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Original article

Modeling the effect of temperature on survival rate of *Salmonella* Enteritidis in yogurt

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Abstract

The aim of the study was to determine the inactivation rates of *Salmonella* Enteritidis in commercially produced yogurt and to generate primary and secondary mathematical models to predict the behaviour of these bacteria during storage at different temperatures. The samples were inoculated with the mixture of three *S*. Enteritidis strains and stored at 5°C, 10°C, 15°C, 20°C and 25°C for 24 h. The number of salmonellae was determined every two hours. It was found that the number of bacteria decreased linearly with storage time in all samples. Storage temperature and pH of yogurt significantly influenced survival rate of *S*. Enteritidis ($p < 0.05$). In samples kept at 5°C the number of salmonellae decreased at the lowest rate, whereas at 25°C the reduction in number of bacteria was the most dynamic. The natural logarithm of mean inactivation rates of *Salmonella* calculated from primary model was fitted to two secondary models: linear and polynomial. Equations obtained from both secondary models can be applied as a tool for prediction of inactivation rate of *Salmonella* in yogurt stored under temperature range from 5 to 25°C; however, polynomial model gave the better fit to the experimental data.

Key words: yogurt, *Salmonella*, predictive modeling, survival, storage, temperature

Introduction

Salmonella has been the second, after *Campylobacter*, most commonly reported cause of zoonotic disease in Europe. In 2012, a total of 91,034 confirmed cases of human salmonellosis were reported in the European Union. The EU notification rate for confirmed cases was 22.2 per 100,000 population. The EU case-fatality rate was 0.14% as 61 deaths due to non-typhoidal salmonellosis were reported in the EU in 2012 (EFSA 2014).

A total of 5,363 food-borne outbreaks were reported in the EU in 2012, resulting in 55,453 human cases, 5,118 hospitalisations and 41 deaths. Most of the outbreaks (28.6%) were caused by *Salmonella* (EFSA 2014).

In Poland decreasing trend in confirmed salmonellosis cases in humans has been observed during last years, but in spite of that, a total of 8,444 cases were reported in 2012 (EFSA 2014). According to recent official data (Czarkowski et al. 2013), among 10,054 of all cases of bacterial foodborne intoxications

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recorded in Poland, 8,267 (82%) were caused by *Salmonella*.

In foodstuffs, *Salmonella* has been most often detected in fresh broiler meat, minced meat and meat preparations, meat products, as well as live bivalve molluscs (EFSA 2014).

Salmonella was also isolated from dairy products; however, there are no available data to estimate the share of yogurt and other fermented beverages in the total number of non-compliant samples of milk products.

Yogurt and other fermented milk products are the most growing segment of the dairy production in Poland. From 2005 to 2012 consumption of yogurt and milk-based beverages per capita increased by approx. 36% (GUS 2014).

The occurrence of *Salmonella* in yogurt may be a result of exceptionally heavy raw milk contamination, inadequate heat treatment of milk and secondary contamination arising from various food additives and lack of hygiene during packaging. Based on literature data it can be concluded that *Salmonella* cells have unfavorable conditions for growth in yogurt. However, these bacteria may survive in final product for some time dependent on the type of product, its pH, storage temperature and other environmental conditions. Cirone et al. (2013) presented evidence that pathogenic bacteria like *Mycobacterium avium* subsp. *paratuberculosis*, *E. coli*, and *S.* Enteritidis could survive yogurt fermentation conditions and low pH levels followed by refrigerated storage for at least 20 days. Thus, when these pathogens are present in raw materials they might reach consumers.

In 2012, as in previous years, *Salmonella* Enteritidis was the most frequently reported serovar (41.3%) of all known reported serovars in human cases in the EU (EFSA 2014). In Poland, *S.* Enteritidis was also the most often isolated serovar (75.2%) from humans (Czarkowski et al. 2014). Therefore, understanding the behaviour of *Salmonella* Enteritidis in fermented milk products seems to be important for microbiological risk assessment. The data on survival of these bacteria during storage of yogurt at different temperature can be described mathematically and utilized for mathematical models construction (Gibson et al. 1988).

