pISSN 1229-1153 / eISSN 2465-9223 *J. Food Hyg. Saf.*
Vol. 34, No. 4, pp. 374~379 (2019)
https://doi.org/10.13103/JFHS.2019.34.4.374

Journal of Food Hygiene and Safety
Available online at http://www.foodhygiene.or.kr

Predictive Modeling for the Growth of *Salmonella* Enterica Serovar Typhimurium on Lettuce Washed with Combined Chlorine and Ultrasound During Storage

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(Received May 23, 2019/Revised July 16, 2019/Accepted July 26, 2019)

ABSTRACT - This study developed predictive growth models of *Salmonella enterica* Serovar Typhimurium on lettuce washed with chlorine (100~300 ppm) and ultrasound (US, 37 kHz, 380 W) treatment and stored at different temperatures (10~25°C) using a polynomial equation. The primary model of specific growth rate (SGR) and lag time (LT) showed a good fit ($R^2 \geq 0.92$) with a Gompertz equation. A secondary model was obtained using a quadratic polynomial equation. The appropriateness of the secondary SGR and LT model was verified by coefficient of determination (R^2=0.98~0.99 for internal validation, 0.97~0.98 for external validation), mean square error (MSE=-0.0071~0.0057 for internal validation, -0.0118~0.0176 for external validation), bias factor (B_f=0.9918~1.0066 for internal validation, 0.9865~1.0205 for external validation), and accuracy factor (A_f=0.9935~1.0082 for internal validation, 0.9799~1.0137 for external validation). The newly developed models for *S.* Typhimurium could be incorporated into a tertiary modeling program to predict the growth of *S.* Typhimurium as a function of combined chlorine and US during the storage. These new models may also be useful to predict potential *S.* Typhimurium growth on lettuce, which is important for food safety purposes during the overall supply chain of lettuce from farm to table. Finally, the models may offer reliable and useful information of growth kinetics for the quantification microbial risk assessment of *S.* Typhimurium on washed lettuce.

Key words : Lettuce, *Salmonella* Typhimurium, Predictive Growth Model, Chlorine, Ultrasound

The consumption of fresh produce has consistently increased over the last decades due to its health advantages such as lowering blood cholesterol, reducing the risk of heart disease, and preventing colon and breast cancers[1]. Lettuce as a main green leafy vegetable is widely consumed as the raw material for Western-style salads and sandwiches in many countries including Europe and USA. It is also consumed as the Korean traditional vegetable products "*Sangchussam"* (*sangchu,* meaning lettuce, and *ssam*, meaning wrapped with condiments), and "*Sangchugeotjeori"* (*sangchu,* meaning lettuce, and *geotjeori*, meaning partially pickled vegetable) in Korea.

However, fresh produce can be contaminated with food-borne pathogens at any point of the food supply chain, which could increase the risk of food-borne disease. Salmonellosis, traditionally accounting for consumption of poultry origin, has increasingly been linked to fresh produce[2]. Outbreaks caused by fresh produce result in considerable economic losses to farmers, distributors and people who engaged in food industry[3]. *Salmonella* can be contaminated in fresh produce during any stage from farm to table through cross contamination by washing water, handling workers, and food contact surfaces[4]. *Salmonella enterica* Serovar Typhimurium is one of the most frequently isolated pathogens accounting for food-borne outbreaks throughout the world[5,6]. The involving 124 illnesses caused by the consumption of lettuce contaminated with *S.* Typhimurium were reported[7]. Therefore, *S.* Typhimurium has attracted attention as a human pathogen associated with fresh vegetables that is considered to significantly impact public health.

Since fresh produce is usually consumed raw, the current control methods usually depend on washing with chemical sanitizing solution, such as chlorine, electrolyzed water, hydrogen peroxide, organic acid, and ethanol.

Traditionally, chlorine (50~200 ppm) is the most widely used sanitizer to wash produce on a commercial scale to improve the safety and preservation of these products[8]. Ultrasound (US) is a longitudinal wave having a frequency above 20 kHz and the frequency used in the food industry ranges from 20 kHz to 10 MHz[9]. Ultrasound can potentially be used for the treatment of contaminated fresh produce, but this has not been adopted because of the perceived adverse

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effect on food quality.

