Characterization of the growth of *Lactobacillus rhamnosus* GG in milk at suboptimal temperatures

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Summary

The effect of the incubation temperature on the growth of *Lactobacillus rhamnosus* GG in milk was modelled and characterized in relation to the growth rate and the lag phase. Lag phase duration of *L. rhamnosus* GG was increased with decreasing incubation temperature under the Daughtry model $\ln(1/\text{lag}) = 93.082T^2 - 44.403T + 0.999$ ($R^2 = 0.951$). The growth rate was significantly linearly related to the increase in the incubation temperature as follows the equation in accordance with the Ratkowsky model: $\sqrt{Gr} = 0.0235(T - T_{\min}) + 0.1081$ ($R^2_{Gr} = 0.9981$) in the temperature range from 6 °C to 41 °C. The conjunction of pH lag phase and rate of pH decrease ($\text{lag}_{\text{pH}} \times \text{rate}_{\text{pH}}$) representing the physiological state of the culture showed maximum at the temperature of 40.5 °C that can be considered as optimal for acid production.

Keywords

Lactobacillus rhamnosus GG; growth parameters; growth modelling

Lactobacillus rhamnosus is a Gram-positive, non-sporeforming, facultatively anaerobic or microaerophilic, non-motile and catalase-negative bacterium. It belongs to mesophilic organisms, but in dependence on the strain, its cultures may grow at temperatures lower than 15 °C or higher than 40 °C. To grow, it requires a lot of vitamins including folic acid, riboflavin, niacin, pantothenic acid and mineral calcium [1]. Optimal initial pH value for the growth is in the range from 6.4 to 4.5. It grows as rods, single rods or in short chains. The dimension of the cells is from 0.8 to 1.0 μm in width and from 2.0 to 4.0 μm in length [2].

Metabolism of *L. rhamnosus* is facultatively heterofermentative (lactobacilli Group 2). It converts hexoses into L(+)-lactic acid, according to the Embden-Meyerhof pathway, and due to aldolase and phosphoketolase, pentoses are also fermented. Lactic acid is usually produced up to 1.5% in the glucose medium. In the absence of glucose, it produces lactic acid, acetic acid, formic acid and ethanol [3, 4]. *L. rhamnosus* cannot convert pyruvate to lactate at a fast rate to match the rate of glycolysis. Therefore, pyruvate is broken down into various other metabolites, such as acetate, diacetyl and acetoin [3, 5]. *L. rhamnosus* gives a high yield for diacetyl, 64 mg of diacetyl per 1 g of glucose [3]. According to ØSTLIE et al. [6], *L. rhamnosus* produced 0.6 mg.kg^{-1} diacetyl after 8 h of incubation at 37 °C and, at 37 °C after a 72 h incubation, it produced 221 mg.kg^{-1} carbon dioxide and 15 mg.kg^{-1} ethanol [6].

According to JYOTI et al. [3], possible catabolite repression of lactate by glucose and citrate may lead to the diauxic growth on glucose and citrate. This demonstrates that lactate uptake is regulated at the genetic level. Therefore, glucose and citrate are the preferred substrates for *L. rhamnosus* cultivation [3, 7].

L. rhamnosus GG represents a probiotic strain which was clinically studied and was found to enhance human natural resistance and healthy digestive system [8]. The strain was able to inhibit adhesion of *Clostridium histolyticum, Cl. difficile* and *Salmonella enterica*. The combination of *L. rhamnosus* GG with *L. rhamnosus* LC705 inhibited growth of *Staphylococcus aureus, E. coli* and *S. enterica*. However, to inhibit *Cl. difficile*, the best combination was *L. rhamnosus* GG, *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* JS [9]. The combination of *L. rhamnosus* GG, *P. freudenreichii* and *L. plantarum* inhibited clostridia and *Listeria monocytogenes* [10]. Strain *L. rhamnosus* LC705 suppressed growth of some yeasts and

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moulds. The strains of *L. rhamnosus*, GG and LC705, were found to bind aflatoxins [11].

