

Effects of Temperature and Packaging on the Growth Kinetics of *Clostridium perfringens* in Ready-to-eat *Jokbal* (Pig's Trotters)

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Abstract

Ready-to-eat (RTE) *Jokbal* (Pig's trotter), which consists of pig's feet cooked in soy sauce and various spices, is a very popular and widely sold in Korean retail markets. Commercially, the anaerobically packed *Jokbal* have also become a popular RTE food in several convenience stores. This study evaluates the effects of storage temperature and packaging methods for the growth of *C. perfringens* in *Jokbal*. Growth kinetic parameters of *C. perfringens* in aerobically and anaerobically packed *Jokbals* are determined at each temperature by the modified Gompertz equation. The lag time, specific growth rate, and maximum population density of *C. perfringens* are being analyzed as a function of temperature and packaging method. The minimum growth temperature of *C. perfringens* in aerobically and anaerobically packed *Jokbal* is 24°C and 18°C, respectively. The *C. perfringens* in *Jokbal* did not grow under conditions of over 50°C regardless of the packaging methods, indicating that the holding temperature of *Jokbal* in markets must be maintained at above 50°C or below 18°C. Growth of *C. perfringens* in anaerobically packed *Jokbal* is faster than in aerobically packed *Jokbal* when stored under the same conditions. This indicates that there are a higher risks associated with *C. perfringens* for anaerobically packed meat products.

Key words: *Clostridium perfringens*, RTE *Jokbal*, growth model, temperature, packaging method

Introduction

Recently foodborne outbreaks associated with *Clostridium perfringens* have been steadily increasing. During the past three years in Korea, there have been 25 outbreaks and 792 cases reported as *C. perfringens* associated with foodborne illness (MFDS, 2013). Most of the cases of *C. perfringens* associated with food poisoning have occurred in institutions and food service establishments where large amounts of food are cooked in advance prior to serving. Thus, food poisoning outbreak due to *C. perfringens* was reported with the highest number of case per outbreak.

C. perfringens is mainly found in protein-rich foods with 75% of foodborne outbreaks being traced to meat and meat products (Brynstad and Granum, 2002; Johnson and Gerding, 1997). Vehicles for *C. perfringens* outbreaks include roast beef, turkey, meat-containing Mexican food,

stew, salmon, lasagna, reindeer, and anaerobically packed pork (Bryan, 1988; Hatheway, 1990).

Meat products that are thermally processed and do not require further heating by consumers are classified as RTE foods. Golden *et al.* (2009) conducted risk assessment for *C. perfringens* in Ready-to-eat (RTE) and in partially cooked meat products. The major risk factor (91%) for illnesses associated with *C. perfringens* is growth during retail and consumer storage. Another risk factor (7.6%) for illnesses related to *C. perfringens* is that of improper initial heating. *C. perfringens* has the ability to form heat-resistant endospores which allows it to survive in improperly cooked food. Furthermore these spores grow quickly in protein-rich foods, which allows *C. perfringens* to rapidly replicate to the level necessary to initiate infection even with low contamination (Labbe and Harmon, 1992). Thus it is important to control *C. perfringens* growth at retail markets as well as in food service establishments in order to reduce outbreaks associated with *C. perfringens*.

RTE *Jokbal* consists of pig's trotter cooked with soy sauce and spices, which is a very popular and widely sold in Korean retail markets. Recently a food consumption

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survey study was conducted on 50 potentially hazardous foods (PHF) in Korea (Park *et al.*, 2013). About 36% of the respondents ate *Jokbal* more than once per month. Amount consumed in a single sitting was 170.6 g, which is 170.6% of the recommended serving. 76.7% of the respondents purchased *Jokbal* in a restaurant, which was followed by traditional markets (9.7%), supermarkets (6.8%), and retail markets (5.5%). 80.1% of the respondents bought the *Jokbal* stored at room temperature.

