

## Predictive modeling of *Bifidobacterium animalis* subsp. *lactis* Bb-12 growth in cow's, goat's and soy milk

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### Summary

The aim of this study was to use a predictive model to analyse the growth of a probiotic strain *Bifidobacterium animalis* subsp. *lactis* Bb-12 in cow's, goat's and soy milk. The Gompertz model was used, and the suitability of the model was estimated by the Schnute algorithm. Except for the analysis of *Bifidobacterium animalis* subsp. *lactis* Bb-12 growth, the Gompertz model was also used for the analysis of pH changes during the fermentation process. Experimental results, as well as the values of kinetic parameters obtained in this study, showed that the highest growth rate of *Bifidobacterium animalis* subsp. *lactis* Bb-12 was obtained in goat's milk, and the lowest in soy milk. Contrary to the growth of *Bifidobacterium animalis* subsp. *lactis* Bb-12, pH decreased faster in soy milk than in cow's milk. The highest rate of pH decrease was also observed in goat's milk, which is in correspondence with results of various previous studies. The Gompertz model proved to be highly suitable for analysing the course and the fermentation kinetics in these three kinds of milk, and might be used to analyse the growth kinetics of other probiotic and starter cultures in milk.

*Key words:* bacterial growth rate, fermentation kinetics, cow's milk, goat's milk, soy milk, *Bifidobacterium animalis* subsp. *lactis* Bb-12

### Introduction

The incorporation of probiotic bacteria in food products increased over the past two decades (Salazar et al., 2009). A probiotic is a live microbial food or feed supplement, which beneficially affects the host organism by improving its intestinal microbial balance when administered in proper amounts (Brown and Valiere, 2004). Multiple reports have described the probiotic health benefits on gastrointestinal infections, antimicrobial activity, improvement in lactose metabolism, anti-diarrheal properties, immune system stimulation, improvement in inflammatory bowel disease and other health disorders (Saarela et al., 2000; Shah, 2007). Fermented food with probiotic bacteria today has an important

place in the overall food marketing (Parvez et al. 2006).

Viability of probiotic bacteria to high counts (at least  $10^6$  CFU/g) is recognized as an important requirement during the manufacture and marketing of probiotic foods in order to achieve the claimed health benefits. Bifidobacteria are anaerobes with special nutritional requirements, thus often slowly grow in milk during fermentation (Tamime et al., 1995). Except the mechanism of action of the bifidobacteria in the intestine, much research has been taken in an attempt to elucidate the growth kinetics of bifidobacteria in milk. For fermented product to have a therapeutic value, it is necessary to achieve a sufficient viable cell count of bifidobacteria at a certain time of fermentation (Blažić et al., 2011).

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Traditionally, probiotics have been added to yoghurt and other fermented dairy products, such as cow's and goat's probiotic fermented drinks (Penna et al., 2007). Today there is an increasing interest for non-dairy based probiotic products so probiotic cultures are being incorporated into fermented drinks or marketed as supplements (Božanić et al., 2008; Rivera-Espinosa and Gallardo-Navarro, 2010). Soymilk is suitable for the growth of lactic acid bacteria, especially bifidobacteria (Božanić et al., 2008). Being free of cholesterol, gluten and lactose, soymilk is suitable for lactose-intolerant consumers, vegetarians and patients suffering from milk-allergy. Soy-based foods may provide a range of health benefits for consumers due to their hypolipidemic, anticholesterolemic and antiatherogenic properties, as well as the reduced allergenicity (Donkor et al., 2007).

Predictive modeling is an important field of food microbiology. Models allow the prediction of parameters such as the microbial safety, the product shelf life, the detection of critical parts in the production and distribution process, as well as the optimization of production and distribution chains (Zwietering et al., 1990). A number of growth models can be found in the literature, like the model of Gompertz, Stannard, Richards, Schnute, the logistic model and others (Gibson et al., 1987; Bratchel et al., 1989). Accurate determination of the growth curve is particularly important for the fermentation by probiotic starters, since probiotics grow slowly in milk and it is necessary to determine the key points of the fermentation process (Stanton et al., 2005). Furthermore, based on the results of the predictive analysis, the growth kinetics of probiotic strains in different types of substrates can be compared, but also the optimal content of a prebiotic supplement can be determined.

