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Validation of Growth Models for *Listeria monocytogenes and Yersinia enterocolitica* in Cooked Chicken Meat

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ABSTRACT

In this study, growth rate models of the *Listeria monocytogenes* and *Yersinia enterocolitica* were compared in cooked chicken meat. The chicken meat was inoculated with the pure culture or mixed cultures of these pathogens and stored at 4-34˚C. After the growth curves of these two pathogens were obtained, the modified Gompertz function was then fitted to the experimental curves. The parameter of specific growth rate was described by the Zwietering model. The models predicting the growth for these pathogens in chicken meat and parameters generated by pathogens modeling program (PMP) were then compared using the mathematical and statistical parameters such as mean square error (MSE), regression coefficient (R^2), bias factor, and accuracy factor. The Zwietering model was found to be suitable for predicting the specific growth rate of *L. monocytogenes* and *Y. enterocolitica* in the chicken meat and parameters generated by PMP. In addition, the predicted value for the pathogens in the PMP was higher than that in the chicken meat. No growth was observed when *L. monocytogenes* was inoculated in chicken meat and stored at 4˚C. In the presence of *Y. enterocolitica*, the growth of *L. monocytogenes* was competitively inhibited in chicken meat at the temperatures ranging from 10-34˚C .

Key words: prediction, *L. monocytogenes*, *Y. enterocolitica*, cooked chicken meat, PMP

INTRODUCTION

Microbial contaminations of food are often attributed to the microflora residing in the ingredients that comprise the food, or originated from the food manufacturing practices during processing and storage. The proliferation of resident microorganisms not only causes food spoilage, but more seriously, results in food poisoning. From the clinical point of view, pathogenic bacteria can be categorized into four classes: (1) Bacteria that cause infections *per se*, such as *Salmonella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, *Listeria monocytogenes*, certain *Escherichia coli*, and *Aeromonas* spp.; (2) Bacteria that produce toxins after interactions with the hosts, such as *Vibrio cholerae* and enterotoxigenic *E. coli* (ETEC); (3) Bacteria that release bacterial toxins in the hosts directly without any interaction with the hosts, such as *Bacillus cereus* and *Clostridium perfringens*; and (4) Bacteria that produce toxins within food, such as *Clostridium botulinum* and emetic type *Bacillus cereus* and *Staphylococcus aureus*[1]. Among these, the infectious *L. monocytogenes* and *Y. enterocolitica* are the commonly seen psychrotrophic microorganisms[2,3]. Listeriosis, an infection resulted from *L. monocytogenes*, would result in serious adverse health conditions and diseases like miscarriage, septicemia, and encephalitis. It thus increases risks of mortality to expectant women, infants, and immune-compromised populations who are more susceptible to such infection[3,4]. *Yersinia enterocolitica*, which causes Yersiniosis, would induce severe gastrointestinal infections to human[5,6].

Methods of conventional testing are often time-consuming and lack efficiency to meet the urgency of time. Consequently, conventional methods for detecting food pathogens would not instantly reflect the potential risks presented by careless human errors during the process from manufacturing till consumption. This, as a result, augments the uncertainty of food safety. Predictive microbiology provides an option for fast detection of microbial contamination. The establishment of predictive microbiology models, depending on their development and applications, can be classified to[7]: (1) Primary models. Parameters such as specific growth rate and lag time are fitted to experimentally generated growth curves. Examples of primary models include Gompertz model[8,9] and Barrnyi model[10]; (2) Secondary models. These models are derived from a primary model to describe the growth parameters as a function of specific environmental factors. Examples include Bělegrádek [11,12], Arrhenius[13], or the two-variable polynomial equation derived from the Response Surface Methodology design[14]; (3) Tertiary models. These models are created by combining two or more secondary models to provide predictions of the growth of microorganisms under specific environmental conditions. The US Department of Agriculture-developed Pathogens Modeling Program (PMP) and the British Department of Agriculture-established Food MicroModel (FMM) fall into this category[8].

