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A PREDICTIVE MODEL FOR *LISTERIA MONOCYTOGENES* IN UHT DAIRY PRODUCTS WITH VARIOUS FAT CONTENT DURING COLD STORAGE

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ABSTRACT

The growth of *Listeria monocytogenes* was determined in Ultra-High-Temperature (UHT) dairy products (2% milk, 12 and 30% cream) at temperature range of 3–15C. Microbiological data were fitted to primary models (the Baranyi model, the modified Gompertz and logistic functions). The goodness-of-fit of primary models was analyzed by calculating mean square error and Akaike's information criterion. Baranyi model yielded the most accurate adjustment and the growth rates generated by this model were used for further mathematical analyses. Analysis of variance was used to check if the fat content significantly ($P < 0.05$) influences the behavior of *L. monocytogenes*. No statistical differences were noted in the behavior of the pathogen. Microbiological growth data were combined and secondary modeling was performed using Ratkowsky, Arrhenius and polynomial models. The latter model gave the best description and was further validated using accuracy (A_f) and bias (B_f) factors, as well as data from ComBase database and COMBASE Predictor.

PRACTICAL APPLICATIONS

Mathematical models that describe the behavior of microorganisms, especially foodborne pathogens, in a particular product or group of food products with similar characteristics, pose a perspective of using predictive microbiology in order to increase the food safety. Application of predictive models is in agreement with Codex Alimentarius Commission and UE regulations in a risk analysis area. Presented results can be used by food manufacturers in food product development process, as well as a tool to support food safety assurance systems. Moreover, predictive models find practical application in Hazard Analysis and Critical Control Point (HACCP) plans providing useful information on the determination of critical control points (CCPs) and the estimation of critical limits at CCPs. The assessment and management of safety, quality and shelf life of food products can be facilitated by application of mathematical predictive models.

INTRODUCTION

In recent decades, intensive development of computerized techniques and the necessity to quantitatively assess microbiological risks in the food chain have prompted an increased interest in mathematical models to describe microbial growth as a function of environmental factors (such as temperature, water activity, pH, oxygen availability and others). Predictive microbiology is a discipline that develops the models that predict bacterial behavior. Its main assumption specifies that the response of a microbial population to environmental factors is repeatable and that, by describing the environment with the factors that have the biggest impact on microbial growth and survival, it becomes possible to predict the microbial response in a similar environment. This knowledge may be described and depicted as a mathematical model that will be implemented for quantitative behavior prediction (e.g., death, growth, toxin production) in a microbial population in food (Devlieghere *et al.* 2006; Black and Davidson 2008).

A microbial response is measured under defined and controlled environmental conditions, often in liquid media, yet it is being more commonly postulated in the literature that models should be developed based on studies carried out with food products (Dalgaard and Jorgensen 1998).

Listeria monocytogenes is a psychrotrophic microorganism capable of multiplying in food stored under refrigerating conditions. This property implies that ready-to-eat food with prolonged shelf-life requires special attention. This bacterium is particularly dangerous for people with impaired immunity, and the mortality rate because of listeriosis is very high in this group (ca. 30%). Contrary to other foodborne pathogens, *L. monocytogenes* is relatively resistant to acids and high salt concentrations. In addition, *L. monocytogenes* is capable of producing a biofilm, which allows this bacterium to survive for a prolonged period of time in food-manufacturing machines (Gombas *et al*. 2002; Millet *et al*. 2006; Swaminathan and Gerner-Smidt 2007). The above-mentioned properties of *L. monocytogenes* make this microorganism one of the most dangerous foodborne pathogens.

There have been a few published predictive models focused in particular on milk (Murphy *et al*. 1996; Xanthiakos *et al*. 2006). Although the models were successfully validated, no information is provided regarding the product characteristic in terms of fat content. Models include such extrinsic parameters as pH, temperature and salt concentration (Murphy *et al*. 1996) or only temperature (Xanthiakos *et al*. 2006). Moreover, studies conducted by Rosenow and Marth (1987) indicate differences in the growth of *L. monocytogenes* in whipping cream and skim milk, possibly because of the high fat level relative to the water phase.

