Systematic Studies on Growth Kinetics and Prediction of *Salmonella* Enteritidis in Ground Chicken and Liquid Egg Products

(鶏ひき肉および液卵製品におけるサルモネラエンテリティディスの増殖速度と予測に関する体系的研究**)**

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The United graduate school of Veterinary Sciences

Gifu University

(Tokyo University of Agriculture and Technology)

Mohammad Zaher Sakha

Contents

Chapter 2

Growth kinetics and prediction of *Salmonella* Enteritidis in liquid egg products

Chapter 3

Development of predictive program for *Salmonella* Enteritidis growth in raw ground chicken and whole liquid egg products

General introduction

Food is basically obtained from two sources, plants and animal. Those sources are also a good medium for microorganism growth from food safety aspect. Improper handling of food might be a root to allow growth of harmful microorganisms. By observation of some factors in food science the food risk would be prevented. Those are intrinsic factors such as pH, content of moisture, oxidation-reduction potential, nutrient, antimicrobial agent, biological frame, and extrinsic factors like temperature, relative humidity, percentage of gas and presence of microorganism (28). People desire to buy food products, which do not contain food additives from the view point of food safety. Also, the production and distribution of food under sanitary situation is an important requirement to decrease the microbial foodborne prevalence.

It is personal and group responsibility to provide safe food for customers. The food and drug administration, US Department of Agriculture, Centre for Disease Control and Prevention, food processors, consumers and others are responsible for prevention of foodborne disease (52).

Salmonellosis is one of the important diseases and causes many incidences annually worldwide. *Salmonella* spp. cause around 61.8 -131.6 million of gastroenteritis cases every year, among these huge number 80.3 million occurrences were reported due to foodborne infection (33). *Salmonella* infection is still concerning issue in both developing and developed countries (33).

Poultry is used in a wide range over the world, because poultry meat is cheap, easily digestible and has low calorie. Beside its benefit, poultry meat often found contaminated with pathogenic microorganisms such as *Salmonella*, *Campylobacter*, *E.coli*, *Staphylococcus* and other species. Many researchers think that poultry meat is one large source of the foodborne outbreaks to human. Beside poultry meat, egg is also an important food protein. Recently consumption of egg is increasing as the form of liquid egg, yolk, albumin, egg blend, and ready to eat food anywhere especially in developed countries (10).

In the US, *Salmonella* still causes one million foodborne diseases annually and *Salmonella* Enteritidis is a common serotype according to the Active surveillance network (foodnet) (5). The network concluded that the chicken and eggs are important source of *Salmonella* Enteritidis (5).

The ministry of health and welfare of Japan has reported the incidences of *Salmonella* originated outbreaks. *Salmonella* is one of the measure causative foodborne diseases in Japan. Thus the *Salmonella* positive rate in ground chicken is high, more caution is recommended by authorities to producers, processors and consumers (www. idsc.nih.go.jp/iasr. Japan). The incidences of Salmonellosis through egg also have been seen in many studies.

One part of modern food microbiology is the predictive microbiology, which shows the microbial reaction kinetics against the environmental factors under ascertained conditions, the results of this reaction would be a mathematical equation set, thus these equations predict for non-tested materials (47). Predictive microbiology is a powerful tool that prevents human from exposure to

food pathogens. Its response is immediate for improving of the food safety, and also gives us qualitative and quantitative data of contaminated food from production to a consumption point (48). Likewise predictive microbiology is software package that allows users to approximate population of specific organism by determination of conditions. So far many models have been developed by some researchers based on laboratory data. They used broth or real food to estimate the growth of specific organism with different factors such as pH, temperature and other parameters (www.campdenbri.co.uk). For practical aims the best will be the model that developed by using real food. There are several classifications of models. Whiting and Buchanan (53) defined the models in three level, primary, secondary and tertiary models.

Primary model is for microbial growth at constant condition factors against time. The factors can be temperature, pH, and water activity (a_w) etc.

Secondary model explains the effect of the environmental factors such as temperature, pH, a_w and other factors on the parameters at the primary model for example growth rate (53). The Square root model, the Arrhenius model and a polynomial equation are some examples of this model.

The final shape of model is tertiary model that is obtained from integration of the primary and secondary model parameters, as software packages. The model is a valuable tool to all food section and manufactories to predict microbial growth or inactivation values (34).

There are several well-known models to describe microbial growth such as the Baranyi model, the modified Gompertz model, and the three-phase linear model (3, 4, 20). Among them the Baranyi model is used most frequently by researchers worldwide.

Fujikawa et al. (11) developed and improved a model from the original logistic model; they called it the new logistic NL model. This model can predict the growth of bacteria at a variety of temperature. The performance of the model was the same as Baranyi model (13). Some predictive programs based on the NL model for growth of *Escherichia .coli*, *Staphylococcus aureus and Vibrio paraheamolyticus,* have been made so far using sterile food and media (15, 18). The NL model revealed its compatibility in some studies as well as other models. The NL model is a descriptive model, similar to the original logistic model.

Salmonella Enteritidis often contaminates chicken and egg, and occasionally causes outbreaks among people. So, I concerned to conduct a systematic study of *Salmonella* Enteritidis growth in raw chicken and liquid products, pasteurized and unpasteurized to make a data set of lag time, growth rate and maximum population parameters and then predict *Salmonella* growth in those products at a variety of temperature. The final goal was the development of a program to predict *Salmonella* Enteritidis in those products. Users can input the temperature history, select food sample and get predicted growth curves and kinetics values automatically with the program.

Chapter 1

Suppressive impact of native microflora in raw ground chicken on the *Salmonella* Enteritidis growth kinetics

1.1 Introduction

Temperature is a valuable factor for microbial food safety namely, for storage of food and microbial growth in food chain. If foods are subjected to undesired temperatures during handling, the native microflora (NM) can grow. Once NM grows in the food, they will not decrease in number even when the food is then cooled in a refrigerator. If NM includes pathogens, food poisoning outbreak can occur. Also, even if there are no pathogens, food spoilage can occur with an increased number of NM. Thus, the prediction of microbial growth in foods at various temperatures is considerably important for food safety and quality.

Many of the papers published on predictive food microbiology have dealt with the growth of pathogens in sterile foods and media. The growth kinetics of *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus* in sterile foods and media has been successfully described with NL model (11, 12, 13, 17). However, most retail foods and food materials are not sterilized. Competition between a pathogen and NM in a food would greatly influence the growth of the pathogen. But NL model has not been validated for such a food yet.

A few investigators have studied the growth kinetics of a pathogen as well as NM in a food (21, 22, 32). Studies on microbial growth kinetics in a food with NM are becoming important for the next stage of predictive food microbiology. Therefore, we need to analyze and predict growth of pathogens and NM in a lot of kinds of food and apply the obtained data to microbial food safety.

Salmonella enterica serovar Enteritidis often contaminates chicken and egg and has caused serious food poisoning outbreaks worldwide. In this study, I conducted a systematic study of the growth kinetics and prediction of *Salmonella* Enteritidis and NM in raw ground chicken. Further, I studied the *Salmonella* growth kinetics in sterilized ground chicken to evaluate the effect of NM on *Salmonella* growth in ground chicken. Here I selected a test strain that showed the average growth characteristics among four *Salmonella* Enteritids strains.

1.2 Materials and methods

1.2.1 *Salmonella* Cell preparations.

A *Salmonella* Enteritidis strain 04-137, an isolate from a food poisoning outbreak in Tokyo, Japan, was activated on a nutrient agar plate (Nissui Pharmaceuticals, Tokyo, Japan) at 35˚C for 24 h. Cells of several well-grown colonies on the plate were incubated in Trypticase soy broth (Oxoid, Basingstoke, UK) with shaking at 80 rpm and at 35˚C for 24 h. Cultured cells (1 ml) were washed with saline (0.85% (w/v) sodium chloride solution) by centrifugation at 13,000 x *g* and at 4˚C for 15 min. Cells were thoroughly suspended in saline (1 ml) and then diluted to 1:10^4 with saline, yielding a cell suspension of about 10^5 CFU/ml. In the experiment at different initial cell doses, the cell suspension was diluted to corresponding ratios with saline.

12.2 Chicken.

The ground chicken which was made of young chicken breast meat and packed in a plastic tray was bought at a retail store in Tokyo. That ground chicken was examined for *Salmonella* spp. contamination before use (1). Ground chicken (6 kg) was purchased and then two ground chicken samples with low and high levels of NM were prepared for experiments at various constant and dynamic temperature patterns. Namely, the low NM sample was composed of 3 kg untreated ground chicken purchased at the store and the high NM sample was prepared by incubating 3 kg purchased ground chicken at 30˚C for 10 h to increase the level of NM. After thorough mixing, about 250 g of each ground chicken sample was placed in sterile plastic cups. The cups were

frozen at -20˚C until use and then thawed at <10˚C overnight for use. Retail ground chicken was also sterilized at 121˚C for 15 min for experiments with sterile chicken.

1.2.3 *Salmonella* spiking and storage.

Salmonella cell after making dilution (2 ml/100 g chicken) was inoculated into ground chicken and mixed gently, then distributed into sterilized glass bottles with caps (10 g/ bot). The glass bottles were stored in either a water bath unit (SM-05R; Taitech, Koshigaya, Japan) for trials at a constant temperature above room temperature or a programmable incubator (SU-221; Espec Co., Osaka, Japan) for trials at constant temperature below room temperature. The time for the sample in the bottle to reach the designated temperature (i.e., 30 min) in each incubator was measured with a digital thermometer (AM-7002; Anritsu Meter Co., Tokyo) and was taken into consideration during the experiment (12, 13, 17). Immediately after incubation, each sample (one bottle per data point) was taken from the incubator and cooled in ice water. At least two trials were performed at each constant temperature.

For a dynamic temperature experiment, the glass bottles were placed in the programmable incubator and the sample temperature was measured in duplicate every 30 sec throughout the experiment with the digital thermometer (11, 12, 17). Immediately after each incubation period, the sample in duplicate was taken from the incubator and cooled in ice water. One trial was performed for each dynamic temperature pattern.

1.2.4 Bacterial cell counts.

To obtain 10% food homogenate, buffered sodium chloride peptone water (90 ml) (Nissui Pharmaceuticals) was added into the bottles contained chicken meat, then mixed well and was transferred to sterile plastic bag. The homogenate was thoroughly mixed in a stomacher (SH-IIM; Elmex, Tokyo) for one minute. Each sample was then serially 10-fold diluted with saline without peptone, to suppress microbial growth in the diluted samples (1). Total aerobic bacteria counts of the sample were enumerated in duplicate with the surface-plating method using standard method agar plates (Nissui Pharmaceuticals) after incubation at 35˚C for 48 h (1). *Salmonella* counts of the sample were enumerated in duplicate with the surface-plating method using desoxycholate-hydrogen sulfide-lactose (DHL) agar plates (Nissui Pharmaceuticals) or XLD agar plates (Oxoid). For *Salmonella* counts, agar plates were incubated at 42˚C for 24 h to suppress the growth of microorganisms other than the test strain. Suspected colonies were examined for identification with a serological test using antiserum to *Salmonella* O antigens (Denka-Seiken, Tokyo) on a glass slide or a real-time PCR method (26). The average bacterial count with two plates for each data point was calculated for *Salmonella* and total bacteria.

Salmonella cells at a very low dose were enumerated with the 5-tube (MPN) method (1). Namely, 3 dilutions consisting of 10, 1, and 0.1 ml of a 10% food homogenate of a sample were cultured in each 5 tubes containing Enterobacteriaceae Enrichment Mannitol broth (Merck, Darmstadt, Germany). The tubes was incubated at 37^{o}C for 24 h and then isolated on a DHL or XLD plate. Suspected colonies were then tested as described above.

1.2.5 Growth model and statistical analysis.

In this experiment at constant and dynamic temperatures two trials were performed. The *Salmonella* and total bacteria count average were enumerated and analyzed with the NL model, which is expressed as follows (11):

$$\frac{dN}{dt} = rN\{1-(\frac{N}{N_{\max}})^m\}\{1-(\frac{N_{\min}}{N})^n\} \quad [1]$$

Here N is the population of a microorganism (CFU/g) at time t (h), r is the rate constant of growth (1/h), N_{max} is the maximum population (CFU/g), and N_{min} is the initial population (CFU/g). m and n (>0) are parameters related to the curvature of the deceleration phase and the period of the lag phase, respectively. The equation was solved numerically with the 4th-order Runge-Kutta method. Numerical data of microbial counts were analyzed with a computer program to fit to the growth model, which was developed using a spread sheet software program, Microsoft Excel (17) Microbial populations estimated with the model (CFU/g) were then transformed to logarithm to make a growth curve (17).

Growth at a dynamic temperature was predicted using the values of parameters in Eq. [1] studied at constant temperatures (11, 12, 13, 17). The value of r at the measured temperature of the time interval during an experiment was obtained from the square root model (35).

The mean of the square error, MSE, between log-transformed cell concentrations predicted with the model and observed at the observation points

was calculated. Statistical analysis of data including regression analyses was performed with Microsoft Excel. The lag period in a growth curve, lag, defined as the period between the initial point and the point where the regression line for the exponential phase intersected the horizontal line passing through the initial point on the semi-logarithmic plot was estimated with the Excel program (17).

1.3 Results

1.3.1 Growth kinetics at various initial doses.

Salmonella Enteritidis with different initial doses was studied in ground chickens. The *Salmonella* was spiked into high level of NM ($10^{7.1}$ CFU/g) with *Salmonella* initial doses of (10^4 to 10^1 CFU/g) and was incubated at designed temperature. *Salmonella* growth curves were similar during the storage (Fig. 1A). The values of the rate constant of growth, or *r* were similar among them, being 0.53, 0.51, 0.55, and 0.46 (1/h) at the initial doses of $10^{4.2}$, $10^{3.2}$, $10^{2.0}$, and $10^{1.1}$ CFU/g, respectively. Also, values of *lag* in the all growth curves were very short (< 1 h). However, in the stationary phase, N_{max} was higher at the higher initial doses (Fig. 1A). Values for N_{max} were $10^{8.5}$, $10^{8.0}$, $10^{7.6}$, and $10^{7.0}$ CFU/g at the initial doses of $10^{4.2}$, $10^{3.2}$, $10^{2.0}$, and $10^{1.1}$ CFU/g, respectively. No *Salmonella* cells were detected in the original ground chicken.

Growth of total bacteria in the ground chicken spiked at $10^{2.0}$ CFU/g of *Salmonella* was measured for comparison (Fig. 1A). Since the ground chicken used in this experiment was originally contaminated with a high level of NM ($10^{7.1}$ CFU/g), growth of NM was much less than that of *Salmonella*.

Similar phenomena on the growth kinetics were observed in ground chicken with a low level of NM ($10^{4.9}$ CFU/g) (Fig. 1B). N_{max} for *Salmonella* was higher at the higher initial doses; values for N_{max} were $10^{8.6}$, $10^{8.0}$, $10^{7.4}$, and $10^{6.5}$ CFU/g at the initial doses of $10^{4.3}$, $10^{3.3}$, $10^{2.3}$, and $10^{1.4}$ CFU/g, respectively. Also, values for *r* were similar and values of *lag* were short for those all growth curves. No *Salmonella* cells were detected in the original ground chicken. It is

interesting that N_{max} for *Salmonella* was dependent on the initial dose in the ground chicken with the high and low NM levels (Fig. 1A, B).

1.3.2 Growth kinetics at constant temperatures.

Since the initial dose of *Salmonella* Enteritidis did not remarkably affect its growth characteristics except for N_{max}, growth of the pathogen at various constant temperatures was then studied at a given initial dose. Ground chicken with low and high levels of NM ($10^{4.7}$ and $10^{6.8}$ CFU/g, respectively), were spiked with *Salmonella* at about $10^{3.2}$ CFU/g and then stored at temperatures ranging from 8 to 32˚C to study the effect of NM on the growth of the pathogen. No *Salmonella* cells were detected in the original ground chicken.

Growth curves of *Salmonella* and total bacteria in the ground chicken with the low and high levels of NM stored at the constant temperatures were all sigmoidal and well described with the NL model. No *Salmonella* growth was observed in the chickens with the low and high levels of NM at 8˚C. Growth parameter values obtained for *Salmonella* and total bacteria in the two ground chicken samples were shown in Table 1. Values for the parameters in the table were then studied as follows.

Value of N_{max} for *Salmonella* increased, depending on the temperature in both low and high NM chicken samples and the value in low NM chicken was higher than that in high NM chicken at <32˚C (Fig. 2). The values of N_{max} for both low and high NM levels were precisely described with the following polynomial equations [2] and [3] with high values for coefficient of determination of 0.976 and 0.945, respectively (Fig. 2).

$$N_{max} = -0.0073T^2 + 0.4756T + 1.592 \quad [2]$$

$$N_{max} = -0.0047T^2 + 0.4343T + 1.592 \quad [3]$$

Values of N_{max} for total bacteria in the two ground chicken samples at these temperatures were both constant with the same averages of $10^{9.4}$ CFU/g (Table 1, Fig. 2).