Abundant research projects involving predictive models for growth of pure cultures in culture media and food systems have been reported. The investigated factors affecting

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microorganism growth include holding temperature, water activity, pH, and gas atmospheres. However, multiple species and strains of microorganisms usually co-exist in a single food system. Growth competition or antagonism between species is a potential factor that affects their growth as well. The objective of the present study was to establish growth models of pure or mixed cultures of *Listeria monocytogenes* and *Yersinia enterocolitica* inoculated in chicken meat. Further, the Gompertz function was fitted for the prediction of the growth curves. The relationship between the obtained specific growth rate and temperature was described by the Zwietering model. The goodness-of-fit of the established model was validated by mathematical and statistical parameters.

MATERIAL AND METHODS

I. *Preparation of Test Strains*

Listeria monocytogenes (ATCC 19114) and *Y. enterocolitica* (ATCC 23715) were obtained from the Culture Collection and Research Center (CCRC) of Food Industy Research and Development Institute (Hsinchu, Taiwan). Before use, one loopful of the bacterial strains was inoculated into 5 mL of tryptic soy broth (TSB, Difco Laboratories, Detroit, MI., U.S.A) at 30˚C for 24 hr to activate. The test strains were prepared by individually inoculating *L. monocytogenes* or *Y. enterocolitica* to 50 mL of TSB at 1% (v/v) inoculum each, or co-inoculating of *L. monocytogenes* and *Y. enterocolitica* at equal concentrations.

II. *Bacteria Enumeration*

Freshly slaughtered chickens were purchased from traditional markets in Taiwan and immediately shipped back to our laboratory at chilled temperature. The chicken meat was autoclaved at 121˚C for 15 min for sterilization then cooled on an aseptic platform. Samples of 10-gram chicken meat (approximately 2.5 × 2.5 cm in size) were prepared and surface-inoculated with 100 μL of serial-diluted strains of individual *L. monocytogenes*, *Y. enterocolitica*, or 1:1 mixture of these two bacteria to achieve an initial population of 10^3-10^4 CFU/g. Controls were uninoculated chicken meat. Samples were packed in sterile bags (Nasco, Whirl-Pak Filter, B01318) and stored at various temperatures (4˚C, 10˚C, 16˚C, 22˚C, 28˚C, or 34˚C). At varying time intervals, samples incubated at each storage temperature were collected, diluted 1:10 with sterile deionized water, and homogenized with a Stomacher (Model 400, Seward Medical, London, UK). The samples were subjected to microbiological analysis by total bacteria count to determine the growth of *L. monocytogenes* and *Y. enterocolitica*[15].

III. *Growth Curve Fitting*

(I) Bacterial growth curves were generated from the experimental data using the Gompertz equation modified by Zwietering et al.[16], defined using the following equations.

$$\ln\frac{N}{N_0} = A\times\exp\left\{-\exp\left[\frac{\mu_m\times 2.718}{A}\times(\lambda - t)+1\right]\right\}$$

where A (asymptote) is the maximum numbers of bacteria reached at the stationary phase of the growth curve, representative of the maximum bacterial count; μ_m is the slope of the log phase, indicative of the maximum specific growth rate (hr^{-1}); λ is the x-axis intercept of the linear potion of the log phase with the inflection point, representing the lag time (hr); N (CFU/g) is the microbial population at time t (hr); and N_0 is the initial microbial population (CFU/g). The maximum specific growth rate (μ_m) was optimized by nonlinear regression of the experimental growth rate data modeled with the modified Gompertz model.

(II) The dependence of the maximum specific growth rate (μ_m) related to temperature was described by the following Zwietering model:

Zwietering model

$$\mu_m = \left[b\times(T - T_{\min})\right]^2\times\left\{1-\exp\left[c\times(T - T_{\max})\right]\right\}$$

where T_{min} and T_{max} are the minimum and maximum temperatures (˚C), respectively; b and c are constants of proportionality.