The aim of this study was to evaluate differences in fermentation kinetics and growth rates of *Bifidobacterium animalis* subsp. *lactis* Bb-12 between goat's, cow's and soy milk using the Gompertz model. The Gompertz model was selected as the most appropriate one based on the results of our previous research and was created in such a manner to contain microbiologically relevant parameters.

## Materials and methods

### Sample preparation

Commercial UHT cow's and goat's milk standardized on 3.2 % (w/v) of milk fat were used for the fermentation of samples ("Vindija" Dairy Industry, Varaždin, Croatia). Soymilk was prepared according to the method of Mulimani and Ramalingam (1995). Soybean seeds were ground to flour. The soybean flour was defatted with hexane (1:1 w/v) (Prashnat and Mulimani, 2005), suspended in 10 volume of distilled water and heated to boiling. The undissolved residue was separated from soymilk by centrifugation for 5 min at 5000 rpm (Multifuge 3L-R, Heraeus, Buckinghamshire, England). The supernatant containing soymilk was used for fermentation with bifidobacteria.

### Fermentation of cow's, goat's and soy milk

The monoculture *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Chr. Hansen, Copenhagen, Denmark) was used to inoculate the samples of goat, cow and soy milk at 37 °C for 25 h (method suggested by Tamime and Marshall, 1997).

### Analyzes during fermentation

The viable cell count of *Bifidobacterium animalis* subsp. *lactis* Bb-12 was determined on modified *Bifidobacterium* medium (according to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) in anaerobic jars at 37 °C for 48 h (Tamime et al., 1995). Modification was performed by adding 13.5 g/100 mL Bacteriological Agar (Agar Bios Special LL, Biolife, Milano, Italy) and 3 g/100 mL LiCl (Slačanac et al., 2004). The viable cell count of *Bifidobacterium animalis* subsp. *lactis* Bb-12 and pH values were determined every 5 h during the fermentation process. All measurements were performed in 5 replicates.

### Model description

Bacterial growth often shows a phase in which the specific growth rate starts at a value of zero and then accelerates to a maximum value ( $\mu_m$ ) in a certain period of time, resulting in a stationary phase. In addition, the growth decreases and finally reaches zero, so that an asymptote ( $A$ ) is formed. When

the growth curve is defined as the logarithm of the number of organisms plotted against time, these growth rate changes result in a sigmoidal curve, with a lag phase just after  $t = 0$  followed by an exponential phase and then by a stationary phase (Zwietering et al., 1990). The three phases of the growth curve can be described by three parameters: the maximum specific growth rate ( $\mu_m$ ) is defined as the tangent in the inflection point; the lag time ( $\lambda$ ) is defined as the x-axis intercept of the tangent; and the asymptote ( $A = N_\infty/N_0$ ) is the maximal value reached. The Gompertz equation is written as follows:

$$y = a \times \exp[-\exp(b-ct)] \quad (1)$$

where  $a$ ,  $b$  and  $c$  are mathematical parameters.

The parameter conversion of the Gompertz equation to parameters with microbiological meaning ( $\mu_m$ ,  $\lambda$  and  $A$ ) was described by Zwietering et al. (1990).

The modified Gompertz equation has the following form:

$$y = A \exp\left\{-\exp\left[\frac{\mu_m - c}{A}(\lambda - t) + 1\right]\right\} \quad (1).$$

Parameters  $A$ ,  $\mu_m$  and  $\lambda$  were calculated and the same model was used to analyse trends of pH changes during fermentation of goat's and cow's milk.

### Statistics

The mean value and standard deviations of four measurements were calculated in STATISTICA 8.0 (StatSoft). Influence of type of milk was analysed by the Fisher's Least Significance Differences test (LSD). The nonlinear equations were fitted to growth data by the nonlinear regression with a Marquand algorithm (Marquand, 1963). The algorithm

calculates the set of parameters with the lowest sum of squares (RSS) and their 95 % confidence interval (Schnute, 1981).

### Results and discussion

The aim of this study was to evaluate differences in fermentation kinetics and growth rates of *Bifidobacterium animalis* subsp. *lactis* Bb-12 between goat's, cow's and soy milk using the Gompertz model. pH values decreased more rapid (Figure 1) and larger number of viable cells of *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Figure 2) were found in goat milk than in cow and soy milk during the fermentation. Some authors have previously indicated that goat milk might be a better substrate for lactobacilli and bifidobacteria growth in comparison to cow milk (Haenlein, 2004; Slačanac et al., 2010). Results obtained in this research supported that claims.