The value of *r* for *Salmonella* also increased, depending on the temperature in both NM levels and the value was higher in low NM chicken than high NM chicken (Fig. 3A). Values of *r* for *Salmonella* at the storage temperatures for both NM levels were successfully described with the square root model (Fig. 3A). Linear regression lines for *r* in both low and high NM chicken were described by equations [4] and [5], respectively.

$$\sqrt{r} = 0.0399(T - 5.11) \quad [4]$$

$$\sqrt{r} = 0.0285(T - 4.18) \quad [5]$$

The coefficients of determination for the low and high NM chicken were 0.976 and 0.986, respectively. The steeper slope of *r* for low NM chicken indicated that it was more sensitive to temperature. Values for *r* in both NM levels at 8˚C, which were measured to be almost zero, obviously deviated from the regression lines (Fig. 3A).

Values of *r* for total bacteria were also well described with the square root

model (Fig. 3B). The regression lines for low and high NM chicken were described with equations [6] and [7], respectively.

$$\sqrt{r} = 0.0277(T - 4.40) \quad [6]$$

$$\sqrt{r} = 0.0238(T - 1.45) \quad [7]$$

The coefficients of determination for low and high NM chicken samples were 0.989 and 0.986, respectively. Interestingly, the value for *r* was higher in low NM chicken than high NM chicken in the temperature range of this study, similar to *Salmonella* (Fig. 3B).

The value of *lag* for *Salmonella* tended to be longer at lower temperatures for both NM levels of ground chicken (Table 1). This was also seen for total bacteria in low NM chicken, while values for *lag* were all small (i.e., <1h) for total bacteria in high NM chicken (Table 1).

When total bacteria counts of a chicken sample reached 10^9 CFU/g, the chicken was found to spoil with an offensive smell, but the storage experiments were continued until the *Salmonella* population reached the maximum level.

1.3.3 Growth prediction at dynamic temperatures.

The growths of *Salmonella* Enteritidis and total bacteria in ground chicken with two NM levels at various dynamic temperatures were predicted by NL model. The data which were used for prediction were obtained from *Salmonella* and NM growth parameters at the constant temperatures.

Namely, equations [2] and [3] for *Salmonella,* and the average of N_{max} for total

bacteria was introduced for N_{max} in the growth model. Equations [4] to [7] were also introduced for *r* in the growth model. For *m* and *n* in equation [1], the averages obtained at constant temperatures for both NM levels of ground chicken shown in Table 2 were used for prediction.

The growth model succeeded in predicting the growth of *Salmonella* and total bacteria in both NM levels of chicken stored at various dynamic temperatures (Figs. 4 and 5). Here, the dynamic temperatures included high (A) and low (B) temperature ranges. Similar results were obtained in ground chicken exposed at a wider temperature range between 11.4˚C and 30.5˚C (data not shown). Values of *MSE* for those predictions were very small. The averages of *MSE* for *Salmonella* and total bacteria were 0.049±0.021 and 0.075±0.070 log units in low NM chicken and 0.18±0.059 and 0.10±0.055 log units in high NM chicken, respectively (n=3).

1.3.4 Growth kinetics in sterile chicken.

Salmonella Enteritidis growth in sterilized ground chicken at various constant temperatures between 8˚C and 32˚C was studied for comparison with that in ground chicken with low and high NM levels. An example of the growth curve at 24˚C is shown in Fig. 6A. *Salmonella* growth, characterized by both N_{max} and *r*, was highest in sterilized chicken, followed by chicken with the low NM level; the values of N_{max} for sterilized chicken, low NM chicken, and high NM chicken were $10^{10.3}$, $10^{8.6}$, and $10^{8.0}$ CFU/g, and values of *r* were 0.85, 0.59, and 0.37 1/h, respectively. Values of all *lag* were small, <1 h. Similar results were observed at other temperatures. These results showed that the level of NM in ground chicken influenced *Salmonella* growth kinetics. While the ground

chicken sterilized by heating might have developed better nutrients for the bacteria growth, the results showed potential growth of the bacteria under optimal conditions without any competitors.

The slope in the log phase, or *r* was greater at higher temperatures in sterilized ground chicken, similar to that in the low and high NM ground chicken (Fig. 6B). However, the most striking characteristic of growth was that the values of N_{max} between 12˚C and 32˚C were almost constant, being around 10^{10} CFU/g (Fig. 6B) and were distinctly different from those in the ground chicken with NM (Table 1, Fig. 2). At 12˚C, *Salmonella* finally grew to 10^{10} CFU/g after 240 h of storage data not shown.

No *Salmonella* growth in the sterilized chicken was also observed at 8˚C for about 7 days, similar to that in the low and high NM chicken. This means that non-growth at 8˚C would be due to a physiological characteristic of the organism, not by microbial competition in chicken.

1.4 Discussion

While NM in raw ground chicken is thought to consist of many kinds of microorganisms such as bacteria, yeast, and mold, the level of NM was evaluated with standard method agar plates in this study. And the level of NM was evaluated before spiking with *Salmonella* cells. On the other hand, total bacteria of ground chicken samples spiked with *Salmonella* cells consisted of NM and *Salmonella*. Therefore, total bacteria and NM were used with these corresponding meanings in this study.

NM was always the dominant in the ground chicken samples during the storage in this study, as compared with spiked *Salmonella* cells. Ratios of *Salmonella* counts to total bacteria counts in most samples at the constant temperatures were very low, being 2-3% at the maximum even in ground chicken with low NM level. At higher temperatures, the ratio became higher as the storage period proceeded; the maximum ratio reached 40% at the stationary phase at 32˚C for ground chicken with both NM levels (Table 1).

The present study clarified several growth characteristics of *Salmonella* Enteritidis in ground chicken with NM. Three parameters of microbial growth kinetics including the period of lag, the rate constant of growth, and N_{max} are considered important to characterize its growth. Especially, the value of N_{max} for a microbe of concern is necessary for a mathematical model that describes and predicts microbial growth over a given time. But so far, only a few researchers have focused on values of N_{max} for pathogens in food with NM when they modeled microbial growth (30, 43, 44). In the present study, I therefore determined values of N_{max} for the *Salmonella* strain in raw ground

chicken according to temperature (Fig. 2), initial *Salmonella* level (Fig. 1), and level of NM (Figs. 2, 6A). The results in Fig. 2 are similar to those of studies (30), who studied the growth of *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* Enteritidis and Typhimurium on lettuce leaves. In their paper, N_{max} for pathogens increased at higher temperatures. Oscar (43, 44) also reported that N_{max} for *Salmonella* Typhimurium differed with the initial level of the strain and temperature. However, in their studies (30, 43, 44), they used foods with a single NM level and did not studied the growth kinetics of NM in the foods. In the present study, I found that a higher level of NM yielded a smaller value of N_{max} for *Salmonella* Enteritidis (Figs. 2 and 6A). As shown in Fig. 1 A, B, as total bacteria in ground chicken increased to the maximum population, *Salmonella* growth was suppressed, resulting in a lowered maximum population. I infer that this is due to microbial competition between *Salmonella* and NM in the ground chicken. This phenomenon is known as the Jameson effect and has been observed for several microorganisms (9, 22, 27, 48).

In this study I also found that values of N_{max} for *Salmonella* Enteritidis in sterile ground chicken at various constant temperatures were almost constant (Fig. 6B), in contrast to the raw ground chicken with NM (Fig. 2). The result in sterile ground chicken is similar to reported results of *E. coli* grown in broth and *Salmonella* Typhimurium grown on the sterile chicken (12, 42). Values of N_{max} for *E. coli* grown at various initial cell doses in broth are also constant (12). In my preliminary study, values of N_{max} for *Salmonella* Enteritidis at various initial doses in sterile chicken were also constant at a given temperature, in contrast

to raw chicken with NM (Fig. 1A, B). These results suggest that this difference in N_{max} would also due to the Jameson effect by NM in ground chicken.

No remarkable differences in values of *r* and *lag* in raw ground chicken were observed at the various initial *Salmonella* doses in my study (Fig. 1A, B). Mackey and Kerridge (32) inoculated *Salmonella* cells at 10^4 and 40 CFU/g into minced beef and reported that the maximum growth rate and lag period of *Salmonella* spp. in minced beef are unaffected by the inoculum size. Oscar (44) also reported no remarkable differences in the maximum specific growth rate and lag period for *Salmonella* at $10^{1.12}$ CFU/g and $10^{3.7}$ CFU/g in ground chicken. Also, Gimenez and Dalgaard (21) reported similar results of *L. monocytogenes* in cold-smoked salmon. While the contamination level of *Salmonella* in retail chicken is generally very low, with <1 MPN/g in most positive samples (43, 51, 54), the above papers suggest that the initial dose would not affect the growth kinetics of the organism. Thus, for parameters other than value of N_{max}, I believe that the analysis and prediction methods used in this study can be extended to *Salmonella* growth in foods with very low initial levels of *Salmonella*.

The present study also showed the effect of NM levels on the growth parameter *r* for *Salmonella* Enteritidis. Value of *r* at a given temperature was smaller at the higher level of NM (Figs. 3A, 6A). This suggests that NM suppresses *Salmonella* growth during the log phase. On the other hand, no obvious effect on the value for *lag* for *Salmonella* was observed (Table 1). I hypothesize that the population of NM in the ground chicken was not enough to suppress *Salmonella* growth at beginning of storage in this study.

The results were obtained from two trials for each experiment, so there might be some changes in the values for the growth parameters in the model when the number of trials increased. However, I believe that the findings in this present study such as the Jameson effect and others would not be unique, but common.

There are some methods for studying microbial growth of multiple species. I studied microbial growth in raw food with NM, similar to previous studies (30, 43, 44). Guillier et al. (22) studied microbial competition by inoculating sterilized food with both a pathogen (*L. monocytogenes*) and natural biofilm microflora. Other investigators studied microbial growth for co-culture in broth with specific microorganisms (6, 38, 41). Each method has advantages and disadvantages. This method with real foods can obtain applicable, microbial data for the tested food, but it is difficult to adjust the populations of NM to desired levels; by trial and error I was able to prepare ground chicken with higher NM population with a difference of two orders of magnitude by incubating the original sample at 30˚C for 10 h.

Strain 04-137 used in this study was selected from a survey of four *Salmonella* Enteritidis strains with different origins in Trypticase soy broth (Oxoid). The growth curves of the four strains were very similar to each other (data not shown). Thus, the results obtained in this study are thought to be applicable to other *Salmonella* Enteritidis strains.

For enumeration of *Salmonella* cells in foods with NM, some investigators have used selective agar media specific to the target organism (31, 39); however, it is difficult to select *Salmonella* colonies on agar plates where a

number of bacterial colonies grow. Suspected colonies need to be examined for further microbiological identification. Other investigators spiked antibiotic-resistant target cells or target cells artificially transformed with fluorescent proteins (25, 32, 43, 44). In this study, I incubated food samples inoculated on DHL or XLD agar plates at a high temperature of 42˚C for *Salmonella* enumeration. This high temperature suppressed the growth of most NM in chicken on selective agar plates; thus, it became much easier to count the *Salmonella* colonies. In preliminary experiments, I confirmed that the *Salmonella* counts of samples incubated at 42˚C were the same as those at 35 or 37˚C. This method is based on the physiological characteristics of *Salmonella* for temperature and did not require any special *Salmonella* cells described above.

For *Salmonella* enumeration, I used DHL agar plate as well as XLD agar plate in this study. The DHL agar plate is an official selective medium for *Salmonella* in Japan (1). In a preliminary study, it confirmed that the DHL agar plate was equivalent to the XLD agar plate; *Salmonella* counts of samples on DHL agar plates were the same as those on XLD agar plates.

The predominant species of microorganims in ground chicken might change with the storage period and temperature, but I believe that the growth model can describe and predict *Salmonella* Enteritidis as well as total bacteria in ground chicken at various temperature patterns. To my knowledge, no researchers have yet successfully reported growth prediction for both pathogen and total bacteria in food at various temperature patterns. Delignett-Muller et al. (9) showed first-order simulations of growth of *L. monocytogenes* and food flora

in cold-smoked salmon at 4˚C, but they did not confirm their results with microbial experiments. On the other hand, competition among microbial species within a food has not been introduced in NL model.

1.5 Summary

In this chapter the ground chicken NM effect on the growth kinetics of *Salmonella* Enteritidis were studied. First, ground chicken with high and low levels of NM ($10^{7.1}$ and $10^{4.9}$ CFU/g, respectively) was spiked with *Salmonella* at doses ranging from 10^{1} to 10^{4} CFU/g, and the growth kinetics, including the rate constant of growth, *r*, and the lag period were similar, but the maximum cell level, N_{max}, was higher at higher initial *Salmonella* doses for both NM levels. Second, ground chicken with high and low NM levels ($10^{6.8}$ and $10^{4.7}$ CFU/g, respectively) were spiked with *Salmonella* and then stored at various constant temperatures ranging from 8˚C to 32˚C. Both N_{max} and *r* for *Salmonella* were higher at higher temperatures in both NM levels. Although *r* for total bacteria which consisted of NM and *Salmonella* was also higher at higher temperatures, N_{max} was constant at all temperatures for both NM levels. Further, *Salmonella* growth was compared among ground chicken with high and low NM levels and sterilized chicken. *Salmonella* growth, characterized by both N_{max} and *r*, was highest in sterilized chicken, followed by chicken with the low NM level. Growth of *Salmonella* and total bacteria in chicken at the constant temperatures was successfully described and analyzed with NL model. Using obtained data, growth of *Salmonella* and total bacteria in ground chicken stored at dynamic temperatures was also well predicted with the NL model. This study clarified the effects of NM at different doses in ground chicken on growth kinetics of the *Salmonella* and the usability of the growth model for foods with NM.

Table 1. Growth characteristics of *Salmonella* Enteritidis and total bacteria in ground chicken with high and low NM levels at various constant temperatures.

	Ground chicken with high NM level					
	Salmonella			Total bacteria		
Temp (˚C)	*r* (1/h)	*lag* (h)	N_{max} (log CFU/g)	*r* (1/h)	*lag* (h)	N_{max}(log CFU/g)
32	0.59	<1	9.4	0.60	<1	9.6
28	0.44	<1	8.5	0.53	<1	9.3
24	0.37	<1	8.0	0.34	<1	9.8
20	0.20	1.3	6.4	0.28	<1	9.4
16	0.10	2.8	6.5	0.17	<1	9.2
12	0.038	5.5	4.3	0.088	<1	9.2
	Ground chicken with low NM level					
32	1.0	<1	9.4	0.99	1.2	9.6
28	0.96	<1	9.1	0.84	1.0	9.7
24	0.59	<1	8.6	0.65	1.4	9.3
20	0.36	2.0	8.1	0.38	2.2	9.3
16	0.16	<1	7.6	0.34	5.3	9.5
12	0.055	8.0	5.9	0.19	5.8	9.3

Parameters *r*, *lag*, and N_{max} are the rate constant of growth, the lag period, and the maximum cell population, respectively. Values are the averages of two trials. No *Salmonella* growth was observed in both ground chicken samples at 8 ˚C.

Table 2. Values for parameters *m* and *n* of the NL model for *Salmonella* Enteritidis and total bacteria in ground chicken with low and high NM levels.

	Level of native microflora			
	Low		High	
Parameter	*Salmonella*	Total bacteria	*Salmonella*	Total bacteria
m	0.28	0.45	0.67	0.68
n	62	14	67	100

Values are the averages for *m* and *n* of the model (Eq. 1) in ground chicken samples with low and high NM levels that were stored at the constant temperatures ranging from 8˚C to 32˚C and then used for growth prediction at dynamic temperatures.

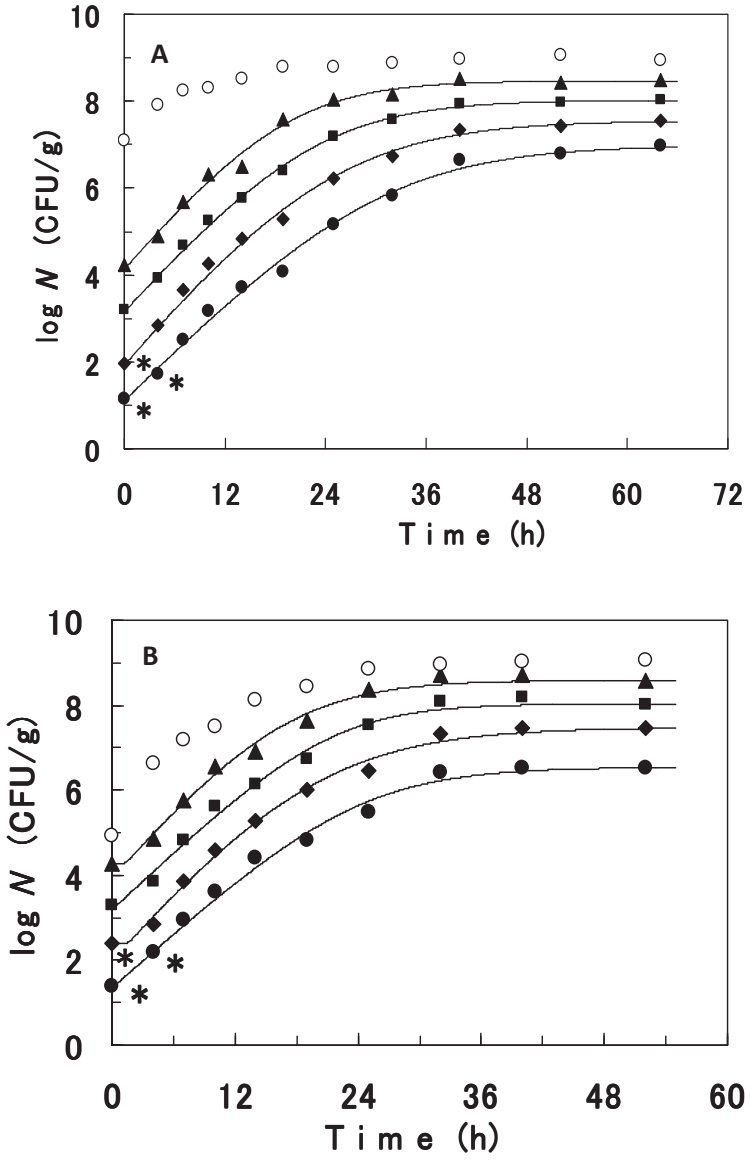

Fig. 1. Growth curves of *Salmonella* Enteritidis in ground chicken spiked at various initial doses of 10^4, 10^3, 10^2, and 10^1 CFU/g. Ground chicken samples with (A) the high level ($10^{7.1}$ CFU/g) and (B) the low level ($10^{4.9}$ CFU/g) of NM were stored at 24˚C. Solid symbols show *Salmonella* counts at the initial doses of 10^4 (▲), 10^3 (■), 10^2 (♦), and 10^1 (●) CFU/g. Open circles are total bacteria counts of ground chicken at the initial dose of 10^2 CFU/g (♦) of *Salmonella.* Asterisks show the counts enumerated with the MPN method. Growth curves are described with the NL model.