Predictive food microbiology can be used to predict the levels of microorganism contamination in food at the time of consumption, as well as the food distribution period. So, it can be used in "quantitative microbial risk assessment"[10] and shelf-life prediction[11]. Microorganisms growth behavior has relations between intrinsic (a_w, pH, nutrient component, etc.) and extrinsic factors (storage temperature, chemical washing treatment, etc.), and these relations can be described by mathematical quantitative models, which provide great benefit for food industry in the respect of cost and time saving. There were no predictive models on the growth of *S.* Typhimurium in fresh produce treated with any chemical solutions and US as the washing treatment.

Therefore, the current study was performed to develop predictive models of *S.* Typhimurium growth kinetics in fresh lettuce (*Lactuca sativa var. capitata*) washed with combined chlorine (100, 200, and 300 ppm) and US (37 kHz, 380 W) under different storage temperatures (10, 15, 20, and 25°C) using a polynomial model.

Materials and Methods

Preparation of bacterial strain

A poultry isolate of *Salmonella* Typhimurium, having novobiocin (Saint Louis, MO, USA) and nalidixic acid (NA, Sigma) resistance, was used in this study. The stock culture was stored at -70°C in 0.1 mL of tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) containing 50% (vol/vol) glycerol (Fisher Scientific, Itasca, IL). To obtain a working culture, fresh cultures were successively cultured twice at 37°C for 24 h in tryptic soy broth. The cells were allowed to grow to a target concentration of 10^9 CFU/mL, which was measured by plating on tryptic soy agar (TSA, Difco Laboratories). The resulting cells were centrifuged at 8,000×*g* for 10 min at 4°C and suspended in 10 mL of 0.1% peptone water (PW, Oxoid, Basingstoke, Hampshire, UK). Bacterial loads were determined by plating on brilliant green agar (BGA, Difco Laboratories) containing 25 μg/mL of NO and 25 μg/mL of NA and incubating at 37°C for 24 h.

Preparation of samples and inoculation

Iceberg lettuce (*Lactuca sativa* var. *capitata*) was purchased from a local market in Anseong, Korea. The edible part of the lettuce was cut into 5-g pieces with sterile knife. One hundred microliters of culture suspension was inoculated by the spot-inoculation method on each sample to obtain a final concentration of 10^5 CFU/mL. After inoculation, samples were dried in a clean bench for 30 min to allow the microorganisms attach to the surface of lettuce.

Chorine and US treatment and storage temperature

Chlorine in the form of sodium hypochlorite (NaOCl, 12%, Shimadzu Co., Kyoto, Japan) was used in this study due to its widespread use in processing plants of various foods, and it was prepared with sterile distilled water. The US (P 300 h model, 230V, Hucom system Co., Elmasonic, Germany) was used as the physical treatment for detaching bacteria from the surface of lettuce. For the US treatment, the chamber was filled with 5 L of sterile distilled water (control) or chlorine (100, 200, and 300 ppm) and used at samples were completely immersed in sterile distilled water or chlorine for 10 min at room temperature (20 ± 2°C). The treated samples were stored at 10, 15, 20 and 25ºC before the analysis of microbial growth at different time interval.

Microbial analysis

The treated sample (5 g) was homogenized for 60 sec in a sterile stomacher bag containing 45 ml of sterile 0.1% PW using a stomacher (Bag Mixer® 400; Interscience Co., France), and then diluted in 0.1% PW. One hundred microliters of the serially diluted sample was spread-plated in duplicate on BGA plates containing 25 μg/mL of NO and 25 μg/mL of NA and incubated at 37ºC for 24 h. The colonies were then counted and reported as log colony-forming units per gram ($\log_{10}$CFU/g).

