

GROWTH OF *LACTOBACILLUS ACIDOPHILUS* NCFM IN DEPENDENCE ON TEMPERATURE

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Environmental factors, including temperature and nutrient composition, have considerable impact on the growth dynamic of each microbial species; moreover it is strongly dependent on the selected strain. Therefore, the aim of this study was to describe and analyse the growth dynamics of the strain *Lactobacillus acidophilus* NCFM (Howaru) by predictive microbiology tools. The intensity of *Lb. acidophilus* NCFM growth in MRS broth and in milk was significantly affected by the incubation temperature described by the Gibson's model, from which the optimal temperature for the *Lb. acidophilus* growth of 40.5 °C in MRS broth and 40.1 °C in milk was calculated. These cardinal temperatures were verified with the CTMI model providing also other cardinal (minimal T_{min} , maximal T_{max} and optimal T_{opt}) values for *Lb. acidophilus* NCFM growth T_{opt} = 40.2 °C, T_{min} = 15.4 °C, T_{max} = 46.0 °C and T_{opt} = 40.3 °C, T_{min} = 14.3 °C, T_{max} = 46.6 °C in MRS broth and in milk, respectively.

Keywords: *Lactobacillus acidophilus* NCFM, growth parameters, predictive microbiology

Many intrinsic and extrinsic factors affect the growth and metabolism of microorganisms. According to the classical definition of Monod from 1949, “the growth of bacterial cultures, despite the immense complexity of the phenomena to which it testifies, generally obeys relatively simple laws”. So, the responses of a microbial population to environmental factors are reproducible and are bases of the predictive microbiology (ROSS & McMEEKIN, 1994). The predictive microbiology would not only enable to focus on foodborne and spoilage pathogens, but it should also predict the behaviour of lactic acid bacteria during fermentation, which can ensure the food safety, quality, and functionality not only of the dairy products. Since the temperature is one of the most important factors in the microorganism growth, in control of bioprocesses, describing the temperature effect on the microbial growth parameters is required.

The bacterial culture *Lactobacillus acidophilus* NCFM consists of the probiotic strain that was isolated from human gastrointestinal tract in 1900. It is a homofermentative lactic acid bacterium. Depending on the strain and other nutritional conditions, e.g. oxygen tension, amount of fermentable carbohydrates, proteins, vitamins of the B-complex, minerals, etc., *Lb. acidophilus* grows optimally at 37–42 °C (ALTERMANN et al., 2005).

NCFM strain has been widely used in yogurts, milk products and beverages over the last 40 years. As it is recommended by its producer DANISCO (2015), it is also added to many toddler formulas and dietary supplements in a form of capsules, powder, or tablets. The EFSA Panel on NDA (EFSA, 2011) confirmed that *Lb. acidophilus* NCFM helps to strengthen the natural defences of the human body and contributes to enhance its resistance against

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infectious agents. Its beneficial properties on human health are in detail collected by MANČUŠKOVÁ and co-workers (2013). It is obvious that the NCFM helps to enhance the gut health of patients suffering from functional bowel disorders, diarrhoea, and food intolerance. It was determined that it can increase prostaglandin E2 concentrations, resulting in an improvement of physiological gastrointestinal functions and cytoprotection against NSAID-induced injury. A correlation between the consumption of NCFM and the levels of IL-10 and IL-12 was also observed. Moreover, consumption of NCFM resulted the retardation of the growth of tumour volume and the enhancement of the apoptosis of tumour cells.

Despite the fact that the culture has been characterised by a large number of in vitro and in vivo studies, data describing its response to changes of environmental factors are not generally available. That is why this work deals with the quantification of temperature effect on the growth of *Lb. acidophilus* NCFM in real and synthetic growth media. Mathematical modelling coupled with experimental analysis allows understanding growth dynamics of studied strains under specific conditions. This data will help to optimise the processing conditions, when the probiotic strain will be used as a part of a starter culture in dairy products or as a dietetic supplement.

1. Materials and methods

1.1. Microorganism

The strain of *Lb. acidophilus* NCFM (Howaru; Danisco A/S, Copenhagen, Denmark) was provided by the Rajo, Inc. Company (Bratislava, Slovakia). Its identification and the monoculture composition was confirmed by Gram-staining, microscopic examination, API 50CHL test (BioMerieux, Marcy-l'Étoile, France), and by the PCR analysis of the 16S rRNA gene (not shown).

