Population changes and growth modeling of *Salmonella enterica* during alfalfa seed germination and early sprout development

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Abstract This study examined the effects of alfalfa seed germination on growth of *Salmonella enterica*. We investigated the population changes of *S. enterica* during early sprout development. We found that the population density of *S. enterica*, which was inoculated on alfalfa seeds was increased during sprout development under all experimental temperatures, whereas a significant reduction was observed when *S. enterica* was inoculated on fully germinated sprouts. To establish a model for predicting *S. enterica* growth during alfalfa sprout development, the kinetic growth data under isothermal conditions were collected and evaluated based on Baranyi model as a primary model for growth data. To elucidate the influence of temperature on *S. enterica* growth rates, three secondary models were compared and found that the Arrhenius-type model was more suitable than others. We believe that our model can be utilized to predict *S. enterica* behavior in alfalfa sprout and to conduct microbial risk assessments.

Keywords Predictive microbiology · Seed sprout safety · Salmonellosis

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Introduction

Over the past few decades, a number of outbreaks of foodborne illnesses associated with the consumption of raw seed sprouts have been reported (Callejon et al., 2015; Nsoesie et al., 2014; Taormina et al., 1999). Since the first report of an outbreak due to the consumption of raw sprouts contaminated with *Bacillus cereus* in 1976, there have been consistent occurrences of foodborne illnesses associated with its consumption (CDC, 2018; Portnoy et al., 1976). Alfalfa sprouts, which are the sprouts most commonly available worldwide, were implicated in most of the outbreaks. *Salmonella* spp. have been largely responsible for these outbreaks worldwide since the year 2000 (Yang et al. 2013). The levels of foodborne pathogens including *Salmonella* spp. in naturally contaminated seeds are generally very low (Wu et al., 2001). *Salmonella* spp. can grow significantly over the initial density during sprout production (Castro-Rosas and Escartin, 2000). Within 2 days of seed sprouting, microbial density on seed sprouts reaches approximately 7–8 log cfu/g (Splittstoesser et al., 1983). Human pathogenic bacteria such as *S. enterica* can grow at an average of 3.7 log cfu/g during alfalfa seed sprouting at room temperature (Charkowski et al., 2002) and were reported to reach a maximum population of approximately 5.7 log cfu/g on alfalfa sprouts within 48 h (Howard and Hutcheson, 2003).

Quantitative microbiological risk assessment has been used as a tool to estimate foodborne illness and to establish safety management goals (McNab, 1998). Estimates of the risk of foodborne illness generally depend on the number of microorganisms present on food at the time of consumption (Ross and McMeekin, 2003). Predictive mathematical models have been used to predict changes in bacterial populations at any given time and temperature of

the processing operations from production to distribution in the quantitative microbial risk assessment (Baranyi and Roberts, 1995). In the literature, there are reports showing that *S. enterica* on alfalfa seed sprouts can grow rapidly within a few days, indicating that the seed germination stage is when *S. enterica* grows most profusely and that there is a need to develop a growth model to estimate the risk associated with the production of alfalfa sprouts. Although foodborne illness associated with *Salmonella* contamination on alfalfa sprouts has occurred worldwide, quantitative microbial risk assessment has not been conducted as a tool for enhancing the safety of food. This led us to assess the microbial growth prediction model of *S. enterica* during alfalfa seed germination and early sprout development. The aim of this study was to elucidate and develop a prediction model of *S. enterica* growth during alfalfa sprout development for quantitative microbial risk assessment of *S. enterica*. In this study, we evaluated predictive secondary models for the quantitative prediction of *S. enterica* growth during alfalfa seed germination and early sprout development. We believe these results provide insight into population changes of *S. enterica* and microbial risk assessment in alfalfa sprout production.