Primary modeling

Growth curves were created by a Gompertz equation (Prism, ver. 4.0, GraphPad Software, San Diego, CA, USA) to determine specific growth rates (SGRs, $\log_{10}$CFU/h) and lag times (LTs, h) at each combined treatments under different storage temperatures. The Gompertz equation, $Y = N_0 + C*\exp[\exp\{(2.718*SGR/C)*LT - X) + 1\}]$, which was described by Gibson *et al.*[12], was used. Y = log cell number, X = incubation time, N_0 = log initial number of cells, C = difference between initial and final cell numbers, LT = lag time before growth, same units as X, SGR = maximum specific growth rate.

Secondary modeling

A polynomial model as a function of the combined chlorine and US treatment under different storage temperatures was calculated based on the growth of *S.* Typhimurium on the lettuce. The SGR and LT were determined by least squares analysis using PROC GLM of SAS version 8.1 (SAS Institute). The equations of polynomial models contained the following format:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1x_2 + b_5x_1x_3 + b_6x_2x_3 + b_7x_1^2 + b_8x_2^2 + b_9x_3^2$$

Where Y is the predicted value (SGR or LT); x_1 is the storage temperature; x_2 is the concentration of chlorine, and x_3 is the frequency of the US; b_0 is the intercept; b_1, b_2, and b_3 are the linear coefficients; b_4, b_5, and b_6 are the interaction coefficients; and b_7, b_8, and b_9 are the squared coefficients[12].

Validation of model performance

The coefficient of determination (R^2) is often used as the

overall measure of a prediction attained. R^2 = (1-Σei^2/ Σ(predicted values –average of the predicted values))^2 , where ei is the error of the predictive values. The mean square error (MSE), calculated as the residual sum of squares divided by the number of degrees of freedom, is a measure of the variability that is not accounted for by changes in the independent variable (i.e., temperature). MSE = ΣLog(observed values – predicted values)^2/n, where n is the number of observations. The bias factor (B_f) indicates whether, on average, observed values lie above or below the line of equivalence and by how much. It also indicated structural deviations of the model. B_f = $10^{\Sigma Log(observed\ values/predicted\ values)/n}$, where n is the number of observations. The accuracy factor (A_f) averages the distance between each point and the line of equivalence as a measure of how close, on average, the predictions are to the observed value. $A_f = 10^{\Sigma Log|(predicted\ values/observed\ values)|/n}$, where n is the number of observations.

Results and Discussion

Development of *S.* Typhimurium predictive growth model on fresh lettuce as washed with of combined chlorine and US under different storage temperatures

The Gompertz equation is typically used to fit bacterial growth curves for estimating maximum SGR and LT by U.S. Department of Agriculture (USDA)[13,14]. Moreover, the SGR and LT of *S.* Typhimurium in TSB[15] and in kimbab[16] was determined by the Gompertz equation with R^2>0.90. Based on the Gompertz equation for the primary modeling, the values of the SGR and LT on each treatment are shown in Table 1. The observed SGR values range were from 0.0044 to 0.0125 at 10°C, 0.0250 to 0.0507 at 15°C, 0.0205 to 0.0598 at 20°C, and 0.0225 to 0.0688 at 25°C. The LT values range from 25.86 to 43.49 at 10°C, 17.2 to 31.14 at 15°C, 8.13 to 24.68 at 20°C, and 2.45 to 17.18 at 25°C.The SGR and LT as the growth parameters of *S.* Typhimurium on lettuce had a high goodness of fit, and the curves had a high coefficient determination (R^2 > 0.92) in all experimental combinations (data not shown). Therefore, we concluded that the Gompertz equation was appropriate for the primary modeling.