1.2. Inoculation and cultivation conditions

The strain was kept in MRS broth (Biomark, Pune, India) at 5 ± 1 °C. A standard suspension was prepared from a 24-h old culture grown in the MRS broth at 37 °C and 15% CO₂ (CO₂ incubator, Binder, Tuttlingen, Germany). This culture was inoculated into the pre-tempered ultra-pasteurised milk (1.5% w/v fat content, Rajo, Bratislava, Slovakia) or MRS broth (Biokar Diagnostics, Beauvais, France) in as constant concentration as possible of 10^3 CFU ml⁻¹. The static incubation at appropriately ranked temperatures from 15 to 46 °C was performed in three parallels.

1.3. Numbers of *Lb. acidophilus* NCFM in growth media

In proper time intervals, the required amounts of *Lb. acidophilus* NCFM were taken to determinate its actual density according to ISO 20128:2006 on MRS agar (Biokar Diagnostics, Beauvais, France).

1.4. Fitting the growth curves and calculating the growth parameters

The growth data, curves, and parameters of the strain under study were analysed, fitted, and calculated, using the mechanistic modelling technique of BARANYI and ROBERTS (1994) that is incorporated in the DMFit tools.

1.5. Secondary models

The growth parameters from each individual growth curve were analysed by secondary models. The specific growth rate ($\mu = Gr \times \ln 10$) was modelled as a function of the incubation temperature T . For that purpose the transformation of temperature (T_w): $T_w = \sqrt{(T_{max} - T)}$ was used, where T_{max} was estimated from data points in the high-temperature region as it was recommended by RATKOWSKY and co-workers (1983). Then the natural logarithm of the specific growth rates was modelled by the quadratic function: $\ln \mu = C_0 + C_1 T_w + C_2 T_w^2$, as introduced by GIBSON and co-workers (1994). The coefficients C_0 , C_1 , and C_2 were estimated by linear regression. From these, the optimum value $T_{(opt)} = T_{max} - (C_1/C_2)^2$ for the maximal growth rate was calculated. Finally, predictions for the NCFM growth rates at a given value of the incubation temperature were obtained.

The cardinal temperature model with inflection (CTMI) was introduced to empirically describe the influence of selected environmental factor on the data. The base of this model is a use of three cardinal temperatures directly included as parameters in model. The effect of temperature on the growth rate (G_r) is described by the equation

$$G_r = G_{r,opt} \frac{[(T - T_{max})(T - T_{min})^2]}{(T_{opt} - T_{min})[(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)]^2}$$

where T_{min} ($^{\circ}\text{C}$) is the temperature below which no growth is observed, T_{max} ($^{\circ}\text{C}$) is the temperature above which the growth is not observed, and T_{opt} ($^{\circ}\text{C}$) is the temperature at which the maximal growth ($G_{r,opt}$) is observed (Rosso et al., 1993).

1.6. Validation of the growth parameters

To validate mathematical models that were applied, following mathematical and statistical indices were used: the accuracy (A_p), discrepancy ($\%D_p$), and bias (B_p) factors (BARANYI et al., 1999), standard error of prediction ($\%SEP$; ZURERA-COSANO et al., 2006), a measure of "goodness-of-fit" ($RMSE$; TE GIFFEL & ZWIETERING, 1999), the sum of the squared residuals (RSS ; ZWIETERING et al., 1991), and the per cent variance ($\%V$; DAUGHTRY et al., 1997).