IV. *Prediction of Growth Parameters from the Pathogens Modeling Program (PMP)*

The Pathogens Modeling Program was created by the following steps: First, fit the experimentally obtained growth data to the primary model of the Gompertz equation. Second, growth parameters generated from the primary Gompertz model were used as responses and applied to the secondary model established by the Response Surface Methodology to establish the tertiary model system PMP. Growth parameters such as maximum population density (MPD, log CFU/g), exponential growth rate (EGR, log CFU/g•hr), generation time (GT, hr), and lag phase duration (LPD, hr) can be accordingly yielded from this software by inputting the specific experimental conditions, including temperature, pH, water activity, gas phase, or inoculation density. The present study inoculated the chicken meat with bacteria at a density of 10^3 CFU/g, and cultured at 10, 16, 22, 28, or 34˚C under aerobic condition with the pH and the water activity adjusted to 6.5 and 0.99, respectively. The specific growth rate (μ_m, hr^{-1}) was derived by inversing the duration time obtained from the growth parameters.

V. *Validation*[17]

The goodness-of-fit of the growth models established in the present study was assessed utilizing mathematical and statistical parameters to compare the experimentally obtained and predicted values.

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(I) Mean Square Error (MSE)

$$MSE = \frac{SSR}{n} = \frac{\Sigma(\mu_{observed} - \mu_{predicted})^2}{n}$$

where *SSR* is the regression sum of squares.

(II)Regression Coefficient (R^2)

$$R^2 = \frac{SSR}{SST}$$

where *SSR* is the regression sum of squares; and *SSR* is the sum of squares.

(III) Bias Factor

$$\text{Bias factor} = 10^{\left[\frac{\sum \log\left(\frac{\mu_{obersved}}{\mu_{predicted}}\right)}{n}\right]}$$

where $\mu_{observed}$ is the observed specific growth rate; $\mu_{observed}$ is the predicted specific growth rate; and n is the number of observations.

(IV) Accuracy Factor

$$\text{Accuracy factor} = 10^{\left[\frac{\sum \left|\log\frac{\mu_{obersved}}{\mu_{predicted}}\right|}{n}\right]}$$

where $\mu_{predicted}$ is the observed specific growth rate; $\mu_{predicted}$ is the predicted specific growth rate; and n is the number of observations.

VI. *Statistical Analysis*

The experimental growth parameters were fitted to the Gompertz model in SigmaPlot software (version 4.0, SPSS Science, Chicago, IL, USA) with the Marquardt estimation. Other data were calculated and statically analyzed with Microsoft Excel (version 5.0).

RESULTS AND DISCUSSION

The objective of the present study was to investigate the growth of pure and mixed cultures of *L. monocytogenes* and *Y. enterocolitica* in sterilized chicken meat stored at various temperatures (4, 10, 16, 22, 28, or 34˚C). No growth of *L. monocytogenes* was observed at 4˚C when it was inoculated in chicken meat in the mixed culture. Similar result was reported in chicken meat inoculated with pure culture of *L. monocytogenes*[18]. *Yersinia enterocolitica*, on the contrary, multiplied with time at 4˚C, which demonstrated a growth of approximately 3-4 log CFU/g (data not shown). Nissene *et al.* [19] reported similarly that the growth rate of *L. monocytogenes* inoculated in ground beef at 4˚C was fairly slow. Wake *et al.*[20] found that the bacterial counts of *L. monocytogenes* and *Y. enterocolitica* inoculated in ice cream had 1-log/g and 2-log/g increments after a 10-day storage at 2 and 4˚C,