Predictive food microbiology can be defined as the use of mathematical expressions to describe microbial behavior in food production. This is an efficient way to control the microbiological safety of meat products. Growth or survival predictive models have also been a very useful tool in microbial risk assessment and are thus an important tool used by the Hazard Analysis Critical Control Point (HACCP) program. Previous studies have developed predictive models for *C. perfringens* in various meats (Juneja *et al.*, 2008, 2010; Le Marc *et al.*, 2008), but these models are not appropriate for assessing risk in the popular and widely sold RTE *Jokbal*.

In the present study, the effects of storage temperature and packaging method (aerobic and anaerobic) on the growth kinetics of *C. perfringens* in *Jokbal* was investigated and a predictive growth model of *C. perfringens* was also developed as a function of temperature and packaging method.

Material and Methods

Bacterial strains and sample preparation

An α -toxin producing strain of *C. perfringens* ATCC 13124 was purchased from the Korean Culture Center for Microorganism (Korea). For each experiment, stock cultures of *C. perfringens* were thawed at room temperature. Fifty microliter of thawed stock was added into a sterile polypropylene coning tube (SPL, Korea) containing 5 mL of sterile Reinforced Clostridium medium (RCM) broth. It was placed in a microbial anaerobic jar (Merck, Germany), which was then injected with modified gas (H₂ 5%, CO₂ 10%, and N₂ 85%) by a gas exchange device (Don Whitley Scientific, UK) and incubated at 37°C for 24 h. RTE *Jokbals* were purchased from a retail market in Seoul, Korea. The *Jokbals* were heated in a microwave for 1 min and 10 g of RTE *Jokbals* were aseptically weighed and placed in a sterile petri dish. Each thin slice of RTE *Jokbal* was then uniformly surface inoculated with 100 μ L of the diluted starter culture of *C. perfringens* using a sterile pipette to give a target population of

approximately 2.5-3.5 Log CFU/g. Samples were packed aerobically or anaerobically (Fresh pack FM-03, Eiffel, Korea) and stored at 18, 20, 24, 28, 36, 44, and 50°C.

Microbial analysis

The samples were homogenized with 90 mL of sterile 0.1% peptone water in a stomacher lab blender (Bag Mixer 400, Interscience, France) for 2 min. Serial dilutions were prepared with sterile peptone water and 100 μ L aliquots were taken and plated onto a thin layer (15 mL) of tryptose-sulfite-cycloserine (TSC; Oxoid, UK) agar without egg yolk. A modified plating technique was used (dual-layer pour plating) for pour plating. TSC agar plates were overlaid with an additional 5 mL of TSC agar. All plates were incubated for 24 h at 37°C in a microbial anaerobic jar, which was injected with modified gas (H₂ 5%, CO₂ 10%, and N₂ 85%) by a gas exchange device. Typical black colonies were counted as Log CFU/g (Colony Counter SCAN 1200, Interscience, France).

Primary model development

The growth kinetic parameters including lag time (LT) and specific growth rate (SGR) in the primary model at each temperature were determined by the modified Gompertz equation (Gibson *et al.*, 1987) using GraphPad Prism V4.0 (GraphPad Software, USA).

Gompertz model :

$$Y = N_0 + C \times \exp(-\exp((2.718 \times \text{SGR}/C) \times (T-t) + 1)) \quad (1)$$

where Y is the viable cell count (Log CFU/g) at time t (h), N₀ is the initial log number of cells, C is the difference between the initial and final cell numbers, SGR is the maximum specific growth rate (Log CFU/h), T is the lag time before growth, and t is sampling time. Each experiment was replicated twice. The goodness-of-fit of the data was evaluated based on the coefficient of determination (R²), which was calculated by GraphPad Prism.

Secondary model development

LT, SGR, and MPD values graphed as a function of temperature and then fitted to the Davey, Square-root, and second order polynomial equations, respectively. The Davey model used was as follows (Daughtry *et al.*, 1997):

$$Y = a + (b/T) + (c/T^2) \quad (2)$$

where Y is LT (day), a, b, and c are regression coefficients without biological meaning, and T is the tempera-

ture.

The square-root model used was as follows (Ratkowsky *et al.*, 1982):

$$\sqrt{Y} = b(T - T_{\min}) \quad (3)$$

where Y is SGR (Log CFU/day), b is a regression coefficient, T_{\min} is the cardinal minimum growth temperature.