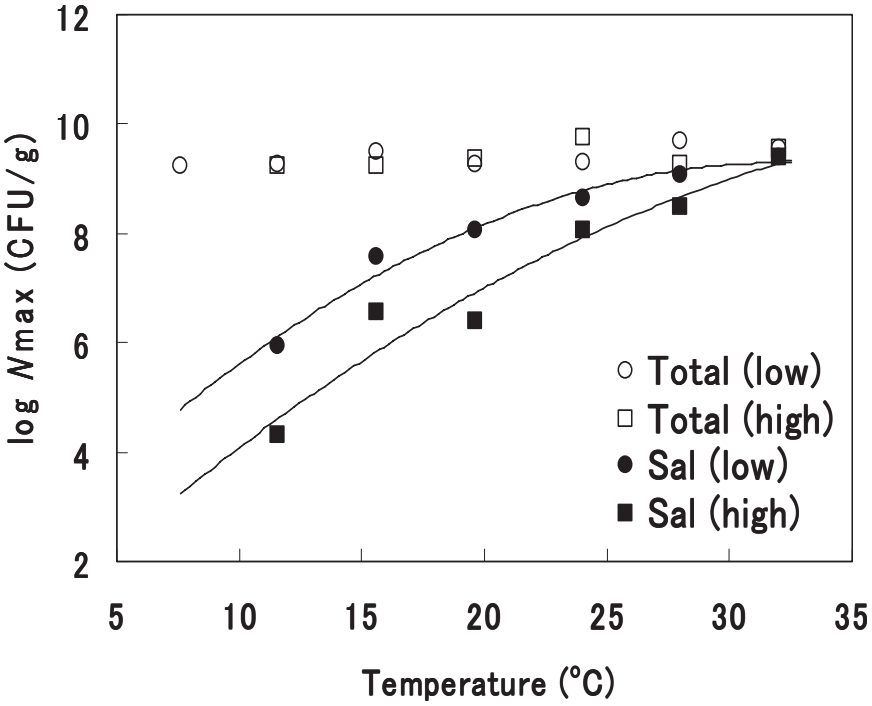

Fig. 2. The N_{max} for *Salmonella* Enteritidis and total bacteria in ground chicken with the low and high levels of NM at various constant temperatures. Total and *Sal* show total bacteria and *Salmonella*, respectively. Curves are depicted with a polynomial equation.

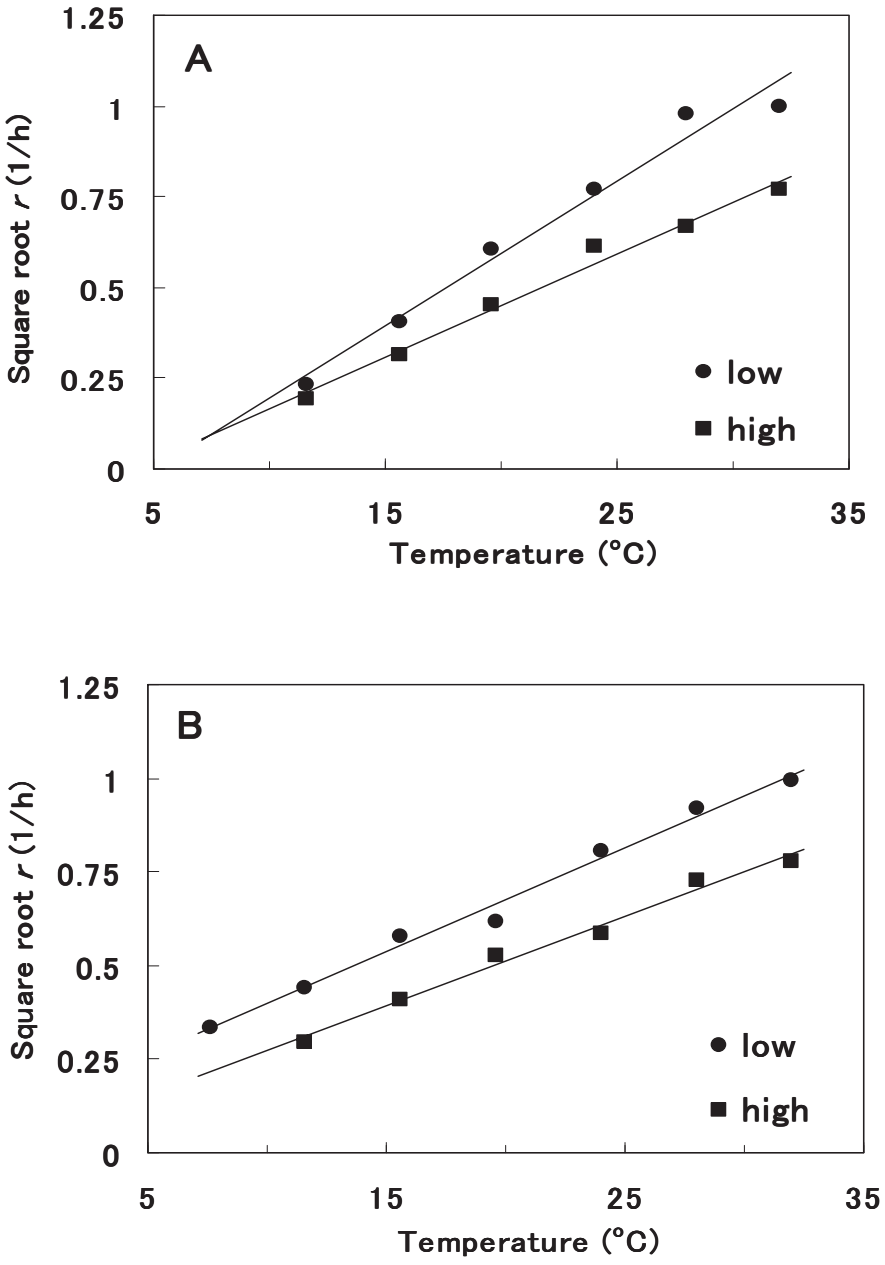

Fig. 3. The rate constant of growth for *Salmonella* Enteritidis (A) and total bacteria (B) in ground chicken with the low and high levels of NM at various constant temperatures. Values are analyzed with the square root model. Straight lines are linear regression lines.

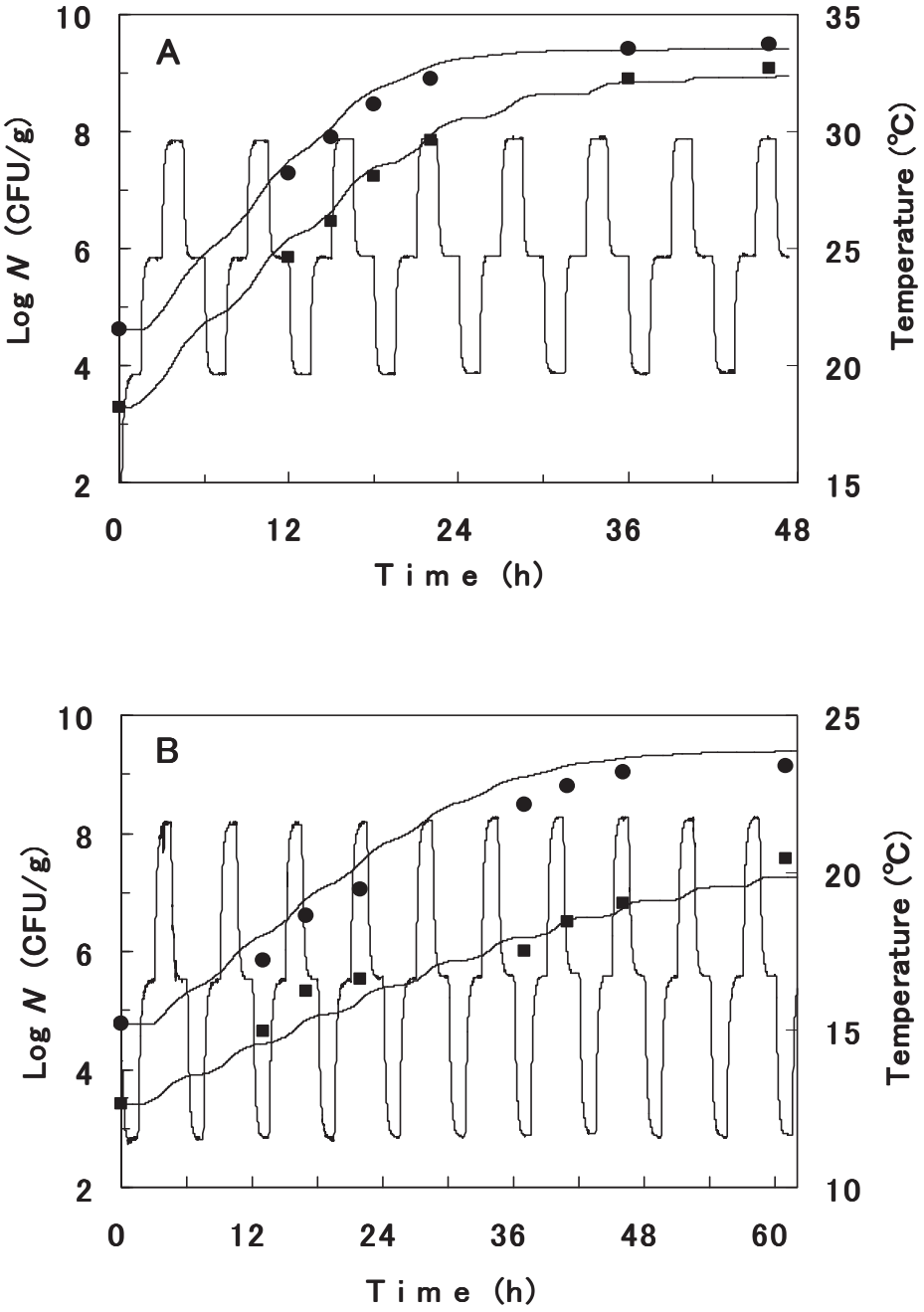

Fig. 4. Predictions of *Salmonella* Enteritidis and total bacteria in ground chicken with the low level of NM at dynamic temperature patterns A and B. Symbols; ■: *Salmonella*, ●: total bacteria. Growth curves are predicted with NL growth model. Regularly changing curves during the storage period are the measured temperature of the chicken.

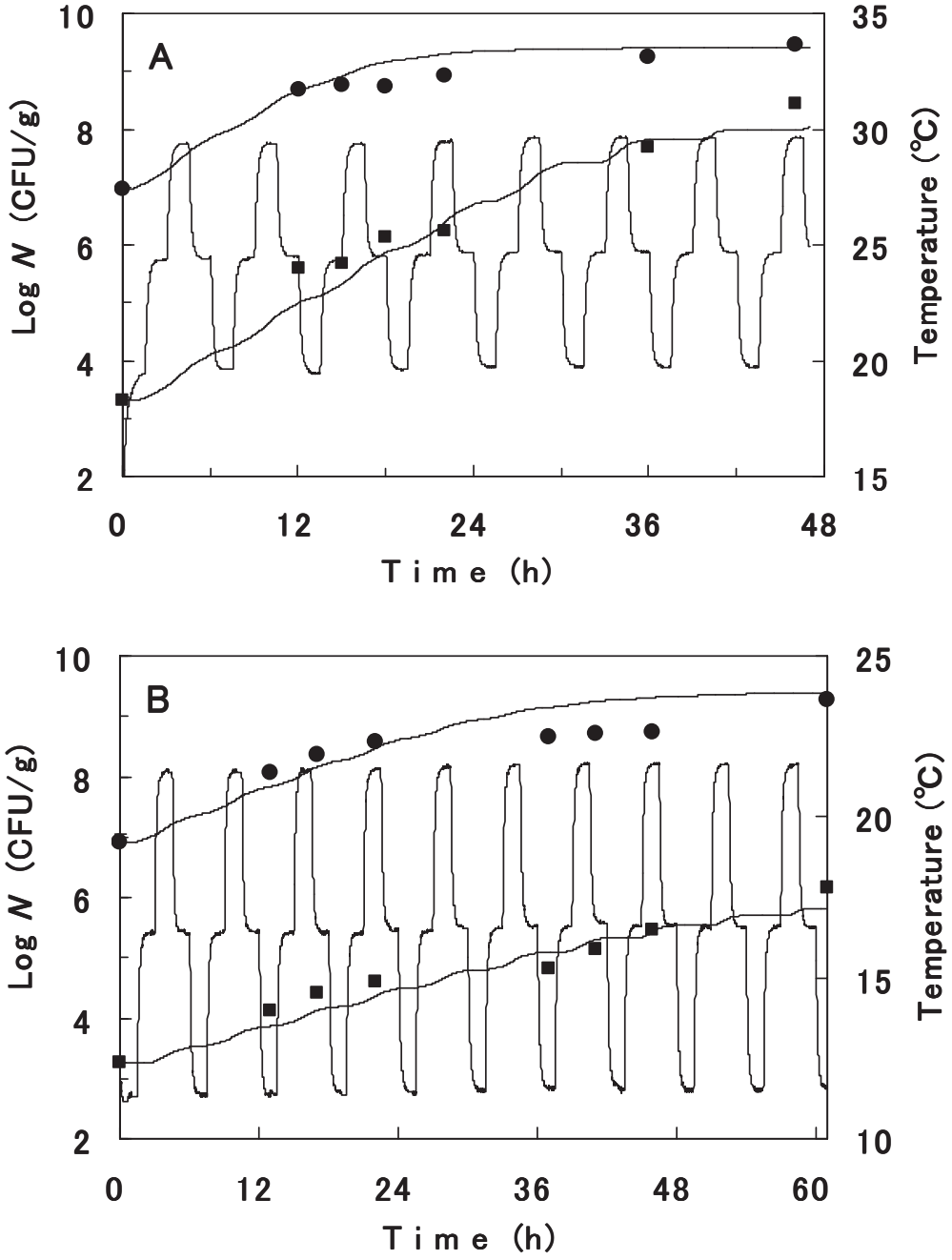

Fig. 5. Predictions of *Salmonella* Enteritidis and total bacteria in ground chicken with the high level of NM at dynamic temperature patterns A and B. Symbols; ■: *Salmonella*, ●: total bacteria. Growth curves are predicted with NL growth model. Regularly changing curves during the storage period are the measured temperature of the chicken.

