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Original article

Modeling the effect of temperature on survival rate of *Listeria monocytogenes* in yogurt

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Abstract

The aim of the study was to (i) evaluate the behavior of *Listeria monocytogenes* in a commercially produced yogurt, (ii) determine the survival/inactivation rates of *L. monocytogenes* during cold storage of yogurt and (iii) to generate primary and secondary mathematical models to predict the behavior of these bacteria during storage at different temperatures.

The samples of yogurt were inoculated with the mixture of three *L. monocytogenes* strains and stored at 3, 6, 9, 12 and 15°C for 16 days. The number of listeriae was determined after 0, 1, 2, 3, 5, 7, 9, 12, 14 and 16 days of storage. From each sample a series of decimal dilutions were prepared and plated onto ALOA agar (agar for Listeria according to Ottaviani and Agosti).

It was found that applied temperature and storage time significantly influenced the survival rate of listeriae ($p<0.01$). The number of *L. monocytogenes* in all the samples decreased linearly with storage time. The slowest decrease in the number of the bacteria was found in the samples stored at 6°C (D-10 value = 243.9 h), whereas the highest reduction in the number of the bacteria was observed in the samples stored at 15°C (D-10 value = 87.0 h). The number of *L. monocytogenes* was correlated with the pH value of the samples ($p<0.01$). The natural logarithm of the mean survival/inactivation rates of *L. monocytogenes* calculated from the primary model was fitted to two secondary models, namely linear and polynomial. Mathematical equations obtained from both secondary models can be applied as a tool for the prediction of the survival/inactivation rate of *L. monocytogenes* in yogurt stored under temperature range from 3 to 15°C, however, the polynomial model gave a better fit to the experimental data.

Key words: yogurt, *Listeria monocytogenes*, predictive modeling, survival, storage, temperature

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Introduction

Listeria monocytogenes causes listeriosis, a disease especially dangerous and highly fatal (30-40%) to fetuses, newborns, infants, pregnant women, the elderly and immunocompromised individuals such as those with cancer, renal diseases, heart diseases, and AIDS. People receiving organ transplants and treated with immunosuppressive drugs are also susceptible to listeriosis (Ray and Bhunia 2007).

In the European Union (EU) 1,763 confirmed human cases of listeriosis were reported in 2013 by 27 member states (MS). The EU notification rate was 0.44 cases per 100,000 population which represented an 8.6 % increase compared to 2012. There was a statistically significant increasing trend of listeriosis in the EU and European Economic Area (EEA) over the period 2009-2013. A total of 191 deaths due to listeriosis were reported in 2013. The highest number of fatal cases (64) was reported in France. Mortality rate in the EU was established at 15.6% among cases with known outcome. In 2013, a total of 13 outbreaks caused by *L. monocytogenes* were reported by seven MS and one non-MS. It was observed that the number of listeriosis in 2013 was slightly higher than in the previous years (EFSA 2015).

An increasing trend in the number of human listeriosis outbreaks has been also observed in Poland. A calculated median of listeriosis cases in Poland in years 2007-2011 was 43 with an average incidence rate 0.11 per 100 000 population. Fifty eight cases of listeriosis with incidence rate 0.15 were reported in Poland in 2013 (Sadkowska et al. 2015).

According to the last European Food Safety Authority (EFSA) report from 2015 (EFSA 2015) the non-compliance in 2013 for different categories of ready-to-eat (RTE) food was generally at a comparable level to that observed in previous years. Nevertheless, the higher level of non-compliance was observed in case of fishery products at a processing plant level (mainly smoked fish). Consistent with the results of the baseline study on the prevalence of *Listeria monocytogenes* in certain ready-to-eat foods in the EU in 2010-2011 (EFSA 2013) the proportion of positive samples at retail was highest in fishery products, followed by soft and semi-soft cheeses, RTE meat products and hard cheeses. The study did not include data estimating the share of yogurt and other fermented beverages in the total number of non-compliant samples in the category of dairy products (EFSA 2013).

Yogurt and other fermented dairy products constitute an important segment of the dairy production in Poland. In 2013 the production of yogurt in Poland amounted to 450 000 tonnes with an average monthly consumption per capita 0.51 kg (GUS 2014).

The presence of *Listeria* in yogurt may be a result of very bad quality of raw milk, inadequate heat treatment of milk or re-contamination as a result of using contaminated additives and poor hygiene during processing and packaging (Dzwolak et al. 2000). *Listeria monocytogenes* is a psychrotrophic microorganism (able to grow and multiply during cold storage) and even a few cells present in the final product can multiply to a level that is dangerous to consumers (Posfay-Barbe and Wald 2009).