L. rhamnosus GG showed a high tolerance to the acidic conditions of the stomach [11, 12], survived intestinal passage [13], was able to adhere to intestinal mucus [9, 11, 14] and transiently colonized the gastrointestinal tract after three days of treatment [11–13]. Clinical effects of *L. rhamnosus* GG were represented by an increasing resistance to respiratory and gastrointestinal infections, decreasing occurrence of fever and a healing effect to atopic eczema [13].

Because of its probiotic and antimicrobial activities, *L. rhamnosus* GG is used in food industry not only as probiotics but also as a protective culture in fermented and non-fermented dairy products, beverages, ready-to-eat foods, dry sausages and salads [10, 15]. To have a beneficial effect on the gut microbiota, probiotics have to be in such an excess (approximately 10^8–10^9 CFU.ml^{-1}) so as their amounts were higher than 10^6 CFU.ml^{-1} at the end of the shelf-life [4, 6].

In spite of the broad usage of probiotics in the food industry, the knowledge on specific strains is usually restricted only to the producers. That is why the aim of our work was to describe growth dynamics of the probiotic strain *L. rhamnosus* GG in milk.

MATERIAL AND METHODS

Microorganisms

The strain of *Lactobacillus rhamnosus* GG was provided by Dr. Salminen (University of Turku, Turku, Finland) through the mediation of Dr. Lauková (State Veterinary and Food Institute, Košice, Slovakia).

Inoculation and cultivation conditions

The strain of *L. rhamnosus* GG was kept in de Man - Rogosa - Sharpe broth (MRS; Biomark, Pune, India) at (5 ± 1) °C. The standard suspension of the microorganism was prepared from a 18 h culture grown in MRS broth at 37 °C. This culture was inoculated to the ultra-pasteurized (UHT) milk (Rajo, Bratislava, Slovakia) in a concentration of approximately 10^3 CFU.ml^{-1}. Two parallel static aerobic cultivations of milk samples were carried out at appropriately ranked temperatures from 6 to 50 °C.

Numbers of *L. rhamnosus* GG in milk

In relevant time intervals, the required amounts of *L. rhamnosus* GG were withdrawn to determine CFU on MRS agar according to the Slovak Technical Standard STN ISO 15214 [16]. Typical colonies were white, round to oblate, convex and their morphology was confirmed microscopically.

Determination of active acidity

In the same time as the microbiological determination, pH values were measured using the WTW 720 pH meter (Inolab, Weilheim, Germany).

Fitting growth curves and calculation of the growth parameters

Growth curves of *L. rhamnosus* GG at each temperature were modelled with a mechanistic model of Baranyi et al. [17]. Growth parameters (lag phase duration, growth rate and others) were calculated from each curve and analysed in the secondary phase of microbial growth modelling.

Secondary models

Growth rate (*Gr*) as a function of suboptimal growth temperature has been described with two models. The square root model [18]:

$$\sqrt{Gr} = b\left(T - T_{\min}\right) \tag{1}$$

where T_{min} (°C) is defined as the theoretical minimum growth temperature, *Gr* (log CFU.ml^{-1}.h^{-1}) is the growth rate of a microorganism at suboptimal temperature and *b* (log CFU.ml$^{-0.5}$.h$^{-0.5}$.°C^{-1}) is the regression coefficient.

Alternatively, models developed by Daughtry et al. [19] and based on the Arrhenius model [20] were used for description of *Gr* and lag phase dependence on temperature:

$$\ln Gr = C_0 + \frac{C_1}{T} + C_2 \ln T \tag{2}$$

$$\ln\left(\frac{1}{\text{lag}}\right) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} \tag{3}$$

C_0, C_1, C_2 are coefficients, T is temperature (°C) as a sole environmental factor.

Validation of the growth parameters

The accuracy, bias and discrepancy factors were calculated as defined by Baranyi et al. [21] to validate the used mathematical models describing the growth response to various incubation temperatures:

$$A_f = \exp\left(\sqrt{\frac{\sum_{k=1}^{n}\left(\ln f\left(Gr^k\right) - \ln Gr^k\right)^2}{n}}\right) \tag{4}$$

$$B_f = \exp\left(\frac{\sum_{k=1}^{n}\left(\ln f\left(Gr^k\right) - \ln Gr^k\right)}{n}\right) \quad (5)$$

$$\%D_f = (A_f - 1) \times 100 \quad (6)$$

where Gr is growth rate obtained from the growth curve, $f(Gr)$ – growth rate calculated from the model f that fitted experimental values, n – number of measurements, A_f – accuracy factor, B_f – bias factor and $\%D_f$ is percent discrepancy.