Materials and methods

Bacterial culture

A spontaneous rifampicin-resistant mutant of *S. enterica* subsp. *enterica* serovar Enteritidis strain ATCC 4931 was used in this study. The strain was preserved at -80°C and activated by transferring onto tryptic soy agar (TSA; BD, Sparks, MD, USA) plus rifampicin ($50\text{ }\mu\text{g/mL}$) and incubating for 24 h at 35°C . A single colony was inoculated into 3 mL of tryptic soy broth (TSB; BD, Sparks) plus rifampicin ($50\text{ }\mu\text{g/mL}$) and incubated for 18–21 h at 35°C . Bacterial suspension was centrifuged at 4000 rpm for 10 min at 4°C (1736R; Labogene, Seoul, Republic of Korea). The bacterial pellet was resuspended twice in 3 mL of sterile water. The final bacterial suspension was serially diluted (1:10) in sterile water to obtain appropriate concentrations for inoculation.

S. enterica inoculation on fully germinated alfalfa sprouts

Prior to inoculation, the alfalfa seeds were soaked in a sterile water and cultured at 25°C for 3 days to obtain fully germinated sprouts. Bacterial inoculum was prepared as described above. Approximately 10^4 cfu/mL of bacterial inoculum was added and homogenized for 3 min. After removing the excess suspension, 30 g of sprouts were put

on a Petri plate with 2.5 mL of sterile water. The alfalfa sprouts were then incubated at isothermal temperatures of 10, 15, 20, 25, and 30°C .

S. enterica inoculation on alfalfa seeds

Alfalfa seeds were purchased from Nongwoo Bio Co., Ltd. (Suwon, Republic of Korea). Ninety grams of alfalfa seeds were placed in a sterile sample bag (BO1365WA; WHIRL-PAK®, Fort Atkinson, WI, USA) with 180 mL of sterile water after which 30 mL of bacterial suspension containing approximately 10^4 CFU/mL was added, and gently mixed for 3 min. The alfalfa seeds were soaked in the bacterial suspension for 5 h at 25°C . Then, after removing the excess suspension, approximately 30 g of seeds were put on a Petri plate with 2.5 mL of sterile water. The seeds were then incubated at isothermal temperatures of 10, 15, 20, 25, and 30°C .

Enumeration of *S. enterica* population

Three subsamples were examined at each time point for each experiment, and all experiments were repeated three times. To determine the number of *S. enterica* cfu per gram of seeds or sprouts, 2 g of seeds or sprouts from random positions within the batch were added to 18 mL of sterile water and then vortexed to release adherent *S. enterica* cells. The suspensions were serially diluted and plated on TSA plus rifampicin ($50\text{ }\mu\text{g/mL}$). The colonies grown on medium were enumerated manually after incubation at 35°C for 24 h.

Primary and secondary models

Bacterial counts for each temperature were converted to $\log_{10}$ values, means and standard deviations plotted in Excel spreadsheets. The Baranyi model (Eqs. 1, 2; Baranyi and Roberts, 1995) was fitted to the data to estimate values for primary kinetic parameters, including specific growth rate [μ_{max} (log cfu/h)].

$$Y(t) = Y_0 + \mu_{\text{max}} * A(t) - \ln\{1 + \exp[\mu_{\text{max}} * A(t)] - 1/\exp(Y_{\text{max}} - Y_0)\} \quad (1)$$

$$A(t) = t + 1/\mu_{\text{max}} * \ln[\exp(-\mu_{\text{max}} * t) + \exp(-h_0) - \exp(-\mu_{\text{max}} * t - h_0)] \quad (2)$$

where Y_0 , Y_{max} , and $Y(t)$ are the bacterial populations, in log cfu of bacteria count, initially, at maximum, and at time t ; μ_{max} is the specific growth rate; $A(t)$ is time variable; and h_0 is the physiological state of the microorganism under consideration.