On the basis of literature data (Szczawiński et al. 1998, Lefoka 2009) it can be concluded that *L. monocytogenes* has unfavorable conditions for growth in yogurt. However, these bacteria may survive in the final product for definite time depending on the type of product, its characteristics (e.g. pH, competitive microflora), storage conditions (temperature) and other environmental circumstances. Cirone et al. (2013) proved that pathogenic bacteria like *Mycobacterium avium* subsp. *paratuberculosis*, *E. coli*, and *S.* Enteritidis could survive the fermentation process during yogurt production and were not inactivated by low pH levels followed by cold storage for at least 20 days. This implies that when *L. monocytogenes* is present in a large number in raw milk some cells may survive the production process of yogurt and pose a serious hazard for consumers health.

Therefore, understanding the behavior of *Listeria monocytogenes* in fermented dairy products constitutes a crucial knowledge for Microbiological Risk Assessment (MRA) process, as well as Hazard Analysis and Critical Control Point (HACCP) system. The experimental data regarding survival or inactivation of this foodborne pathogen during production and cold storage of yogurt at different temperatures can be described mathematically by predictive models (Gibson et al. 1988, Baranyi and Roberts 1994, Hoang et al. 2012). Predictive microbiology is based on the premise that the response of microorganisms to environmental factors is reproducible. By defining the parameters that have the strongest effect on the behavior of microorganisms, it is possible to predict the response of microorganisms based on the performed observations (Devlieghere et al. 2006, Black and Davidson 2008).

In recent years predictive microbiology has become an important tool supporting food safety management systems. Moreover, predictive microbial models are being commonly used by the food producers, as well as food inspectors in their routine work (Baranyi and Roberts 1994, Hoang et al. 2012, Szczawiński 2012).

The aim of present study was to determine and compare inactivation rates of *Listeria monocytogenes*

in the 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 behavior of *L. monocytogenes* in yogurt during its storage.

Materials and Methods

Three following reference strains of *Listeria monocytogenes* were used in the study: ATCC® No. 7644 (isolated from human), ATCC® No. 15313 (isolated from rabbit) and ATCC® No. 19112 (isolated from human spinal fluid). To prepare the inoculum, the organisms were cultured in 10 mL nutrient broth at 37°C for 24 h.

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 onto ALOA – Agar Listeria Ottavani & Agosti Medium (Merck®).

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

Inoculation and storage of inoculated samples. Samples of yogurt (10 g) were placed in polyethylene pouches and inoculated with the mixture of three *L. monocytogenes* strains by adding 0.1 cm^3 bacterial suspension into each pouch. Immediately after inoculation the samples were placed in incubators and stored at 3, 6, 9, 12 and 15°C for 16 days.

Bacteriological examination. The number of listeriae was determined after 0, 1, 2, 3, 5, 7, 9, 12, 14 and 16 days of storage. After homogenization (2 min) of the samples ten-fold dilution series were prepared followed by surface plating (0.5 ml) on ALOA medium. The plates were incubated at 37°C for 48 h under aerobic conditions. The colonies were counted and the number of bacteria (colony-forming units) was calculated.

pH measurement. The pH of uncontaminated samples of yogurt stored at 3, 6, 9, 12 and 15°C was determined using a digital pH meter Schott® Instruments equipped with a temperature sensor. The influence of temperature on pH values was taken into account during calibration of the equipment and further measurements.

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

Primary modelling – curve fitting. Obtained on the basis of experimental data microbiological survival curves were fitted to general linear regression model (1) according to the following equation:

$$y = a + bx \tag{1}$$

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 mathematical analysis a mean log cfu/cm^3 from 5 replications for each temperature was calculated and used for secondary modeling.

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

Secondary modeling. The natural logarithm of mean survival rates of *L. monocytogenes* calculated from general linear regression model was fitted to two secondary models, namely linear (2) and polynomial (3) according to the following general functions:

$$\ln(x) = a_0 + a_1 * temp \tag{2}$$

$$\ln(x) = a_0 + a_1 * temp + a_2 * temp^2 \tag{3}$$

where:
ln – natural logarithm, x – survival/inactivation rate, a_0 and a_1 – adjustment factors, temp – temperature (°C).