In this study, the final pH value was selected as a key parameter to control the fermentation process. It was necessary to determine the time required for the acidity of fermented milk to reach the isoelectric point of casein (pH = 4.6). At that point the fermentation was terminated in order to avoid over-acidification of the fermented milk. Since the pH value during milk fermentation also decreased exponentially ( $y = \ln(pH/pH_0)$ ), by analogy, in this paper the Gompertz model was used for the analysis of changes in pH value. Table 1 shows the values of kinetic parameters for pH changes during fermentation of cow's, goat's and soy milk. The maximum rate of change in pH ( $v(pH)_m$ ) and the time required to reach pH 5.0 ( $t_{pH=5.0}$ ) and 4.6 ( $t_{pH=4.6}$ ) in the fermented milk were selected as key parameters. The

Table 1. Kinetic parameters of pH changes during fermentation of cow's, goat's and soy milk by *Bifidobacterium animalis* subsp. *lactis* Bb-12\*

Sample	Kinetic parameter				
	$A$	$v_{max}$ (h <sup>-1</sup> )	$\lambda$ (h)	$t_{pH=5.0}$ (h)	$t_{pH=4.6}$ (h)
CM	1.71±0.17 <sup>a</sup>	0.15±0.002 <sup>a</sup>	0.19±0.12 <sup>a</sup>	20.91±4.82 <sup>c</sup>	33.14±7.41 <sup>c</sup>
GM	2.05±0.14 <sup>ab</sup>	0.19±0.01 <sup>ab</sup>	0.27±0.16 <sup>ab</sup>	10.18±2.19 <sup>a</sup>	17.99±3.83 <sup>a</sup>
SM	2.26±0.06 <sup>ab</sup>	0.27±0.05 <sup>b</sup>	0.50±0.23 <sup>c</sup>	14.03±2.94 <sup>b</sup>	22.95±4.47 <sup>b</sup>

\* - mean values of 5 determinations

CM - cow's milk; GM - goat's milk; SM - soy milk

$A$  - asymptote;  $v_{max}$  - maximal rate of pH decrease;  $\lambda$  - lag phase;  $t_{pH}$  - time required to reach a certain pH value

<sup>abc</sup> - Mean values followed by the same letter in the same column are not significantly different ( $p < 0.05$ )

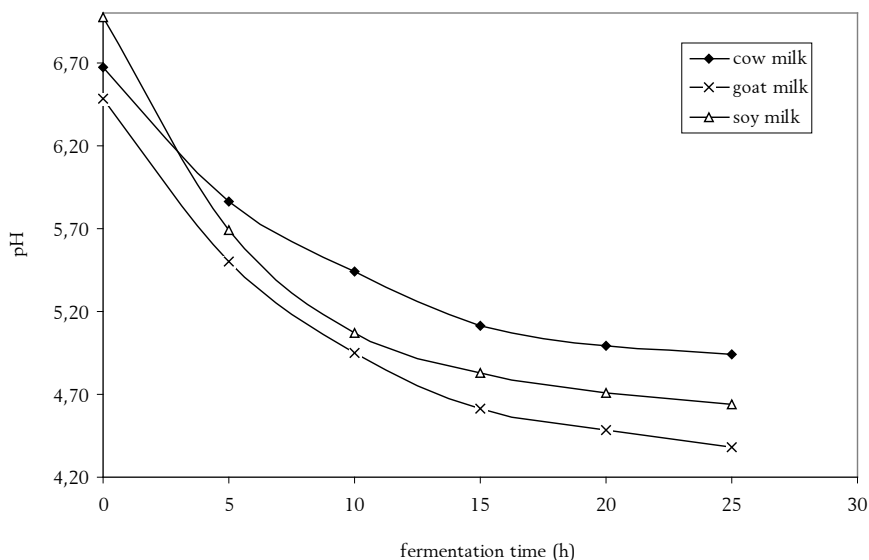


Figure 1. Changes in pH during fermentation of cow's, goat's and soy milk by *Bifidobacterium animalis* subsp. *lactis* Bb-12