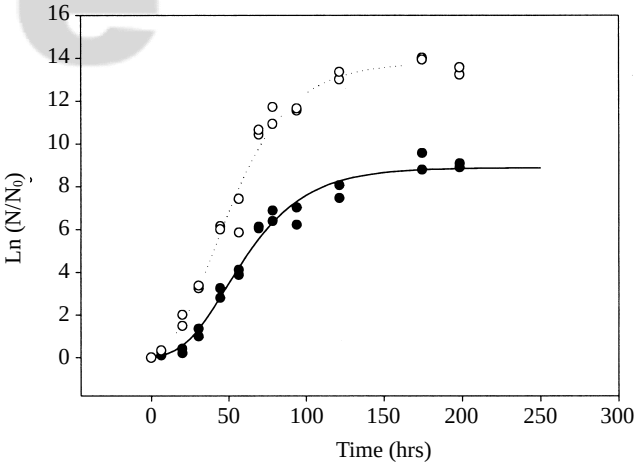

Figure 1. The growth curve for the mixed culture of *L. monocytogenes* and *Y. enterocolitica* inoculated on chicken meat at 10˚C and predicted growth curves fitted with the Gompertz model. Symbols: ..., *Y. enterocolitica* predicted; ○, *Y. enterocolitica* observed; –, *L. monocytogenes* predicted; ●, *L. monocytogenes* observed.

respectively. The Gompertz equation, derived from an empirical model, was developed for sigmoidal growth curves. It is therefore not suitable for fitting the growth of *L. monocytogenes* at 4˚C where no growth was observed [8]. The present study fitted the growth of bacteria inoculated in chicken meat samples stored at the range of 10-34˚C to the Gompertz model by nonlinear regression. The data showed that the correlation coefficients (R^2) of the growth of these two pathogens were greater than 0.90, indicating an excellent fit of the Gompertz model with the growth of *L. monocytogenes* and *Y. enterocolitica*. Figure 1 shows the experimental growth data and predicted growth curves fitted with the Gompertz model for the mixed culture of *L. monocytogenes* and *Y. enterocolitica* inoculated in chicken meat at 10˚C. The growth of *L. monocytogenes* was significantly slower than *Y. enterocolitica*; in addition, the maximum bacteria count of the *Y. enterocolitica* was significantly greater than that of *L. monocytogenes*. The growth parameters of the pure cultures of *L. monocytogenes* and *Y. enterocolitica*, and their 1:1 mixed culture obtained from the Gompertz model and the PMP are shown in Table 1. The specific growth rates of *L. monocytogenes* and *Y. enterocolitica* in chicken meat were estimated by the Zwietering model, which predicted the temperature dependence of the specific growth rate and parameters generated by the Gompertz model and the PMP. Of these, the PMP database was created by fitting the growth parameters to the Gompertz equation based on experiment-derived growth curves of pathogen cultured in media. Minimum and maximum growth temperatures estimated by nonlinear regression of the Zwietering model were found to be -4˚C and 36˚C, respectively, for both the pure or mixed cultures of *L. monocytogenes* in chicken meat. The minimum growth temperature for both the pure and mixed cultures of *Y. enterocolitica* was about -4˚C; the maximum temperatures for the pure and mixed cultures of this bacterium were 38˚C and 36˚C, respectively. Because the PMP database does not contain growth parameters of mixed cultures, only parameters of

Table 1. Parameter values from Zwietering model for predicting the effect of temperature on the specific growth rate of *L. monocytogenes* and *Y. enterocolitica* in chicken meats and parameters generated by PMP

Parameter	Organisms					
	L. monocytogenes			*Y. enterocolitica*		
	Pure	Mixed	PMP	Pure	Mixed	PMP
b	0.03	0.02	0.05	0.03	0.03	0.08
T_{min} (˚C)	- 4.62	- 3.99	1.70	- 4.96	- 4.10	0.17
c	1.95	1.46	1.60	0.18	0.44	0.01
T_{max} (˚C)	36.05	36.62	35.62	38.63	36.05	55.46