In recent years predictive microbiology has become an important supporting tool in food chain risk management and microbial models have been increasingly used by food producers as well as food inspectors in their routine work (Baranyi and Roberts 1994, Hoang et. al. 2012, Szczawiński 2012).

The aim of the study was to determine and compare the inactivation rates of *Salmonella* Enteritidis in the samples of commercially produced yogurt stored at different temperatures, as well as to generate primary and secondary mathematical models to describe the microbiological data and to predict the behaviour of *S.* Enteritidis in yogurt during its storage.

Materials and Methods

Salmonella enterica subsp. enterica serovar Enteritidis was used in the study. Bacterial colonies taken from nutrient agar plates were suspended in a nutrient broth (BTL®) and incubated for 24 h at 37°C. The following strains were used in the experiments: No. 1592/08 isolated from turkey, No. 2419/07 isolated from poultry (both obtained from the National Veterinary Research Institute in Pulawy, Poland) and reference strain ATCC® No. 13076 (MicroBiologics, Minnesota, USA).

Inoculum preparation

Mixture of the three cultures in the nutrient broth in the stationary phase of growth was used for inoculum preparation. The density of bacterial suspension (approx. 10^4 cfu/cm^3) was determined by surface plating on Brilliant Green Agar (Merck®).

Material

Plain yogurt (without sugar addition) packed in plastic cups (370 g, Danone) was used in the experiments.

Inoculation and storage of samples

10 g samples of yogurt were placed in polyethylene pouches and inoculated with the mixture of *Salmonella* strains by adding 0.1 cm^3 bacterial suspension into yogurt. Immediately after inoculation the samples were placed in incubators and stored at 5°C, 10°C, 15°C, 20°C, and 25°C for 24 h.

Bacteriological examination

Number of salmonellae was determined every 2 h. After homogenization (2 min) ten-fold dilution series were prepared followed by surface plating (0.5 ml) on BGA. The plates were incubated at 37°C for 24 h under aerobic conditions.

The colonies were counted and the number of bacteria (colony-forming units) was calculated.

pH measurement

The pH of uninoculated samples of yogurt stored at 5°C, 10°C, 15°C, 20°C, and 25°C was determined every 2 h using a digital pH meter Schott® Instruments. The pH was measured with a temperature sensor. Temperature dependence of the pH values was taken into account during calibration and measurements.

Statistical calculations

The experiment was performed in five replications. Bacterial counts, transformed into decimal and natural logarithms, were used for calculations using the Microsoft® Office Excel 2007, the General Linear Models supplied through IBM SPSS Statistics 20 and Statistica 10 (StatSoft, Polska).

Curve fitting – primary model

Obtained survival curves of bacteria were fitted to linear regression model:

$$y = a + bx \quad (1)$$

where: a = y intercept (point where x = 0 and the line passes through the y-axis); b = slope of the line (y_2-y_1/x_2-x_1). Linear regression equations were generated separately for each replication. For further analysis a mean of 5 replications for each temperature was calculated and used for secondary modeling.

For each survival curve D-value, time (hours) required for decimal reduction of bacterial cells during storage at given temperature (1/-b), was calculated.

Curve fitting – secondary models

The natural logarithm of mean inactivation rates of *Salmonella* calculated from primary model were fitted to two secondary models, namely linear (2) and polynomial (3) according to the following general functions:

$$\ln(\text{inactivation rate}) = a_0 + a_1 \cdot \text{temp} \quad (2)$$

$$\ln(\text{inactivation\_rate}) = a_0 + a_1 \cdot \text{temp} + a_2 \cdot \text{temp}^2 \quad (3)$$

where: ln – natural logarithm, a0 and a1 – adjustment factors, temp – temperature.

The internal mathematical validation of obtained secondary models was performed by calculating accuracy (A_f) and bias (B_f) factors suggested by Ross (1996):

$$A_f = 10^{\left(\Sigma \frac{\log \mu_{predicted}}{\mu_{observed}}\right)} \quad (4)$$

$$B_f = 10^{\left(\Sigma \log \frac{\mu_{observed}}{\mu_{predicted}}\right)} \quad (5)$$

where: n – number of observations, $\mu_{predicted}$ – predicted specific growth rate; $\mu_{observed}$ – observed specific growth rate.