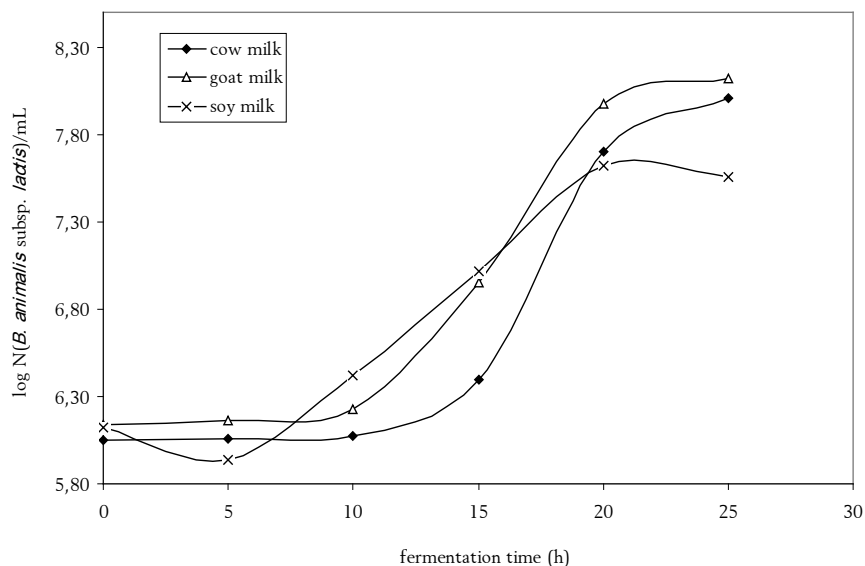


Figure 2. Changes in the viable cell count of *Bifidobacterium animalis* subsp. *lactis* Bb-12 ( $\log(N)$ ) during fermentation of cow's, goat's and soy milk

values of kinetic parameters in Table 1 show different tendencies of pH decrease in cow's, goat's and soy milk. Although the  $v_{\max(\text{pH})}$  was highest for soy milk samples, they reached a pH value of 5.0 four hours later than goat's milk, and pH 4.6 five hours later than goat's milk. Such results may be associated to the longer lag phase ( $\lambda$ ) in soy milk than in goat's milk. The value of the asymptote ( $A$ ) did not

significantly differ between the goat and soy milk. In this case, value  $A$  suggested a minimal pH value achieved to stationary phase. The values of all kinetic parameters of pH decrease during fermentation of cow's milk were lower in comparison to goat's milk and soy milk. Cow's milk did not reach a pH of the isoelectric point of casein (4.6) even after 25 hours of fermentation. The data obtained in this

Table 2. Differences in a viable cell count of *Bifidobacterium animalis* subsp. *lactis* Bb-12 and final pH values after 20 and 25 h of fermentation determined by Fisher's LSD test (influence of a milk type)\*

Sample	log N (BL) <sub>20h</sub>	log N (BL) <sub>25h</sub>	pH <sub>20h</sub>	pH <sub>25h</sub>
CM	7.70 <sup>ab</sup>	8.00 <sup>b</sup>	4.99 <sup>bc</sup>	4.94 <sup>c</sup>
GM	7.98 <sup>b</sup>	8.35 <sup>c</sup>	4.48 <sup>a</sup>	4.38 <sup>a</sup>
SM	7.62 <sup>a</sup>	7.56 <sup>a</sup>	4.71 <sup>b</sup>	4.64 <sup>b</sup>

Mean values followed by the same letter in the same column are not significantly different ( $P < 0.05$ ) - for all samples separately

\*Mean of 5 determinations

CM - cow milk; GM - goat milk; SM - soy milk; BL - *Bifidobacterium animalis* subsp. *lactis* Bb-12

<sup>a,b,c</sup> - samples marked with same letters in the same column are statistically not significantly different on level of significance  $p \leq 0.05$

study are consistent with the following statements about the rapid acidification of goat's milk (Martin Diana et al., 2003), but also can be an indicator of metabolism activity of starter culture in milk during fermentation.