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

$$A_f = 10^{(\Sigma \left| log \mu_{predicted} / \mu_{observed} \right| / n} \tag{4}$$

$$B_f = 10^{\left(\Sigma \log \left(\frac{\mu_{observed}}{\mu_{prewdicted}} \right) / n \right)} \tag{5}$$

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).

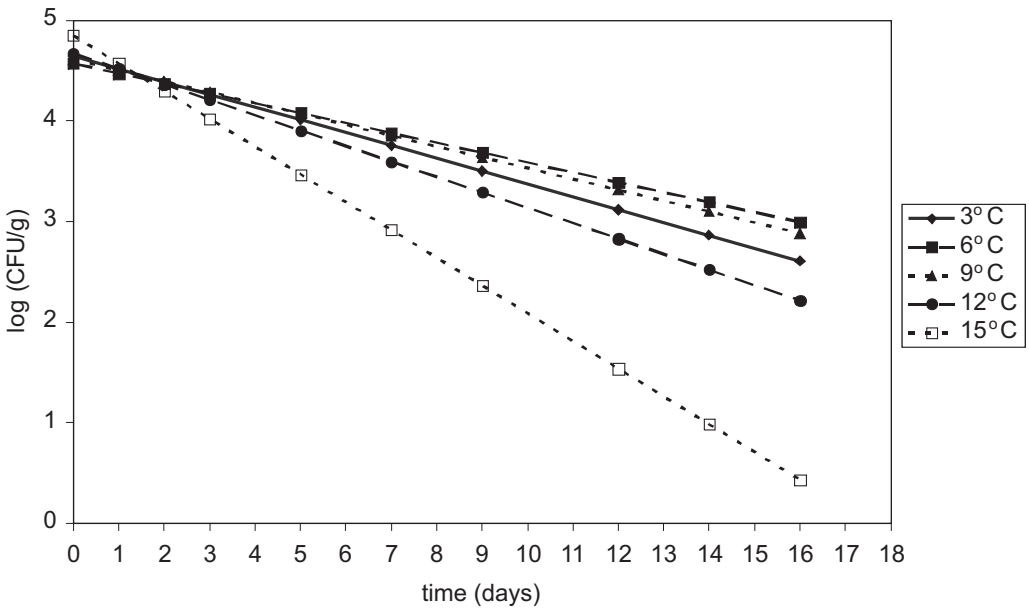

Fig. 1. Survival of *Listeria monocytogenes* in yogurt stored at different temperatures. Experimental data fitted into linear model.

Table 1. Relationship between the number of *L. monocytogenes* in yogurt at different temperatures.

Storage temperature (°C)	Linear regression equation (y=a+bx)	Correlation (R^2)	D-10 value* (1/-b)
3	y = 4.64-0.0053x	-0.978	188.7^{bc}
6	y = 4.57-0.0041x	-0.985	243.9^{c}
9	y = 4.61-0.0045x	-0.987	222.2^{c}
12	y = 4.67-0.0064x	-0.974	156.3^{b}
15	y = 4.85-0.0115x	-0.977	87.0^{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 $p<0.05$

Results

The effect of temperature and storage time on the mean number of *L. monocytogenes* in natural yogurt is shown in Fig. 1.

The presented results (Fig. 1) as well as the statistical analysis indicated that the number of listeriae in yogurt decreased linearly during storage at 3, 6, 9, 12 and 15°C.

The overall analysis of variance (ANOVA) showed that storage temperature and time significantly affected the number of *L. monocytogenes* ($p<0.01$). Statistically significant ($p<0.01$) was also interaction of both those agents (temperature x time).

The linear regression equations describing the relationships between the number of *L. monocytogenes* in yogurt and storage time at different temperatures are shown in Table 1.

D-values, calculated from equations presented in Table 1, show general tendency to decrease along with an increase of the applied storage temperature, however, the differences between decimal reduction times calculated for temperatures 3, 6 and 9°C were not statistically significant. The lowest D-value (87.0 h) was found for listeriae present in yogurt incubated at 15°C.

The effect of temperature on inactivation rates of *L. monocytogenes* in yogurt at a temperature range 3-15°C estimated by the secondary models (linear and polynomial) is shown in Figs. 2 and 3.