The bias factor B_f shows consistent over- and under-prediction, whereas the coefficient of accuracy A_f indicates an average difference between the predicted and observed values (Dalgaard and Jorgensen 1998).

Results

The effects of temperature and storage time on the mean number of *S*. Enteritidis cells in the samples of natural yogurt are shown in Fig. 1.

The presented results (Fig. 1) as well as statistical analysis indicate that the numbers of salmonellae in yogurt decreased linearly with storage time in the samples incubated at all temperatures used in the experiments, i.e. 5°C, 10°C, 15°C, 20°C, and 25°C.

It is shown in Fig. 1 that storage temperature significantly influenced survival rate of *S*. Enteritidis. It is also confirmed by analysis of variance (ANOVA) that showed statistically significant differences ($p \ll 0.05$) between the lowest (5, 10°C) and the highest (20, 25°C) applied storage temperature, as well as between 15-25°C and 20-25°C. In samples stored at 5°C the number of salmonellae decreased at the lowest rate, whereas in samples stored at 25°C the decrease of these bacteria was the most dynamic.

The linear regression equations describing relationships between the numbers of *S*. Enteritidis in the samples of yogurt and storage time at different temperatures are shown in Table 1.

D-values, calculated from these equations, systematically decreased with raising storage temperature, however, the differences between decimal reduction times calculated for temperatures 5°C, 10°C, 15°C were not large enough to be statistically significant. The lowest D-value (7.52 h) was found for salmonellae present in samples incubated at 25°C.

The effect of temperature on inactivation rates estimated from the secondary models is shown in Figs. 2 and 3.

