GROWTH CHARACTERISTICS OF TWENTY *LACTOBACILLUS DELBRUECKII* STRAINS ISOLATED FROM BULGARIAN HOME MADE YOGHURTS

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ABSTRACT
Among other microorganisms twenty Lactobacillus strains were isolated from home made yoghurts from different sources across Bulgaria. 13 strains were obtained from yoghurts prepared from cow milks while the origin of another 4 strains were sheep's milks, and 3 buffalo's milk yoghurts. The strains were previously classified with API tests to belong to the species Lactobacillus delbrueckii. In this study their growth characteristics at different temperatures were obtained, and a simplified mathematical growth model was proposed.

Keywords: *Lactobacillus delbrueckii*, growth modeling, logistic model

Introduction

Nowadays industrial production of yoghurts almost completely replaced the homemade milk products such as yoghurts and cheeses. However in many isolated small villages in the mountain areas such small scale artisan productions persist, especially those of cow, buffalo and sheep yoghurts. It has been shown that they are very rich source of "natural" bacterial isolates of *Lactobacillus delbrueckii* imparting good probiotic and nutritional properties to the made in the house yoghourts. These strains can be of great potential for the milk industry if investigated, in particular by determining their growth characteristics.

Predictive modeling is a promising field of food microbiology. Several models are described in the scientific literature, among them those of Richards (3), Schnute (5), Stannard et al. (6), and logistic model and others (2, 4).

Mathematics modeling

The logistic model. For the description of the *Lactobacilli* growth curve the logistic model was chosen. The three phases of the growth curve can be described by three parameters with biological meaning: the lag time (λ), the maximum specific growth rate (μ_m), and the asymptote (*A*) (**Fig. 1**). λ is defined as the *x*-axis intercept of the inflection point tangent. μ_m represents the tangent in the inflection point. *A* shows the maximum values reached by the model's function.

The logistic model equation describing a sigmoidal growth curve includes mathematical parameters (*a, b, c*) rather than the already mentioned biological parameters. The reparameterization of the growth model was imposed by the fact that it is difficult to estimate start values for the parameters if they have no biological meaning. This was done by deriving an expression of the biological parameters as a function of the parameters of the basic model function, and than substituting them in the equation.

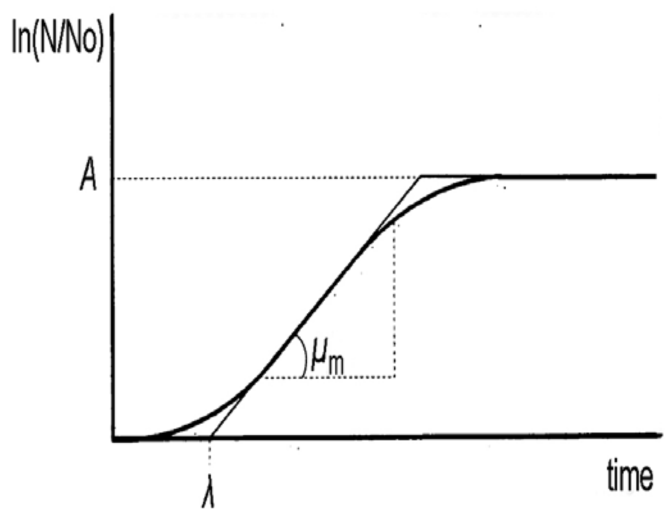

Fig. 1. Description of the growth curve by the three parameters with biological meaning: the lag time (λ), the maximum specific growth rate (μ_m), and the asymptote (*A*)

The logistic model is written as:

$$y=a/[1+\exp(b-cx)]$$

To obtain the inflection point of the curve the second derivative of the function with respect to *t* is calculated:

$$dy/dt = a/(1+\exp(b-ct))^2.c.\exp(b-ct)$$

TABLE 1

Lactobacillus delbrueckii strains used in the experiments and their origins

Origin of the Lactobacillus delbrueckii strains used in the experiments		
Caw yoghourt	Buffalo yoghourt	Sheep yoghourt
K2, K3, K5, K6, K14, K15, K16, K20, K21, K22, K24, K26, K27	B5, B7, B8	O42, O43, O45, O47

$$d^2y/dt^2 = ac^2.\exp(b-ct).(\exp(b-ct)-1)/(1+\exp(b-ct))^3$$

At the inflection point, where $t = t_i$, the second derivative is equal to zero:

$$d^2y/dt^2 = 0 \rightarrow t_i = b/c$$

The maximum specific growth can be derived by calculating the first derivative at the inflection point.

$$\mu_m = (dy/dt)_{ti} = 1/4.a.c$$

The parameter c in the logistic model equation can be substituted with $c = 4.\mu_m/a$