Secondary models enable to predict the inactivation rate of *L. monocytogenes* during storage of yogurt at temperature from 3 to 15°C. The results graphically presented in Figs. 2 and 3 indicate that both secondary models, namely linear and polynomial, can be successfully used to predict the inactivation rate of *L. monocytogenes* in yogurt during storage at temperature range 3-15°C. The accuracy (A_f) and bias (B_f) factors (Ross 1996) presented in Table 2 used for mathematical validation purposes indicated that the

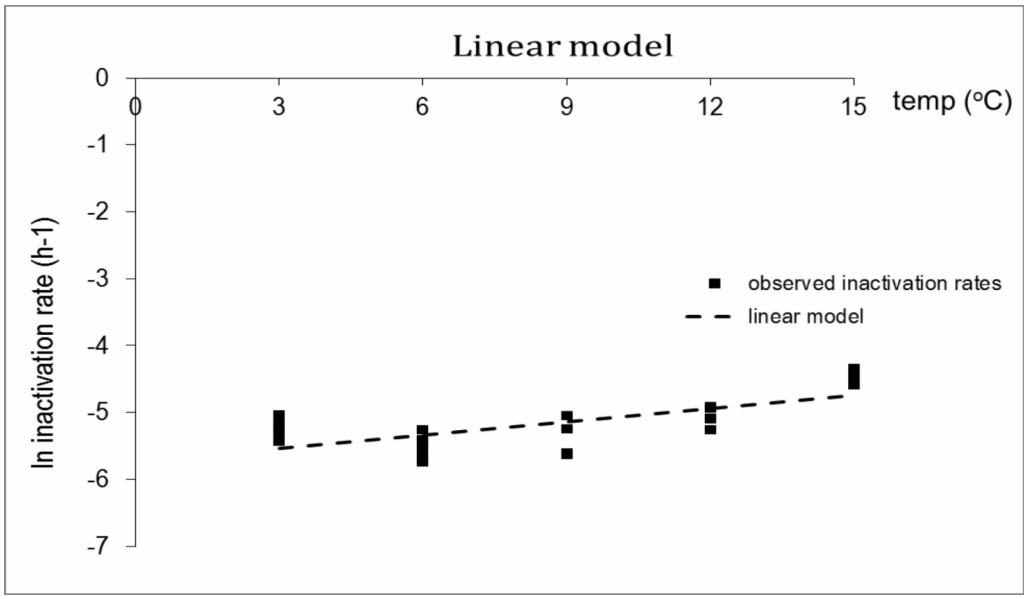

Fig. 2. Effect of storage temperatures on ln inactivation rate of *Listeria monocytogenes* in yogurt – linear model.

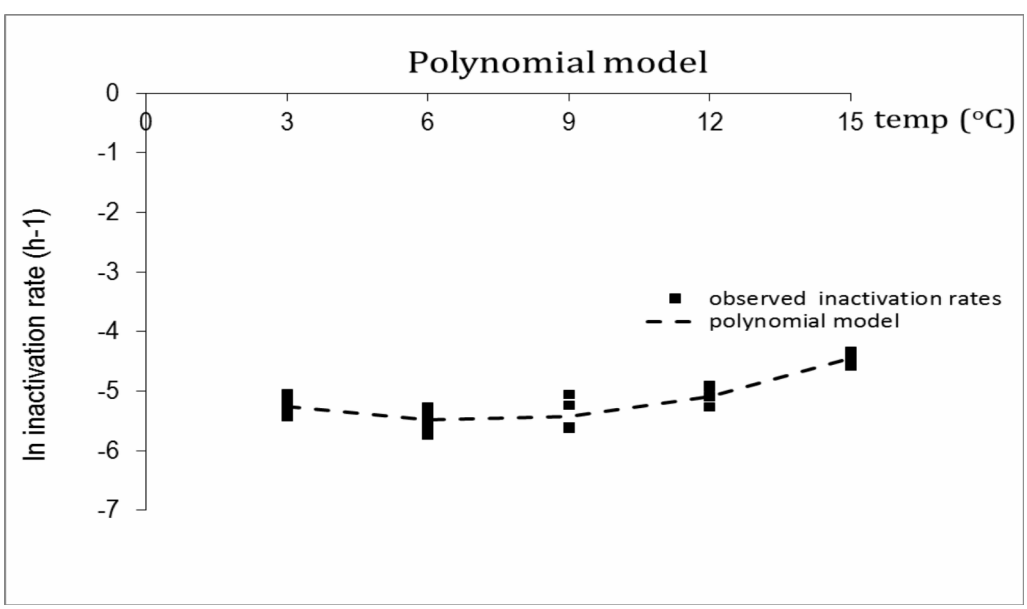

Fig. 3. Effect of storage temperatures on ln inactivation rate of *Listeria monocytogenes* in yogurt – polynomial model.

