Effects of cinnamaldehyde on the germination and growth of *Bacillus cereus* spores in ready-to-eat boiled ground beef

Menghan Zhang, Ruixiang Zhou, Sixu Jin, Yuhang Song, Nuonuo Xu, Yuexia Wang*

College of Life Sciences, Henan Agricultural University, Zhengzhou, China

***Corresponding Author:** Yuexia Wang, College of Life Sciences, Henan Agricultural University, Zhengzhou, Henan, China. Email: yxwang2100@126.com

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RESEARCH ARTICLE

Abstract

The study quantitatively described the effects of cinnamaldehyde on the germination and growth of *Bacillus cereus* spores in boiled ready-to-eat ground beef. With the combination of the concentrations of cinnamaldehyde 0, 0.1, 0.5, and 1.0% vol/wt at temperatures 12, 20, 28, and 36°C, the Huang model was successfully used as the primary model to predict the lag time (λ) and maximum growth rate (μ_{max}). Thereafter, the cubic polynomial models were used to estimate the values of Ln λ and Ln μ_{max} considering both the storage temperature and cinnamaldehyde concentration. The models were highly accurate, because they produced acceptable root mean squared error (RMSE) values that were close to 0, while the determination (R^2), accuracy factor (A_f), and bias factor (B_f) values were all close to 1. As indicated by the fitted models, the supplementary of cinnamaldehyde in samples increased the lag time of *B. cereus* significantly from 17.7 to 75.8 h at 12°C of storage. Increasing the storage temperature from 12 to 36°C led to only 0.80-fold prolongation of lag time from 1.6 to 2.9 h in the sample containing cinnamaldehyde. However, the μ_{max} value declined most obviously at 20°C, while 66% decrease was determined. According to the results, the cinnamaldehyde can be used as a natural antimicrobial agent in boiled ready-to-eat ground beef in staple food industry by inhibiting the germination and growth of *B. cereus*. The results also provided regressing models that can be used for effectively designing the storage temperature and cinnamaldehyde concentration for a specific requirement.

Keywords: *Bacillus cereus*; boiled ready-to-eat beef; cinnamaldehyde; predictive microbiology

Introduction

Bacillus cereus is widely detected in retailed flour (Kindle *et al.*, 2019), rice and rice derivatives (Rodrigo *et al.*, 2021), ultra-high temperature mild (Alonso *et al.*, 2021), pasteurized rice cakes (Park and Yoon, 2019), pasteurized milk, and flour products (Chitov *et al.*, 2008). In China, a recently systematic investigation on the prevalence and characterization of *B. cereus* in meat and meat products showed 26.37% positive contamination rate, and all isolates presented with multiple antibiotic resistance, virulence genes, and genetic diversity (Kong *et al.*, 2021). *B. cereus* is ubiquitous in the environment, especially surviving from non-pressurized thermal treatment by forming endospores and commonly implicated in foodborne outbreaks. Therefore, compared to the raw meat, *B. cereus* is more likely to contaminate or residue in the cooked meat sample, thus can pose a potential public health threat (Tewari *et al.*, 2015).

Ready-to-eat boiled ground beef is a type of meat products that is commonly used to constitute cooked meals along with the staple rice. As the requirement of food safety control, natural antibacterial agents are widely used to inhibit the spore germination or proliferation of foodborne pathogens including *B. cereus* (Cayemitte *et al.*,

2021; Hou *et al.*, 2021). Cinnamaldehyde is a natural agent that exists in *Cinnamomum cassia*, which has exhibited a broad-spectrum antibacterial property in animal feeds and human foods (Friedman, 2017). The cinnamaldehyde has been used as an antifungal agent to inhibit *Aspergillus niger* in bread preservation (Sun *et al.*, 2020). The supplementation of cinnamaldehyde in ground pork facilitated the thermal inactivation of *Salmonella* (Suo *et al.*, 2017b) and *Listeria monocytogenes* (Wang *et al.*, 2020). Application of cinnamaldehyde at high pressures can inactivate *B. cereus* spores in infant formula (Cetin-Karaca and Morgan, 2018). The addition of cinnamaldehyde in carrot broth inhibited the outgrowth from activated spores of *B. cereus* (Valero and Francés, 2006). In minced beef meat, the treatment of cinnamaldehyde increased the relative sensitivity of *B. cereus* spores to radiation, thus inhibited the growth of *B. cereus* during refrigerated storage (Ayari *et al.*, 2012). However, only little information was reported about the effect of cinnamaldehyde on the germination and growth of *B. cereus* spores in ready-to-eat boiled ground beef.