In this study, the SGR values increased and LT values decreased as temperature increased. Moreover, the SCR and LT values decreased and increased, respectively when the combination of the high concentration of chlorine and US were used as a decontamination method against S. Typhimurium on lettuce. This fact was somewhat connected to the previous studies of Kim et al.[16] and Oh et al.[17]. Kim et al.[16] noted that the temperature markedly affected the growth of *S.* Typhimurium; as the temperature increased, the growth of *S.* Typhimurium increased. Oh et al.[17] reported that SGR and LT values of *Listeria monocytogenes o*n the chicken breast meat were significantly decreased and increased, respectively after the meat was exposed to combination treatment of chlorine (50~200 ppm) and ultraviolet radiation. In the study of Yoon et al.[18], the growth and survivability of the bacteria may vary by differences in strains or food matrix. Park et al.[15] predicted the SGR of *S.* Typhimurium in TSB broth at 10 and 20°C was 0.059 and 0.129, respectively. According to Jung et al.[19], the SGR values of non-treated *S.* Typhimurium in BHI broth were 0.38 and 0.83; LT values were 5.55 and 2.60

Table 1. Growth parameters of *S.* Typhimurium on lettuce washed with chlorine and US under different storage temperatures as calculated by the Gompertz equation for the primary modeling

Storage temperature (°C)	Chlorine (ppm)	US (kHz)	SGR (log_{10}CFU/h)	LT (h)
10	0	0	0.0125	25.86
		37	0.0121	26.83
	100	0	0.0109	30.45
		37	0.0106	35.77
	200	0	0.0107	35.15
		37	0.0095	35.84
	300	0	0.0065	40.34
		37	0.0044	43.49
15	0	0	0.0507	17.20
		37	0.0502	18.69
	100	0	0.0418	21.56
		37	0.0411	23.67
	200	0	0.0344	25.99
		37	0.0317	26.79
	300	0	0.0275	30.53
		37	0.0250	31.14
20	0	0	0.0598	8.13
		37	0.0567	10.50
	100	0	0.0475	12.57
		37	0.0432	14.34
	200	0	0.0328	17.55
		37	0.0255	18.56
	300	0	0.0226	23.20
		37	0.0205	24.68
25	0	0	0.0688	2.45
		37	0.0628	4.65
	100	0	0.0530	6.70
		37	0.0500	7.67
	200	0	0.0389	11.57
		37	0.0305	12.37
	300	0	0.0229	16.60
		37	0.0225	17.18

US: ultrasound, SGR: specific growth rate, LT: lag time.
The data present means of three samples with standard deviations (3 samples/treatment).

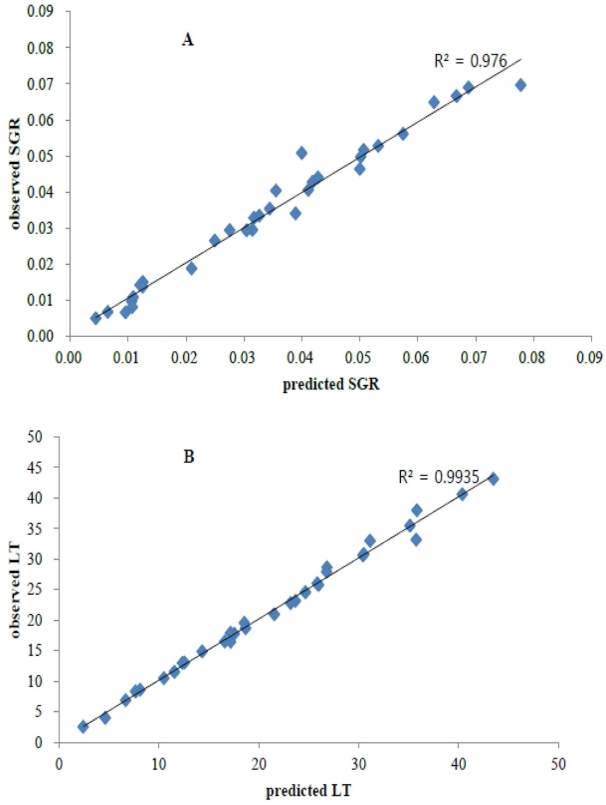

Fig. 1. Comparative plots of the observed and predicted SGR (A) or LT (B) of *S.* Typhimurium on lettuce in the internal validation.