The second order polynomial model used was as follows (McMeekin *et al.*, 1993):

$$Y = a + bT + cT^2 \quad (4)$$

where Y is the maximum population density (Log CFU), a, b, and c are regression coefficients without biological meaning and T is the temperature.

Model performance

The performance of the models was quantified using the ratio method described by Ross (1996).

$$B_f \text{ for LT} = 10^{\log(\text{predicted}/\text{observed})/n} \quad (5)$$

$$A_f \text{ for LT} = 10^{|\log(\text{predicted}/\text{observed})|/n} \quad (6)$$

$$B_f \text{ for SGR or MPD} = 10^{\log(\text{observed}/\text{predicted})/n} \quad (7)$$

$$A_f \text{ for SGR or MPD} = 10^{|\log(\text{observed}/\text{predicted})|/n} \quad (8)$$

where n is the number of prediction cases used in the calculation. B_f values consider whether prediction error is more in the fail safe direction or not, while A_f values do not consider the direction of prediction error. Thus different ratios were used for LT, SGR, and MPD, so that B_f less than 1 would represent fail-safe predictions, and B_f above 1 would represent fail-dangerous predictions (Abou-Zeid *et al.*, 2009)

In addition, relative error (RE) of individual prediction cases was calculated using the following equation (Delignette-Muller *et al.*, 1995).

$$\text{RE for LT} = [(\text{predicted} - \text{observed})/\text{predicted}] \quad (9)$$

$$\text{RE for SGR or MPD} = [(\text{observed} - \text{predicted})/\text{predicted}] \quad (10)$$

The median relative error (MRE) and the mean absolute relative error (MARE) were also used to measure the prediction bias and accuracy of the model, respectively.

Table 1. Growth kinetics of *C. perfringens* in anaerobically and aerobically packed RTE *Jokbals* as a function of temperature

Temperature (°C)	Parameter	Anaerobic	Aerobic	t-value
18	¹ LT	20.13	-	-
	² SGR	0.19	-	-
	³ MPD	8.26	-	-
20	LT	13.26	-	-
	SGR	0.08	-	-
	MPD	8.39	-	-
24	LT	-	12.3	-
	SGR	-	0.06	-
	MPD	-	7.74	-
28	LT	5.46	8.53	-1.53
	SGR	0.56	0.24	1.70
	MPD	8.61	7.94	15.39*
36	LT	3.44	3.10	4.72
	SGR	0.97	0.64	1.22
	MPD	8.76	8.45	3.44
44	LT	1.91	2.16	-0.90
	SGR	1.57	1.24	1.92
	MPD	8.56	8.27	4.72
50	LT	1.29	2.35	-2.29
	SGR	2.21	2.34	-0.28
	MPD	8.16	7.76	9.33*

¹LT: Lag time (h)

²SGR: Specific growth rate (Log CFU/h)

³MPD: Maximum population density (Log CFU)

* $p < 0.05$

Statistics analysis

Experiments were replicated as previously indicated. Data were analyzed with SAS software, version 9.3 (SAS Institute, Inc., USA) with log-transformed data. Analyses of variance were performed using ANOVA and a t-test was also conducted for comparison between samples. Significant ($p < 0.05$) differences between the means of duplicate measurements in each independent trial were determined with Duncan's multiple range test.

Results and Discussion

Effect of storage temperature and packaging on the growth kinetics of *C. perfringens*