Standard error of prediction (*SEP*) was calculated according to ZURERA-COSANO et al. [22]:

$$\%SEP = \frac{100}{Gr_{meanobs}}\sqrt{\sum \frac{\left(Gr_{obs} - Gr_{pred}\right)^2}{n}} \quad (7)$$

where $Gr_{meanobs}$ is arithmetic mean of the observed growth rates Gr_{obs} and Gr_{pred} is the value predicted by the model.

RESULTS AND DISCUSSION

Growth curves of *L. rhamnosus* GG at all temperatures are shown in Fig. 1; at 6, 8, 12, 15 and 18 °C in Fig. 1A, and 21, 30, 35 and 41 °C in Fig. 1B.

The pH values are depicted in the background of the pictures. They did not change during entire experiments at the temperatures lower than 21 °C. This can be explained with the lower β-galactosidase activity in milk [7] and the low ability of *L. rhamnosus* to utilize lactose and convert pyruvate to lactate at a fast rate to match the glycolysis [5]. Inside the cell, β-galactosidase cleaves lactose to form glucose and galactose. The latter is exchanged with lactose via a lactose-galactose antiport system [23]. In a study conducted by GAUDREAU et al. [7], much greater amounts of lactic acid were produced by *L. rhamnosus* R011 in milk supplemented with either crude cellular extract (CCE) containing β-galactosidase and protease activity (20.7 g.l^{-1}), or glucose/peptone (35.0 g.l^{-1}) in comparison to unsupplemented milk (11.0 g.l^{-1} of lactic acid). The addition of CCE significantly improved the acidification rate of the probiotic *L. rhamnosus* R011 in milk. In experiment done by ØSTLIE et al. [6], *L. rhamnosus* GG inoculated into UHT milk but supplemented in advance with 0.75% (w/v) fructose, reached pH of 3.9–4.1 after 24 h of incubation at 37 °C.

Despite the negligible acid production, *L. rhamnosus* GG showed good growth in milk. In the stationary phase, *L. rhamnosus* GG reached higher counts than 10^8 CFU.ml^{-1} at all studied temperatures, except for marginal temperatures 6, 8 and 50 °C. The growth parameters, growth rate *(Gr)*, duration of lag phase, time to double *(t_d)*, initial number of *L. rhamnosus* *(N_0)* and number of *L. rhamnosus* in stationary phase *(N_{max})* at all studied temperatures are summarized in Tab. 1. Cultures of *L. rhamnosus* GG grew, although slowly, even at 6 °C. In this case, the experiment lasted for ten days and growth was still in the exponential phase. At 8 °C, the stationary phase was reached after nine days of incubation, and the density of *L. rhamnosus* was 7.5 log counts. The pH value was stable and so it was supposed that the taste properties of milk were unaltered. As the incubation temperature increased, the duration of lag phase decreased and the growth rate in the exponentially phase increased. At 41 °C, the shortest time necessary to reach the stationary phase (9 h) was achieved and with further increasing the incubation temperature, this time was prolonged. The given temperature was expected to be optimal for *L. rhamnosus.*