Table 2. Mathematical validation of secondary models describing the inactivation rate of *L. monocytogenes* in yogurt.

Secondary models	Equations	A_f	B_f
Linear	ln(inactivation_rate) = -5.74+0.67*temp	1.33	1.09
Polynomial	ln(inactivation_rate) = -4.74-0.22*temp+0.02*temp^2	1.18*	1.03*

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

polynomial secondary model gave a better fit to experimental data describing inactivation rate of *L. monocytogenes* in yogurt in comparison to the linear model (Table 2).

Changes of pH during storage of uncontaminated yogurt at different temperatures for 16 days are shown in Fig. 4. The overall analysis of variance (ANOVA) revealed that time and temperature

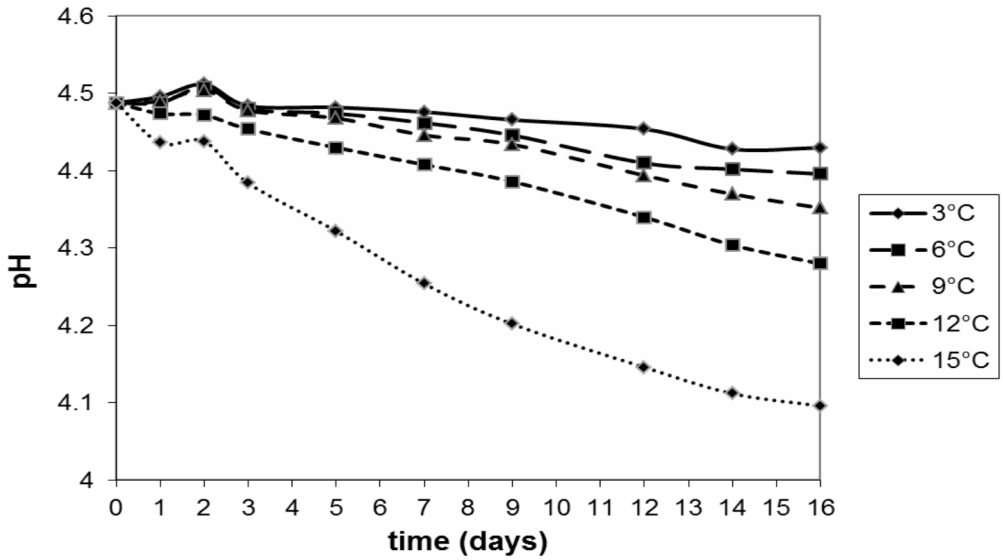

Fig. 4. Effect of time on pH of yogurt samples stored at different temperatures (n=5).

applied during storage significantly influenced ($p<0.01$) the pH value of yogurt. Interaction between time and temperature was also statistically significant ($p<0.01$) indicating that storage time had different influence on pH of yogurt incubated at various temperatures. As shown in Fig. 4, pH of yogurt decreased systematically along with storage time. Moreover, the higher temperature of the storage, the higher was the dynamics of this process. The pH drop was the most noticeable in samples of yogurt stored at 15°C. At the beginning of incubation of yogurt at 15°C an average pH value amounted to 4.5 and dropped do 4.1 after 16 days of storage.

Discussion

An inactivation of pathogenic bacteria in fermented dairy products, including yogurt, has been observed in many studies (Rubin and Vaughan 1979, Rubin et al. 1982, Alm 1983, Kotz et al. 1990, Issa and Ryser 2000, Hlvarez-Ordóñez et al. 2013, Cirone et al. 2013, Szczawińska et al. 2014). The main mechanism of bactericidal effect of yogurt on foodborne pathogens seems to be the decline in pH due to lactose fermentation by *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* added to milk as a starter culture, as well as the production of organic acids, mainly lactic acid (Rubin et al. 1982, Lefoka 2009, Hlvarez-Ordóñez et al. 2013). Kowalik et al. (2013) observed growth of *L. monocytogenes* in all samples of cottage cheese stored at temperature 3, 6, 9, 12 and 15°C. They found that lag phase and growth rate of *Listeria* were affected not only by storage temperature but also by lactic acid concentration and pH of product ranged from 5.2 do 5.8. The same researchers (Kowalik et al. 2012) observed more dynamic growth of *L. monocytogenes* in milk samples, having a pH of 6.6, and stored under conditions identical to those applied for the samples of the cottage cheese. Some authors have suggested that antimicrobial activity of yogurt is not exclusively due to accumulation of lactic acid and may be also the effect of lactic acid and other compounds such as hydrogen peroxide, carbon dioxide, acetaldehyde, polysaccharide and bacteriocins (Minj and Behera 2011). It has been observed in many studies that the growth of *L. monocytogenes* can be controlled by the activity of the lactic acid bacteria such as *L. delbrueckii* subsp. *bulgaricus, L. plantarum, L. lactis, L. lactis ssp. diacetylactis L. cremoris, L. curvatus* and others (Schaack and Marth 1988, Harris et al. 1989, Pitt et al. 2000, Benkerroum et al. 2002).