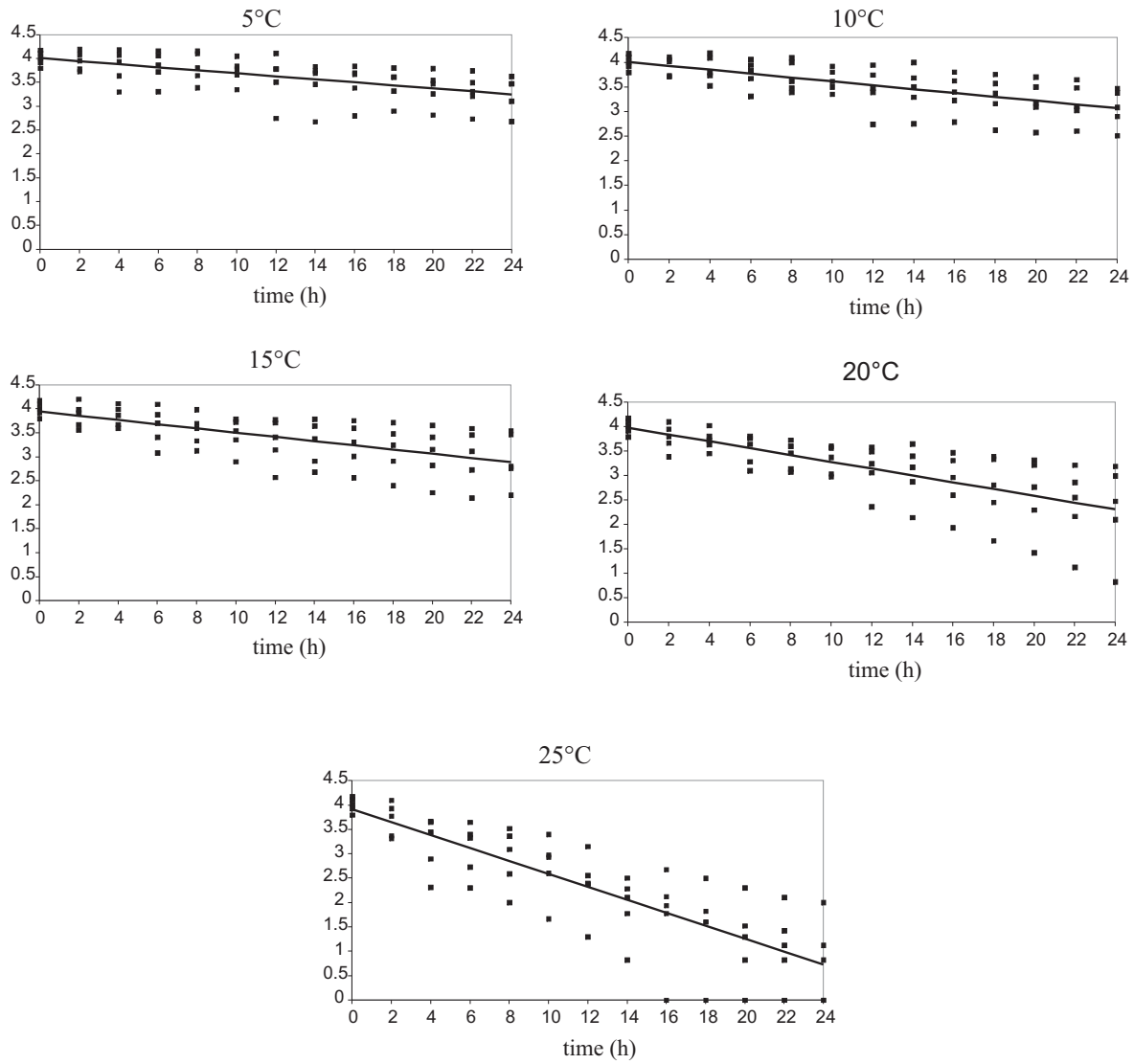

Fig. 1. Survival of *Salmonella* Enteritidis in yogurt stored at different temperatures. Experimental data fitted into linear model. Black squares represent raw data from five replicates.

Table 1. Relationship between the number of *S*. Enteritidis in the samples of yoghurt and storage time at different temperatures.

Storage temperature	Linear regression equation (y = a + bx)	Correlation	D-10 value* (1/-b)
5°C	y = 4.003 – 0.032x	-0.987	31.25[c]
10°C	y = 4.006 – 0.039x	-0.996	25.64[c]
15°C	y = 3.944 – 0.044x	-0.989	22.73[c]
20°C	y = 3.977 – 0.070x	-0.998	14.29[b]
25°C	y = 3.913 – 0.133x	-0.995	7.52[a]

* time (hours) required for decimal reduction of bacterial cells during storage at given temperature

[a, b, c] values bearing various superscripts are different at $p < 0.05$

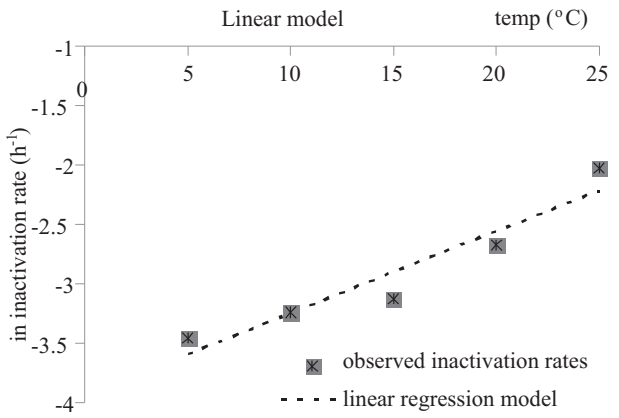

Fig. 2. Effect of storage temperatures on ln inactivation rate of *Salmonella* Enteritidis in yogurt.

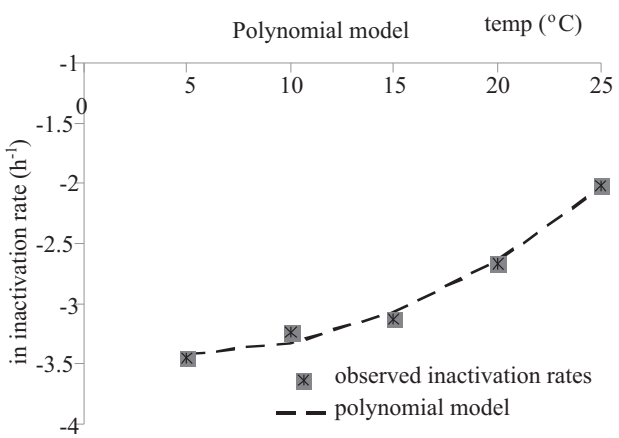

Fig. 3. Effect of storage temperatures on ln inactivation rate of *Salmonella* Enteritidis in yogurt.