Due to the possible health risk and the consideration of cost consumption, the antibacterial agents are normally used in food industry. Even so, the usage of the antibacterial agent is still needed to be well-designed to fulfill the least requirement for inhibiting bacterial growth (Doyle and Stephens, 2019). Predictive microbiology provides a useful tool for describing bacterial growth according to individual food environmental factors, such as the storage temperature and the additive concentration (Omidi-Mirzaei *et al.*, 2020; Wemmenhove *et al.*, 2021). Some studies have predictively modeled the growth of *B. cereus* during the cooling of cooked rice (Hwang and Huang, 2019; Juneja *et al.*, 2019a) and pasta (Juneja *et al.*, 2019b). However, to our knowledge, sparsely primary and secondary models have been developed or suggested to quantitatively describe the effect of cinnamaldehyde on the germination and growth of *B. cereus* spores in ready-to-eat boiled ground beef during storage at different temperatures.

The objective of this study was to assess the effect of cinnamaldehyde on the germination and growth of *B. cereus* in boiled ready-to-eat ground beef. Meanwhile, a secondary model was fitted to describe its effect during storage, which would be helpful for designing the additive concentration and storage temperature, based on the statistically described behavior of *B. cereus*.

Material and Methods

Bacterial strains and spore production

Four strains are used that include *B. cereus* CICC 23828 and three isolates from meat samples. The *B. cereus* CICC 23828 was obtained from China Center of Industrial Culture Collection (CICC), and the isolates were gifted by the Quality Detection Center of Henan Provincial Animal Husbandry Bureau. The isolates were identified using both the partial 16S rRNA gene sequencing and the biochemical identification (Kong *et al.*, 2021). Among the used strains, one isolate is psychrotrophic strain as it can grow slowly at 10°C, while others should be mesophilic strains as non-growth was observed at the same condition within 20 h (Guérin *et al.*, 2016). The strains were stored at −80°C with glycerol.

For sporulation, the cryo-preserved cultures of all strains were plated on tryptic soy agar (TSA, Land Bridge Technology Co. Ltd, Beijing, China), and then a single colony was picked and inoculated into 0.1 mL tryptic soy broth (TSB, Land Bridge Technology Co. Ltd, Beijing, China). The cells were grown at 37°C for 24 h, and 0.1 mL of the enriched culture was spread onto TSA supplemented with 0.05 g/L manganese sulfate ($MnSO_4$; Sinopharm Chemical Reagent Co., Ltd, Shanghai, China), as described by Juneja *et al.* (2018). The cells were incubated at 37°C for 7 days for sporulation according to the method of Kim *et al.* (2017) with minor modifications. Cell sporulation was monitored via a microscope attached with a blood counting chamber until around 85% of the cells were sporulated. The spores on each plate were submerged into 2 mL of 0.1% sterile peptone water (w/v), and then harvested using a sterile loop. The spore suspension harvested from five TSA plates were combined into a conical tube, and then incubated in a water bath at 80°C for 10 min for fully inactivating the residue vegetative cells. The spore pellets were collected by centrifugation at 4000 × *g* for 20 min at 4°C, after which the pellets were washed three times and then resuspended in 0.1% sterile peptone water. The pores from each strain were combined in equal proportions to forming a *B. cereus* spore cocktail of approximately 10^8 spores/mL for meat inoculation.

Preparation of ready-to-eat ground beef and cinnamaldehyde supplementation

The raw ground beef was purchased from a local supermarket and stored at −18°C until use. The ground beef was confirmed to be free from *B. cereus* before spiking by plating on the MYP agar (MYP, Land Bridge Technology Co. Ltd, Beijing, China). Prior to boiling, the frozen meat was thawed overnight at 4°C. The meat was boiled in water containing 5 g/L NaCl and 1 g/L monosodium L-glutamate (food grade, Wamole Food Co., LTD, Shanghai, China) for 15 min. The boiled ground beef was left at room temperature to cool for a period of about an hour. Note that 2 kg of the boiled ground beef was transferred to a sterile plastic bag, and then mixed thoroughly with cinnamaldehyde (Food grade, >99% pure, Guangfu Institute of Superfine Chemical Industry, Tianjin, China) for the final concentrations of 0, 0.1, 0.5,

and 1.0% (vol/wt), respectively. All the bags were stored at −18°C until theirs use within 90 days.

Inoculation

Prior to spore inoculation, the frozen boiled ground beef was thawed overnight at 4°C, and 10 gm of thawed ready-to-eat beef was transferred to a new sterile plastic bag and inoculated with 100 µL of an appropriate dilution of the prepared *B. cereus* spore mixture so that the final concentration was approximately 2.0 log spores/g. Thereafter, the inoculated boiled beef was thoroughly mixed manually in bags to ensure an even distribution of spores. The sample in each bag was adjusted to remove air and form an approximately 1-mm-thick layer by firmly pressing the sample against a flat surface. The bags were immediately sealed prior to bacterial growth assay.

