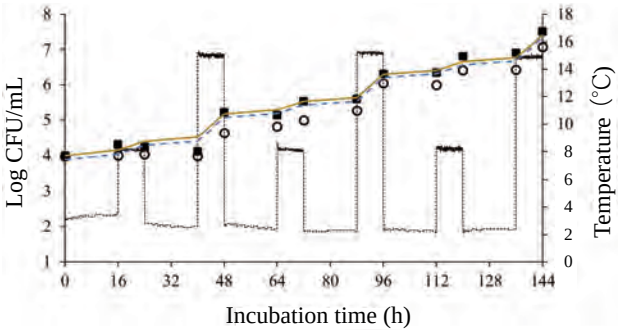

Fig. 3. Growth of *Listeria monocytogenes* in pasteurized cow milk under fluctuating temperature for 16 h at 2°C, 8 h at 8°C, and 8 h at 15°C
Experimental values of *L. monocytogenes* obtained from real-time polymerase chain reaction (PCR) quantification and conventional agar enumeration are represented by the symbols ■ and ○, respectively. The straight line represents *L. monocytogenes* growth predicted from the model constructed using real-time PCR data and the dashed line from the model constructed by conventional agar data, whereas the dotted line represents the temperature profile.

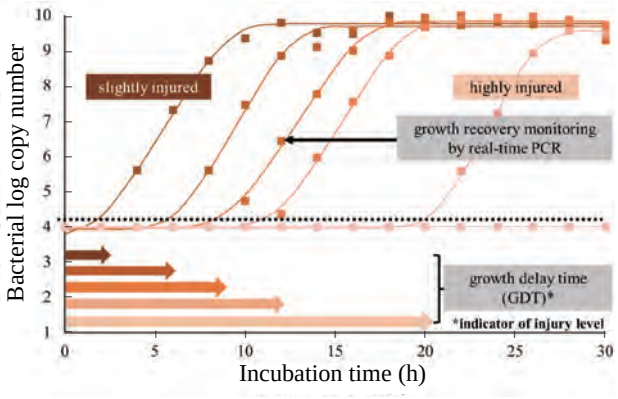

Fig. 4. Conceptual diagram of bacterial injury analysis by the growth delay time method
The actual and predicted values of the recovery monitoring for target pathogenic bacteria after heat treatment at 52.5°C under six different exposure times are represented by the symbol ■ and the solid line, respectively.

quantification assays were applicable for bacterial injury analysis along with other existing methods. Thus, the real-time PCR method can be considered a viable alternative tool for analyzing bacterial injury levels following inactivation treatment during different processing steps, such as thermal treatments. Such accurate analysis results will enable food industries, manufacturers, regulatory bodies, and researchers to evaluate risk management in the food production process, thereby facilitating the optimization of food processing technologies.

Future prospective and benefits of real-time PCR application on a broader scale

In recent years, Hazard Analysis and Critical Control Point (HACCP) measures have been widely implemented in the food industry worldwide, for both food safety assurance and promotion of international trade by increasing consumers' trust in food safety (Weinroth et al. 2019). A range of different methods can be used to assess potential microbiological hazards and identify critical points when hazards are present in a particular processing plant. Given that the conventional methods used for microbial detection and quantification are laborious, time-consuming, inaccurate, and expensive, there has been a growing shift toward microbiological analyses based on more rapid and economical methods. Some of these methods are currently at an advanced developmental stage, whereas others are still at a relatively nascent stage and applied only to a limited extent (Law et al. 2015). A successful combination of the HACCP program and rapid methods for microbial analyses may enable food industries to identify efficient innovative approaches that guarantee reliable results and ensure the safety of food products.

Based on the findings of previous studies (Hosotani et al. 2018; Kawasaki et al. 2014, 2018; Noviyanti et al. 2018, 2020, 2021), it is assumed that the real-time PCR method could play a valuable role in the construction of HACCP systems, given its proven efficacy in determining potential hazards arising from microbial growth associated with production practices, transportation, or treatments of food products. This novel technique can replace other methods used in the food industries to monitor the growth behavior of bacterial pathogens after food processing that is often assessed to minimize the significant risk of microbial food poisoning at the time of consumption. Furthermore, real-time PCR quantification can be used to examine bacterial responses to changes or interventions in food processing. In general, food industries perform a few different treatments designed to minimize food spoilage, such as heat treatment for bacterial inactivation. However, there is a growing demand among consumers for more fresh and minimally processed foods to cater to the current changes in eating habits and preferences as consumers seek a more natural and healthier diet. Hence, food industries and manufacturers aim to meet market demands by developing alternative approaches to eliminate microbial loads with minimized influences on the desirable properties of food products. Production of minimally processed foods treated at temperatures lower than those typically used in thermal processing steps has presented new challenges. Recently, there has been a growing concern regarding the newly recognized microbial risks associated with injured bacteria in food processing. Accordingly, food industries should accurately evaluate the influence of intervention processes on the population of injured bacteria. Accurate quantification of bacterial hazards arising in the processing line can contribute to the improvement of bacterial inactivation treatments while maintaining the freshness of foods.

Conclusion

Accurate data regarding microbial behavior provide valuable insights into food safety management along the food chain. A precise determination of pathogenic bacterial response to different environmental conditions will contribute to optimizing food safety from farm to table. Pathogenic bacterial growth monitoring and injury analysis will enable food industries to objectively evaluate the parameters involved in minimizing microbial risks. Moreover, the application of bacterial growth behavior data is not only valuable for food industries but also beneficial for distribution and retail companies. The

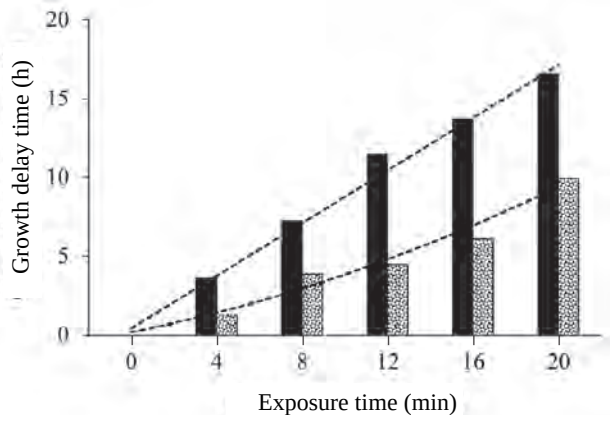

Fig. 5. Comparison of growth delay time for *Salmonella* Enteritidis in phosphate-buffered saline (black bar) and ground beef (speckled bar) following high temperature exposure at 55°C

data are also required in implementing an HACCP system for the improvement of microbial safety and promoting the international trade of food products. Compared to the conventional monitoring techniques, the newly developed real-time PCR quantification method is more practical for the routine assessment of numerous bacterial growth kinetics throughout a product's entire life cycle and will contribute to enhancing food safety from farm to table, playing a significant role in the establishment of food value chains. Finally, food quality and safety improvement will generate direct economic gains, including increased public trust, higher quality food, improved nutritional quality and constituent profiles, improved health, and better-tasting food.

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