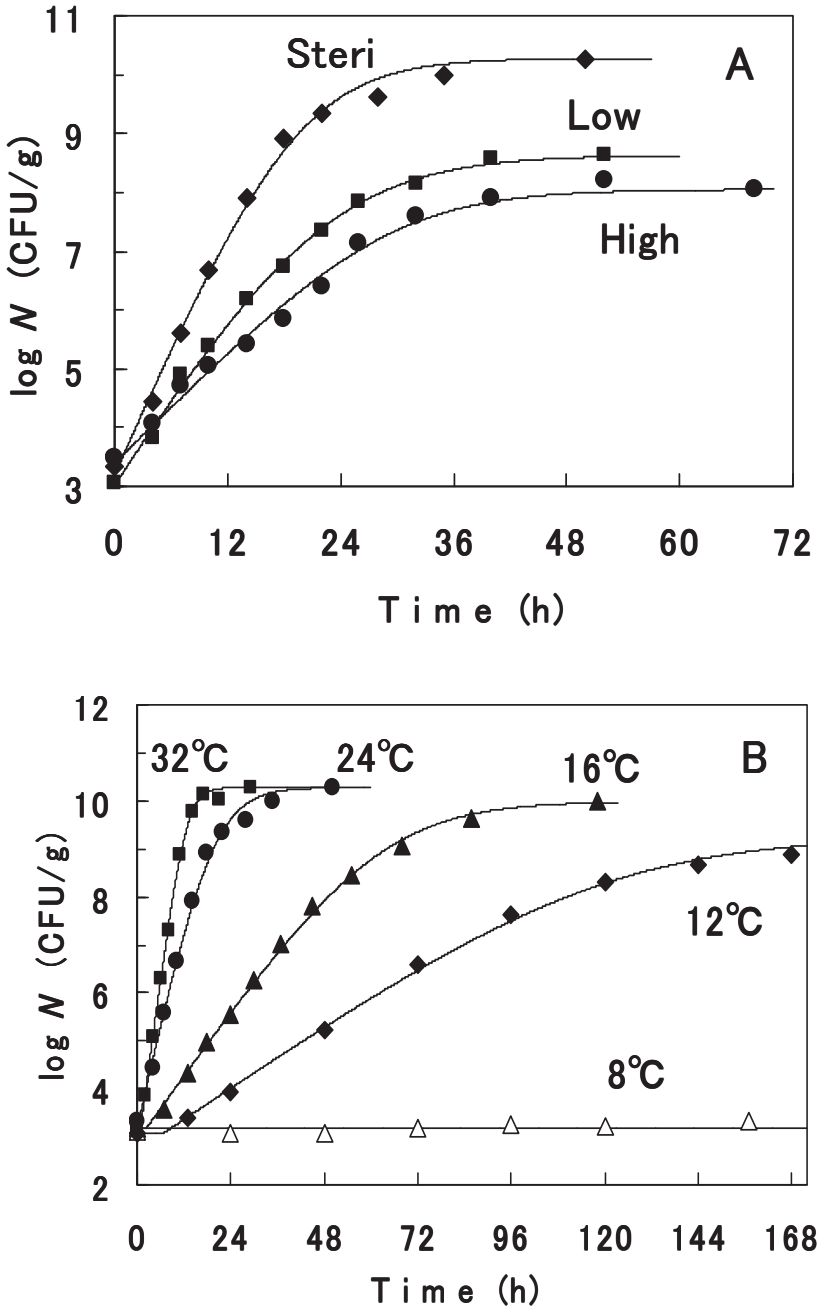

Fig. 6. Growth of *Salmonella* Enteritidis stored in sterilized chicken. A. Growth curves in ground chicken with the low (Low) and high (High) levels of NM and sterilized chicken (Steri) stored at 24˚C. B. Growth curves in sterilized chicken at various temperatures ranging from 8 to 32˚C. Symbols are experimental. Curves are described with growth model.

Chapter 2

Growth kinetics and prediction of *Salmonella* Enteritidis in liquid egg products

2.1 Introduction

According to the report of food and agriculture organization, the use of liquid egg is arising over the world (10). Recently many final food products such as cake, bread, mayonnaise are made with liquid egg, because the usage of liquid egg is easier than shell egg. Liquid egg products are classified into three types, whole liquid egg, egg yolk and white egg or albumin. Those products are pasteurized and unpasteurized. Pasteurized liquid egg is made by heating the raw liquid egg at a given temperature to kill *Salmonella*, which may contaminate eggs. Especially, *Salmonella* serotype Enteritidis, which sometimes contaminates eggs, has caused many food poisoning outbreaks worldwide and is one of the most implicated serotypes among *Salmonella* serotypes (46). Hara-Kudo and Takatori (24) reported that 73.5% of unpasteurized liquid egg products and as high as 1.7% of pasteurized products in Japan were contaminated with *Salmonella*. Unpasteurized liquid egg products are sometimes contaminated with *Salmonella* (24). Thus, when food companies use unpasteurized liquid egg products contaminated with *Salmonella*, *Salmonella* cells must be inactivated during the heating or cooking process of food production. *Salmonella* cells in such “pasteurized” products will grow to a certain level when those products are exposed to abuse temperatures after the pasteurization process. McQuestin et al. (37) described that a greater biosecurity risk is associated with cold-stored, liquid egg products that are

consumed uncooked or “lightly cooked”, typically as ingredients in meringue, home-made ice cream, and so on. Therefore, information on the growth kinetics of *Salmonella* in both pasteurized and unpasteurized liquid egg products is very important to prevent food poisoning outbreaks originating from liquid egg products contaminated with *Salmonella*.

Likewise, commercially available unsterilized food and food materials are contaminated with the NM. Competition between a pathogen and NM in a food would influence the growth of the pathogen in the food. Recently the kinetics of microbial competition in food has attracted the interest of many researchers. Those researchers have studied growth kinetics of food-borne pathogens in actual food with NM by using mathematical growth models. I also have succeeded in describing the growth of a *Salmonella* Enteritidis and NM in raw ground chicken with the NL model.

There are some papers on the growth kinetics of *Salmonella* in liquid egg. For example, McQuestin et al. (37) studied the kinetics of the growth of *Salmonella* Typhymurium DT104 in pasteurized liquid eggs. Gurtler and Conner (23) reported on the growth of *Salmonella* Enteritidis in raw, sterile liquid eggs. However, there are few studies on the growth kinetics of *Salmonella* in pasteurized liquid egg products (37). Moreover, to my knowledge, there seems to be no systematic study on the growth kinetics of *Salmonella*, especially *Salmonella* Enteritidis, in both pasteurized and unpasteurized liquid egg products.

Therefore, in this study, I first studied the growth kinetics of *Salmonella*

Enteritidis in both types of liquid whole egg products that are actually used in food industry with the NL model and evaluated how closely the model fits the actual growth. Then I examine if the NL model could predict the *Salmonella* Enteritidis growth in the above products at dynamic temperatures, because the temperature of a liquid egg product which might be contaminated with *Salmonella* would change through the periods of transportation and storage. I also examine the prediction of *Salmonella* growth by using the Baranyi model for comparison in the present study, as I analyzed the *Salmonella* growth at the constant temperatures with the model. Here I used a cocktail of four *Salmonella* Enteritidis strains to get objective data sets. *Salmonella* growth in raw, sterile liquid eggs was also studied in comparison with the growth in the two types of products. I also studied the growth of *S.aureus* in two liquid egg products and raw sterile liquid egg in comparison with *Salmonella* growth.

2.2 Materials and methods

2.2.1 Bacterial cell preparation

Four *Salmonella* Enteritidis strains SE2, SE3, SE5, and 04-137 were all isolated from independent food poisoning outbreaks in Tokyo, Japan and three *S.aureus* from retail foods in Tokyo. All strains were kindly provided by the Tokyo Metropolitan Research Institute of Public Health in Japan. Cell preparation was done in the same manner as I did for ground chicken study (chapter 1). Briefly, cells of the *Salmonella* strains were activated on XLD agar (Oxoid, Basingstoke, UK). Cells of well-grown colonies on the plates were incubated in Trypticase soy broth (Oxoid) with shaking. Cultured cells (1 ml each) of the strains were washed with saline by centrifugation. Cells of the strains were thoroughly suspended in saline (1 ml) and then mixed together. The mixture was diluted to $1:10^4$ with saline, yielding a cell suspension of about 10^5 CFU/ml. In the experiment of various initial cell doses, the cell suspension was diluted according to the doses.

2.2.2 Liquid egg

Two kinds of liquid egg products including pasteurized (60^oC for 3.5 min) and unpasteurized liquid egg was used in this study. These products were kindly provided by Kewpie Corporation Company in Tokyo. The liquid egg products were tested for several microbiological groups with selective media. The examined groups were total bacterial numbers with standard agar (Nissui Pharmaceuticals, Tokyo, Japan), aerobic spore formers with standard agar (Nissui), psychrophilic bacteria with standard agar (Nissui), the coliform group

with Desoxycholate agar (Nissui), lactic acid bacteria with MRS agar (Oxoid), *Clostridia* with *Clostridia* count agar (Nissui), and fungi with Potato Dextrose agar (Eiken Chemicals, Tokyo) (Anonymous, 2004 (2). *Salmonella* and *Bacillus cereus* in the products were examined with XLD agar and MYP agar, respectively (1).

The liquid egg products were stored in sterile plastic cups (300 ml) at -20˚C until use and before doing the experiment, it thawed at <10˚C overnight in refrigerator. Values for pH of the products were measured with a pH meter (F-52, Horiba, Kyoto, Japan).

A sterile, raw liquid egg sample was also prepared with commercially available shell eggs. Namely, fresh shell eggs were purchased at a market in Tokyo and then immersed in 80% ethanol for 30 min to kill contaminants on the shell surfaces. The shells of the sterilized eggs were then cracked to remove whole egg aseptically. The whole egg was mixed well in a sterile plastic cup. Sterility of the liquid egg was confirmed by plating a total of 1 ml of it on standard agar plates (Nissui).

2.2.3 *Salmonella* cell spiking and storage

Salmonella cell suspension was prepared as described in experiment with ground chicken study (chapter 1). Briefly *Salmonella* cell (2 ml/100 g) was spiked into liquid egg samples. After samples were thoroughly mixed, a portion (10 g) was placed in sterile glass bottles (110 ml) with caps. The glass bottles were stored in an incubator at various constant temperatures. Immediately after incubation, each sample (one bottle per data point) was taken from the

incubator and cooled in ice water. Three trials were performed on samples kept at each constant temperature. *S.aureus* cells preparation, spiking into liquid egg and storage of samples were the same as *Salmonella* method.

2.2.4 Bacterial cell counts

Salmonella cells in spiked liquid egg samples were enumerated as described for ground chicken experiments. Briefly, after 10% food homogenates were prepared for the samples with a buffered sodium chloride peptone solution, 0.1 ml of each 10-fold diluted homogenates was placed on standard method agar plates and XLD agar plates in duplicate for viable bacteria and *Salmonella*, respectively. After incubation, colonies suspected as *Salmonella* were examined for identification with a serological test using the antiserum to *Salmonella* O antigens (Denka-Seiken, Tokyo). The average bacterial number on two plates at a certain dilution for each data point was calculated.

Salmonella cells at a very low dose were enumerated with the 5-tube (MPN) method as is described in chapter one (1). The staphylococcal strains for enumeration were plated on the Baird Parker selective agar plates (Oxoid).

2.2.5 Growth models

Average bacterial counts for each data point in triplicate in the constant temperature experiments were calculated and then analyzed with the NL model, which described and was used for chicken meat analysis in chapter one (11, 12, 13, 14).

The value for *r* was experimentally estimated from the slope of the log phase in a growth curve on the semi-logarithmic plot (11). The period of the lag phase, *lag*, was also experimentally estimated as the period between the initial point

and the point where the regression line for the exponential phase intersects the horizontal line penetrating the initial point on the semi-logarithmic plot (11). Numerical data of microbial counts were analyzed with a computer program fitted to the growth model, which was developed using a spread sheet software program, Microsoft Excel (17). This program estimates *r* and *lag* with the log phase in a log-transformed, sigmoidal growth curve.

The prediction of *Salmonella* Enteritidis in liquid egg products at a dynamic temperature carried out with the NL model by using the values for *r* and N_{max} and parameters *m* and *n*, which were obtained from constant temperature experiments, into the NL model Eq. 1, chapter one (11, 12, 13, 14).

Growth data of *Salmonella* were also analyzed by the Baranyi model. Namely, the data at the constant temperatures was analyzed with a computer program, DMFit (www.ifr.ac.uk/safety/DMFit). For analysis the values for mCurv and h0 in the model were set both 10 (the default values). Values for *mumax*, which corresponds to *r* in NL model and *lag* were obtained for each data set. With the data analyzed by the program, growth curves by the Baranyi model were generated with the 4-order Runge-Kutta method on Microsoft Excel.

Values for *r* at various constant temperatures were analyzed with the square root model, which is an empirical model, but precisely describes the linear relationship between *r* and temperature (35).

2.2.6 Statistical analysis

The standard deviations, SDs, for data points were calculated from triplicate values. Statistical analysis for data including regression analyses was

performed with Microsoft Excel. Likewise performance of the model for each temperature pattern was evaluated with (i) the MSE, between log-transformed cell concentrations estimated with the model (log N_{est}) and those observed (log N_{obs}) at the observation points (12, 13, 14, 29) and (ii) the residual, which is the value of log N_{obs} minus log N_{est}, for each observation point during the growth (45).

2.3 Results

2.3.1 Microbial contamination of the liquid egg products.

The pasteurized liquid egg was examined for either *Salmonella* or other bacterial contamination; consequently the product was free of *Salmonella* and other contaminants. The unpasteurized product contained total bacteria at the level of $10^{7.3}$ CFU/g. This product was also contaminated with psychrophilic bacteria ($10^{7.6}$ CFU/g), the coliform group ($10^{2.3}$ CFU/g), the lactic acid bacteria ($10^{3.6}$ CFU/g), *Clostridia* ($10^{2.8}$ CFU/g), and fungi ($10^{5.4}$ CFU/g). No *Salmonella* or aerobic spore formers including *Bacillus cereus* were found in the unpasteurized product.

Values for pH of the pasteurized and unpasteurized products studied were 7.83 and 6.52, respectively.

2.3.2 Growth kinetics at various initial doses.

Salmonella Enteritidis growth kinetics in the pasteurized and unpasteurized liquid egg products at various initial doses of *Salmonella* was first studied at 24˚C. *Salmonella* growth curves at various initial doses in the pasteurized liquid egg product were well described with the NL model (Fig. 1A). The values for the rate constant of growth, *r* and the periods of lag, *lag* in the growth curves were similar among them (Table 1). The values of the N_{max} in the stationary phase at the various initial doses were constant (Table 1).

Growth curves of *Salmonella* in the unpasteurized product at various initial doses of *Salmonella* were also well described with the growth model (Fig. 1B). The values for *r* and lag in the growth curves were also similar among them in

the unpasteurized product, regardless of the level of microbial contamination in the product (Table 1).

A clear difference in the kinetics between the pasteurized and unpasteurized liquid egg products was found in the value for N_{max}; the N_{max} value for the unpasteurized product was lower at the lower initial level (Table 1). The values for both *r* and *lag* in the growth curves in the unpasteurized product were lower than those in the pasteurized (Table 1). The growth of NM in the unpasteurized liquid egg was limited, because the initial level was already high (Fig. 1B).

2.3.3 Growth kinetics at various constant temperatures

The growth kinetics of the pathogen in the pasteurized and unpasteurized products at constant temperatures ranging from 8 to 36˚C was then studied at the initial dose of about $10^{3.2}$ CFU/g. Growth curves of *Salmonella* in the pasteurized liquid egg stored at the constant temperatures were all sigmoidal and well described with the NL model (Fig. 2A, B). When the Baranyi model was applied to the growth, it well described the *Salmonella* growth at 12 to 36˚C, but it could not describe the growth at 8˚C because of a technical error caused in the DMFit program (Fig. 2A, B).

Growth curves of *Salmonella* in the unpasteurized product stored at the constant temperatures were also all sigmoidal and well described with growth model (Fig. 3A, B). No growth of the pathogen was observed at 8 ˚C (Fig. 3B). The Baranyi model well described the *Salmonella* growth at 16 to 36˚C, but it could not describe the growth at 12˚C because of a technical error in the

analysis program (Fig. 3A,B).

Temperature dependency of *r* in the pasteurized and unpasteurized products was precisely depicted by the square root model with a high value for coefficient of determination of 0.999 and 0.958, respectively (Fig. 4). The regression lines in the pasteurized and unpasteurized products are shown in Equations, 1 and 2, respectively.

$$\sqrt{r} = 0.0473(T - 4.02) \quad [1]$$

$$\sqrt{r} = 0.0313(T - 4.48) \quad [2]$$

Here *T* is temperature (˚C). Values for N_{max} in the pasteurized product were almost constant, being $10^{9.7}$±$10^{0.24}$ CFU/g as the average (Fig. 5). Values for log N_{max} increased linearly with the temperatures in the unpasteurized product and were expressed by Equations [3] with a high value for coefficient of determination of 0.962 (Fig. 5).

$$N_{\max} = 10^{0.906T + 2.92} \quad [3]$$

Parameters *m* and *n* in the growth model, which described in chapter one, at the constant temperatures, were estimated to be 0.42 and 4.9 as the average, respectively in the pasteurized product and 1.5 and 11.5, respectively in the unpasteurized. These values have been used for prediction of *Salmonella*

at dynamic temperatures.

2.3.4 Growth prediction of *Salmonella* Enteritidis at dynamic temperature

With the values for r and N_{max} estimated with equations 1-3 and the above parameter values, the NL model succeeded in predicting the growth of *Salmonella* in both liquid egg products stored at various dynamically changing temperatures (Figs. 6A, B and 7A, B). Here, the dynamic temperature patterns included (A) high and (B) low temperature ranges. Values of MSE in log units for those predictions in the figures were very small, being 0.052 (6A), 0.13 (6B), 0.041 (7A), and 0.014 (7B). The residuals between the observed and predicted populations (in log) for the points in the growth curves in Figs. 6 and 7 were plotted along the time (Fig. 8).

For comparison, the Baranyi model was also examined for its capacity to predict *Salmonella* growth at the dynamic temperatures in the two types of liquid egg products. The values in the temperature range between 20 and 36 ˚C in the pasteurized liquid egg were relatively constant; the average for the value in this range was estimated to be 3.34±0.516. Thus, with this average, I predicted the *Salmonella* growth at the dynamic temperature pattern of the higher range in the present study with the Baranyi model. As a result, the model could well predict the bacterial growth at a changing temperature (Fig. 6A). The *MSE* value (0.075 log) was small, but a little higher than that by the NL model (0.052 log). These results suggested that the Baranyi model could be applied for the growth prediction in a limited temperature range where the numeral product is relatively constant.