2. Results and discussion

To describe the effect of the incubation temperature on the growth of *Lb. acidophilus* NCFM, experiments in the ultra-pasteurized milk and in the MRS broth were carried out. The temperature of 15 $^{\circ}\text{C}$ was not appropriate for the *Lb. acidophilus* NCFM growth. At this condition the strain was not able to adapt to the environmental conditions even after 11 or 13 days of incubation neither in milk nor in MRS broth, and after this period it began to decrease. This is also the reason why the growth parameters at 15 $^{\circ}\text{C}$ were excluded from the secondary modelling. Increase of the incubation temperature with 3 $^{\circ}\text{C}$ resulted in the multiplication of studied strain in both media. The growth was still slow, represented by time to double in milk of 5.7 h and in broth of 11.6 h ($t_d = \ln 2 / 2.303 \times Gr$; BARANYI et al., 1999). Further increase of temperature led to more intensive growth almost in the whole studied temperature range, expect for interval 21–25 $^{\circ}\text{C}$. By comparing the growth parameters at 21 $^{\circ}\text{C}$ and 25 $^{\circ}\text{C}$, the decrease of growth rate by 7.2% in milk and even by 30.8% in broth at the higher temperature was noticed. One of the possible explanations can be the effect of the culture itself. At temperatures below 30 $^{\circ}\text{C}$ the duration of lag phase was longer than 4 days. This time can be

too long, negatively affecting the physiological state of the culture, resulting in the unexpected responses to the change of incubation conditions.

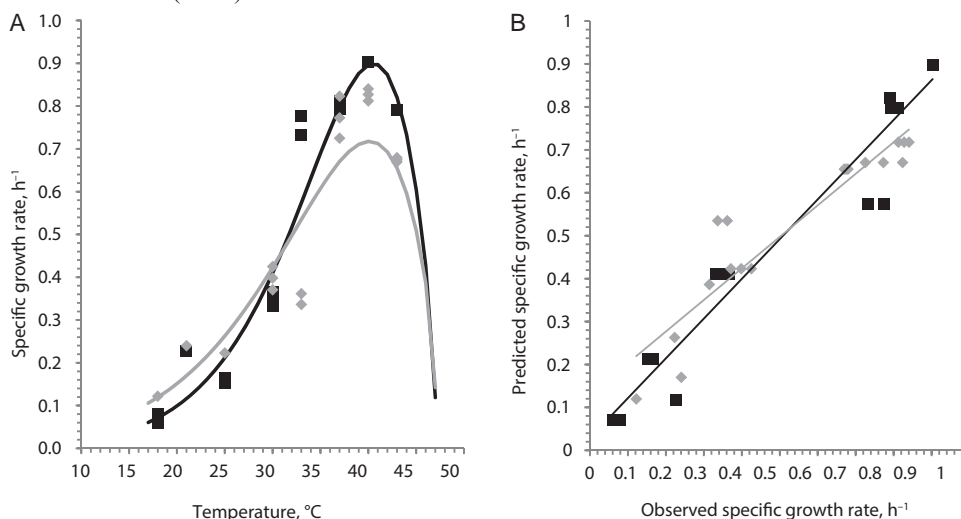
As a break point in lag phase duration, temperature of 30 °C can be assigned, since there was a considerable shortening of the adjusting phase (from 103–138 h to 3.4–4.7 h). Also, the growth in the exponential phase was faster by 33% in milk and about 55% in broth. The fastest growth as expressed by the growth rate (0.392 log CFU ml⁻¹ h⁻¹ in broth and of 0.359 log CFU ml⁻¹ h⁻¹ in milk), was reached by the NCFM strain at 40 °C. Further increasing of the incubation temperature had a negative effect on the growth dynamic.

2.1. The effect of incubation temperature on the growth of NCFM

At temperatures from 18 to 40 °C, the NCFM strain grew from initial counts $N_0=3.39 \pm 0.57$ log CFU ml⁻¹ (%V=16.8) to stationary phase reaching densities $N_{max}=8.91 \pm 0.52$ log CFU ml⁻¹ (%V=5.8). At 43 and 46 °C, weaker growth of NCFM was noticed. Strain 593N used by KASIMOĞLU and co-workers (2004) grew within first 7 days up to 10⁹–10¹⁰ CFU g⁻¹. On the other hand, during the cultivation of *Lb. acidophilus* ACC and IBB801 in MRS broth at 37 °C, lower maximal counts 7.9 log CFU ml⁻¹, were reached (AVONTS et al., 2004). The strains grew 72–89% slower compared to the NCFM strain. It is also remarkable, that contrary to the NCFM, strains in the study of AVONTS and co-workers (2004) were not capable of growing in milk unless it was supplemented with yeast extract.