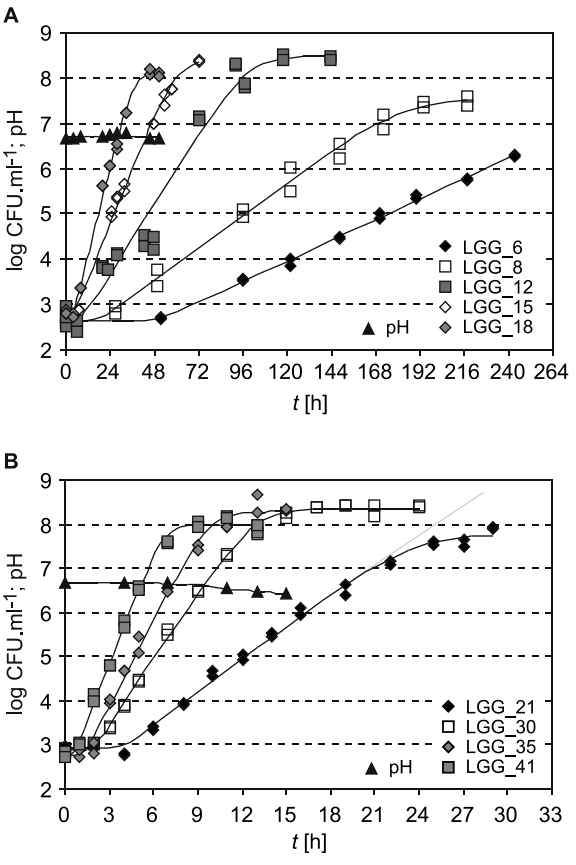

Fig. 1. Growth dynamics of *L. rhamnosus* GG and pH values in milk in relation to incubation temperature.

Tab. 1. Growth parameters of *Lactobacillus rhamnosus* GG at various temperatures.

T [°C]	Gr [log CFU.ml^{-1}.h^{-1}]	lag phase [h]	t_d [h]	N_0 [log CFU.ml^{-1}]	N_{max} [log CFU.ml^{-1}]
6	0.019	51.07	15.93	2.65	-
8	0.030	17.99	10.03	2.64	7.57
12	0.065	9.12	4.65	2.71	8.49
15	0.101	4.80	2.98	2.83	8.39
18	0.161	3.73	1.87	2.76	8.13
21	0.247	3.82	1.22	2.93	7.78
30	0.493	1.78	0.61	2.88	8.36
35	0.653	1.31	0.46	2.77	8.29
41	0.859	0.60	0.35	2.79	8.00

Gr – growth rate, t_d – time to double, N_0 – initial number of *L. rhamnosus*, N_{max} - numbers of *L. rhamnosus* GG in the stationary phase.

In literature, there are some references about *L. rhamnosus* GG growth analyses in various media. In a study of FARNWORTH et al. [24], *L. rhamnosus* GG was inoculated into either cows' milk or soy beverage together with a yoghurt starter culture and incubated for 12 h at 41 °C. The initial numbers of *L. rhamnosus* GG were 10^5 CFU.ml^{-1} and they increased by about 2 log counts, but the growth rate was not determined. In another experiment, *L. rhamnosus* GG was inoculated into a maize porridge at two levels; 6 or 7 log CFU.g^{-1}, and the porridge was fermented for 24 h at 37 °C. *L. rhamnosus* GG reached a maximum population of 7.2 to 8.2 log CFU.g^{-1}, but the growth rate was again not determined [25]. During a batch fermentation in MRS broth at 37 °C, the growth rate of *L. rhamnosus* GG was 0.82 h^{-1} [26]. Because this value was calculated based on optical density, it can not be directly compared with our results.

Temperature influence on the growth rate of Lactobacillus rhamnosus GG

At suboptimal course, the temperature influence on the culture growth rate is characterized by Ratkowsky square root model that linearizes the dependence of the growth rate on the incubation temperature:

$$\sqrt{Gr} = 0.0235\left(T - T_{\min}\right) + 0.1081$$
$$R^2_{\sqrt{Gr}} = 0.9981 \qquad (8)$$

In the temperature range from 6 °C to 41 °C (equation 8, Fig. 2), the growth rate showed a significant positive linear relationship to the incubation temperature with the standard error of 0.017 that was equivalent to coefficient of variation (*CV*) 3.76%. Until the temperature reached the optimum level, the growth rate was increasing linearly with the rising incubation temperature. The maximal growth rate of 0.859 log CFU.h^{-1} was observed at 41 °C and was represented by an average time to double t_d = 21.0 min.