Zwietering model $\mu_m=[b\times(T-T_{min})]^2\times\{1-\exp[c\times(T-T_{max})]\}$

pure cultures can be estimated from this database. The minimum growth temperatures of the pure cultures of *L. monocytogenes* and *Y. enterocolitica* predicted by the PMP database were 1.7˚C and 0.17˚C, respectively; and the predicted maximum growth temperatures were 35.6˚C and 55.46˚C, respectively. The discrepancies of the maximum and minimum growth temperatures of the pure cultures of these two bacterial strains modeled by the Gompertz equation and by the PMP were speculated to result from the compositional difference of chicken meat and the culture media[21]. Figures 2 shows the specific growth rates of *L. monocytogenes* in chicken meat at various temperatures obtained experimentally and predicted by the PMP modeled with the Zwietering equation. The values predicted by the PMP were greater than those obtained from the chicken meat. Additionally, experimental data showed the growth of *L. monocytogenes* in chicken meat was faster in the mixed cultures than in the pure culture. Figure 3 shows the specific growth rates of *Y. enterocolitica* in chicken meat at various temperatures obtained experimentally and predicted by the PMP modeled with the Zwietering equation. The values predicted by the PMP for *Y. enterocolitica* were, again, greater than the values obtained from the chicken meat. However, the specific growth rates of the pure and mixed cultures did not differ. The fitted Zwietering model was validated for the goodness-of-fit by mean square error (MSE), regression coefficient (R^2), bias factor, and accuracy factor (Table 2). The data showed that the mean square errors of *L. monocytogenes* and *Y. enterocolitica* were both very small, and the regression coefficients of them were in the range of 0.93-0.99, indicating that the Zwietering model is appropriate for describing the specific growth rates of *L. monocytogenes* and *Y. enterocolitica* as a function of temperature. Evaluation of the closeness between the predicted values and the observed values can be performed by the bias factors. Except for higher than observed values in the mixed culture, the bias factors of *L. monocytogene* derived from the pure culture and generated from the PMP both approximately equal to 1, indicating the predicted values were representative of the observed values. On the other hand, bias factors for *Y. enterocolitica* in the pure or mixed cultures, or derived from the PMP were all close to 1. The accuracy factors represent the precision of the predictions. The closer the accuracy factor approaches 1, the more precise the predicted value is. The accuracy factors obtained

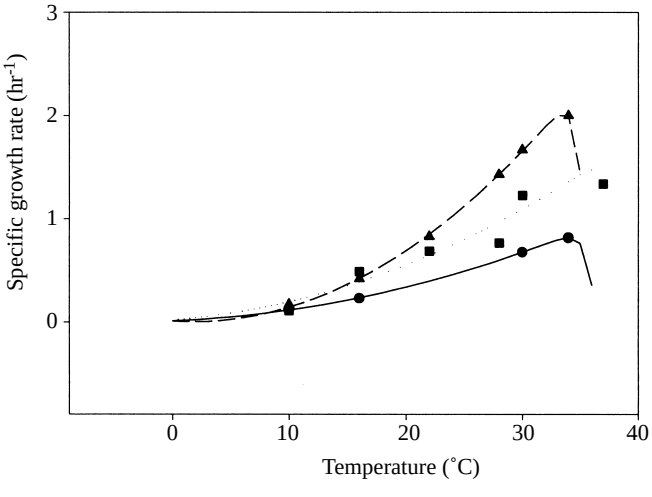

Figure 2. Experimental data of specific growth rate at different temperatures of *L. monocytogenes* in chicken meat (pure or mixed cultures) and parameters generated by PMP, which modeled with Zwietering equation. Symbols: ●, *L. monocytogenes*, pure culture, observed; ■, *L. monocytogenes*, mixed culture, observed; –, *L. monocytogenes*, generated by PMP; ▲, *L. monocytogenes*, pure culture, predicted; ...., *L. monocytogenes*, mixed culture, predicted; – – –, *L. monocytogenes*, parameters from PMP, predicted.