Three secondary models, Suboptimal Ratkowsky square-root model [Eq. (3); Ratkowsky et al., 1983]; Suboptimal Huang square-root model [Eq. (4); Huang et al., 2011]; and Arrhenius-type model [Eq. (5); Huang et al., 2011] were fitted to elucidate the influence of temperature on the growth of *Salmonella* during alfalfa sprout development.

$$\sqrt{\mu_{max}} = a(T - T_{min}) \quad (3)$$

$$\sqrt{\mu_{max}} = a(T - T_{min})^{0.75} \quad (4)$$

where a is a constant, T is the storage temperature (°C), and T_{min} is the theoretical minimum temperature for cell growth.

$$\mu_{max} = a(T + 273.15) \exp\{-[\Delta G'/R(T + 273.15)]^n\} \quad (5)$$

R is a gas constant (8.134 J/mol), $\Delta G'$ is a type of kinetic energy related to bacterial growth, a and n are coefficients, and T is temperature in degrees Celsius.

The parameters of the secondary models were determined and described using the Integrated Pathogen Modeling Program software (Huang, 2014).

Model performance

Statistical criteria were used to assess the fitness of the model, such as the root mean squared error (RMSE) (Chai and Draxler, 2014), accuracy factor (A_f), and bias factor (B_f) (Ross, 1996):

$$RMSE = \sqrt{(O - P)^2 / N - P}$$

$$A_f = 10^{\sum |\log(P/O)| / N}$$

$$B_f = 10^{\sum \log(P/O) / N}$$

where N is the total number of observation in a growth curve; P is the predicted value, and O is the observed value.

Fig. 1 Survival of *Salmonella enterica* inoculated on fully germinated alfalfa sprouts at selected temperatures

Results and discussion

S. enterica survival on alfalfa sprouts

To elucidate the effect of germination of alfalfa seeds on the increase of *S. enterica*, we investigated the changes in the population of *S. enterica*, which was inoculated on fully germinated alfalfa sprouts under isothermal conditions (10, 15, 20, 25, and 30 °C). As shown in Fig. 1, *S. enterica*, which was inoculated on fully germinated alfalfa sprouts showed a reduction under all experimental temperatures. These data indicate that there is a possibility that if the alfalfa seed exudates could be removed by consumption of other microorganisms during seed germination, the outbreaks of *Salmonella* population could be prevented effectively.

Primary modeling of *S. enterica* growth

All the experimental data were obtained in triplicate under isothermal conditions (10, 15, 20, 25, and 30 °C) and were fitted using the Baranyi model. The initial populations of *S. enterica* on alfalfa seed ranged from 3.75 to 4.11 log cfu/g. *S. enterica* was able to grow at all experimental

temperatures and the growth rate increased with increasing temperature. A significant increase in *S. enterica* populations was observed at all temperatures, with populations exceeding 7 log cfu/g at 25 °C. The mean values of estimated kinetic growth parameters, specific growth rate (log cfu/h), lag (h), initial bacterial population (Y_0), and maximum bacterial population (Y_{max}), derived from three trials at each temperature are shown in Table 1. The specific growth rate ranged from 0.034 to 0.468 log cfu/h in the experimental temperature range. The value of lag decreased with a rise in temperature from 15 to 30 °C in the range of 3.86–0.82 h; however, this information was not obtained for 10 °C. The growth of *S. enterica* ranged from 1.61 log cfu/g at 10 °C to 3.70 log cfu/g at 30 °C. *S. enterica* during alfalfa seed germination and early sprout development reached maximum density within 24 h at all experimental temperatures except 10 °C.