2.3.5 Difference among whole liquid eggs.

Salmonella growth kinetics in the pasteurized and unpasteurized products and a raw sterile liquid egg sample were compared at 24˚C (Fig. 10). The growth in the unpasteurized product was greatly suppressed in comparison with that in the pasteurized product; large differences between the two products in N_{max} ($10^{9.76}$ in the pasteurized versus $10^{5.07}$ in the unpasteurized) and the slope of the log phase, r (0.887 versus 0.272) were observed. These differences between the products were also observed at other temperatures, as shown in Figs. 2 and 3. The *Salmonella* growth in the raw, sterile liquid egg was slightly suppressed in comparison with that in the pasteurized product at the same temperature; the growth rate in the raw sample slightly slowed down (r = 0.664), while the N_{max} value in the raw sample ($10^{9.53}$) was close to that in the pasteurized. On the other hand, the growth in the raw liquid egg was much greater than that in the unpasteurized product (Fig. 10).

2.3.6 Growth of *S. aureus* in liquid egg products

For comparison between growth of *S. aureus* and *Salmonella*, the growth of *S. aureus* was studied in the pasteurized, unpasteurized and raw sterile liquid egg. Staphylococcal growth was strongly suppressed in the unpasteurized product, but the *S. aureus* shows a remarkable growth in the pasteurized and raw sterile liquid egg (Fig.11). The growth rate of *S.aureus* was lower in raw sterile liquid egg than pasteurized products, but the N_{max} of both samples are close to each other (Fig.11).

2.4 Discussion

The *Salmonella* Enteritidis growth difference between the pasteurized and unpasteurized liquid egg products was remarkable. A similar difference was seen for *Salmonella* Enteritidis in raw and sterilized ground chicken. The growth characteristics of *Salmonella* Enteritidis in the pasteurized liquid egg at various initial doses and various constant temperatures in the present study were also the same as those of *Escherichia coli* in broth and media (12, 13). The reason for the difference in *Salmonella* growth between the two liquid egg products may be the existence of NM in the unpasteurized product, thus was already discussed in previous study with raw ground chicken. Namely, competition between *Salmonella* and NM in the unpasteurized liquid egg is thought to suppress the *Salmonella* growth.

It is already reported that *Salmonella* growth in the albumen is inhibited due to the existence of bactericidal substances like lysozyme and ovotransferrin, while the yolk is rich in nutrients and has no bactericidal substances (55). Gurtler and Conner (23) studied *Salmonella* Enteritidis growth in sterile liquid egg and reported that the growth of the pathogen was greater in the egg yolk, then the whole egg and albumen at 23 and 37˚C. Schoeni et al. (49) also reported similar results for three serotypes of *Salmonella*. Gast et al. (19) also reported significant differences in *Salmonella* Enteritidis multiplication by inoculation sites (yolk > vitelline membrane > albumen).

The pH value of the pasteurized product (7.83), which was similar to that (7.6) reported by Musgrove et al (40), was not high enough to suppress

bacterial growth. It is known that the pH of albumen is between 7.6 and 7.9, but it gradually increases to more than 9 during storage (40). This increase in pH would also suppress bacterial growth in albumen. The pH value of the unpasteurized product was (6.52), which was close to 7, would be suitable for growth of *Salmonella* and many species of NM in the product (28). The activity of lysozyme is at the optimum pH at 6 - 7 (50), but the suppression of the *Salmonella* growth by the bactericidal substances in the raw, sterile egg was weak in the present study. Thus, the strong suppression of the *Salmonella* growth in the unpasteurized product would possibly be due to the competition between NM and the pathogens, as I found the same phenomenon for *Salmonella* growth in raw ground chicken with NM and sterilized ground chicken.

It is interesting that the deceleration period which is located at the shoulder in the growth curves was generally found at around 15 h of storage for various initial *Salmonella* doses (Fig. 1B). At that time the level of NM (total bacteria counts) had already reached the maximum in the product (Fig. 1B). Similar results were also observed for *Salmonella* growth in raw ground chicken with a high level of NM. This is not likely coincident to the Jameson effect that notes that competing microbial populations stop growing simultaneously (7, 27, 48). Some investigators reported that the effect is not applicable to every interaction of competing microbial populations (7, 38). Unknown factors other than the Jameson effect might affect the *Salmonella* growth in the unpasteurized product in this study.

The concentration of total bacteria (NM) in the unpasteurized liquid egg product ($10^{7.3}$ CFU/g) was higher than I expected. I thought that the contaminants possibly came from the original shell eggs and by the cross contamination during the processes of the production, storage and transportation of the liquid egg products. The product was also highly contaminated with fungi ($10^{5.4}$ CFU/g). Most of the fungi were yeast. *Torula* spp. is known as the yeast often found in eggs (28).

The growth curves of NM in the unpasteurized product at the constant temperatures were not sigmoidal in the present study (Fig. 1B and 3A, B); there were no clear lag phases observed in the curves. This might come from the fact that the initial level of NM in the product was already considerably high. Thus, the growth data of NM at constant temperatures could not be analyzed with growth model; there were very few data points to analyze before the growth reached the stationary phase in those curves (Fig. 1B and 3A,B).

In the prediction curve the acceptable rates in the range of +0.5 and -1.0 log for all data points were 100% (27/27), which were considerably over the level of acceptance of 70%. When a narrower acceptable range was set between +0.5 and -0.5 log, a proposition as high as 92.6% (25/27) was still within the acceptable range. The average of the residuals for all points was -0.138 log, meaning that the observed populations were slightly smaller than the predicted ones. These results showed that the NL model successfully predicts the *Salmonella* growth in the liquid egg products at the changing temperatures. This prediction for *Salmonella* growth in unpasteurized products at dynamic

temperatures would be the first finding to my knowledge.

I could not predict the growth of NM in the unpasteurized liquid egg products with the NL model in this study, while I could do so for the NM in the raw ground chicken. This came from the fact that the growth data of NM at the constant temperatures in the liquid egg could not be analyzed with the NL model, because the initial level of NM in the product was too high to make a sigmoidal growth curve during the incubation. If the initial level of NM of the liquid egg had been low enough to show a sigmoidal curve, the model could have predicted the growth of NM.

For comparison, the Baranyi model was also examined for its capacity to predict *Salmonella* growth at the dynamic temperatures in the two types of liquid egg products. The model is built under the prerequisite condition that the (numerical) products of the maximum specific growth rate (1/h) and the lag period (h) in the growth curves of a given microorganism under various conditions should be constant (3). With these values, one can predict microbial growth under a new condition with this model (3).

Thus I first estimated the numerical products for the *Salmonella* growth curves in the liquid egg samples at the constant temperatures ranging from 8 to 36˚C. The values for the maximum specific growth rate and the lag period for the growth curves were estimated with a computer program, DMFit (www.ifr.ac.uk/safety/DMFit), which analyzes microbial growth data with the Baranyi model. Consequently, the products at the constant temperatures were constant in neither liquid egg product; the values were lower at the lower

temperatures (Fig 9). The reasons for this tendency were can not understood. However, the values in the temperature range between 20 and 36 ˚C in the pasteurized liquid egg were relatively constant; the average for the value in this range was estimated to be 3.34±0.516. Thus, with this average, I predicted the *Salmonella* growth at the dynamic temperature pattern of the higher range in the present study with the Baranyi model. As a result, the model could well predict the bacterial growth at a changing temperature (Fig. 1A). The MSE value (0.075 log) was small, but a little higher than that by the NL model (0.052 log). These results suggested that the Baranyi model could be applied for the growth prediction in a limited temperature range where the numeral product is relatively constant.

The present study showed that the NL growth model successfully described the growth and prediction of *Salmonella* Enteritidis in the pasteurized and unpasteurized liquid egg products at various constant and dynamic temperatures. In my recent study, I succeeded in predicting the growth of the *Salmonella* Enteritidis at dynamic temperatures in raw ground chicken with the values of the variables and the parameters obtained at constant temperatures.

The *Salmonella* growth in the raw sterile liquid egg was slightly slowed down in comparison with the pasteurized product in the present study. As it is described above, it is known that the growth inhibition of *Salmonella* in albumen is possibly due to the existence of bactericidal substances in albumen (55). Actually, the effect of those substances on *Salmonella* growth in the raw whole liquid egg was not bactericidal, but bacteriostatic in the present study; the

growth was just slowed down. Similar results were also observed for *S. aureus* strains a gram-positive pathogen, in the above liquid egg products in my preliminary study. From these results, the bactericidal effect of egg albumen on *Salmonella* and *S. aureus* is thought to be decreased or masked in a mixture of raw whole egg. On the other hand, in commercial, pasteurized liquid egg that is obtained by heating at 60˚C for 3.5 min, some of the lysing enzymes in egg albumen might be inactivated by the heating. This would account for the growth difference between the pasteurized product and the raw, sterile liquid egg.

The program would be a useful tool for those concerned to estimate the bacterial growth in such food materials at the temperature history they measure.

2.5 Summary

The growth of a *Salmonella* Enteritidis was studied in pasteurized and unpasteurized liquid egg products. The NL model was used for analysis of the growth. The unpasteurized product contained the total bacteria at $10^{7.3}$ CFU/g, but no *Salmonella*. When the products were spiked with *Salmonella* at various doses ranging from 10^1 to 10^4 CFU/g, the growth curves of the pathogen at 24˚C were well described with the model. *Salmonella* growth curves at constant temperatures from 8˚C to 36˚C in the two products were also well described with the model. The Baranyi model also described well most of the growth curves. The rate constants of growth for *Salmonella* at various constant temperatures were well described with the square root model. The N_{max} of *Salmonella* was constant at all temperatures in the pasteurized products, while a linear relationship between log N_{max} and temperature was observed in the unpasteurized one. *Salmonella* growth in the unpasteurized product was highly suppressed in comparison with that in the pasteurized.

With the estimated values of the parameters in the model, it successfully predicted the *Salmonella* growth in the liquid egg products at dynamic temperatures in high and low ranges. The Baranyi model, which is well known worldwide, could predict *Salmonella* growth in the pasteurized product at the dynamic temperature conditions in the high range only.

For comparison the *S*. Enteritidis and *S. aureus* growth was studied in raw sterile egg and two liquid egg products too. Both *S*. Enteritidis and *S. aureus* were slightly suppressed in sterile liquid egg in comparison with pasteurized

liquid egg. *S.aureus* could not able to grow in unpasteurized liquid egg whereas *Salmonella* had grown in this product.

This study suggested the suitability of the model for application in the *Salmonella* growth analysis and prediction in pasteurized and unpasteurized liquid egg products.

Table 1. Growth characteristics of *Salmonella* Enteritidis at various initial doses in the pasteurized and unpasteurized liquid egg products.

A. Pasteurized liquid egg

Initial doses (log CFU/g)	*r* (1/h)	*lag* (h)	N_{max} (log CFU/g)
4.3	1.19	4.9	9.8
3.3	0.85	4.0	9.9
2.5	0.91	4.6	9.8
1.5	0.94	4.9	9.8
Av±SD	0.96±0.14	4.6±0.38	9.8±0.032

B. Unpasteurized liquid egg

Initial doses (log CFU/g)	*r* (1/h)	*lag* (h)	N_{max} (log CFU/g)
4.2	0.28	1.9	6.0
3.2	0.29	2.0	5.0
2.3	0.33	2.4	4.1
1.2	0.36	3.1	3.4
Av±SD	0.32±0.038	2.4±0.57	

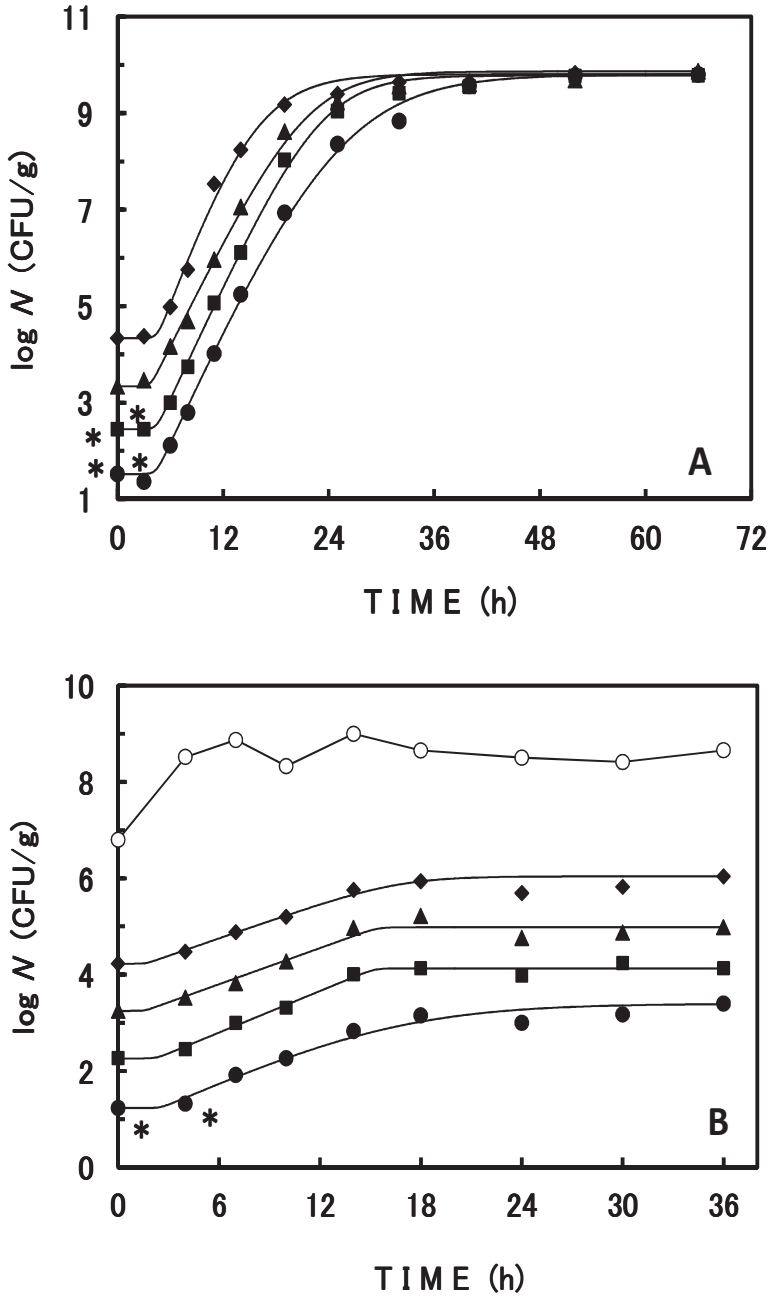

Fig. 1. Growth curves of *Salmonella* Enteritidis in liquid whole egg products spiked at various initial doses. Pasteurized (A) and unpasteurized liquid egg products (B) were spiked with the pathogen and then stored at 24˚C. Symbols: ◆, 10^4 :▲ , 10^3: ■, 10^2, and ●, 10^1 CFU/g. Open circles in (B) show the level of NM of the unpasteurized product. Asterisks show the counts estimated with the MPN method. Growth curves are described with the NL model.

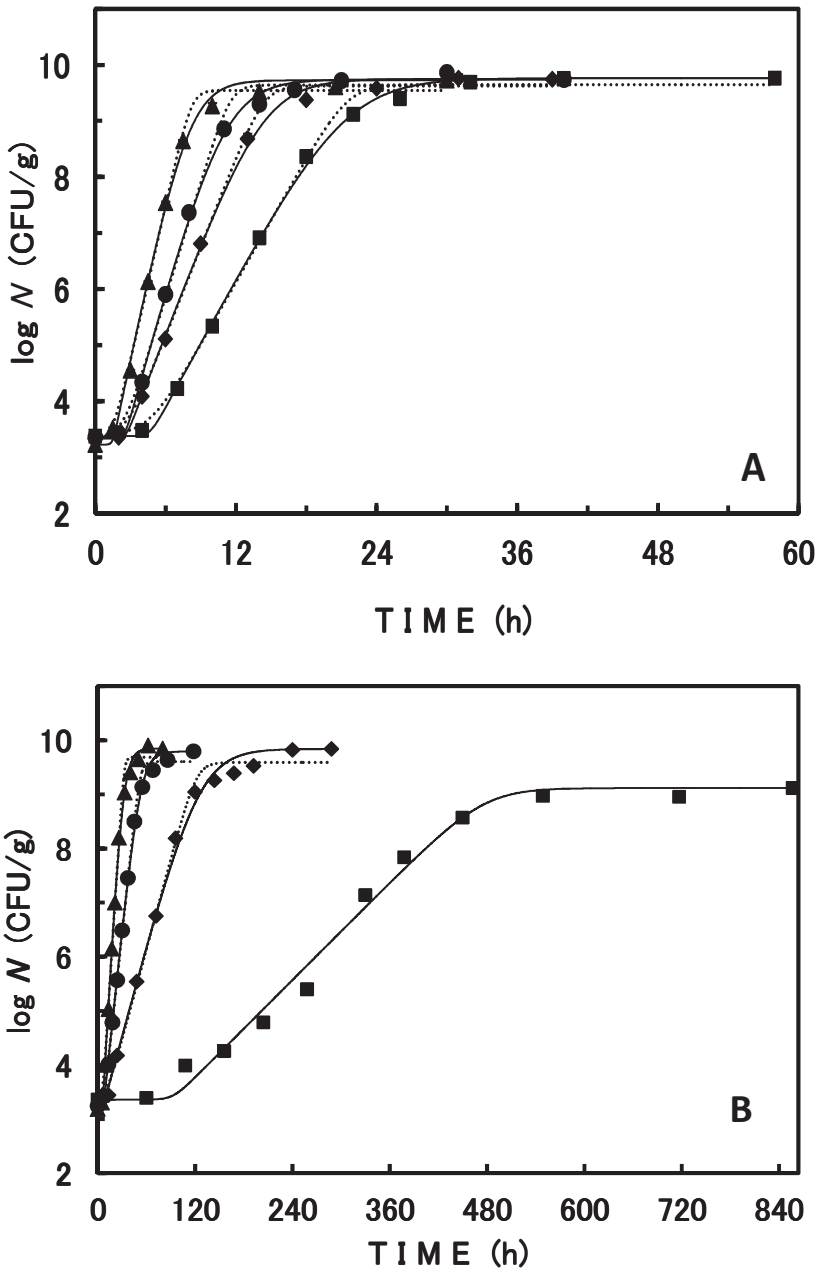

Fig. 2. Growth curves of *Salmonella* Enteritidis in pasteurized products stored at various constant temperatures. Symbols: (A) ▲, 36˚C; ●, 32˚C; ◆, 28˚C; ■, 24˚C; (B) ▲, 20˚C; ●, 16˚C; ◆, 12˚C; ■, 8˚C. Bars which indicate SDs at data points are too short to appear in the figure. Growth curves are described with the NL model (solid lines) and the Baranyi model (dotted lines).