Heat resistance of *L. monocytogenes* varies depending on strain, but in many scientific publications, it was proven that *L. monocytogenes* is not a heat-resistant microorganism and does not survive the heat treatment processes (Doyle *et al*. 2001; Sergelidis and Abrahim 2009). On the other hand, one cannot eliminate the risk of reinfection or cross-contamination as a result of errors in the production process and even few cells of *L. monocytogenes* that enter the product are able to multiply during storage to a high level and pose a serious threat to consumer health. Furthermore, a potential risk of UHT products contamination after opening the packaging at household cannot be excluded. Moreover, the purposefulness of present publication has a cognitive character in terms of mathematical modeling and predictive microbiology aspects.

The aims of present work were as follows:

1. analyze the behavior of *L. monocytogenes* in 2% UHT milk and 12 and 30% UHT creams during storage at temperature range 3–15C;
2. evaluate and compare the goodness-of-fit of the primary models (Baranyi and Roberts model, modified Gompertz and logistic equations);
3. check if fat content influences the behavior of pathogen in UHT dairy products;
4. generate secondary models according to Arrhenius, Ratkowsky and polynomial equations; and
5. perform mathematical and graphical validation of generated secondary models.

MATERIAL AND METHODS

Isolation and Preparation of the *L. monocytogenes* Culture

Two strains of *L. monocytogenes* were isolated from raw milk at the Chair of Industrial Microbiology, Faculty of Food Sciences, University of Warmia and Mazury in Olsztyn, Poland, and were registered in the Collection of Strains of the Chair of Industrial and Food Microbiology as *L. monocytogenes* 3 and 38. The isolation of the strains was carried out in compliance with the standard PN-EN ISO 11290-2:2000.

The stock cultures of *L. monocytogenes* were maintained on Tryptic Soy Agar (Merck, Warsaw, Poland) slopes incubated at 30C for 48h, subsequently stored at 2C and subcultured monthly. Prior to use for the tests, 100 μL of both cultures was transferred into 10 mL of Tryptic Soy Broth (Merck) at 37C for 18 h. Cultures were grown until late exponential phase; the count of *L. monocytogenes* cocktail after passage was ~9.0 log cfu/mL.

An independent study was performed in order to determine the number of *L. monocytogenes* 3 and 38 in monoculture by plating on selective agar medium. Both strains exhibited the number of cells at the level ca. 9 log cfu/mL.

Preparation of UHT Milk and Cream Samples for Tests, Inoculation and Determination of the *L. monocytogenes* Count

Commercial UHT dairy products (2% UHT milk, 12% UHT cream, 30% UHT cream) were purchased in a grocery store. Prior to use for the tests, the dairy products were analyzed for *L. monocytogenes* presence using Fraser broth (Merck) in accordance with PN-EN ISO 11290-2:2000. Milk and cream was aseptically transferred to sterile bottles and the culture of *L. monocytogenes* was added to reach a contamination level of 10^3 cfu/mL. Contaminated samples of milk and cream were incubated at 3, 6, 9, 12 and 15C to reach full growth curves (lag, exponential and late stationary phase) of *L. monocytogenes*.

At appropriate time intervals, depending on the storage temperature, the samples were diluted in a peptone water (Merck) and the number of *Listeria* was checked using

selective agar for *Listeria* according to Ottaviani and Agosti (Merck). The mean from three platings of each sampling point was used to establish the growth kinetics. The experiment was performed in three replicates for each temperature.

Mathematical Analysis

Primary Modeling and Goodness-of-Fit. The following primary models were used in order to fit the obtained microbiological data:

• A modified logistic (Eq. 1) and Gompertz (Eq. 2) functions (Gibson *et al.* 1987)

$$\log x(t) = A + \frac{C}{\left(1 + e^{(-B(t-M))}\right)} \tag{1}$$

$$\log x(t) = A + Cexp\{-exp[-B(t-M)]\} \tag{2}$$

where $x(t)$ – cell concentration at time t, A – asymptotic count as t decreases to zero, C – difference in value of the upper and lower asymptote, B – relative growth rate at M, M – time at which the absolute growth rate is maximum.