2.2. The effect of incubation temperature on the growth rate of NCFM

In an empirical approach to model the effect of incubation temperature on the *Lb. acidophilus* NCFM growth rate, the Gibson's model was used with the temperature transformation (see above). Its actual graphical representation is shown in Figure 1A. The maximal temperature of 47 °C (required by the model) was derived based on the recommendation of RATKOWSKY and co-workers (1983).



Figs. 1A and 1B. Plots of the specific growth rates (μ) versus temperature (T) for *Lb. acidophilus* NCFM in milk (\diamond) and in MRS broth (\blacksquare) are presented in 1A. Symbols indicate the μ calculated from the growth curves at each incubation temperature. The continuous lines indicate the fitted μ versus T function, where $\ln \mu = C_0 + C_1 T_w + C_2 T_w^2$. The graphical comparison of observed and predicted values is shown in 1B with $R^2=0.943$ for MRS broth and $R^2=0.867$ for milk

Although there is no consensus on the minimum amount of probiotics in a product to ensure health benefits for consumers, the food product should contain a physiologically maintained active probiotic culture with the concentration of at least 10^6 – 10^8 CFU ml⁻¹ (VALÍK et al., 2013). The practical application of the Gibson's model is the prediction of time necessary for increasing the culture density at the selected temperature of the process. For that purpose, the time t_x (in hours) needed for the increase of *Lb. acidophilus* NCFM counts at selected temperature by x logarithmic counts ($x=1, 2, \dots$) can be predicted according to the equation $\ln t_x = x/\mu$, where $\mu = \exp^{(C_0 + C_1 T_0 + C_2 T_0^2)}$. For example, if the initial concentration of the probiotic culture had been 3 log counts, the NCFM culture would have increased by at least 4 log counts to reach the density of 10^7 CFU ml⁻¹.

The optimal temperature (T_{opt}) for *Lb. acidophilus* NCFM growth in MRS broth of 40.5 °C and in milk of 40.1 °C was calculated by the use of Gibson's model. To confirm these optimal temperatures, also the CTMI model was used. Since the settings of the parameters of the CTMI model are based on their biological interpretation and due to the lack of structural correlation between parameters, the simple and accurate estimation of cardinal temperature values is allowed (Rosso et al., 1993). So, in the MRS broth the most optimal conditions for NCFM growth are expected at 40.2 °C and in milk at 40.3 °C.

Under the optimal temperature conditions, the 4th parameter of the CTMI model provides the maximal growth rate of 0.396 log CFU ml⁻¹ h⁻¹ in MRS broth and of 0.346 log CFU ml⁻¹ h⁻¹ in milk. This can be used by dairy technologists and microbiologists in dairy practices after its recalculation to time, to double the culture counts in 52 or 46 min in milk and broth, respectively.

Moreover, by using the CTMI model, also the other cardinal temperatures were estimated: theoretical minimal temperature (no growth occurs below) is 15.4 °C in broth and 14.3 °C in milk. The temperature of 46.0 °C and 46.6 °C is the maximal temperature that allows the NCFM strain to grow in MRS broth and milk, respectively. These findings also confirmed that our previous estimations of the maximal temperature of 47 °C were correct.

With respect to different modelling techniques, the narrow range of each cardinal temperature for NCFM strain is expected even in such different media as milk and synthetic broth. Moreover, the NCFM strain should grow under conditions (affected by temperature regime, media composition, etc.) in processing plant with defined errors in expectation, taking into account the discrepancies calculated in the validation process.

2.3. Validation

The validation of a model is an inevitable part of the mathematical prediction of microorganism growth in specific environmental conditions. As there was a lack of comparable growth data of *Lb. acidophilus* NCFM in literature, only an internal validation was performed. First of all, the graphical comparison of observed and predicted values of specific growth rates of the NCFM strain in milk and in broth was performed with the responding multiple regression coefficients (R^2 ; Fig. 1B). Subsequently, the mathematical validation of values observed and predicted by Gibson's or CTMI model was carried out with the data summarized in Table 1. The model equations with validation indices are summarized in Table 2.