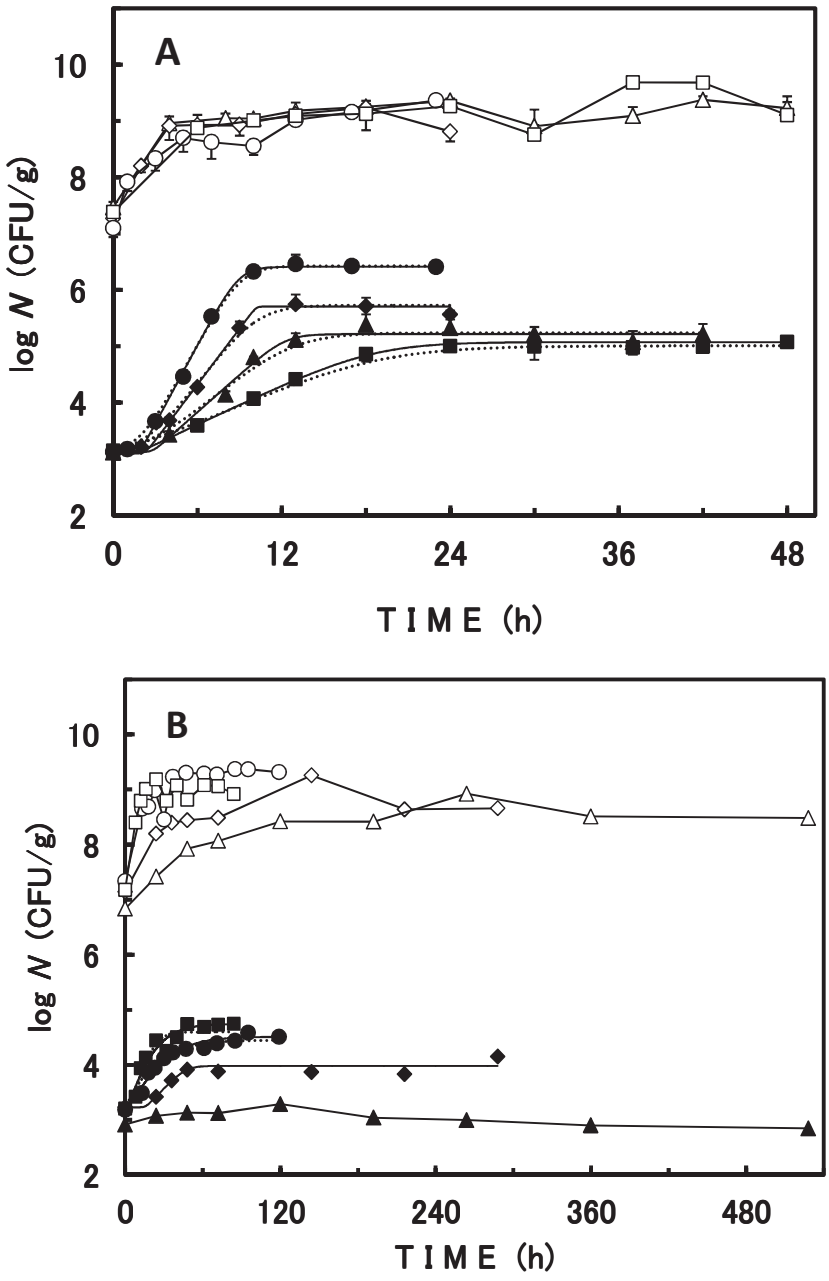

Fig. 3. Growth curves of *Salmonella* Enteritidis in unpasteurized products stored at various constant temperatures. Symbols: (A) ●, 36˚C; ◆, 32˚C; ▲, 28˚C; ■, 24˚C: (B) ■, 20˚C; ●, 16˚C; ◆, 12˚C; ▲, 8˚C. Open symbols show the levels of NM at corresponding temperatures. Bars indicate SDs at data points. Growth curves are described with the NL model (solid lines) and the Baranyi model (dotted lines).

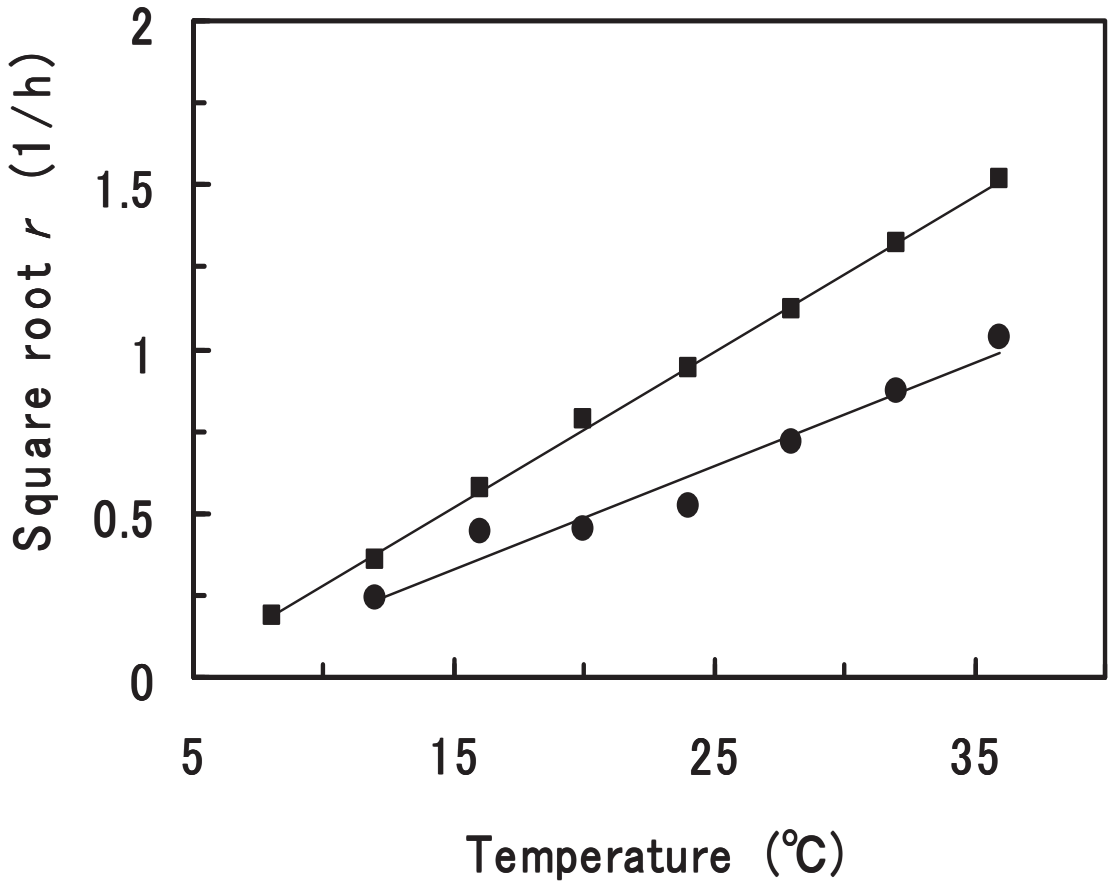

Fig. 4. The rate constant of growth for *Salmonella* Enteritidis in liquid egg products at various constant temperatures. Symbols: ■, pasteurized liquid egg; ●, unpasteurized liquid egg. Values are analyzed with the square root model. Straight lines are linear regression lines.

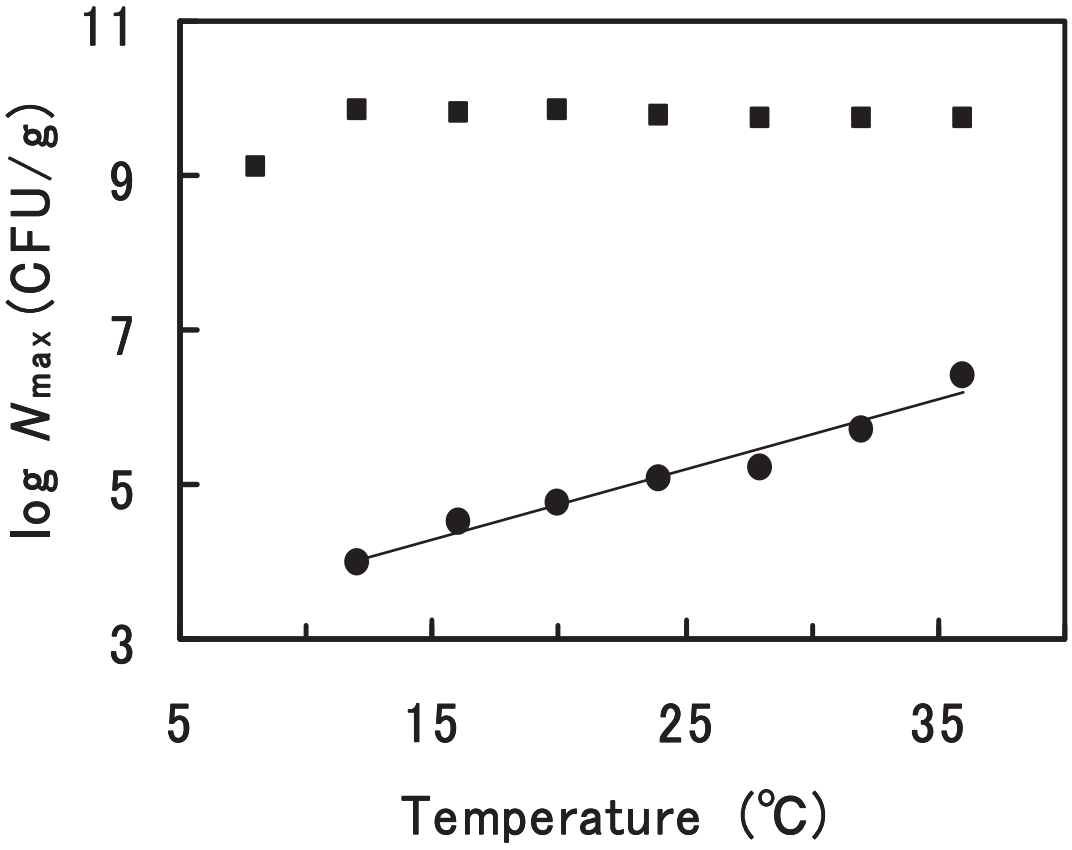

Fig. 5. Values for N_{max} at various constant temperatures in liquid egg products. Symbols: ■, pasteurized liquid egg; ●, unpasteurized liquid egg. The straight line is a linear regression line.

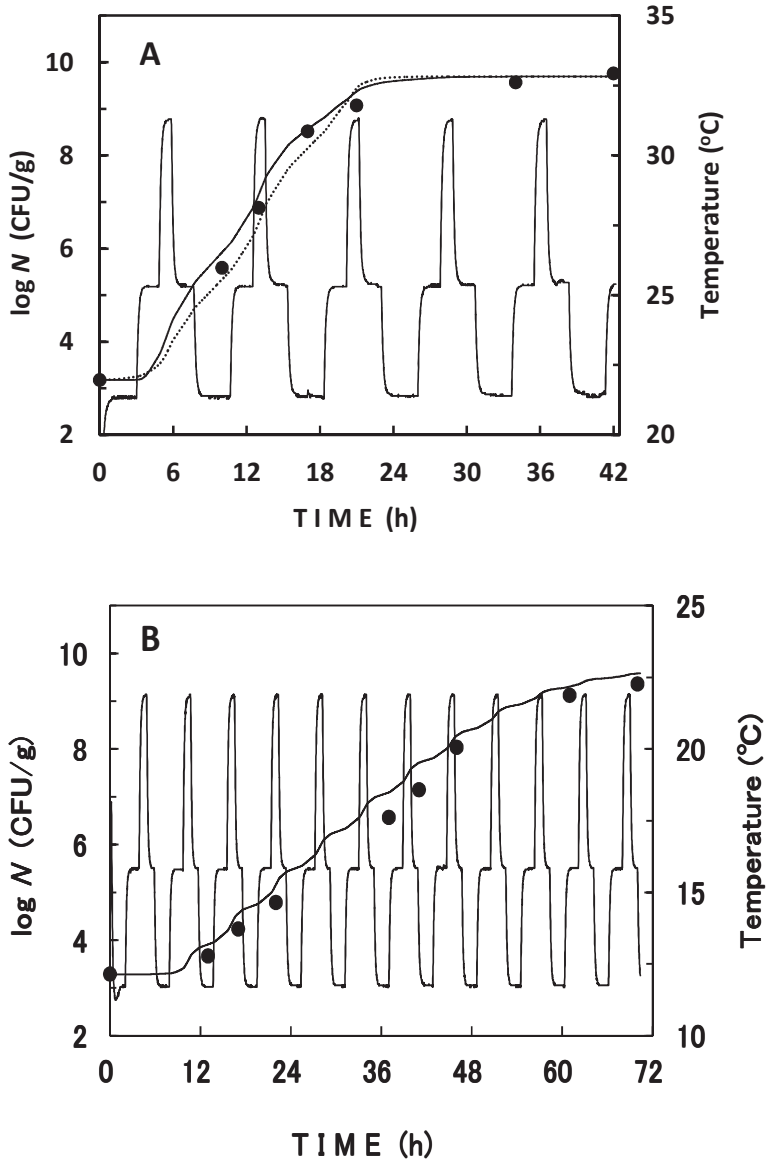

Fig. 6. Prediction of *Salmonella* Enteritidis growth in pasteurized liquid egg at dynamic temperature patterns in the high range (A) and the low range (B). Closed circles are the measured *Salmonella* population. Bars indicate SDs at data points, but they are too short to show in the figures. Growth curves are predicted from the measured temperatures (regularly changing curves) during the storage period with the NL model (solid lines) and the Baranyi model (a dotted line). Regularly changing curves during the storage period are the measured temperature of the product.

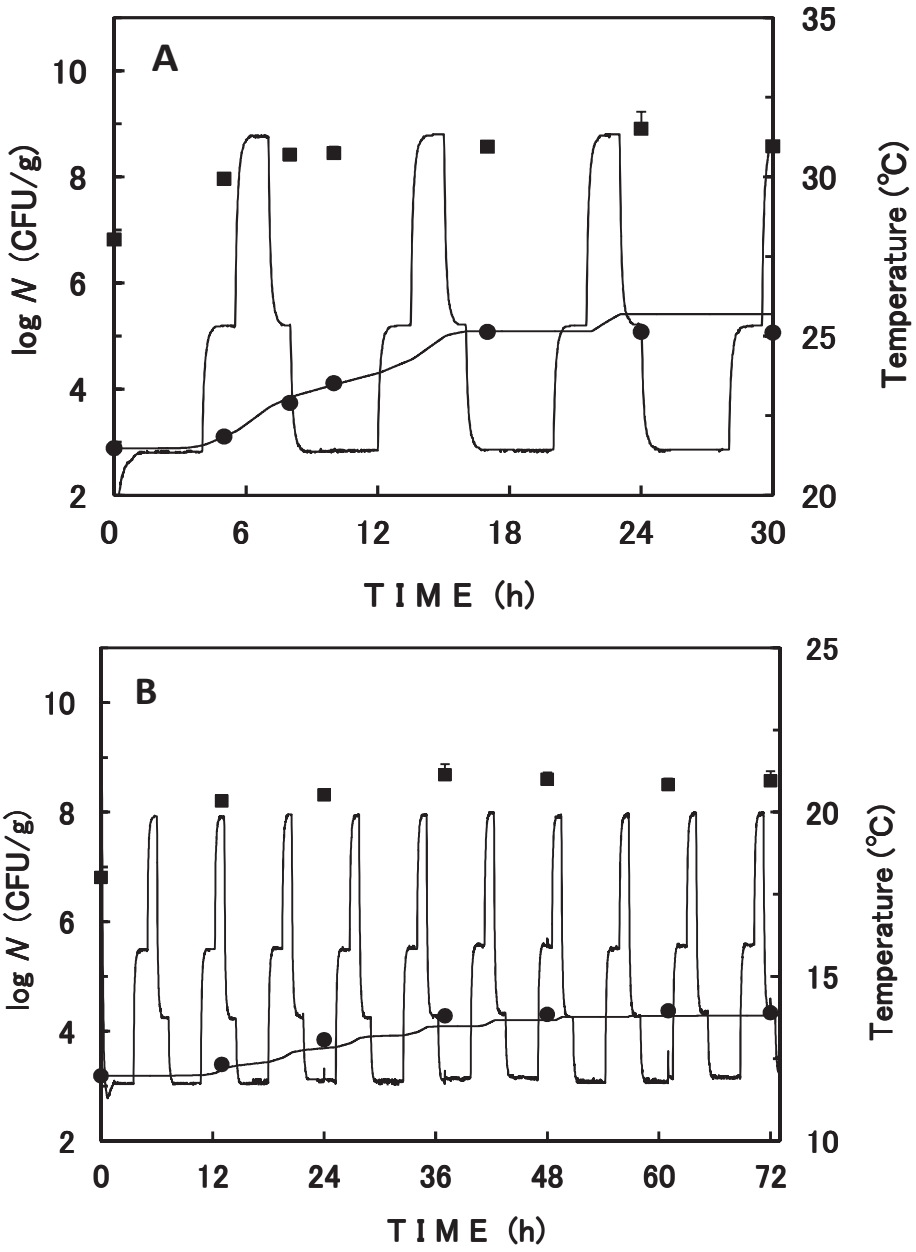

Fig. 7. Prediction of *Salmonella* Enteritidis growth in unpasteurized liquid egg at dynamic temperature patterns in the high range (A) and the low range (B). Closed circles (●) and squares (■) are the measured populations of *Salmonella* and NM, respectively. Bars indicate SDs at data points. Growth curves are predicted with the NL model. Regularly changing curves during the storage period are the measured temperatures of the product.