Growth study and enumeration

The inoculated boiled ground beef samples were incubated at static temperatures of 12, 20, 28, and 36°C in an incubator (LHP-250, Hongdu Electronic Technology Co. Ltd, Shanghai, China). The samples were removed from the incubators for *B. cereus* enumeration at specific time intervals corresponding to the cinnamaldehyde concentration and incubation temperature. Prior to cell enumeration, each sample was mixed in duplicate with 20 mL of sterile 0.1% peptone water (PW, Land Bridge Technology Co. Ltd, Beijing, China) and homogenized for 4 min in a stomacher (SCIENTZ-11, Xinzhi Biological Technology Co. Ltd, Ningbo, China). Thereafter, 100 µL of the liquid portion of the stomached samples were plated either directly or after serial dilution in 0.85% NaCl (w/v) on MYP plate. Three independent growth experiments were performed for each condition. The bacterial colonies were counted and expressed as log CFU/g after incubation at 30°C for 24 h.

Mathematical Modeling of Bacterial Growth

Primary modeling

In the primary modeling analysis, all 16 data combinations were used to fit the inactivation curves by employing the integrated Pathogen Modeling Program (IPMP) 2013 (Huang, 2014). After thoroughly evaluating the parameters SSE, MSE, and RMSE, the growth of *B. cereus* was described by the Huang model that included lag, exponential, and stationary phases (Equation 1) (Huang, 2008, 2013). The equation can be expressed as

$$Y(t) = Y_0 + Y_{max} - \ln\left\{e^{Y_0} + \left[e^{Y_{max}} - e^{Y_0}\right]e^{-\mu_{max}B(t)}\right\},$$

$$B(t) = t + \frac{1}{4}\ln\frac{1+e^{-4(t-\lambda)}}{1+e^{4\lambda}}. \quad (1)$$

The dependent variable used in the regressions is the nature logarithm of the observed number of cells *Y(t)* at storage time *t*, where Y_0 and Y_{max} were the initial and maximal number of cells, respectively. μ_{max} is the specific growth rate and λ is the lag phase duration.

Secondary modeling

Three independent variables, the cinnamaldehyde concentration, temperature, and time, were considered for regressing the secondary modeling of *B. cereus* growth during the storage period. Thereafter, a cubic polynomial model was used to estimate the values of the lag phase duration (λ) and the specific growth rate (μ_{max}) as described by Equations (2) and (3), which are expressed as

$$\text{Ln}\,\lambda = a + b(eT + fC) + c(eT + fC)^2 + d(eT+fC)^3, \quad (2)$$

$$\text{Ln}\,\mu_{max} = a + b(eT + fC) + c(eT + fC)^2 + d(eT + fC)^3. \quad (3)$$

In Equations (2) and (3), *T* is the storage temperature and *C* is the concentration of cinnamaldehyde.

Model evaluation

The coefficient of the determination (R^2), accuracy factor (A_f), bias factor (B_f), and root mean squared error (RMSE) were calculated according to Equations (4)–(7), respectively. These parameters were employed to evaluate the performance of the regressed model based on the difference between the predictive and experimental values.

$$R^2 = 1 - \left(\frac{\sum e_i^2}{\sum (y_i - y')^2}\right) \quad (4)$$

where e_i is the error of predictive data, y_i is the predictive data, and y′ is the average of predictive data.

$$A_f = 10^{\frac{\sum|\log_{10}(\text{predicted/observed})|}{n}} \quad (5)$$

$$B_f = 10^{\frac{\sum|\log_{10}(\text{predicted/observed})|}{n}} \quad (6)$$

$$\text{RMSE} = \sqrt{\frac{\sum(\text{observed values} - \text{predicted values})^2}{n}} \quad (7)$$

where *n* represents the number of trials.

Furthermore, the difference between the predictions and observations (observation − model) was calculated using

the SPSS software (version 19.0, IBM SPSS Statistics, Chicago, IL) and the Microsoft Excel 2016 to determine the distribution of the residual errors and the linear fit chart, respectively.