Table 1. Values of observed specific growth rate (μ) of *Lb. acidophilus* NCFM in milk and in MRS broth in dependence on incubation temperature and predicted values calculated according to CTMI and Gibson's model

T	Milk				MRS broth			
	μ_{obs}	μ_{predCTMI}	$\mu_{\text{predGibson}}$	λ	μ_{obs}	μ_{predCTMI}	$\mu_{\text{predGibson}}$	λ
15	-0.005	*	*	273.4	-0.002	*	*	206.1
18	0.122	0.025	0.120	107.0	0.059	0.016	0.072	154.9
21	0.239	0.081	0.170	135.4	0.228	0.072	0.116	164.9
25	0.223	0.203	0.263	138.0	0.156	0.206	0.212	103.5
30	0.334	0.412	0.423	4.7	0.348	0.450	0.411	3.5
33	0.336	0.528	0.535	–	0.732	0.619	0.573	5.1
37	0.771	0.726	0.670	2.9	0.801	0.829	0.797	2.3
40	0.827	0.795	0.718	1.2	0.903	0.911	0.896	1.5
43	0.675	0.712	0.655	2.2	0.792	0.788	0.820	–
46	0.315	0.200	0.386	–	1.052	*	*	18.8

T: incubation temperature (°C); μ : specific growth rate (h^{-1}); μ_{obs} : observed values; μ_{pred} : predicted values; μ_{CTMI} : values calculated according to CTMI model; μ_{Gibson} : values calculated according to Gibson's model; *: values excluded from the secondary modelling; λ : observed values of lag phase

Table 2. The model equations of the Gibson's model or the coefficients of the CTMI model and the indices of the internal validation for the *Lb. acidophilus* NCFM growth

Equation/ Coefficients of the equation							
A_f	B_f	$\%D_f$	R^2	$\%V$	RSS	RMSE	$\%SEP$
Gibson's model, broth; $\ln \mu_{\text{MRS}} = -0.313 T_w^2 + 1.592 T_w - 2.128$							
1.246	1.001	24.6	0.941	93.8	0.024	0.033	14.4
Gibson's model, broth; $\ln \mu_{\text{MRS}} = -0.236 T_w^2 + 1.233 T_w - 1.950$							
1.220	1.007	22.0	0.867	85.9	0.029	0.041	17.6
CTMI model, MRS broth							
1.188	1.065	18.8	0.935	93.2	0.024	0.033	14.2
CTMI model, milk							
1.256	1.045	25.6	0.903	89.8	0.019	0.031	15.2

Taken into account that if $B_f > 1$, the over prediction of growth rates of about 4.5–6.5% will be expected according to the CTMI model. With agreement of MELLEFONT and co-workers (2003), it can still be considered as acceptable. Better bias factors were calculated for the Gibson's model, so the model can be considered as good. In the study of ZURERA-COSANO and co-workers (2006), the bias factor of 0.95–1.01 for *L. mesenteroides* broth culture was found. Almost perfect agreement between predictions and observations, as expressed by the bias factors of 0.999–1.0, was achieved for *Lb. rhamnosus* GG growth in milk (VALÍK et al., 2008).

The best estimated error in prediction is 10% per independent variable (MELLEFONT et al., 2003). So, taking the % D_f and % SEP values into account, the predictions of growth rate of NCFM strain can be still considered as acceptable. However, the thought-out interpretation of the obtained data is as important as their achievement. Also, the more measures of goodness of fit of the model are used, the more precise validation of the data is achieved.

3. Conclusions

Since the growth of microorganisms is strain dependent, it is necessary to know growth dynamics of specific culture in dependence on environmental factors. So, the growth responses of the probiotic *Lb. acidophilus* NCFM in milk and in MRS broth were studied as affected by the incubation temperature. For that purpose, predictive models were used that allow estimation of the cardinal growth temperatures. Based on the results, dairy practise and research may utilize the following findings: the theoretical minimal temperature of *Lb. acidophilus* NCFM is in the range 14.3–15.4 °C, the maximal temperature is in the range from 46.0 to 46.6 °C. The fastest growth in exponential phase with time to double of 46–52 minutes can be expected at the optimal temperature of 40.1–40.5 °C in dependence on the growth medium. It seems that maximal growth rates reached in the used media at optimal temperature were not as high as expected. We suppose the growth rate will be higher in a co-culture with other starter culture of lactic acid bacteria applied in the production of fermented dairy products. Verifying this hypothesis will be the subject of our next work.

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