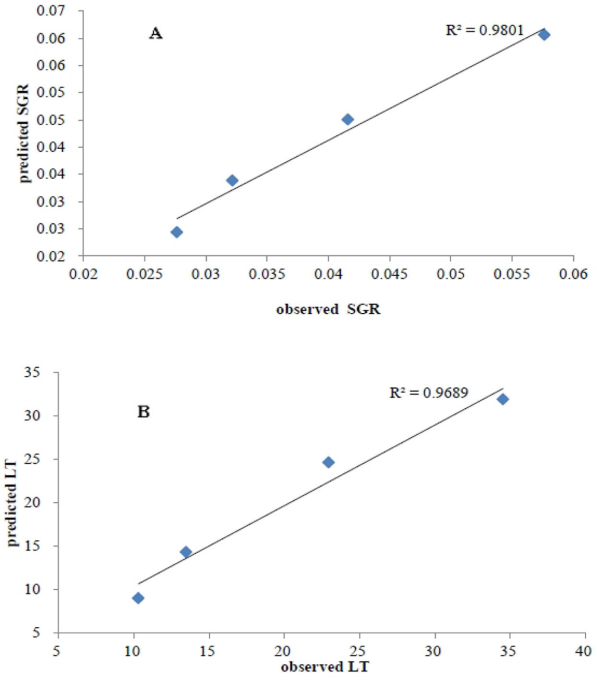

Fig. 2. Comparative plots of the observed and predicted SGR or LT (B) of *S.* Typhimurium on lettuce in the external validation.

at 24, and 35°C, respectively.

The SGR and LT polynomial quadratic equation for the predictive secondary modeling of *S.* Typhimurium on lettuce treated with chlorine (100~300 ppm) and fixed US (37 kHz) under different storage temperatures (10~25°C) produced the following equations:

$$\text{SGR model: } Y = -0.1142110225 + 0.0166451495x_1 + 0.0000433995x_2 + 0.0000372507x_3 - 0.0000092996x_1x_3 - 0.0000059149x_2x_3 - 0.0000000883x_2x_3 - 0.0003728375\ x_1^2 + 0.0000000729\ x_2^2$$

$$\text{LT model: } Y = 50.37079125 - 2.78467575x_1 + 0.04535541x_2 + 0.0905902x_3 - 0.00014007x_1x_3 - 0.00202365x_2x_3 + 0.00007182\ x_2x_3 + 0.03481875\ x_1^2 + 0.00001548x_2^2$$

According to the developed equation, we can know that temperature largely affected the values of SRG and LT. However, chlorine and US treatment also assist with the temperature to effectively inhibit the growth of *S.* Typhimurium in lettuce. R^2 values as the coefficient of determination of the polynomial quadratic secondary model were 0.976 for SGR and 0.9994 for LT (Fig. 1 and 2), showing that the SGR and LT values exploited in this study displayed an overall good fit with the secondary model.

Validation of *S.* Typhimurium predictive growth model on lettuce

The reliability of the secondary model requires mathematical evaluation as both internal and external validation prior to its practical application. For the internal validation, the scatter plot of the observed versus predicted data can be used to assess the success of the model predictions. Most points were relatively close to the 100% correlation (y=x) line, indicating satisfactory performance of the predictive model (Fig. 1). Four additional experimental conditions were selected to verify the reliability of the developed model. For the external validation, the observed and predicted SGR and LT values are shown in Table 2, and the values are compared in Fig. 2.

The plotted points for SGR and LT values were close to the line (R^2>0.969) indicating that the predicted values were similar to the observed values. MSE, B_f and A_f were calculated to evaluate the reliability of the predictive model for *S.* Typhimurium. A higher R^2 value is indicative of an accurate prediction by the model[20], and the lower the value of the MSE, the more adequate the model[21]. B_f indicates the average bias of predictions to verify the performance of the predictive model, and A_f provides an indication of the average estimation accuracy of the predictive model. When $A_f = B_f = 1$, the model is ideally perfect[22]. When B_f is 0.9–1.05, the model is considered "good", 0.7–0.9 or 1.06–1.15 is considered "acceptable", and < 0.7 or > 1.5 is considered "unacceptable"[23,24]. As shown in Table 3, for the model-dependent data, MSE, B_f and A_f for SGR values were 0.0057, 1.0066 and 0.9935, respectively, and MSE, B_f and A_f for LT values were –0.0071, 0.9918 and 1.0082, respectively. For the additional experimental