Results and Discussion

Primary growth model development

This study investigated the possible effect of the adding cinnamaldehyde and storage temperature on the germination and growth of *B. cereus* spores in ready-to-eat boiled beef. The fitting curves described by Huang model in IPMP program are represented in Figure 1, while the goodness-of-fit are shown in Table 1 as indicated by the values of RMSE, R^2, A_f, and B_f. The results showed that the values of RMSE ranged from 0.048 to 0.331, while only 1 within 16 treatment combinations were higher than 0.3. It is known that lower RMSE value normally represents a better goodness-of-fit of the models (Wang *et al.*, 2017), although value 0.4 of RMSE is still acceptable (Jia *et al.*, 2020). The values of A_f are of the range 1.005 to 1.043 while B_f range is 0.998 to 1.004, both ranges close to 1. The A_f provides an average accuracy of prediction by calling off under or over estimations. B_f value provides an overall objective indication of model performance by describing the consistency between experimental value and predicted data (Ye *et al.*, 2013). Other studies have suggested that models with B_f values of 0.7–1.15 can be regarded as accepted for describing a pathogen growth rate (Oscar, 2005), and the growth model is regarded as quite good if B_f value determined in the range of 0.9–1.05 (Zhao *et al.*, 2020). The determined R^2 values were all higher than 0.98. Therefore, the result indicated that the RMSE, R^2, A_f, and B_f values were all acceptable when Huang model was used to describe the primary growth curves of *B. cereus* in boiled ground beef supplemented with cinnamaldehyde. Comparatively, previous reports on the *B. cereus* in cooked beans (Juneja *et al.*, 2018) and cooked pasta (Juneja *et al.*, 2019b) showed that the Baranyi model had a better performance than Huang model in describing the bacterial growth. It should be noted that both the Huang and Baranyi models are mechanistic models that allows characterizing the transition from lag phase to exponential phase, accounting for biological factors that bacteria encounter during adaption and growth (Baranyi *et al.*, 1999; Huang, 2008).

Secondary model regression and validation

To describe the synergistic effect of temperature and cinnamaldehyde concentration on the growth of *B. cereus* in boiled ground beef, secondary models were regressed to predict the natural logarithm values of the lag phase

Table 1. Experimental parameters of cinnamaldehyde inhibiting *Bacillus cereus* spore germination and growth in boiled ready-to-eat beef simulated by Huang model.

Growth temperature (°C)	Concentration of cinnamaldehyde (vol/wt) (%)	λ (h)	μ_{max}(logCFU/g per h)	Y_{max}(logCFU/g)	RMSE	A_f	B_f	R^2
12	0.0	17.730	0.034	7.780	0.067	1.009	1.000	0.999
12	0.1	38.200	0.024	7.723	0.107	1.015	0.998	0.998
12	0.5	58.189	0.025	7.416	0.119	1.023	1.000	0.998
12	1.0	75.758	0.017	7.120	0.184	1.024	1.000	0.993
20	0.0	5.221	0.242	8.285	0.331	1.040	1.000	0.991
20	0.1	8.290	0.132	8.039	0.134	1.017	1.001	0.998
20	0.5	11.194	0.110	7.685	0.244	1.030	1.000	0.993
20	1.0	19.393	0.082	7.680	0.274	1.041	1.004	0.989
28	0.0	1.466	0.551	8.086	0.048	1.005	1.000	0.999
28	0.1	2.641	0.494	7.886	0.141	1.018	1.001	0.998
28	0.5	4.254	0.342	7.588	0.150	1.017	1.002	0.997
28	1.0	4.616	0.210	7.438	0.079	1.012	1.001	0.999
36	0.0	1.626	1.042	8.113	0.119	1.015	1.001	0.999
36	0.1	2.085	0.724	7.955	0.153	1.016	1.001	0.998
36	0.5	2.524	0.571	7.594	0.152	1.014	1.000	0.997
36	1	2.923	0.459	7.035	0.294	1.043	1.002	0.987