Effect of storage temperature and packaging method on the growth kinetics of *C. perfringens*, including lag time (LT), specific growth rate (SGR), and maximum population density (MPD) were studied in RTE *Jokbal* (Table 1). In addition, primary growth models for *C. perfringens* in

anaerobically and aerobically packed *Jokbals* were developed at each storage temperature and are shown in Fig. 1. The growth curves in RTE *Jokbal* fitted well to a modified Gompertz equation ($R^2 = 0.9689$ to 0.9958). Overall, the growth of *C. perfringens* in anaerobically packed *Jokbal* was more rapid than that in aerobically packed *Jokbal*. The minimum growth temperatures were also different depending on packaging method. *C. perfringens* in anaerobically packed *Jokbal* was grown at the temperature range of 18 to 50°C, while the growth of *C. perfringens* in aerobically packed *Jokbal* was not observed at the temperature lower than 24°C. Overall, LT values of *C. perfringens* in aerobically packed *Jokbal* were longer than those in anaerobically packed *Jokbal*. The LT of *C. perfringens* in anaerobically packed *Jokbal* stored at 18°C was approximately 15 times longer than that at 50°C. SGR value of *C. perfringens* in anaerobically packed RTE *Jokbal* stored at 18°C and 36°C was 11.9 and 2.3 times slower, respectively, compared to that at 50°C. Also the SGR of

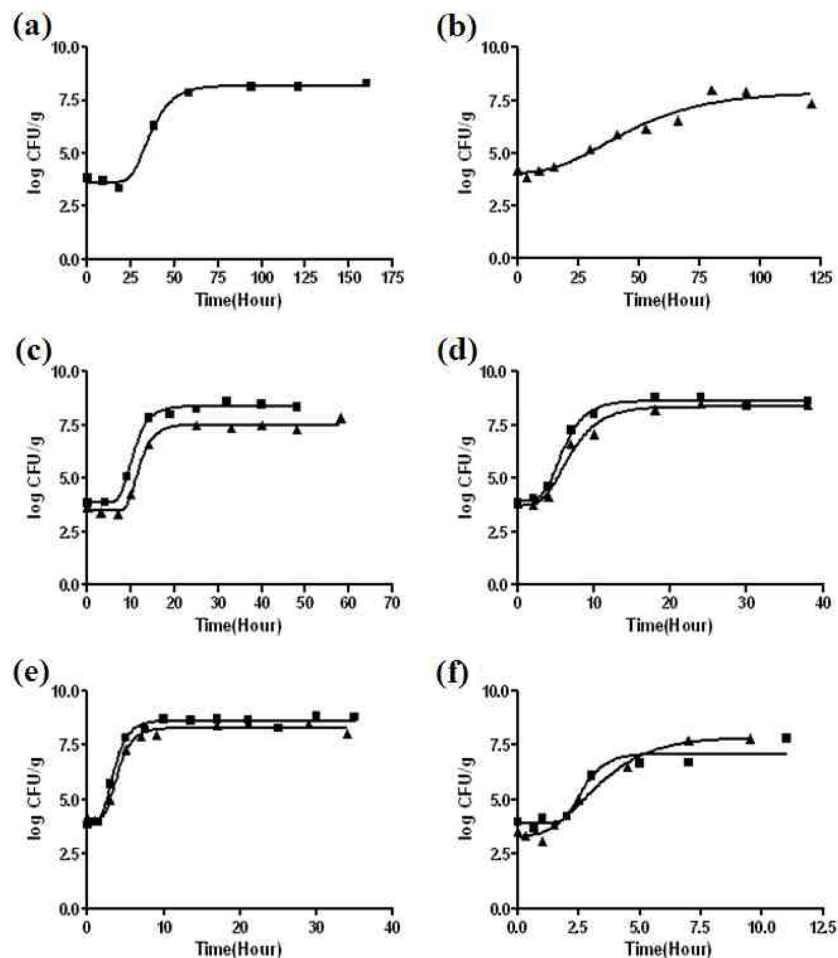


Fig. 1. Primary growth models of *C. perfringens* in anaerobically (■) and aerobically (▲) packed RTE *Jokbals* at 18°C (a), 24°C (b), 28°C (c), 36°C (d), 44°C (e), and 50°C (f), respectively.