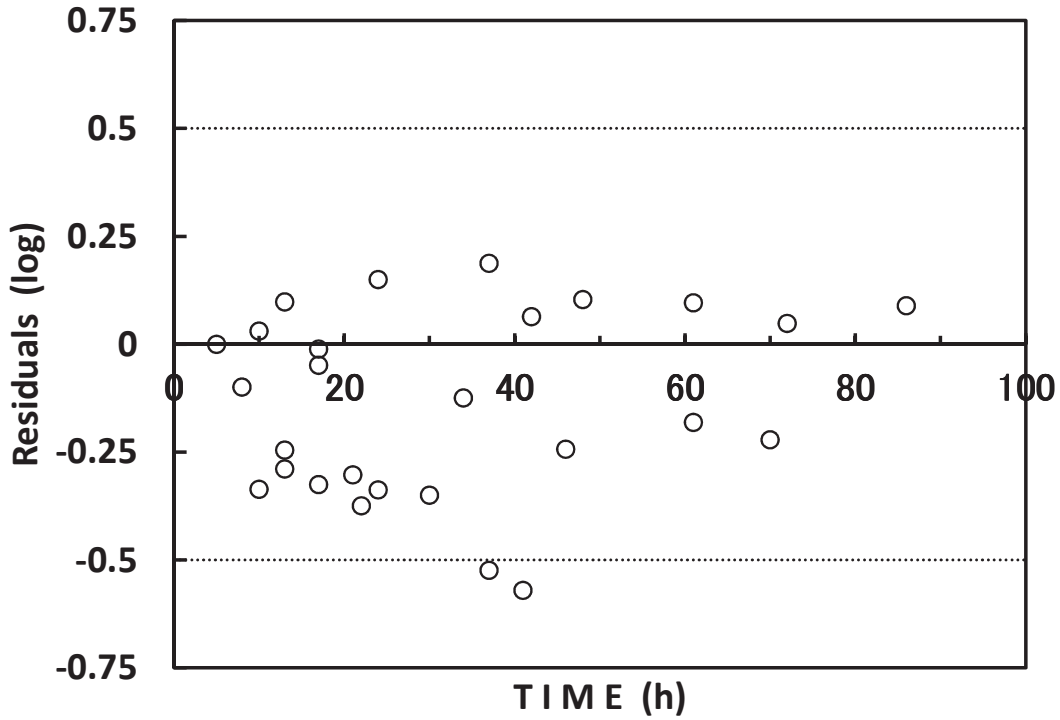

Fig. 8. Residual plots for the populations by the NL model along through the storage period. All residuals between observed and estimated values shown in Figs. 1 and 2 are plotted. Dotted lines show the boundaries of acceptance.

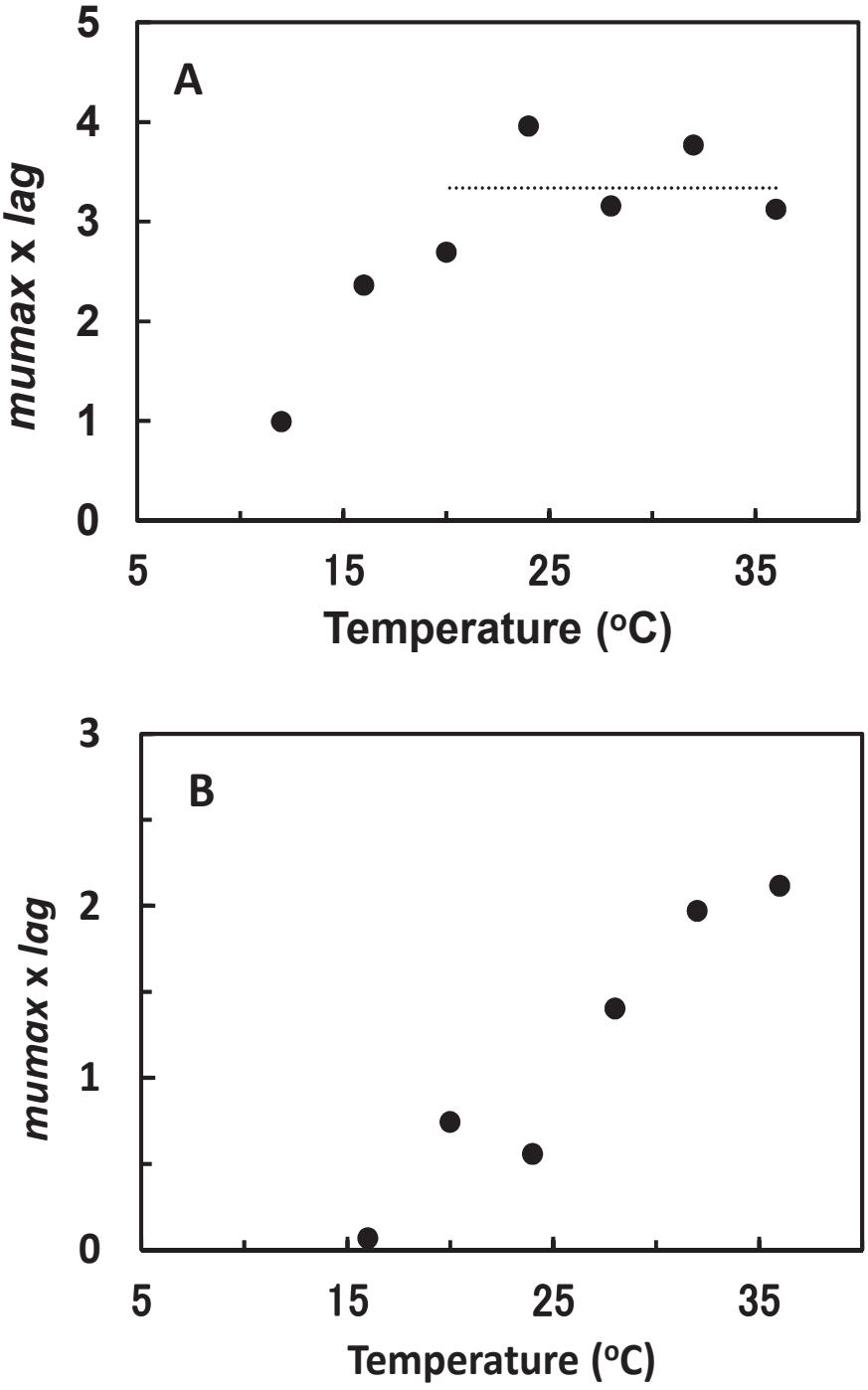

Fig. 9. *Salmonella* growth products of mumax and lag at constant temperatures in pasteurized liquid egg (A) and unpasteurized liquid egg (B).

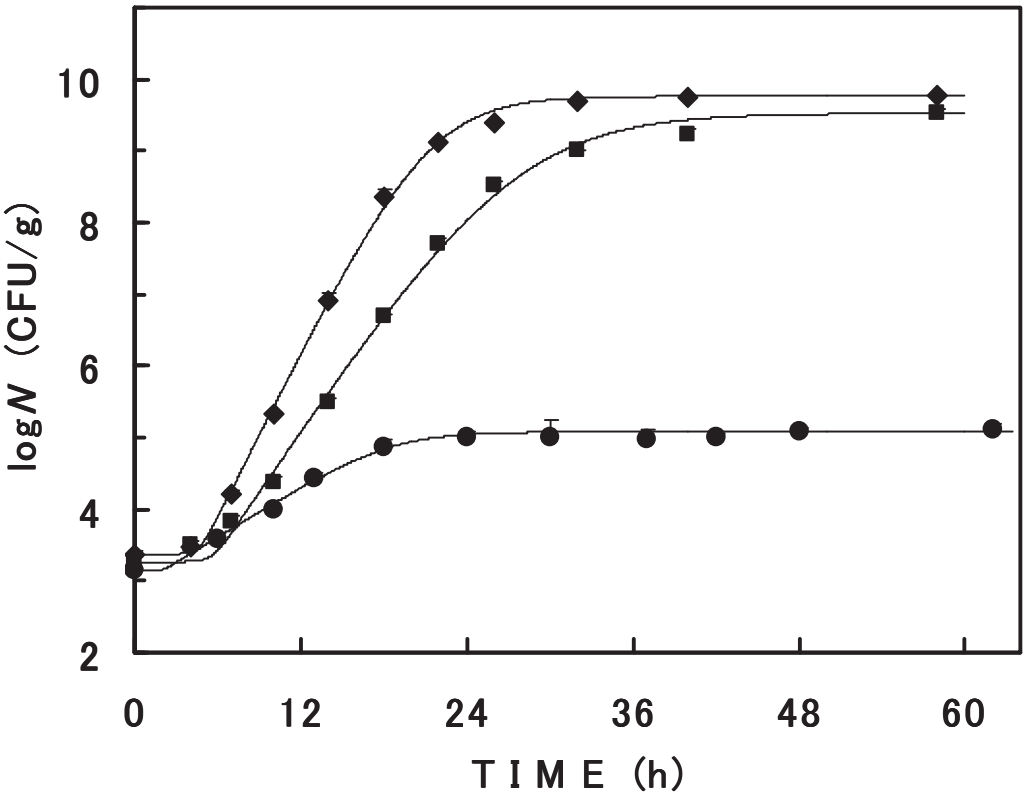

Fig.10. Difference in *Salmonella* Enteritidis growth among pasteurized and unpasteurized products and raw, sterile liquid egg stored at 24°C. Symbols:◆ pasteurized liquid egg ■, sterile raw liquid egg and●unpasteurized liquid egg. Bars shows SDs at each data point.

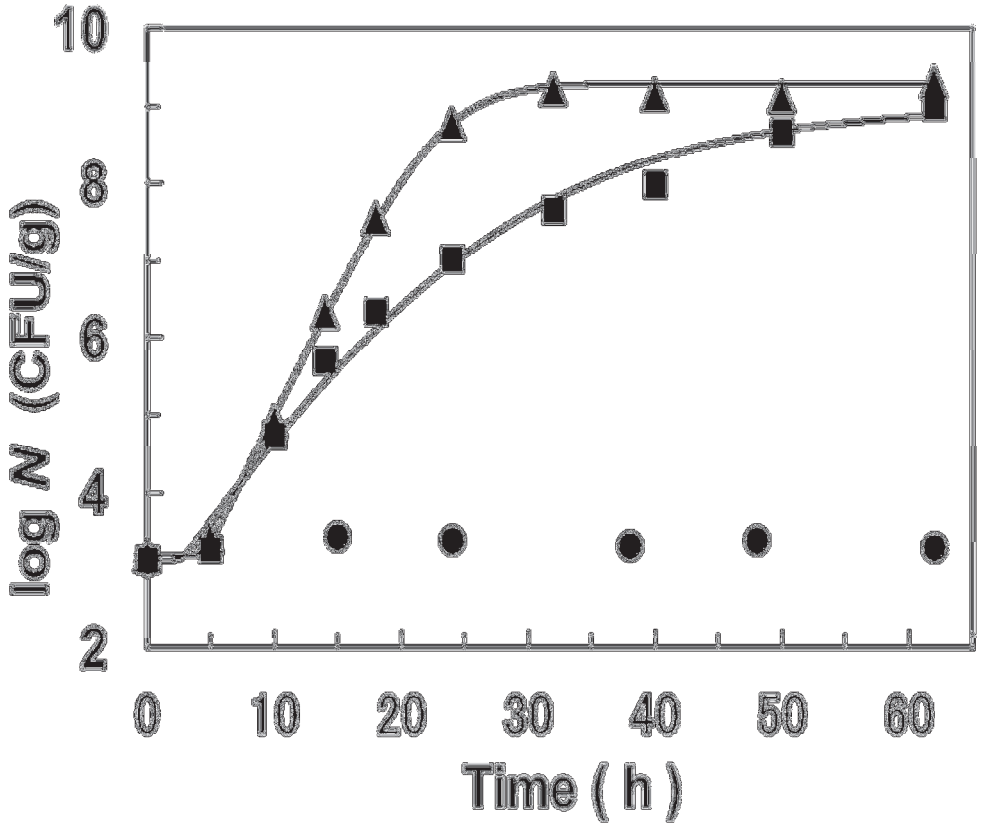

Fig.11. Difference in *S. aureus* growth among pasteurized, unpasteurized products and raw, sterile liquid egg stored at 24°C.

Symbols: ▲ pasteurized liquid egg ■, sterile raw liquid egg and●unpasteurized liquid egg.

Chapter 3

Development of predictive program for *Salmonella* Enteritidis growth in ground chicken and whole liquid egg products

3.1 Introduction

Predictive microbiology models can be a tool to reduce microbial food poisoning outbreaks. In predictive microbiology, expert computer programs have been developed for users in food industry. When users input environmental data such as temperature, pH, and water activity into the program, it instantly predicts microbial growth and/or death on a computer screen. Pathogen Modeling Program (PMP) is one of the representative examples of these programs (http://pmp.arserrc.gov/PMPHome.aspx). Combase is also a database program for predictive microbiology (http://wyndmoor.arserrc.gov/combase/). Some predictive programs have been developed for the growth of *E.coli*, *S.aureus*, and *Vibrio parahaemolyticus* using a commercial spread-sheet calculation program, Microsoft Excel (15, 18). The predictive programs can be provided free from the Japan Food Industry Center (http://www.shokusan.or.jp/haccp/news/index_18.html) (15, 18).

However NL predictive programs are for growth of the target microorganisms in culture media and pasteurized food (milk). PMP and Combase are also mostly based on the microbial growth data in culture media and sterilized food. On the other hand, many of commercial foods and food materials are contaminated with various kinds of microorganisms. Growth of pathogens like *Salmonella* and *S. aureus* in food is often suppressed by other

contaminants in it by microbial competition and/or antimicrobial substance production (28). Thus, it is required to estimate the growth of those harmful microorganisms in real foods and food materials with contaminants for the food safety as the second stage of predictive microbiology.

Among the pathogens related to food poisoning, *Salmonella* is one of the most important one. It often contaminates food materials including egg, meat, chicken, and vegetable (28). Especially, there were world-wide, serious *Salmonella* outbreaks with serotype Enteritidis (46). Thus, we recently reported the prediction of growth of *S.* Enteritidis and NM in ground chicken and liquid egg products with the NL model. In the present study, therefore, we report a predictive program developed for the pathogen in the food materials. The language used on the screen of this program is English; users easily operate and predict the *Salmonella* growth on the screen. This is similar to other NL predictive programs which are described above (15, 18).

3.2 Method and materials

The NL model was used to predict the growth of *Salmonella* Enteritidis and NM in ground chicken and liquid egg products at various temperature patterns, to develop a new predictive program. The new logistic model with its related parameters has been completely described in chapter 1.

The values for *m* and *n* were obtained from the data on the food materials such as raw ground chicken and liquid egg products. The experiments on those products carried out and explained in chapter 1 and 2.

The prediction procedures of this program are demonstrated as follows. When users predict the growth of *Salmonella* and NM in the food materials with the program, they first choose the food material among three ground chicken samples and two liquid egg products on the screen of the program. The ground chicken samples are the samples containing low and high levels of NM which were $10^{4.7}$ and $10^{6.8}$ CFU/g, respectively and sterilized one. The liquid egg products in the program are pasteurized and unpasteurized one with NM of $10^{7.3}$ CFU/g (53).

Next, users input the temperature history of the food material on the screen for analysis. The program provides two ways to input the temperature history of the food. That is, one way is by manually inputting the temperatures history on the screen. Users input the temperature and its period for each step of the temperature history; one example is shown in Fig. 1. The temperature history input in this manner is an outlined one, as shown in the figure 1. Another way is done by pasting the sequential data set of temperature and time recorded with a digital thermometer, which is shown afterwards in the present study.

Users then select the contamination level of the NM of the food material. With the prediction button users can get the predicted growth curves of *Salmonella* and the NM under that condition.

3.3 Results and discussion

Here is an example of the program prediction by selecting food sample and inserting temperature history into the program.