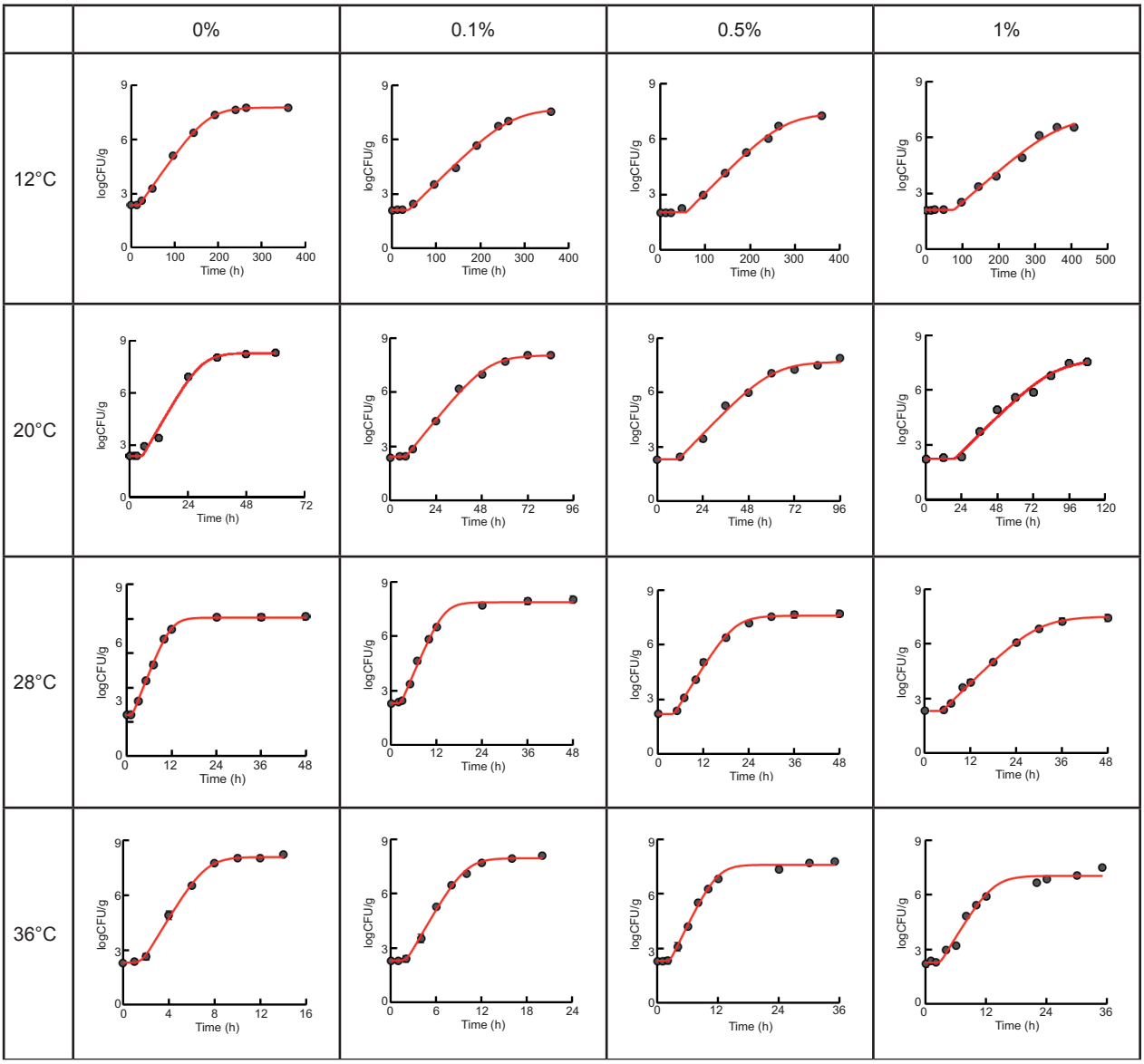

Figure 1. Fitting curve of Huang model for germination and growth of *Bacillus cereus* spores in ready-to-eat beef with cinnamaldehyde at different temperatures.

duration (λ) and specific growth rate (μ_{max}). The cubic polynomial models are presented as Equations (8) and (9) and they are

$$\text{Ln}\,\lambda = 5.220 - 1.966 \times (0.072T - 0.516C) - 0.717 \times (0.072T - 0.516C)^2 + 0.309 \times (0.072T - 0.516C)^3, \quad (8)$$

$$\text{Ln}\,\mu_{max} = -5.905 + 4.415 \times (0.053T - 0.248C) - 0.305 \times (0.053T - 0.248C)^2 - 0.239 \times (0.053T - 0.248C)^3 \quad (9)$$

where Ln λ and Ln μ_{max} represent the natural logarithms of the lag phase duration and the specific growth rate, respectively. T is the treatment temperature and C is the concentration of cinnamaldehyde concentration supplemented in the boiled ready-to-eat beef.

As shown in Figure 2, the two factors including the temperature and cinnamaldehyde concentration significantly influenced the growth of *B. cereus* during storage. The interaction between T and C was also significant, whereby an increase in cinnamaldehyde concentration required higher temperature for an equal cell population, substantiating a synergistic effect of the two parameters on the bacterial growth. Comparatively, more significant downslopes in Ln λ and upslopes in Ln μ_{max} were observed when temperature increased from 12 to 36°C, than when the cinnamaldehyde concentration increased from 0 to 1.0%, indicating that temperature was the most