• Baranyi and Roberts function (Baranyi and Roberts 1994)

$$y(t) = y_0 + \mu_{max} F(t) - \ln\left(1 + \frac{e^{\mu_{max} F(t)} - 1}{e^{(y_{max} - y_0)}}\right) \tag{3}$$

$$F(t) = t + \frac{1}{v}\ln\left(e^{-vt} + e^{-h_0} - e^{(-vt-h_0)}\right) \tag{4}$$

where $y(t)$ – cell concentration at time t (ln cfu/g), y_0 – initial cell concentration (ln cfu/g), y_{max} – maximum cell concentration (ln cfu/g), μ_{max} – maximum specific growth rate (h^{-1}), v – rate of increase of the limiting substrate, h_0 – is a product of $\mu_{max}\lambda$.

The goodness-of-fit of elaborated models was evaluated by calculating the following mathematical parameters:

• The mean square error (*MSE*):

$$MSE = \frac{RSE}{n} \tag{5}$$

where *RSE* – residual sum of errors, n – the number of degrees of freedom (Sutherland *et al.* 1994).

• Akaike's information criterion (*AIC*) with bias adjustment for small sample size

$$AIC_c = -2 * \ln(\text{likelihood}) + 2 * K + (2 * K * (K+1))/(n-K-1) \tag{6}$$

where n – number of observations, K – number of the model's parameters (Burnham and Anderson 2002).

Analysis of variance (ANOVA) was used to check if the fat content (2, 12, 30%) of UHT dairy products significantly ($P < 0.05$) influences the growth of *L. monocytogenes*.

Secondary Modeling and Validation. The square root model described by Ratkowsky (Eq. 7), polynomial model (Eq. 8) and Arrhenius (Eq. 9) models were used within secondary modeling according to the following equations:

$$\sqrt{\mu_{max}} = b(T - T_{min}) \tag{7}$$

where μ_{max} – maximum growth rate (h^{-1}), b – coefficient determined during the modeling process, T – temperature (C), T_{min} – determined minimum temperature for the growth of microorganisms (C) (Ratkowsky *et al.* 1982).

$$\ln y = a_1 + a_2 x_1 + a_3 x_1^2 \tag{8}$$

where y – response of microorganisms (e.g., specific growth rate [h^{-1}]); a_1, a_2, a_3 – adjustment factors; x_1 – temperature (C).

$$k = Ae^{\frac{-Ea}{RT}} \tag{9}$$

where k – growth rate (h^{-1}), A – "collision" factor (h^{-1}), Ea – activation energy of the reaction system (kJ mol/K), T – absolute temperature (K).

Validation of generated secondary models was performed in three manners:

(1) Mathematical validation by calculating A_f and B_f factors (Ross 1996)

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

$$B_f = 10^{\left(\sum \log\left(\frac{\mu_{predicted}}{\mu_{observed}}\right)/n\right)} \tag{11}$$

where n – number of observations, $\mu_{predicted}$ – predicted specific growth rate, $\mu_{observed}$ – observed specific growth rate. A model that gave the best agreement (A_f and B_f close to 1) between observations and predictions was chosen and used for further validation with independent data.

(2) Mathematical validation according to data obtained from ComBase database (www.combase.cc): data describing behavior of *L. monocytogenes* in liquid dairy products (whole, semi-skimmed and whole UHT and pasteurized milk, chocolate milk, reconstituted nonfat milk solids, cream) obtained in the temperature range from 3–15C without the addition of any preservatives, organic acids etc.

(3) Mathematical validation according to simulations from ComBase Predictor (www.modelling.combase.cc): simulations of the behavior of *L. monocytogenes* were performed by entering the following initial parameters: storage temperature – 3, 6, 9, 12, 15C; pH = 6.7; initial level = 3 log cfu/mL; NaCl/lactic = 0.

RESULTS

Behavior of *Listeria monocytogenes* in UHT Dairy Products during Cold Storage

All purchased UHT dairy products used in present study did not show the presence of *L. monocytogenes* before the artificial contamination step.