Since probiotic yogurt was concerned, the number of bacteria as well as their viability during the storage period were the basic parameters of the therapeutic value. Differences in the number of bacteria in cow's, goat's and soy milk after a certain period of fermentation are shown in Table 2. The growth curves of *Bifidobacterium animalis* subsp. *lactis* Bb-12 in cow's, goat's and soy milk are shown in Figure 2. Data obtained in this study confirm the thesis of our previous studies (Slačanac et al., 2010) as well as studies conducted by other authors (Kongo et al., 2006; Herrero and Requena, 2006). Like some other previously tested probiotic starters, *Bifidobacterium animalis* subsp. *lactis* Bb-12 grew faster

in goat's than in cow's milk. Data presented in Figure 2 show that the number of *Bifidobacterium animalis* subsp. *lactis* Bb-12 was significantly higher in goat's than in cow's and soy milk during overall fermentation process. However, the final bacteria number which was after 20 h of fermentation showed a high growth potential of *Bifidobacterium animalis* subsp. *lactis* Bb-12 in all three types of milk. The highest number of *Bifidobacterium animalis* subsp. *lactis* Bb-12 was observed in soymilk after 20-25 h of fermentation (Figure 2). However, that number was still above the required minimum necessary to obtain healthy effects as a probiotic ( $10^6$ - $10^8$  CFU/g) (FAO/WHO, 2002). Data in Table 1 and Figure 2 also indicated a significant increase of *Bifidobacterium animalis* subsp. *lactis* Bb-12 viable cells count in goat's and cow's milk between the 20<sup>th</sup> and the 25<sup>th</sup> h of fermentation. In soy milk, a viable cell count of *Bifidobacterium animalis* subsp. *lactis* Bb-12 decreased between the 20<sup>th</sup> and the 25<sup>th</sup> h.

The use of predictive models is extremely useful for monitoring and optimization of lactic acid fermentation by probiotic strains (Saxelin et al., 2000). Probiotic strains in most cases slowly ferment milk and have special requirements for growth. Therefore, growth stimulators have been often added to milk before fermentation. The values of kinetic parameters calculated by the Gompertz model (Table 3) confirmed the experimental data shown in Table 1 and Figure 2. The highest maximum growth rate ( $\mu_{max}$ ) of *Bifidobacterium animalis* subsp. *lactis* Bb-12 was in goat's milk, and the lowest in soy milk. Value  $A$ , generation time ( $t_g$ ) and the length of lag phase ( $\lambda$ ) did not differ significantly in goat's and

Table 3. Kinetic parameters of *Bifidobacterium animalis* subsp. *lactis* Bb-12 growth in cow's, goat's and soy milk obtained by the Gompertz model\*

Sample	Kinetic parameter			
	$A$	$\mu_{max}$ (h <sup>-1</sup> )	$\lambda$ (h)	$t_g$ (h)
CM	1.94±0.19 <sup>ab</sup>	0.34±0.06 <sup>b</sup>	14.36±0.61 <sup>b</sup>	2.11±0.35 <sup>a</sup>
GM	1.86±0.05 <sup>ab</sup>	0.70±0.07 <sup>c</sup>	13.08±1.56 <sup>b</sup>	2.13±1.71 <sup>a</sup>
SM	1.59±0.15 <sup>a</sup>	0.15±0.04 <sup>a</sup>	7.98±2.72 <sup>a</sup>	4.81±1.35 <sup>b</sup>

\* - mean values of 5 determinations

CM - cow's milk; GM - goat's milk; SM - soy milk

$A$  - asymptote;  $\mu_{max}$  - maximal growth rate;  $\lambda$  - lag phase;  $t_g$  - generation time

<sup>abc</sup> - Mean values followed by the same letter in the same column are not significantly different ( $p < 0.05$ )