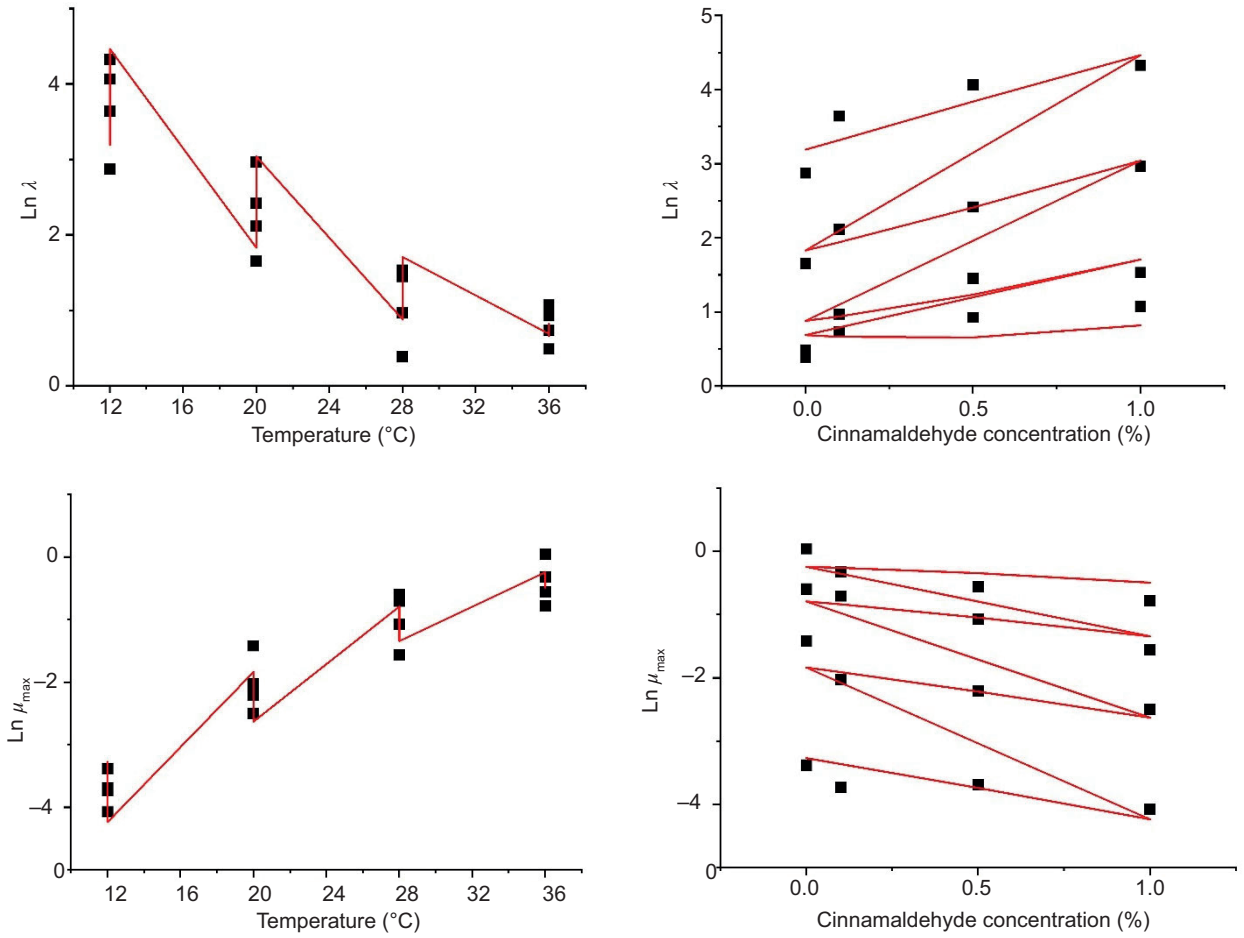

Figure 2. Fitting diagram of temperature and cinnamaldehyde concentration with the lag time (Ln λ) and maximum specific growth rate (Ln μ_{max}) of *Bacillus cereus* spores in boiled ready-to-eat beef.

important variable influencing the *B. cereus* growth in boiled ground beef.

As shown in Table 2, both the models had an acceptable goodness because the RMSE values were 0.230 and 0.203 for Ln λ and Ln μ_{max}, respectively. The R^2 values respectively were 0.965 and 0.974 (Figure 3), suggesting both equations have a satisfactory correlation between the experimental data and regression model predictions for the dependent variable of *B. cereus* growth. The A_f values were 1.185 and 1.291 for Ln λ and Ln μ_{max}, respectively, suggesting the predicted data were acceptably close to the experimental data. The B_f values were 1.023 and 1.086, indicating the predictions were within an acceptable range in this study (Nyhan *et al.*, 2018).