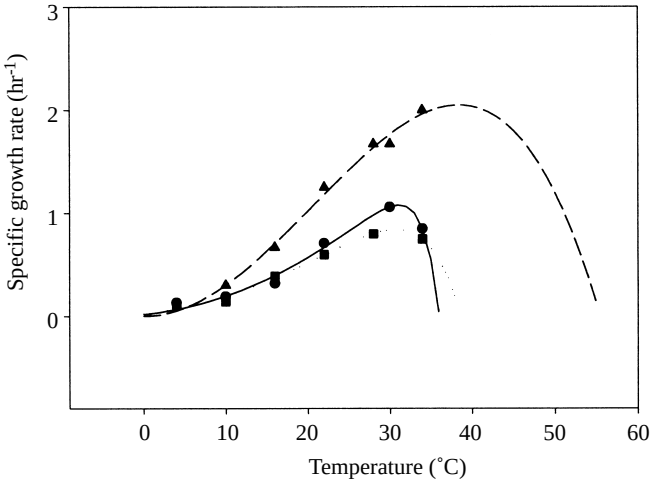

Figure 3. Experimental data of specific growth rate at different temperatures of *Y. enterocolitica* in chicken meat (pure or mixed cultures) and parameters generated by PMP, which modeled with Zwietering equation. Symbols: ●, *Y. enterocolitica*, pure culture, observed; ■, *Y. enterocolitica*, mixed culture, observed; ▲, *Y. enterocolitica*, generated by PMP; –, *Y. enterocolitica*, pure culture, predicted; ...., *Y. enterocolitica*, mixed culture, predicted; – – –, *Y. enterocolitica*, parameters from PMP, predicted.

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Table 2. Compared of Zwietering models for predicting the specific growth rate of *L. monocytogenes* and *Y. enterocolitica* on chicken meat and parameters generated by PMP according to mathematical/statistical characteristics

Organisms	Mathematical/statistical characteristics											
	MSE[a]			R^{2b}			Bias[c]			Accuracy[d]		
	Pure	Mix	PMP	Pure	Mix	PMP	Pure	Mix	PMP	Pure	Mix	PMP
L. monocytogenes	0.01308	8.22E-06	0.00024	0.93	0.99	0.99	0.94	1.00	1.02	1.22	1.01	1.04
Y. enterocolitica	0.000781	0.00200	0.00293	0.99	0.98	0.99	1.03	1.09	0.99	1.15	1.18	1.04

[a]$MSE=RSS/n = \Sigma(\mu_{observed}-\mu_{predicted})^2/n$.
[b]R^2= Regression coefficient.
[c]$Bias = 10^{\Sigma(\log(\mu_{observed}/\mu_{predicted}))/n)}$.
[d]$Accuracy = 10^{(\Sigma|\log(\mu_{observed}/\mu_{predicted})|/n)}$.

from *L. monocytogenes* in the pure culture and *Y. enterocolitica* in both the pure and mixed cultures were closer to 1 than that obtained from *L. monocytogenes* in the mixed culture. All of the accuracy factors derived from the PMP were very close to 1, possibly due to the homogeneity of the experimental conditions that the PMP data derived from. The food systems, on the other hand, are complex and heterogeneous. Therefore the growth of the bacteria varies greatly. Further investigations are required for testing the hypothesis.