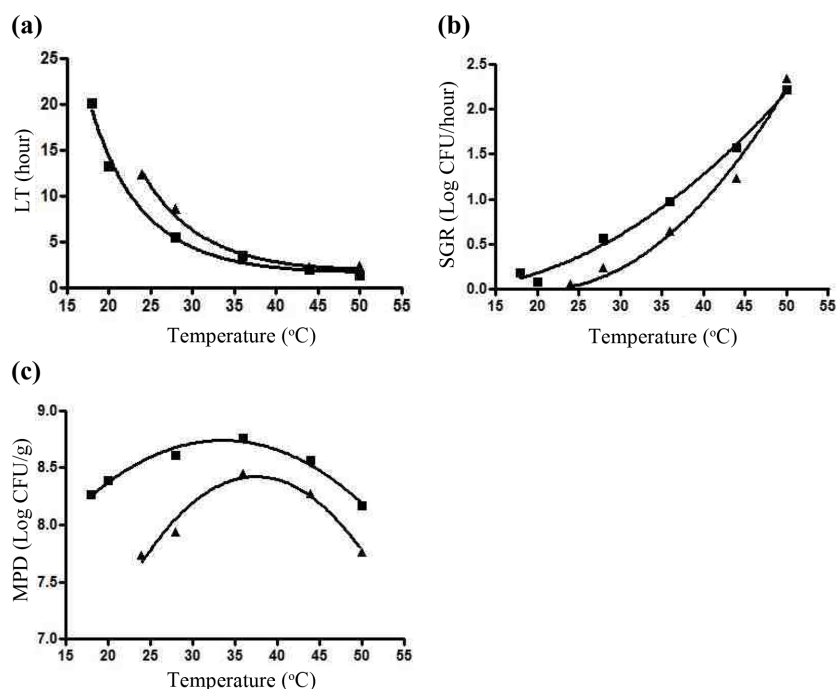
Table 2. Secondary model for lag time (LT), specific growth rate (SGR) and maximum population density (MPD) of *C. perfringens* in *Jokbals* by packaging method

Packaging	Parameter	Equation	R ²
anaerobically	LT	¹ $Y = 6.72 + (-516.5/T) + (13398/T^2)$	0.991
	SGR	² $\sqrt{Y} = \{0.0352(T-7.912)\}$	0.994
	MPD	³ $Y = 6.47 + 0.1353T - 0.0020T^2$	0.964
aerobically	LT	$Y = 9.23 + (-764.9/T) + (20304/T^2)$	0.988
	SGR	$\sqrt{Y} = \{0.0509(T-20.61)\}$	0.984
	MPD	$Y = 2.63 + 0.3085T - 0.0041T^2$	0.954

¹ $Y = a + (b/T) + (c/T^2)$

² $\sqrt{Y} = \{b(T - T_{min})\}$

³ $Y = a + bT + cT^2$

**Fig. 2. Secondary models of the LT (a), SGR (b), and MPD (c) of *C. perfringens* in *Jokbals* as a function of temperature ■ ; anaerobically packaging, ▲ ; aerobically packaging.**

anaerobically packed *Jokbal* was faster than that of aerobically packed *Jokbal* at all tested temperatures. Significant differences in MPD values were also observed between anaerobically and aerobically packed *Jokbals* at 28°C and 50°C ($p < 0.05$). These results indicate that the packaging method influences the growth kinetics of *C. perfringens* in *Jokbals*.

Optimal growth conditions for *C. perfringens* have been reported in previous works (Labbe and Juneja, 2001; McClane, 1997). *C. perfringens* growth has been shown at temperatures as high as 50°C, while slowly arresting near 6°C and below (McClane, 1997). Labbe and Juneja (2001) also reported 43–45°C as an optimal growth temperature range with less than 10 min generation time. In addition, *C. perfringens* is capable of growth at relatively low water

activities (A_w) in the range of 0.93–0.97 (Bartsch and Walker, 1982). Optimum pH for growth is between pH 6.0 and 7.0 and most strains are inhibited by 5–6.5% salt, but the organism has been observed to grow at up to 8% NaCl in foods (Johnson, 1990). The water activity of RTE *Jokbal* used in this study was 0.96, which was optimum level for growth of *C. perfringens*. However, the pH of RTE *Jokbal* was 8.25, which seems to be a limiting factor for the growth of *C. perfringens* at temperatures below 18°C in the present study. This outcome differs from the results of a previous study (McClane, 1997). Additionally, packaging method might influence the behavior of *C. perfringens* in this study. Modification of the atmosphere surrounding the food may provide a ‘hurdle’ that helps restrict the growth of an aerobic microorganism. Modified