Based on the conducted microbiological studies, the behaviour of *Listeria monocytogenes* in UHT milk and UHT cream with, respectively, 2, 12 and 30% fat content was determined during storage at the following temperatures: 3, 6, 9, 12 and 15C (Fig. 1).

UHT milk constitutes a very good substrate for *L. monocytogenes* growth and the maximum cell density at the stationary phase increased together with storage temperature, reaching on average 8.45 log cfu/mL (8.15–8.75 log cfu/mL). The adaptive period, i.e., duration of lag phase, in UHT milk was 170, 75, 17, 13 and 10 h for 3, 6, 9, 12 and 15C, respectively.

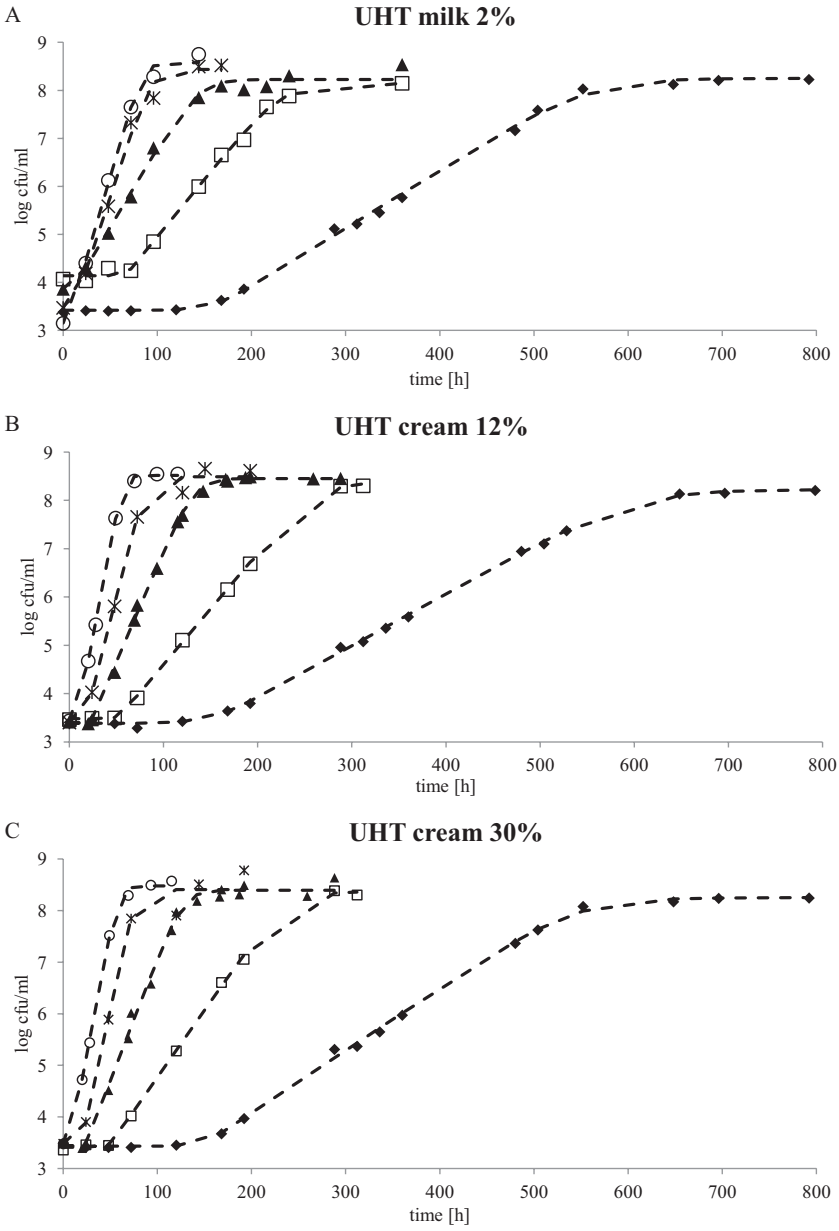

FIG. 1. PRIMARY MODELING (DOTTED LINE – BARANYI MODEL) OF *LISTERIA MONOCYTOGENES* BEHAVIOR IN (A) UHT MILK 2%, (B) UHT CREAM 12%, (C) UHT CREAM 30% AT 3 (♦), 6 (□), 9 (▲), 12 (*) AND 15C (○)