The growth prediction of *Salmonella* and the NM in the ground chicken with the high level of the NM is shown in Fig. 2. The program also demonstrates the populations of *Salmonella* and the NM predicted at the time of interest; the predicted populations for *Salmonella* and the NM after 12 hours of storage in this example are $10^{4.6}$ and $10^{8.0}$ CFU/g, respectively, in Fig. 2.

For the liquid egg products, the program can also show the growth prediction of *Salmonella,* as shown in Fig. 3. The temperature history was manually input into the program for prediction. Here the levels of NM are high and zero (sterile) for selection of liquid egg NM. Also, prediction is for *Salmonella* only in the program, because the growth of the NM in those products, whose initial level was already very high ($10^{7.3}$ CFU/g), did not show sigmoidal curves.

Another way of inputting the temperature history with the recorded data sets of time and temperature also gives prediction. The temperature data is input in the table of Excel sheet (Fig .4), it is an example of the temperature history of ground chicken with this manner; a periodic red line in the graph represents the temperature history measured with a digital recorder. With the prediction button users then can get the predicted growth curves of *Salmonella* and the NM in the food of interest. Figure 4 shows the predicted curves in the ground chicken with the low NM level at the recorded temperature pattern. The program also demonstrates the populations of *Salmonella* and the NM predicted at the time of

interest Fig. 4.

An example of the *Salmonella* growth prediction in the liquid egg products with recorded digital data is shown in Fig. 5. This way of inputting the temperature data would be applied much often in food industry, because users mostly record the temperature history of the food materials with digital thermometers.

The present program could be applied to confirm the microbiological food safety in the processes of production and transportation of the food materials by giving substantial, quantitative information to users. Namely, when the prediction with this program at the measured temperature history is an increase of *Salmonella*, users should take some options to prevent a food poisoning outbreak by the pathogen. Since the program is based on the previous data, *Salmonella* growth at different initial populations is not able to predict at present. In the near future, we would like to have this opportunity to improve the program by obtaining more experimental data.

This program would be useful tool to food processors, food microbiology members, customers and who related to food science. They can obtain *Salmonella* Enteritidis growth values by using that program.

3.4 Summary

A predictive program for *Salmonella* Enteritidis growth was developed by us. The *Salmonella* was grown on ground chicken and liquid egg product at different temperature series in this study. Here the ground chicken samples were sterilized chicken and raw chicken containing high and low levels of NM, and the liquid egg products were pasteurized and unpasteurized ones. Microbial growth data published in previous papers were used for prediction with NL model. The program for the bacterial growth in those food materials was developed on a commercial, spread-sheet program. Users can instantly predict the *Salmonella* growth in those chicken and egg products by inputting their temperature histories. The growth of NM in the chicken products can also predict with the program. This program could be a useful tool to ensure the microbial safety of those materials for *Salmonella* Enteritidis growth.

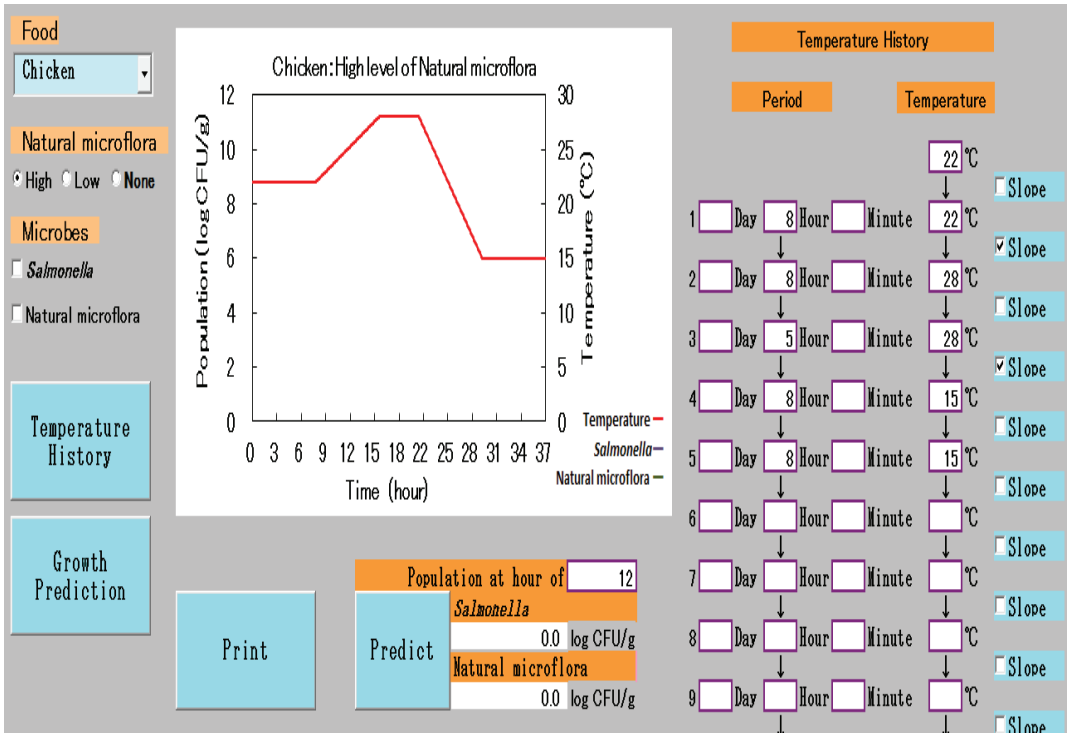

Fig. 1. Input of the temperature history manually. The temperature data of the temperature and period at each temperature step are input in the right-handed boxes. An example of temperature history, which is consisted of five steps, is input into the boxes. The whole temperature pattern is then shown in the graph.

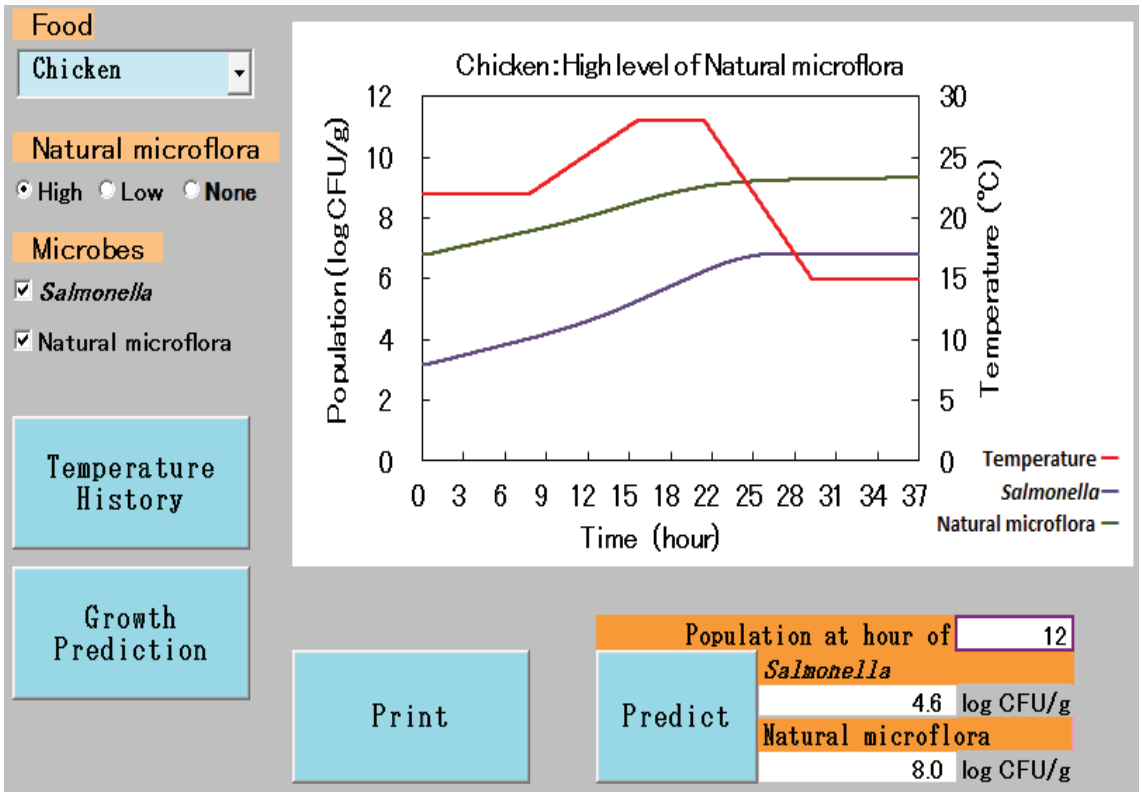

Fig. 2. Growth prediction of *Salmonella* Enteritidis and NM in ground chicken with a high level of NM. The upper and lower curves show predicted growths of the NM and *Salmonella*, respectively.

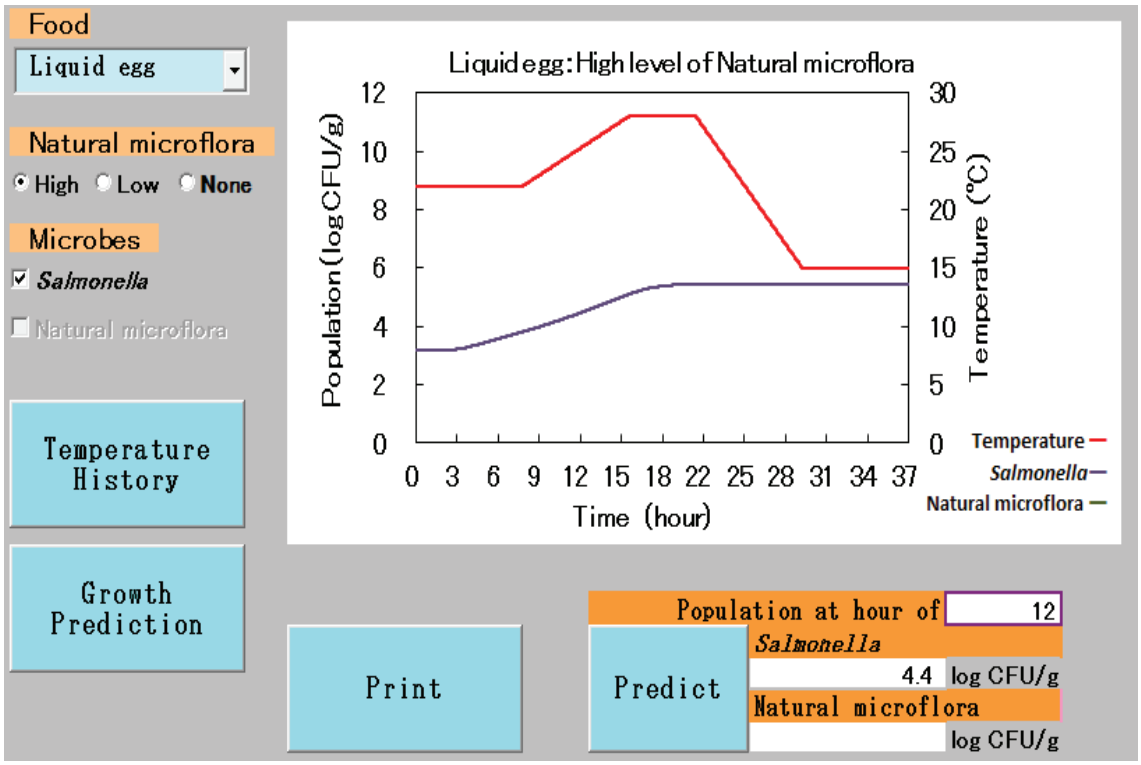

Fig. 3. *Salmonella* growth prediction in liquid egg product with the high level of NM. Predicted population of *Salmonella* at a given time (12 h) of storage is also shown below.

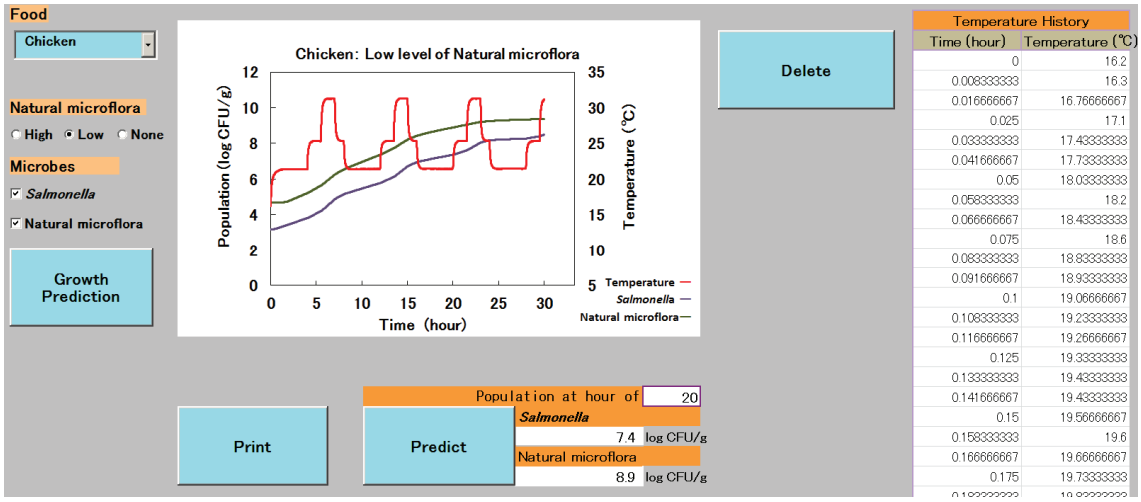

Fig. 4. Growth prediction of *Salmonella* Enteritidis and NM in ground chicken with the low level of the NM. The recorded temperature data is pasted on the right-handed table. The upper and lower curves show predicted growths of the microflora and *Salmonella*, respectively. Predicted populations of *Salmonella* and the microflora at a given time (20 h) of storage are also shown below.

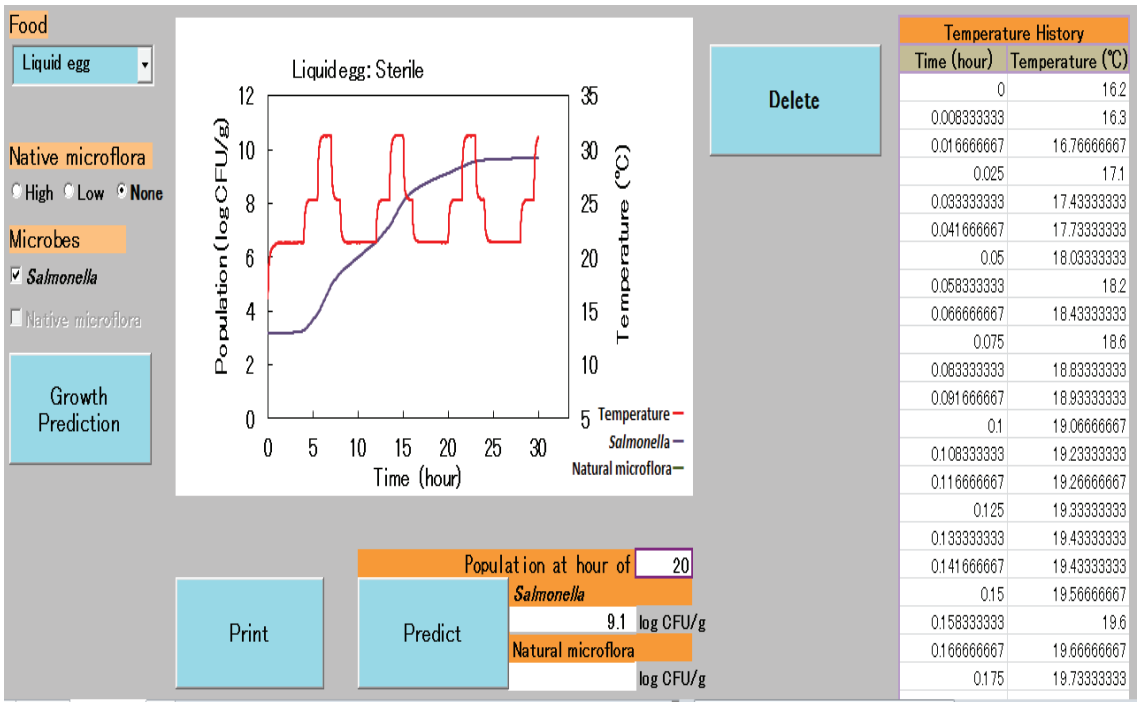

Fig. 5. *Salmonella* growth prediction in the pasteurized liquid egg product. A predicted growth curve of *Salmonella* is described with the recorded temperature data that is pasted on the table. Predicted population of *Salmonella* at a given time (20 h) of storage is shown.

Conclusion

A systematic study of the growth of *Salmonella* Enteritidis, one of the most important pathogens in food-borne poisoning, in raw ground chicken and liquid egg products was performed in the thesis.

The *Salmonella* growth kinetics including the growth rate and the maximum population in raw ground chickens (low and high level of NM) changed with temperature and were suppressed by NM in the chicken. The NL model could well predict the growth of *Salmonella* and NM in the ground chicken at dynamic temperatures.

The growth rate and the maximum population of *Salmonella* growth in the pasteurized liquid egg product were higher than those in the unpasteurized product. *Salmonella* growth was well predicted with the NL model at various patterns of dynamic temperatures in both liquid egg products.

Based on these results a prediction software program for the pathogen growth in chicken and liquid egg products was developed with Microsoft Excel. The program will help users to estimate the growth of the pathogen in those products during the production and distribution processes.

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I would also like to express my great thanks to my respectful parent, my family and my friends for their support and encouragement due to my study.

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