The goodness of the regressed secondary cubic polynomial model was further evaluated by the residue errors. As shown in Figure 4, the difference between the observation and prediction values for Ln λ and Ln μ_{max} both followed normal distributions, because the skewness and kurtosis values were all less than 1, in that the two values were 0.628 and −0.876 for Ln λ, and −0.495 and −1.076 for Ln μ_{max}, respectively. The means of the residual errors were 3.89×10^{-16} and -5.97×10^{-16} for Ln λ and Ln μ_{max}, respectively, while the Standard deviation (SD) value of both is 0.966. Moreover, 94% values (15 in a total of 16 observed combinations) for Ln λ and 100% values (all observed combinations) for Ln μ_{max} of the regression standardized residual are between ±2, indicating that the predictive models are sufficiently accurate in predicting the lag phase duration and the specific growth rate of *B. cereus* in boiled ready-to-eat ground beef supplemented with cinnamaldehyde.

Table 2. Goodness of fitting results of the secondary polynomial models.

Model parameters	RMSE	*F*	R^2	A_f	B_f
Ln λ	0.230	55.641	0.965	1.185	1.023
Ln μ_{max}	0.203	76.167	0.974	1.291	1.086

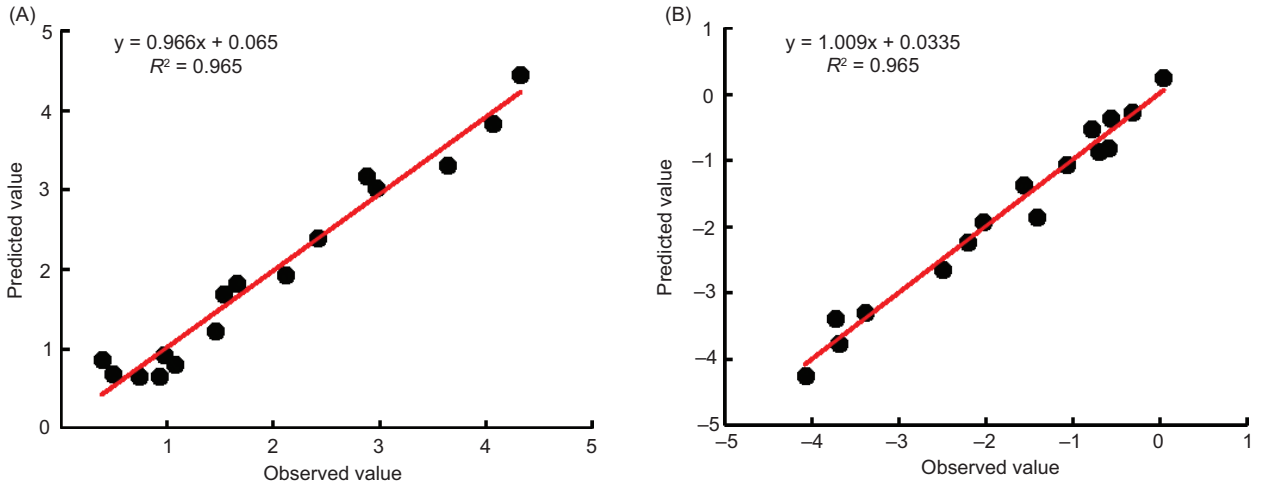

Figure 3. Predicted versus observed values of Ln λ (A) and Ln μ_{max} (B) for the germination and growth of *Bacillus cereus* spores in boiled ready-to-eat beef supplemented with cinnamaldehyde.

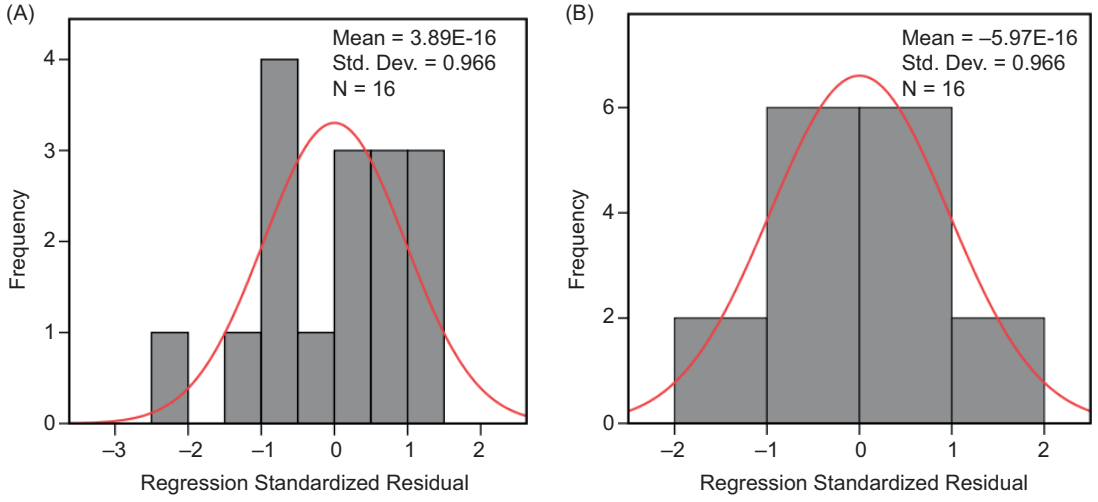

Figure 4. Histogram of residual distribution of Ln λ (A) and Ln μ_{max} (B).