TABLE 1. GOODNESS-OF-FIT OF PRIMARY MODELS ACCORDING TO AKAIKE'S INFORMATION CRITERION (AIC) AND MEAN SQUARE ERROR (MSE)

		MSE			AIC		
Product	Temp (C)	Modified Gompertz function	Logistic function	Baranyi model	Modified Gompertz function	Logistic function	Baranyi model
UHT milk 2%	3	0.013	0.012	**0.005**	–65.193	–66.912	**–80.436**
	6	0.011	0.01	**0.007**	–41.867	–43.083	**–44.78**
	9	**0.011**	0.011	0.019	**–41.486**	–41.175	–33.532
	12	**0.01**	0.01	0.028	**–24.547**	–24.5	–15.011
	15	0.003	**0.001**	0.014	–27.666	**–32.261**	–15.585
UHT cream 12%	3	0.006	0.011	**0.002**	–78.080	–68.644	**–94.414**
	6	0.007	0.012	**0.001**	–36.482	–31.597	**–54.215**
	9	0.007	0.010	**0.004**	–70.895	–64.959	**–77.788**
	12	0.020	**0.019**	0.022	–19.311	**–19.839**	–16.739
	15	0.006	**0.001**	0.002	–28.136	**–40.028**	–33.342
UHT cream 30%	3	0.012	0.013	**0.003**	–67.885	–66.345	**–86.651**
	6	0.006	0.011	**0.003**	–38.368	–32.717	**–43.802**
	9	0.020	0.024	**0.016**	–54.439	–51.698	**–56.471**
	12	0.058	**0.056**	0.058	–11.921	**–12.140**	–9.963
	15	0.003	**0.001**	0.005	–31.669	**–41.912**	–27.456

Values on bold indicates: The smaller values of MSE and AIC, the better fit of the applied model.

In both varieties of UHT cream, *L. monocytogenes* multiplied efficiently with the growth rate coefficient μ (h^{-1}) depending on storage temperature. The duration of adaptive phase was 200, 100, 40, 20 and 15 h for 3, 6, 9, 12 and 15C, respectively. The maximum cell density at the stationary phase was similar in both products, amounting to 8.3 log cfu/mL for 30% cream and 8.4 log cfu/mL for 12% UHT cream. This parameter was only lower in 30% UHT cream at 15C in comparison with other storage temperatures and reached 7.5 log cfu/mL.

The growth curves for *L. monocytogenes* in UHT dairy products were used for mathematical analyses, which included the primary and secondary models and their mathematical validation as well as a comparison of generated models with independent data from the ComBase database and with ComBase Predictor predictions.

Primary Modeling

Primary modeling aims at determining the basic parameters that describe the growth kinetics of microorganisms: growth rate coefficient μ (h^{-1}) and duration of lag phase (h). In practice, these parameters are predominantly used for further mathematical analysis.

The growth curves of *L. monocytogenes* in UHT milk and cream varieties were subjected to primary modeling with three growth models: modified Gompertz's function, logistic function and Baranyi and Robert's model. Two coefficients were used to determine goodness-of-fit of the primary models: MSE and AIC. The values of the calculated MSE and AIC parameters for the individual primary models are presented in Table 1. While interpreting these indicators, it may be concluded that the highest accuracy of matching was detected for Baranyi's model (MSE and AIC were lowest in over 50% of the analyzed cases) and the growth rate coefficients μ determined based on this model were used for secondary modeling (Table 2). The changes in the counts of *L. monocytogenes* in UHT milk and cream varieties during storage and fitted Baranyi model within the temperature range of 3–15C are depicted on Fig. 1.