Alternatively, the model developed by DAUGHTRY et al. [19], which is based on the Arrhenius model [20], was also applied to the growth data with the following result:

$$\ln Gr = -8.964 + 2.369 \ln T + 4.301\left(\frac{1}{T}\right)$$
$$R^2_{Gr} = 0.9964 \qquad (9)$$

At the suboptimal growth temperature range, this model also showed the statistically significant linearity between dependent variable represented by ln Gr and temperature. Despite this model was originally intended to model the growth of moulds, we found it suitable also to model the growth of bacterial cultures.

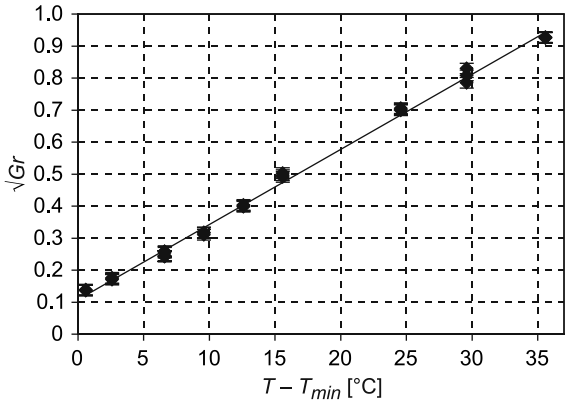

Fig. 2. Ratkowsky model as applied to the growth rate (Gr) of *Lactobacillus rhamnosus* GG in milk.
Y error bars represent the standard error at the 95% confidence interval.

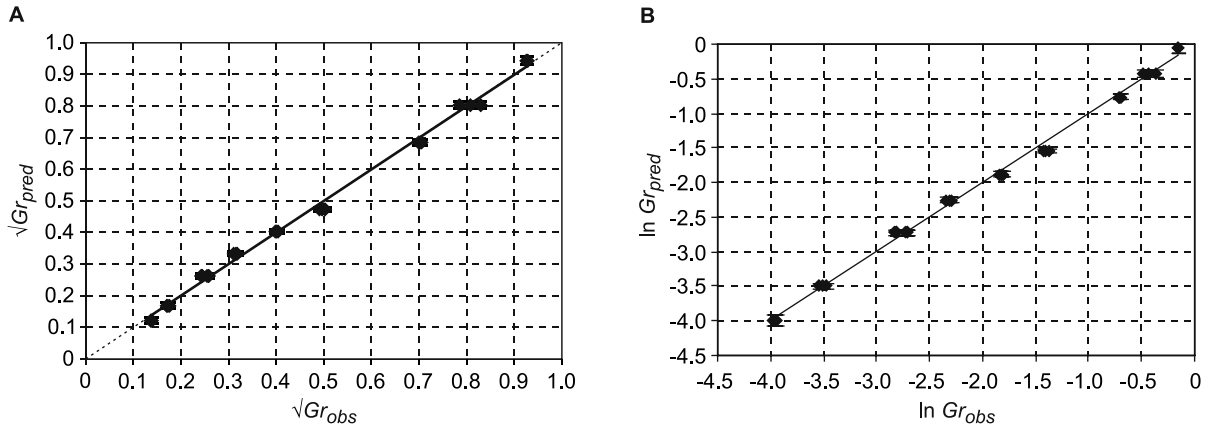

Fig. 3. Comparison of observed and predicted values of the growth rate of *Lactobacillus rhamnosus* GG in milk for Ratkowsky (A) and Arrhenius (B) models.
Y error bars represent the standard error at the 95% confidence interval. Gr_{pred} - predicted growth rate, Gr_{obs} - observed growth rate.

Tab. 2. Results of validation for secondary models of growth of *Lactobacillus rhamnosus* GG as influenced by temperature.

Model	Coefficients			A_f	B_f	$\%D_f$	%SEP	R^2
	C_0	C_1	C_2					
Equation 1	0.1081	0.0235	–	1.116	0.994	11.63	6.85	0.998
Equation 2	–8.964	4.301	2.369	1,076	1.000	7.63	11.64	0.996
Equation 3	0.999	–44.403	93.082	1.328	1.000	32.79	27.56	0.951

Gr – growth rate, C_0, C_1, C_2 – coefficients, A_f - accuracy factor, B_f - bias factor, %*Df* is percent discrepancy, %SEP - standard error of prediction of the model, R^2 – squared coefficient of correlation.