packaging methods resulted in more effective and safer storage regimes and longer shelf-life for meat products (Phillips, 2003). From the USDA-FSIS survey, 78% of consumers store opened packages of deli meats for less than a week and around 10% of consumers keep them for 1-3 wk in their refrigerators (USDA-FSIS, 2003). Juneja *et al.* (1994) evaluated the potential for growth of *C. perfringens* in aerobically and anaerobically packed cooked ground turkey. At 28°C, The organism grew up to 7 logs anaerobically within 9 h and 24 h under aerobic conditions. In addition the lag time of anaerobically and aerobically packed ground turkey at 28°C was 2.57 h and 7.47 h, respectively. The result of the present study is similar to their work. In this study, *C. perfringens* grew up to 8 logs within 9 h anaerobically and 12.5 h aerobically at 36°C. Thus anaerobic microorganisms such as *C. perfringens* have potential hazards under anaerobic packaging method. Especially, temperature abuse of precooked, anaerobic packaging products, such as *Jokbal*, for rela-

tively long periods may lead to high and dangerous numbers of *C. perfringens* at retail markets. According to the results of the survey study (Part *et al.*, 2013), 80.1% of consumers responded that they bought *Jokbal* that was stored at room temperature.

Secondary growth model of *C. perfringens* in *Jokbal* and model performance

Secondary models were also developed to describe the effect of storage temperature and packaging method on the primary model parameters, including LT and SGR, and MPD (Table 2). Fig. 2 shows a comparison of the secondary growth models of *C. perfringens* in anaerobic and aerobically packed RTE *Jokbal*. The Davey model for LT (Fig. 2(a)), square root model for SGR (Fig. 2(b)), and polynomial model for MPD (Fig. 2(c)) had a high goodness-of fit for the parameters of the primary growth model of *C. perfringens* in RTE *Jokbal*.