The statistical analysis with Fisher's test ($P < 0.05$) demonstrated that the fat content (2, 12 and 30%) did not have any significant statistical impact on the growth of

TABLE 2. GROWTH RATES M (H^{-1}) OF *LISTERIA MONOCYTOGENES* IN UHT DAIRY PRODUCTS AT TEMPERATURES 3, 6, 9, 12 AND 15C

Product	Temp (C)	μ (h^{-1})
UHT milk 2%	3	0.012
	6	0.024
	9	0.033
	12	0.060
	15	0.067
UHT cream 12%	3	0.011
	6	0.023
	9	0.045
	12	0.077
	15	0.104
UHT cream 30%	3	0.012
	6	0.026
	9	0.047
	12	0.085
	15	0.098

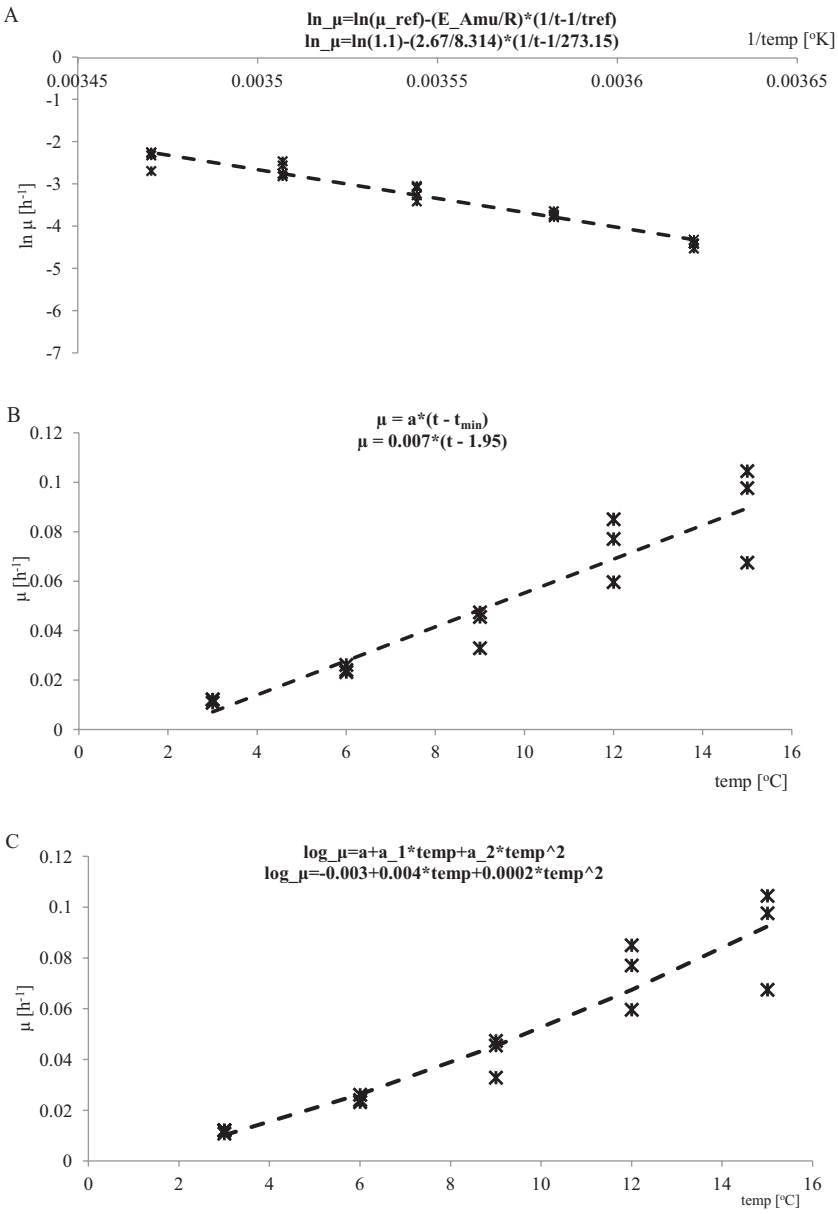

FIG. 2. SECONDARY COMBINED MODEL FOR UHT DAIRY PRODUCTS ACCORDING TO (A) ARRHENIUS, (B) SQUARE ROOT AND (C) POLYNOMIAL FUNCTIONS

L. monocytogenes in UHT milk and cream. For this reason, all microbiological data were combined and treated as one data set representing the behavior of *L. monocytogenes* in UHT dairy products.