Both models, Ratkowsky square root and Arrhenius modified by DAUGHTRY et al. [19], showed a good prediction. The minimal discrepancies between the predicted and observed growth rate are shown in Fig. 3A and 3B. This status was also confirmed by validation parameters summarized in Tab. 2.

Temperature influence on the lag phase duration of *L. rhamnosus* GG

The cell numbers during the microorganism population lag phase remain practically stable. The lag phase of the microbial culture is influenced by many factors including the physiological state of and manipulation with the inoculum, cell adaptation to new environment, and cell growth and multiplication.

Under the statistically high relevant results, the model developed by DAUGHTRY et al. [19] represented with equation 6 and graphical illustration in the Fig. 4 was used in the range from 6 to 41 °C. In real values, the lag phase duration of *L. rhamnosus* GG in milk was increased with a decrease of the incubation temperature. This was connected with faster cell adapting to environmental conditions. The performance of this model represented with mutual comparison of predicted and observed values is shown in Fig. 5.

$$\ln\left(\frac{1}{\text{lag}}\right) = 93.082T^2 - 44.403T + 0.999$$
$$R^2 = 0.951 \qquad (10)$$

Verification of the optimal growth temperature of *L. rhamnosus* GG in milk

Besides following the growth of *L. rhamnosus*, changes in pH values of milk were also analysed. Although these were imperceptible with application according to BARANYI et al. [17], parameters such a pH lag phase (time, when pH value is stable – lag_{pH}) and rate of pH decrease ($rate_{pH}$) can be calculated during the growth of the tested culture in milk. The product of these parameters will point out for *L. rhamnosus* GG metabolism convenience in milk, which in case of growth, BARANYI

et al. [17] defined as a physiological state of the culture. In case of dependence on temperature, the product of lag_{pH} and $rate_{pH}$ values represented quadratic shape of the curve with a maximal zone range around the temperature 40 °C. Based on mutual collation of these two temperature-dependent variables, the range around 40 °C represents the optimal temperature (T_{opt}) for *L. rhamnosus* GG metabolism and thus confirms the previous growth data. The optimal temperature for acid production of the organism under study, T_{opt} = 40.5 °C (CV = 6.3%), is demonstrated by a graphical illustration in Fig. 6 or it was calculated using "Solver" (Microsoft Excel; Eq. 11). Both optimal temperatures, calculated for growth and metabolism, are near to each other and we could claim that optimal temperature for the growth and metabolism of *L. rhamnosus* GG was in the range from 40.5 °C to 41.1 °C.

$$lag_{pH} \times rate_{pH} = -0.003T^2 + 0.243T - 5.0091$$
$$R^2_{(lag_{pH} \times rate_{pH})} = 0.9918 \quad (11)$$

Validation of the secondary models

The validation of the used secondary models was performed in accordance with BARANYI et al. [21] and additionally by *%SEP* parameter [22]. As there was a lack of comparable growth data for this commercial strain in the literature, e. g. because a different medium with supplements or because different methods of determination of microbial numbers were used or data were not suitable for deriving kinetic parameters of validation, an internal validation was carried out [27]. Mathematical and statistical comparisons of the performance of the prediction of growth *L. rhamnosus* GG in milk are presented in Tab. 2.

The indices bias and accuracy provide an objective indication of the model performance. Bias and accuracy factor test the hypothesis that the model under evaluation predicts the true mean or represents it better than another model. A bias factor < 1 indicates that the model is, in general, fail-safe but still for the bias factor 0.90–1.05, the model performance is considered good [28, 29]. As shown in Tab. 2, the calculated values of bias and accuracy factor for the different models are equal or very close to 1. The accuracy factors based on the experimental growth rates of this study indicate that, on average, the predictions differ from the observation by less or about 8%. For the growth rate of *Leuconostoc mesenteroides* as a broth culture, the accuracy factors of 1.09–1.16 and a bias factor of 0.95–1.01 were found by ZURERA-COSANO et al. [22]. However, for example, accuracy factors