The present study showed that the growth of *L. monocytogenes* was competitively inhibited by *Y. enterocolitica* when co-inoculated to and cultured in chicken meat at low storage temperatures. These results are consistent with those reported by Budu-Amoako *et al*.[22]. The specific growth rates of these two pathogens predicted by the PMP were greater than those observed in chicken meat, similar to the results previously reported by Gill *et al*.[21] who found that the growth of *A. hydrophila* and *L. monocytogenes* was better when cultivated in the adipose tissues than in the TSB, which is in contrast with the growth of this pathogen cultivated in the muscle tissues of pork. The reasons for the discrepancies were possibly due to the trace nutrients presented in the adipose tissues but not in the TSB, or due to the presence of inhibitory components in the muscle tissues but not in the TSB. Other studies indicated the co-existence of *L. monocytogenes* and *Y. enterocolitica* in various foods such as ice cream[20], milk[22], dairy products[23], vegetables[24], and ground beef[19]. Many factors, such as holding temperature, biochemical composition of food, pH, gas atmosphere, and packaging, affect the growth of microorganisms in foods. Comparing the growth of *L. monocytogenes* in dairy products with the same water activity and pH, the substrate that contains appropriate levels of fat promoted the growth of *L. monocytogenes*[23]. The growth of mixed culture of *L. monocytogenes* and *Y. enterocolitica* differed in whole milk and skim milk. In whole milk, growth of *L. monocytogenes* was competitively suppressed by *Y. enterocolitica* at 10˚C and 22˚C in mixed culture. This phenomenon was not observed when skim milk was the culture medium[22]. Further, vacuum packaging was not effective in retarding the growth of *L. monocytogenes* and *Y. enterocolitica* in pork chops, likely due to the aerotolerate anaerobic nature of these two pathogens[15,25]. Jacxsens *et al*.[24] showed that the kinds of vegetables played a more significant role in influencing the growth of *L. monocytogenes* and *Aeromonas* spp. than the compositions of atmospheres. Taken together, the model applied in the present study can adequately predict the growth of *L. monocytogenes* and *Y. enterocolitica* observed in cooked chicken meat, either in pure or mixed cultures. Since temperature reduction is one of the common practices for shelf life extension[26] and the compositions of foods vary from one to another, the development of predictive growth models for the psychrotrophic *L. monocytogenes* and *Y. enterocolitica* is essential for the purpose of understanding the risks of potential food poisoning resulting from these two food-spoilage pathogens. Although there is no incidence of food poisoning being linked to *L. monocytogenes* or *Y. enterocolitica* in Taiwan thus far, there have been outbreaks of food poisonings associated with *L. monocytogenes* and/or *Y. enterocolitic* elsewhere[6,27]. After Taiwan joins the World Trade Organization (WTO), food safety issues as a consequence of increasing volumes of imported goods may pose threats to public health. Thereafter, the establishment of predictive food microbiology models is of importance not only to the manufacturer for ensuring the quality monitoring, but also to the consumers for safeguarding food safety.

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李斯特菌與耶爾辛斯菌於熟雞肉系統中預測生長模式之驗證

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摘　要

本研究主要利用Gompertz模式分別配適*Listeria monocytogenes*及*Yersinia enterocolitica*於不同溫度貯存（4-34˚C）之熟雞肉系統中，以純菌或混菌接種後之生長曲線，再由生長曲線所獲得比生長速率之參數值，並與病原菌模式程式（Pathogens Modeling Program; PMP）系統所得比生長速率之參數值，利用Zwietering模式描述其比生長速率與溫度之關連性，並利用殘差均方（MSE）、迴歸係數（R^2）、偏估因子（bias factor）及準確因子（accuracy factor）驗證模式的適用性。研究結果顯示，利用Zwietering模式可適當描述*L. monocytogenes*及*Y. enterocolitica*於雞肉系統中以純菌、混菌接種及PMP系統中其比生長速率與溫度之關連性。此兩種病原菌藉PMP所得比生長速率之預測值皆高於接種於純菌或混菌之雞肉系統中之預測值。*L. monocytogenes*於4˚C貯存之雞肉中幾乎沒有生長，而於10-34˚C貯存之混菌雞肉系統中，*Y. enterocolitica*則會抑制*L. monocytogenes*之生長，致使*L. monocytogenes*的最大菌量較於純菌系統時為低。

關鍵詞：預測，李斯特菌，耶爾辛斯菌，熟雞肉，病原菌模式程式