Secondary Modeling and Validation

In the process of secondary modeling, the square root model by Ratkowsky, the Arrhenius equation and polynomial model were used to determine the impact of temperature (independent variable) on the growth rate of *L. monocytogenes* (dependent variable) in UHT dairy products.

Figure 2 presents a graphic and mathematical depiction of the secondary models according to Ratkowsky, Arrhenius and polynomial model for UHT dairy products. The generated secondary models accurately described the impact of temperature on the growth of pathogen and the R^2 for UHT dairy product reached 0.90, 0.90 and 0.94 for the Ratkowsky, Arrhenius and polynomial models, respectively.

TABLE 3. MATHEMATICAL VALIDATION OF GENERATED RATKOWSKY, ARRHENIUS AND POLYNOMIAL MODELS DESCRIBING THE GROWTH OF *LISTERIA MONOCYTOGENES* IN UHT DAIRY PRODUCTS

	UHT dairy product	
Model	A_f	B_f
Ratkowsky	1.313	1.077
Arrhenius	1.203	1.035
Polynomial	**1.177**	**1.027**

A_f, accuracy factor; B_f, bias factor.
Values in bold indicates: If the values of Af and Bf are closer to 1, then there is a better fit of the applied model.

Since the applicability of predictive models depends on a good validation process, the models describing the behavior of *L. monocytogenes* were subjected to mathematical validation, which included calculating the accuracy A_f and bias B_f factors (Table 3). It allowed for an evaluation of the predictive accuracy of the generated models. Based on the calculations, it was found that the polynomial model most accurately described the impact of temperature on the growth rate of *L. monocytogenes* in UHT dairy products and this model was further subjected to mathematical validation based on external data from ComBase and simulation from ComBase Predictor.

Within the process of external validation, the growth of *L. monocytogenes* in the UHT dairy product during cold storage was compared with independent data from the ComBase database and simulations generated in ComBase Predictor. In order to determine the applicability of the constructed polynomial model for predicting the growth of pathogen in UHT dairy products, the accuracy A_f and deviation B_f factors were calculated. Figure 3 presents the results of external validation based on independent microbiological data and simulations with ComBase Predictor. Table 4 contains the calculated values of A_f and B_f factors.

The simulations of *L. monocytogenes* growth performed with ComBase Predictor for UHT dairy products demonstrated their satisfactory applicability for describing the growth of *L. monocytogenes*. The application precisely determined the behavior of the pathogen within 3–15C range. It is further confirmed with the calculated accuracy A_f and deviation B_f coefficients, which were 1.159 and 1.021, respectively. For a validation process based on external microbiological data, the values of the A_f and B_f coefficients were 1.510 and 1.185, respectively.

DISCUSSION

In the literature, some reports indicate that milk is an excellent medium for *L. monocytogenes* growth and recontaminations after pasteurization may result in substantial multiplication of this pathogen, even if milk is stored at 4C.

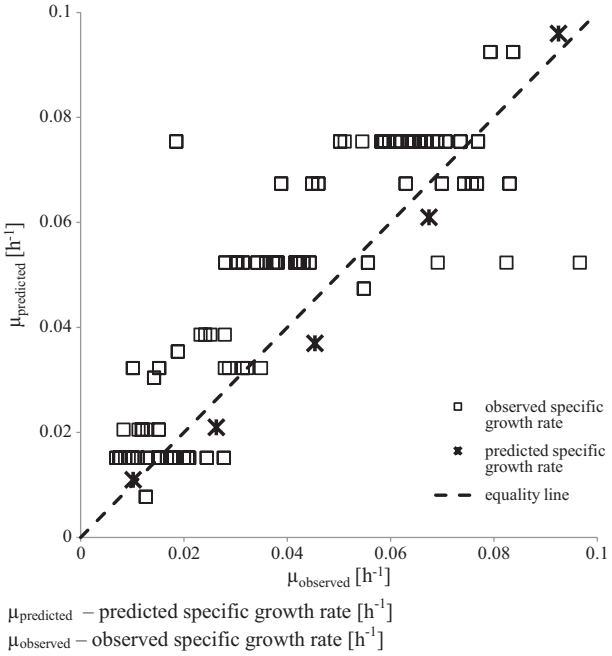

FIG. 3. GRAPHICAL VALIDATION OF GENERATED POLYNOMIAL MODEL FOR UHT DAIRY PRODUCTS ACCORDING TO INDEPENDENT DATA FROM ComBase AND PREDICTIONS FROM ComBase PREDICTOR