Analysis of the lag time and growth patterns

B. cereus is a foodborne pathogen and the psychrotrophic strains *B. cereus* could grow under low temperatures, such as at 8°C in BHI broth in aerobiosis (Guérin *et al.*, 2016), even at 7°C in TSB broth (Samapundo *et al.*, 2011) or TSA agar (Park *et al.*, 2020). Comparatively, mesophilic *B. cereus* more likely grow at 12°C or higher temperature in a broth (Baranyi *et al.*, 2017; Le Marc *et al.*, 2021). Comparatively, in food assessment, the minimum growth temperature of *B. cereus* was 8.2°C from spores in cooked rice (Hwang and Huang, 2019). Another study proposed that the growth of *B. cereus* was only happened when the temperature was higher than 13°C during cooling of cooked rice (Juneja *et al.*, 2019a). However, *B. cereus* can only grow slightly at a low temperature as 5°C in natural carrot substrate (Valero *et al.*, 2000). In this study, *B. cereus* can grow to the maximum population at 12°C in boiled ready-to-eat ground beef, which should be attributed that the food-derived ingredient, such as fatty acids, offer a protection on the membrane, thus improving the growth of cells under cold conditions (de Sarrau *et al.*, 2013).

The determination of primary and second model parameters provided statistical information about the effects of the environmental factors, including the temperature and cinnamaldehyde concentration, on the lag time and growth rate of *B. cereus* in boiled beef during storage. As shown, the microbial growth was temperature dependent. Higher storage temperatures facilitated the growth of *B. cereus* as evidenced by the increasing values of μ_{max} and Y_{max}, and lower λ value, compared to their corresponding 12°C treatments. For example, the

determined μ_{max}, Y_{max}, and λ values were 0.034 log CFU/g per h, 7.780 log CFU/g, and 17.730 h, respectively, at 12°C-treated sample without cinnamaldehyde supplementary. However, the values of the corresponding samples were 1.042 log CFU/g per h, 8.113 log CFU/g, and 1.626 h when treated at 36°C. The highest specific growth rate observed at 36°C-treated sample without cinnamaldehyde supplementary was comparable to the model evaluated result of 0.96 log CFU/g per h in cooked rice at 37.6°C (Hwang and Huang, 2019). The agreed results are another indication that the growth rates of *B. cereus* were similar between the independent data observed in meat- and cereal- based matrices (Ellouze *et al.*, 2021).

Additives are normally used restrictively in food products, and then researchers keep optimizing their usage and dose to pursue their maximum antibacterial effect. Nonoptimal low temperatures normally impose additional damage on bacterial cells, and thus more effectively inhibit the growth of bacterial cells (Suo *et al.*, 2017a). In present study, compared to the lower storage temperature (12°C), along with the augment of cinnamaldehyde concentration from 0 to 1%, the increase of λ value was more significant than that individually stored at higher temperature, while the highest increase of 3.27-fold was observed at 12°C. The μ_{max} value declined most obviously at 20°C, while 66% decrease was determined. The result indicated that the additive cinnamaldehyde functioned as an inhibitor on *B. cereus* germination and growth most effectively at suboptimal temperature. In detail, the most effective contribution of cinnamaldehyde in boiled ground beef at 12°C is supposed to prolong the lag time of *B. cereus*, while is to inhibit its growth at 20°C. In carrot broth, according to the regressed Baranyi model, the cinnamaldehyde prolonged 33–301% lag phase of *B. cereus* at 16°C (Valero and Francés, 2006).

Conclusions

The study quantitatively described the effect of the cinnamaldehyde on the germination and growth of *B. cereus* spores in boiled ready-to-eat ground beef. The Huang model can be used to predict the lag time and maximum growth rate with high accuracy. The supplementary of cinnamaldehyde in samples increased the lag time of *B. cereus*, significantly from 17.7 to 75.8 h at 12°C of storage. The cubic polynomial models were successfully used to estimate the values of Ln λ and Ln μ_{max} considering both the storage temperature and cinnamaldehyde concentration. According to the results, the cinnamaldehyde can be used as a natural antimicrobial agent in boiled ready-to-eat ground beef in staple food industry which inhibits the germination and growth of *B. cereus*. The results also provided regressing models that can be used effectively for designing the storage temperature and cinnamaldehyde concentration for a specific requirement.

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Conflict of interest

There is no conflict of interest.

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