Secondary models allow to predict the inactivation rate of *Salmonella* during storage of yogurt at every temperature from 5°C to 25°C. The results graphically presented in Figs. 2 and 3 indicate that both secondary models chosen for this study can be used to fit the obtained experimental microbiological data. Calculated R^2 factors for linear and polynomial models were 0.91 and 0.99, respectively. The results of calculation presented in Table 2 enabled more detailed comparison of the models. The goodness-of-fit was estimated by calculating the accuracy (A_f) and bias (B_f) factors (Ross 1996).

Both A_f and B_f indicated that polynomial model gave the better fit to experimental data describing inactivation rate of *S.* Enteritidis in yogurt then linear one (Table 2).

The overall analysis of variance indicated that time and temperature of storage significantly influenced ($P < 0.01$) pH of yogurt. Interaction time x temperature was also statistically significant ($P < 0.01$) indicating that storage time had different influence on pH of samples incubated at different temperatures. As shown in Fig. 4, pH values of yogurt samples stored at 5°C, 10°C and 15°C remained almost on the same level during storage for 24 h. However, pH of samples incubated at 20°C and 25°C decreased significantly with storage time from initial value 4.52 to 4.30 and 4.21, respectively, found at the end of observations (Fig. 4).

Discussion

Inactivation of pathogenic bacteria in fermented milk products, including yogurt, was found by many authors (Rubin and Vaughan 1979, Rubin et al. 1982, Alm 1983, Kotz et al. 1990, Issa and Ryser 2000, Álvarez-Ordóñez et al. 2013, Cirone et al. 2013). The main mechanism of bactericidal effect of yogurt on *Salmonella* and other pathogens seems to be the decline in pH due to lactose fermentation and the production of organic acids, mainly lactic acid, by *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* added to the milk in starter culture before fermentation (Rubin et al. 1982, Lefoka 2009, Álvarez-Ordóñez et al. 2013). Some authors suggest that antimicrobial activity of the yogurt is not exclusively due to the accumulation of lactic acid and may be the effect of lactic acid and other compounds such as hydrogen peroxide, carbon dioxide, acetaldehyde, polysaccharide, bacteriocins (Minj and Behera 2011).

The obtained results showed similar regularities to those found in our previous studies on behavior of *Listeria monocytogenes* in fermented milk products. Comparing survival of listeria in kefir, yogurt and sour milk during storage at 6°C and 20°C the linear reduction of listeria, particularly dynamic in samples of yo-

Table 2. Comparison of statistics obtained from secondary models.

Secondary models	Equations	A_f	B_f
Linear	ln(inactivation-rate) = -3.933 + 0.07 · temp	1.17	1.02
Polynomial	ln(inactivation_rate) = -3.33 – 0.035 · temp + 0.003 · temp^2	1.05*	1.00*

* The best fitted model (the value of A_f and B_f closer to 1.0).