In Europe, outbreaks of listeriosis were ca. 50% associated with dairy products, and therefore, the products of this category require special control measures to detect *Listeria monocytogenes* (Rosenow and Marth 1987; Alavi *et al.* 1999; Lundén *et al.* 2004).

The results indicate that *L. monocytogenes* is capable of growing in UHT milk as well as in 30 and 12% UHT cream at 3–15C. There was no correlation between storage temperature and the maximum cell density. A similar lack of correlation was reported by Xanthiakos *et al.* (2006) who examined the behavior of *L. monocytogenes* in pasteurized milk. The differential equation by Baranyi and Roberts (1994) is a commonly applied primary model of growth used to describe the cell count over time (Fernandez *et al.* 1997; McClure *et al.* 1997; Xanthiakos *et al.* 2006; Juneja *et al.* 2007; Koseki *et al.* 2011). This model assumes that

TABLE 4. MATHEMATICAL VALIDATION OF GENERATED POLYNOMIAL MODEL ACCORDING TO DATA FROM ComBase DATABASE AND PREDICTIONS FROM ComBase PREDICTOR

	A_f	B_f
ComBase	1.510	1.185
ComBase Predictor	**1.159**	**1.021**

A_f, accuracy factor; B_f, bias factor.
Values in bold indicates: If the values of Af and Bf are closer to 1, then there is a better fit of the applied model.

during the lag phase, the specific growth rate depends on the needs of an individual cell for synthesis of a certain intracellular substance (e.g., RNA or other cytoplasmatic component such as ribosomes), which is essential for growth and, under constant environmental conditions, the duration of lag phase λ is inversely proportional to the maximum specific growth rate μ_{max} (Baranyi and Roberts 1994).

Thanks to the ANOVA, it was determined that the fat content in UHT dairy products (2, 12 and 30%) does not have any impact on *L. monocytogenes* growth during storage at 3–15C. Augustin and Carlier (2000) analyzed the value of growth rate coefficient and duration of lag phase of *L. monocytogenes* in different groups of food products. They did not observe any statistically significant differences ($P > 0.17$) between the growth rate coefficient of *L. monocytogenes* in skimmed and full-fat milk.

The polynomial model used in secondary modeling precisely described the correlation between the growth rate coefficient μ and storage temperature. Te Giffel and Zwietering (1999) demonstrated that the models, which did not include interactions between environmental factors, were, in numerous cases, sufficiently accurate to predict the growth rate of *L. monocytogenes* in food. It was confirmed with the value of B_f coefficient, and according to Ross (1996), predictions for the growth of pathogenic microorganisms should be assumed satisfactory when the value of bias factor B_f ranges from 0.9 to 1.15.

The majority of tertiary models are constructed based on microbiological data generated in liquid media with a known and easily modified chemical composition. These are the so-called "securing" models, which have a wide safety margin. In food production, however, predictions generated with these models are often laden with significant errors, making the practical implementation of these models impossible. An alternative, increasingly common and more effective approach consists in creating models based on data derived directly from food (Cheroutre-Vialette and Lebert 2000; Augustin *et al*. 2005; Xanthiakos *et al*. 2006).

To sum up, it may be concluded that the created combined model for UHT dairy products accurately predicted the behavior of *L. monocytogenes* in UHT dairy products at 3–15C. The developed predictive model may be a tool used in hazards analyzing during HACCP implementation, as well as for estimating exposure in the process of microbiological risk assessment and in supporting decisions in many aspects of microbiological food and safety quality management.

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