A comparison of the performance of primary models such as modified Gompertz, logistic, and Baranyi models showed that the specific growth rate (μ_{max}) obtained from the Baranyi model provided the best fit for the secondary model. The highest fitness of mean squared errors, and RMSEs was found in the Baranyi model compared with that of those in the Huang, and Gompertz models (data not

Table 1 The mean values of three trials at each temperature after fitting the *Salmonella enterica* growth data on alfalfa sprout to the Baranyi model

Temperatures (°C)	Kinetic growth parameters			
	Specific growth rate (log cfu/h)	lag (h)	Initial bacterial population (Y_0)	Maximum bacterial population (Y_{max})
10	0.03 ± 0.012	–	4.00 ± 0.393	5.61 ± 0.364
15	0.13 ± 0.007	3.86 ± 2.508	4.11 ± 0.733	6.12 ± 0.448
20	0.21 ± 0.038	1.58 ± 0.822	3.83 ± 0.569	6.86 ± 0.435
25	0.37 ± 0.033	1.52 ± 0.578	4.07 ± 0.712	7.56 ± 0.260
30	0.47 ± 0.060	0.82 ± 0.440	3.75 ± 0.268	7.52 ± 0.075

Table 2 Estimated values and performance of the secondary models for the specific growth rates of *Salmonella enterica* during alfalfa sprout development

Model	Parameter	Estimate	Standard-error	<i>p</i> value	RMSE ^a	A_f^b	B_f^c
Suboptimal Ratkowsky square-root model	a	0.02	0.001	6.58×10^{-4}	0.02	1.17	0.91
	T_{min} (°C)	9.01	0.878	1.97×10^{-3}			
Suboptimal Huang square-root model	A	0.05	0.004	10.00×10^{-4}	0.04	1.23	0.95
	T_{min} (°C)	9.76	0.796	1.17×10^{-3}			
Suboptimal Arrhenius-type model	A	0.003	0.001	9.22×10^{-2}	0.02	1.08	1.02
	Alpha	26.44	9.428	1.07×10^{-1}			
	Ea	2450.50	34.663	2.00×10^{-4}			

^aRoot mean squared errors; ^bAccuracy factor; ^cBias factor

shown). Therefore, we used the Baranyi model to fit the data of *S. enterica* growth during alfalfa sprout development.

Secondary model and verification

Since seed sprouts are mainly cultivated at room temperature, we fitted suboptimal secondary models with a range of 10–30 °C. The estimated specific growth rate from the primary model was consequently modeled as a function of temperature with three secondary models (Ratkowsky square-root, Huang square-root, and Arrhenius-type models). The prediction curves of all three secondary models showed noticeably different shapes (Supplementary Fig. S1). The parameter values for each secondary model are listed in Table 2. The minimum growth temperatures estimated by the Ratkowsky and Huang square-root models were 9.0 and 9.8 °C, respectively. The Arrhenius-type model was the best suited to describe the effect of temperature on μ_{max} of *S. enterica*, which was inoculated on alfalfa seeds in this study. The values of RMSE, namely, the difference between predicted and observed values, were 0.02, 0.04, and 0.02 in the three secondary models (Ratkowsky square-root, Huang square-root, and Arrhenius-type models, respectively) (Table 2). The utility of the three secondary models used in this study were verified using the accuracy (A_f) and bias factor (B_f) as indications of model performance. The values of A_f and B_f of the models used for specific growth rates are shown in Table 2. The ranges of A_f and B_f in the Ratkowsky square-root, Huang square-root, and Arrhenius-type models were 1.08–1.23, and 0.91–1.02, respectively. The value of A_f indicates that the predicted values in the three models differ from the observed values by 16.6, 23.1, and 8.1%, respectively. Because the value of B_f in the range 0.9–1.05 could be considered as good (Ross, 1996), three secondary models fitted in this study were acceptable to describe the influence of temperature on microbial growth rate. Thus, the three secondary models used in this study were acceptable for describing *S. enterica* growth rate during alfalfa sprout development.

To the best of our knowledge, no prediction model for the growth of *Salmonella* spp. in alfalfa sprouts has been previously developed, although foodborne illness associated with *Salmonella* contamination on alfalfa sprouts has occurred worldwide. We believe these results provide insight into population changes of *S. enterica* and microbial risk assessment in alfalfa sprout production.

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