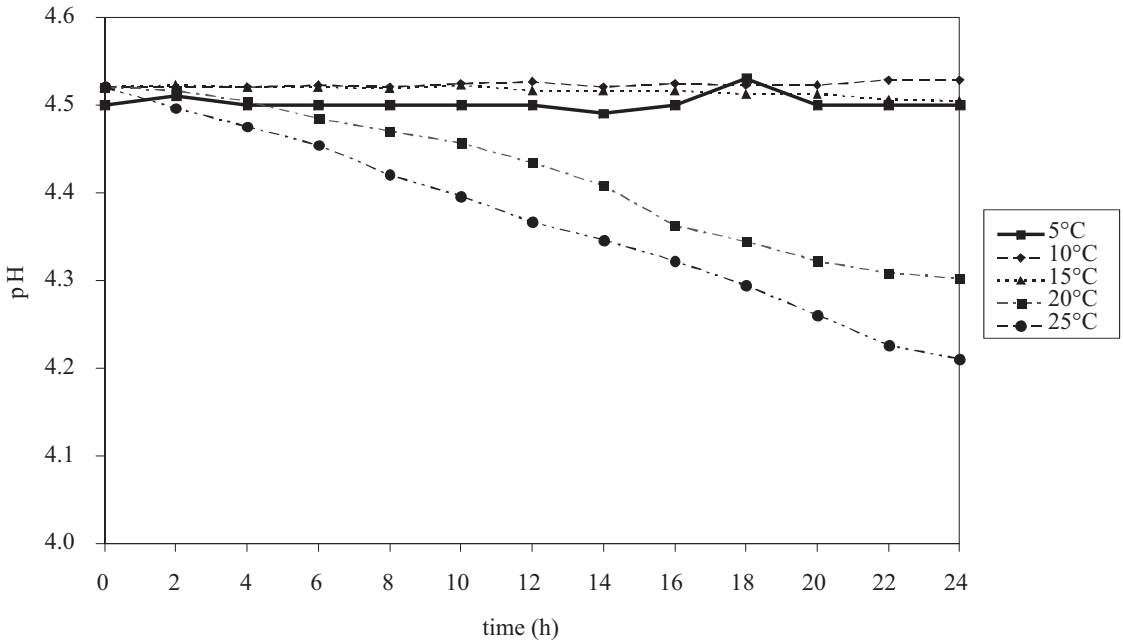

Fig. 4. Effect of time on pH of yogurt samples stored at different temperatures (n=5).

gurt incubated at higher temperature, i.e. at 20°C was observed (Szczawiński et al. 1998). Evidence found in other studies also demonstrated that refrigeration temperatures protect *Salmonella* from inactivation in fermented milk products and a higher bacterial inactivation was observed during storage of yogurt under temperature abuse (Lefoka, 2009, Álvarez-Ordóñez et al. 2013).

It was interesting to compare the D-10 values obtained in present study for *S*. Enteritidis in yogurt (Table 1) to theoretical values obtained from Pathogen Modeling Program (PMP) 6.1 for survival of *Salmonella* in laboratory culture medium with initial pH 4.5, similar like in this experiment. The pH of 4.5 is usually considered as minimum value permitting the growth of *Samonella* in food (Ray and Bhunia 2013); however, pH minimum as low as 3.8 has been reported when acidulants other than acetic acid or equivalent were used (Corlett 1980).

The decimal reduction times obtained from Pathogen Modeling Program (PMP) 6.1 are much shorter than those observed in yogurt and are: 7.02 h for samples incubated at 5°C, 6.74 h at 10°C, 5.83 h at 15°C, 4.55 h at 20°C and 3.20 h at 25°C, respectively. The results obtained from PMP confirm the observation (Table 1) that salmonellae are inactivated faster at higher temperature when pH is 4.5 or lower. They also support the opinions that low pH of the environment is the most important factor responsible for the reduction of *Salmonella* population in yogurt during storage.

Comparison of the *Salmonella* survival curves (Fig. 1) to the curves of pH changes in yogurt (Fig. 4) leads to similar conclusion. In the overall analysis of variance statistically significant effect ($P < 0.01$) of pH on *Salmonella* counts was found.

In our previous study on the effect of temperature on the growth kinetics of *S.* Enteritidis in cooked ham, conducted with the same mixture of *Salmonella* strains on samples having pH of 5.9-6.1, the growth of tested microorganisms was inhibited at 5°C and 10°C, but was observed in samples stored at 15°C, 20°C and 25°C (Szczawińska et al. 2014).

The detailed comparison of results of mathematical analyses conducted in present studies with the data given by other research workers is difficult, because there is very little information in available literature on modeling survival rate of *S*. Enteritidis in yogurt stored under similar temperature conditions.

Conclusions

1. The results obtained demonstrate that the number of *Salmonella* Enteritidis in samples of plain yogurt systematically decreases during storage in the temperature range from 5°C to 25°C. The reduction rate is related to the temperature of storage. The strongest reduction was observed in the samples of yogurt stored at 25°C and the weakest one in the samples stored at 5°C.

2. D-values calculated in these studies from linear model enable to predict behavior of *Salmonella* in yogurt during storage at temperatures of 5, 10, 15, 20, and 25°C.

3. Equation calculated in this research from secondary polynomial model seems to be useful for prediction of inactivation rate of *Salmonella* Enteritidis in yogurt stored under each temperature from 5 to 25°C.

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