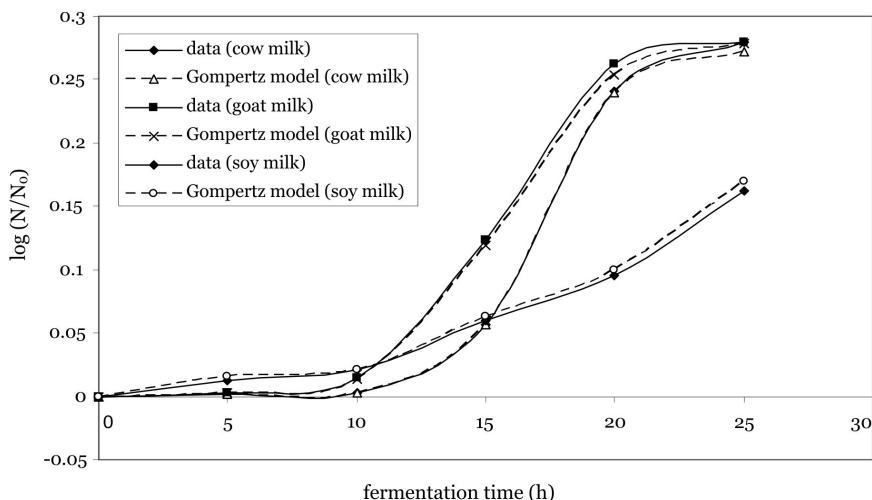


Figure 3. Growth curve of *Bifidobacterium animalis* subsp. *lactis* Bb-12 in cow's, goat's and soy milk fitted with the Gompertz model

cow's milk, while the values of all these parameters were significantly lower in soy milk. Analysis of the values of all kinetic parameters showed that maximum growth rate is a key parameter, which was in goat's milk 51.43 % higher than in cow's milk. The maximum growth rate of *Bifidobacterium animalis* subsp. *lactis* Bb-12 in soy milk was even twice lower than in cow's milk. These data suggest a possible need to add some bifidobacteria growth stimulators to soy milk before fermentation.

Figure 3 shows the growth curves of *Bifidobacterium animalis* subsp. *lactis* Bb-12 fitted by the Gompertz model. The Schnute RSS values were: 1.24 for cow's milk, 1.25 for goat's milk and 1.38 for soy milk, while the  $RSS_{crit}$ , done by Schnute, was 10. Accordingly, the Gompertz model was highly suitable for the analysis of the fermentation kinetics by *Bifidobacterium animalis* subsp. *lactis* Bb-12 in all three types of milk.

## Conclusion

The Gompertz model proved to be very suitable for analysing the kinetics of lactic acid fermentation in different types of milk. Microbiological parameters of the Gompertz model provided an insight into the trends of *Bifidobacterium animalis* subsp. *lactis* Bb-12 growth in cow's, goat's and soy milk. Furthermore,

the analysis of pH changes by the Gompertz model also proved to be useful for optimizing the fermentation process by *Bifidobacterium animalis* subsp. *lactis* Bb-12 in cow's, goat's and soy milk. Based on the results obtained by the Gompertz model, key points of fermentation process in cow's, goat's and soy milk might be assumed even in the absence of specific experimental data. From the results of this study it is obvious that the Gompertz model could be suitable for the analysis of the growth of other probiotic and starter cultures in these three types of milk.

## *Upotreba prediktivnih modela za analizu rasta Bifidobacterium animalis subsp. lactis Bb-12 u kravljem, kozjem i sojinom mlijeku*

### Sažetak

Cilj rada bio je upotrijebiti jedan od prediktivnih modela rasta bakterija za analizu rasta probiotičkog soja *Bifidobacterium animalis* subsp. *lactis* Bb-12 u kravljem, kozjem i sojinom mlijeku. Korišten je Gompertzov model, a prikladnost modela je procijenjena Schnuteovim algoritmom. Osim za analizu rasta *Bifidobacterium animalis* subsp. *lactis* Bb-12, Gompertzov model korišten je i za analizu kretanja pH vrijednosti tijekom fermentacije.

Eksperimentalni rezultati, kao i vrijednosti kinetičkih parametara, dobiveni u ovom radu, pokazali su da je najveća brzina rasta *Bifidobacterium animalis* subsp. *lactis* Bb-12 bila u kozjem mlijeku, a najmanja u sojinom mlijeku. Suprotno u odnosu na rast *Bifidobacterium animalis* subsp. *lactis* Bb-12, pH vrijednost opadala je brže u sojinom mlijeku nego u kravljem. Najveći pad pH bio je također u kozjem mlijeku, što potvrđuju rezultati naših prethodnih studija kao i rezultati drugih autora. Gompertzov model pokazao se pogodnim za analizu tijeka i kinetike fermentacije u ove tri vrste mlijeka i može se koristiti za analizu kinetike rasta drugih probiotičkih i starter kultura u mlijeku.

**Ključne riječi:** brzina rasta bakterija, kinetika fermentacije, modeliranje, kravlje mlijeko, kozje mlijeko, sojino mlijeko, *Bifidobacterium animalis* subsp. *lactis* Bb-12

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