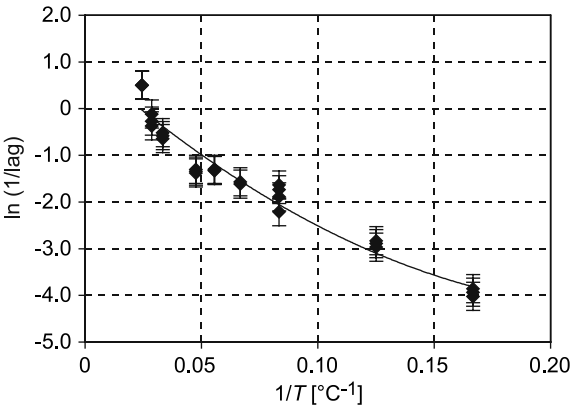

Fig. 4. Dependence of lag phase (lag) duration on the incubation temperature during the growth of *Lactobacillus rhamnosus* GG in milk.
Y error bars represent the standard error.

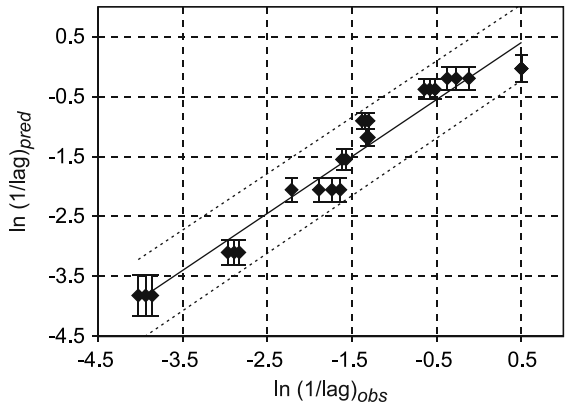

Fig. 5. Comparison of observed and predicted values of lag phase duration for *Lactobacillus rhamnosus* GG in milk for the model by DAUGHTRY et al. [21].
Y error bars represent the standard error. Dashed lines represent the 95% confidence interval. *pred* – predicted, *obs* – observed.

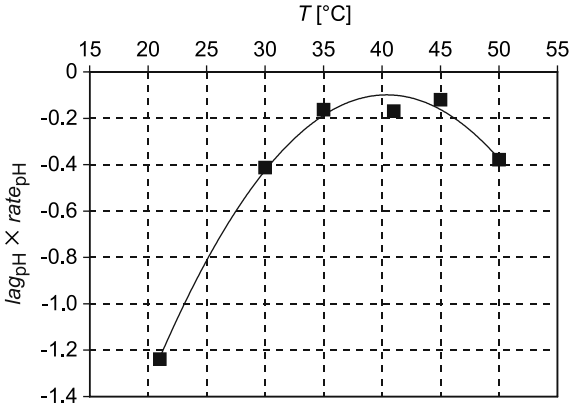

Fig. 6. Dependence of pH decrease rate ($rate_{pH}$) and pH lag phase (lag_{pH}) on the incubation temperature during the growth of *Lactobacillus rhamnosus* GG in milk.

were reported for bacteria and varied from 1.26 for *S. aureus* [28] and from 1.37 to 4.25 for growth of *Listeria monocytogenes* [29]. These experiments were performed in various foods.

For the complexity of lag phase, the accuracy factor based on this growth parameter is naturally higher than the accuracy factor for growth rate. The results for lag phase obtained during our study were in general better or similar to those described by ZURERA-COSANO et al. [22], who obtained adequate values of B_f from 1.10 to 1.18 and A_f from 1.10 to 1.31 in mathematical validation of their models developed for *Leuconostoc mesenteroides*.

CONCLUSION

The strain *Lactobacillus rhamnosus* GG showed very good growth properties in the temperature range from 6 °C to 41 °C. Our results also indicate that the growth potential of this strain at low temperatures could be utilized when added into fermented or non-fermented dairy products. Due to pH stability during its growth in milk media at low incubation temperatures, a culture of *L. rhamnosus* GG could be added also into pasteurized milk. In this case, pasteurized milk could be a carrier of this probiotic organism, because any significant pH changes were not recorded even at temperatures lower than 21 °C. For such a purpose, however, it is important that growth dynamics of the strain used in the application are known.

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