Table 2. The observed and predicted SGT and LT value of *S.* Typhimurium on lettuce in external validation

Storage Temperature (°C)	Chlorine (ppm)	US (kHz)	Observed SGR (log_{10}CFU/g)	Predicted SCR (log_{10}CFU/g)	Observed LT (h)	Predicted LT (h)
13	50	37	0.0322	0.038	22.94	24.63
13	250	0	0.0276	0.0243	34.52	31.91
22	150	37	0.0416	0.0451	13.46	14.31
22	70	0	0.0577	0.0606	10.30	9.00

US: ultrasound, MSE: mean square error, B*f*: bias factor, A*f*: accuracy factor.

Table 3. Internal and external validation of the secondary modeling using mathematical indices for growth parameters of *S.* Typhimurium on lettuce

		Statistical indices for the validation		
		MSE	B_f	A_f
Internal validation	SGR (log_{10}CFU/h)	0.0057	1.0066	0.9935
	LT (h)	–0.0071	0.9918	1.0082
External validation	SGR (log_{10}CFU/h)	–0.0118	0.9865	1.0137
	LT (h)	0.0176	1.0205	0.9799

MSE: mean square error, B*f*: bias factor, A*f*: accuracy factor.

data, MSE, B_f and A_f for SGR values were –0.0118, 0.9865 and 1.01369, respectively, and MSE, B_f and A_f for LT values were 0.0176, 1.0205 and 0.9799, respectively.

The validation results indicated that the developed polynomial model could provide reliable predictions of the effects of temperature, chlorine concentration, and US treatment on the SGR and LT values of *S.* Typhimurium on lettuce. Ultimately, the developed model will be vital for reducing the level of *S.* Typhimurium on leafy vegetables during production, processing, and distribution, thereby engancing the safety of humans in terms of leafy vegetable comsumption. The developed model could show an important function in providing reliable and practical information for the quantitative microbial risk assessment of *S.* Typhimurium on washed lettuce.

Acknowledgments

This work was supported by the Project for Young Researcher group, the Institute of Marine Industry, Gyeongsang National University, 2018. This research was also supported by the Chung-Ang University research grant in 2017.

Conflict of Interest

The authors declare no potential conflicts of interests.

국문요약

본 연구에서는 대표적인 신선 잎채소류인 상추의 세척 단계에서 초음파 (37 kHz) 와 염소 (100~300 ppm) 의 병용처리 후 냉장 ~ 실온저장 (10~25C) 에 따른 이 식품 중의 *Salmonella* Typhimurium 의 성장예측모델을 개발하였다 . 1 차 모델 개발을 위해 Gompertz 방정식을 활용하여 각기 다른 실험 조건에서의 *S.* Typhimurium 의 생육도 (SGR 과 LT) 를 조사했다 . 본 방정식에 의한 1 차 모델 개발시 R^2 가 0.92 이상으로 우수하게 나타났으며 저장온도가 낮을수록 초음파에 사용된 염소의 농도가 높을수록 SGR 값은 감소하였고 LT 값은 증가하였다 . 이를 바탕으로 2 차 polynomial 모델을 개발하여 다양한 통계적 지표 (R^2, MSE, A_f 및 B_f) 를 통해 분석한 결과 개발된 모델의 적합성을 확인할 수 있었다 . 따라서 개발된 모델이 초음파와 염소의 병용 세척에 따른 저장 중 상추에 대한 *S.* Typhimurium 의 성장예측모델로 사용 가능하다고 판단되어지며 , 신선 잎채소류에서의 식중독을 예방하고 미생물학적 위생관리기준을 설정하는데 기초자료로 활용될 수 있을 것으로 사료된다 .

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