Secondary models were also evaluated for their ability

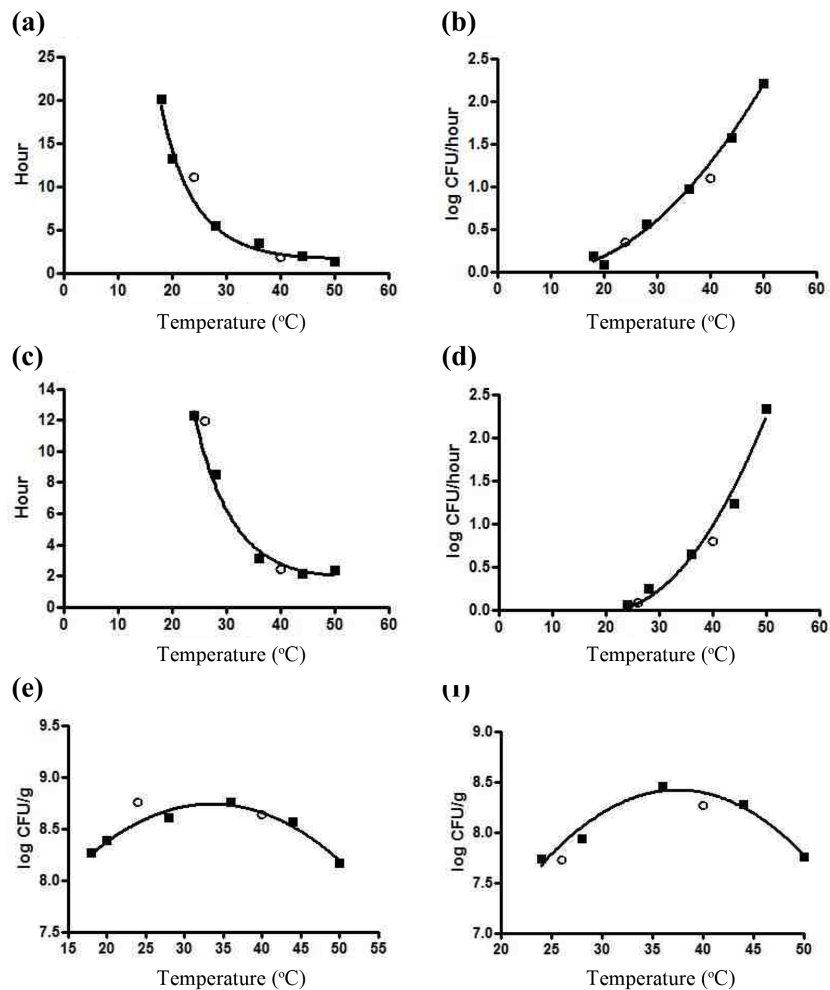


Fig. 3. Secondary LT, SGR, and MPD models for *C. perfringens* in *Jokbals* as a function of packaging and temperature. Anaerobically: (a) LT, (c) SGR, (e) MPD, Aerobically: (b) LT, (d) SGR, (f) MPD. ■ ;Dependent data, ○ ; Independent data.

Table 3. Model Performance of secondary models of *C. perfringens* in *Jokbals*

Packaging	Model	Goodness-of-fit		Prediction bias		Prediction accuracy	
		¹⁾ R ²	²⁾ B _f	³⁾ MRE	⁴⁾ A _f	⁵⁾ MARE	
Anaerobic	LT	0.991	1.00	0.02	1.13	0.12	
	SGR	0.994	1.16	4.99	1.28	0.33	
	MPD	0.964	1.00	0.01	1.01	0.00	
Aerobic	LT	0.988	1.00	-0.01	1.15	0.14	
	SGR	0.984	0.95	0.12	1.23	0.20	
	MPD	0.954	1.00	0.00	1.01	0.01	

¹⁾R²: Coefficient of determination

²⁾B_f: Bias factor

³⁾MRE: Median relative error

⁴⁾A_f: Accuracy factor

⁵⁾MARE: Mean absolute relative error

to interpolate within the response surface using independent temperature data not used in model development (Fig. 3). These data were collected with the same experimental design of the present study at 24 and 40°C for anaerobically packaged *Jokbal* and 26 and 40°C for aerobically packaged *Jokbal*. As shown in Fig. 3, both dependent and independent (interpolation) data fitted well to secondary models. Thus, model performance of secondary models for LT, SGR and MPD of *C. perfringens* in *Jokbal* was validated for interpolation with bias factor (B_f) and accuracy factor (A_f) (Table 3). The acceptable ranges of B_f are 0.7-1.15 and a range of 0.9-1.05 is considered to be good (Ross, 1996). The LT, SGR, and MPD models for anaerobically packed *Jokbal* including interpolation data had the B_f values of 1.00, 1.16, and 1.00, respectively. These results indicated that the developed model for anaerobically packed *Jokbals* predicted a SGR that was 16% lower than the actual experimental values. The LT, SGR, and MPD models for aerobically packed *Jokbal* including interpolation data had the B_f values of 1.00, 0.95, and 1.00, respectively. This result indicates that the developed model for aerobically packed *Jokbals* predicted a SGR that was 5% higher than the actual experimental values.

In conclusion, the growth of *C. perfringens* in anaerobically packed *Jokbal* was more rapid than that in aerobically packed *Jokbal*. Packaging method also influences the minimum growth temperatures of *C. perfringens* in RTE *Jokbal*. Thus anaerobic microorganisms, such as *C. perfringens*, have a potential hazard in RTE anaerobically packed *Jokbal* at retail markets. Temperature abuses between 18 to 50°C may especially lead to dangerous numbers of *C. perfringens* in *Jokbal* at retail markets. This presents a significant risk to public health. In addition, the developed secondary LT, SGR, and MPD models were able to reliably predict the growth of *C. perfringens* in aerobically and anaerobically packed *Jokbal*. This may also provide

useful information for microbial risk assessment of *C. perfringens* in RTE foods containing meat, such as packaged meals called “dosirak,” which are sold in convenience stores.

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