The pH value of 4.6 is usually considered as a minimum pH permitting the growth of *L. monocytogenes* in food (Ray and Bhunia 2007). However, pH minimum as low as 4.39 has also been reported (Corlett and Brown 1980). Comparison of the *L. monocytogenes* survival curves (Fig. 1) to the curves of pH changes in yogurt (Fig. 4) leads to conclusion that low pH of the environment is an important factor responsible for the reduction of *Listeria* population in yogurt. Statistically significant ($p<0.01$) effect of pH on *Listeria* counts was found in the overall analysis of variance. The correlation between the number of *L. monocytogenes* and pH amounted to 0.832 and also proved to be statistically significant ($p<0.01$).

To estimate more precisely the effects of pH and other factors on inactivation of *Listeria* in the experiment performed, the D-10 values obtained in the present study for *L. monocytogenes* in yogurt

(Table 1) were compared to theoretical values obtained from Pathogen Modeling Program (PMP) 6.1. The D-10 values obtained from PMP refer to the survival of *L. monocytogenes* during incubation at 6, 9, 12 and 15°C in optimal laboratory culture medium with initial pH 4.4, 4.3, 4.2 and 4.1. The obtained D-10 values range from 559 to 282 h. The D-10 values for *L. monocytogenes* observed in our study in real yogurt were much shorter and ranged from 244 h to 87 h (Table 1). It suggests that antimicrobial activity of yogurt is not exclusively due to low pH but the presence of various compounds formed during fermentation plays also an important role in inactivation of *L. monocytogenes*.

The simulations obtained from PMP confirm our results (Table 1) showing that *L. monocytogenes* is more intensively inactivated at higher temperatures when pH is in a range of 4.5 – 4.1. It was also found in our previous studies (Szczawiński et al. 1998) on the behavior of *L. monocytogenes* in fermented dairy products. Comparing survival of *L. monocytogenes* in kefir, yogurt and sour milk during storage at 6°C and 20°C the linear reduction of *L. monocytogenes*, particularly dynamic in yogurt incubated at higher temperatures (i.e. 20°C), was observed. Results of other studies also demonstrated that refrigeration temperatures protect pathogenic bacteria from inactivation in fermented dairy products and greater bacteria inactivation was observed during storage of yogurt under temperature abuse (Lefoka 2009, Álvarez-Ordóñez et al. 2013, Szczawiński et al. 2014).

The results of mathematical analyses related to modeling the effect of storage temperature on survival rate of *L. monocytogenes* show similar regularities to those observed in our previous experiments focused on *S*. Enteritidis (Szczawiński et al. 2014). In both studies the number of bacteria linearly decreased during storage and the inactivation rate was related to the applied storage temperature. It was also found that linear and polynomial equations obtained from secondary modeling can be successfully applied for prediction of inactivation/survival rate of both pathogens, nonetheless polynomial model gave slightly better fit to the experimental data. The main difference was that *S.* Enteritidis was inactivated at much higher rate during storage of yogurt than *L. monocytogenes.*

Conclusions

1. The number of *L. monocytogenes* decreases linearly along with storage time in yogurt stored at 3, 6, 9, 12 and 15°C.

2. Temperature and storage time significantly ($p<0.01$) influence the inactivation/survival rate of *L. monocytogenes* in yogurt.

3. The number of *L. monocytogenes* cells is strictly correlated with the pH value of yogurt.

4. Mean inactivation rates of *L. monocytogenes* generated by the primary linear model and fitted to the secondary linear and polynomial model can be successfully applied as a tool for prediction of *L. monocytogenes* inactivation rate in yogurt at the temperature ranging from 3 to 15°C; however, the polynomial model gave a better fit to the experimental data.

5. The results obtained demonstrate that *L. monocytogenes* cells have unfavorable conditions for growth in yogurt, however, these bacteria may survive in the final product for a prolonged time posing a hazard to public health.

Acknowledgments

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