The description of the tangent line through the inflection point is:

$$y = \mu_m.(t - t_0) + y_0$$

The lag time is defined as the x-axis intercept of the tangent through the inflection point:

$$0 = \mu_m.(\lambda - t_0) + y_0$$

Using last three equations and by calculating y_0 yields:

$$\lambda = (b-2)/c$$

The parameter b in the logistic equation can be substituted by:

$$b = (4.\mu_m.\lambda)/a + 2$$

The A value is reached for t approaching infinity:

$$t \rightarrow \infty;\ y \rightarrow a;\ A = a$$

The parameter a can be substituted for by A and logistic model equation was modified as:

$$y = A/\{1 + \exp[4.\mu_m/A.(\lambda - t) + 2]\}$$

Fitting the data. The nonlinear equations were fitted to growth data by nonlinear regression using Marquardt-Levenberg algorithm (1). This algorithm seeks the values of the parameters that minimize the sum of the squared differences between the values of the observed and the predicted values of the dependent variable. The process is iterative – the curve fitter begins with a "guess" at the parameters, checks to see how well the equation fits, than continues to make better guess until the differences between the residual sum of squares no longer decreases significantly.

Starting values needed by the used nonlinear regression algorithm were obtained from literature, as well as from similar models included in the software. The program than calculates the set of parameters with the lowest residual sum of squares (RSS) and their 95% confidence intervals.

Materials and Methods

Bacterial strains and media

All bacterial strains and their suppliers are enumerated in **Table 1**.

All *Lactobacillus delbrueckii* strains were grown on MRS broth, agar or liquid media (*Merck KGaA, Scharlau Chemie S.A.*) at 37 °C for 14-16 hours for stocks preparations, and stored at 4°C for 15 days.

Growth kinetics

Single colony from each strain was inoculated in 3 ml MRS liquid broth and allowed to grow overnight at 37 °C. For the growth kinetics experiments 15 ml liquid cultures in MRS broth in three repeats from each *Lactobacillus delbrueckii* were prepared by inoculating 1% of the overnight cultures. They were allowed to develop at 25°C, 30°C, 33C, 37C, and 42°C till reaching the stationary phase. Meanwhile the optical density at 600 nm (OD_{600}) of each culture was measured every hour after vigorous vortexing for 2-3 seconds in *Ultrospec 1000E* spectrophotometer (*GE Healthcare*). Three independent measurements of the OD_{600} of each culture were performed.

Bacterial cell number/OD_{600} plotting. In order to find the total cell number corresponding to 1 OD_{600} unit a 1% inoculated 15 ml culture of one arbitrary chosen strain was allowed to develop at 37 ºC, and the OD_{600} measured several times in *Ultrospec 1000E* spectrophotometer (*GE Healthcare*) after vigorous vortexing for 2-3 seconds. After each measurement the total cell number in the probes was estimated by the 10-fold serial dilutions method.

Software

For fitting the data with nonlinear regression *Sigma Plot 9.0* was used (*Systat Software Inc*). The solving of the equations and the finding of the derivatives needed for the growth parameters determination were performed with *MatLab 6.5* (*MathWorks*).

Results and Discussion

Estimation of bacterial cell number by measurement of the OD_{600}. The cell number corresponding to the OD_{600} reading was calculated from a calibration curve obtained for the arbitrary chosen strain *Lactobacillus delbrueckii* K24 (**Fig. 2**). It was determined that in average 1 OD_{600} unit correspond to $6,7.10^8$ cfu/ml.

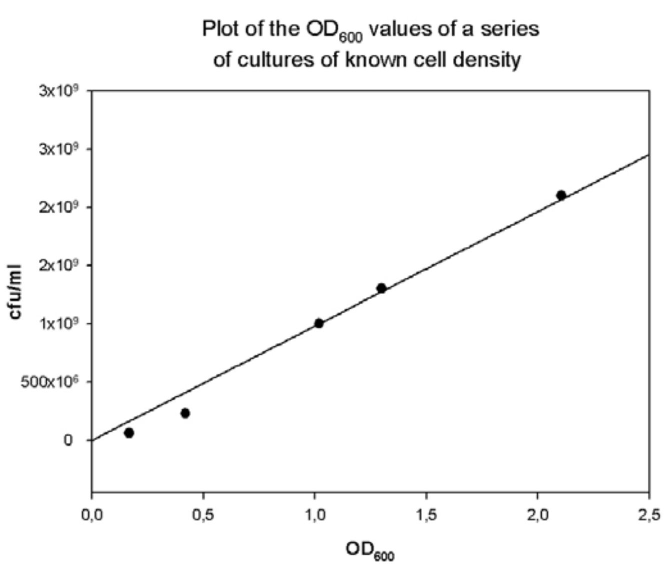

Fig. 2. Calibration curve used for determination of the cell number corresponding to the OD_{600} readings

Growth kinetics of the 20 *Lactobacillus delbrueckii* strains at 5 different temperatures. The total data set included 100 growth curves – 5 curves for each strain at 25 ºC, 30 ºC, 33 ºC, 37ºC, and 42 ºC. The maximal growth rates (μ_m) were estimated (**Table 2**).

TABLE 2

The maximal growth rates, μ_m of the twenty *Lactobacillus delbrueckii* strains at 25 ºC, 30 ºC, 33 ºC, 37 ºC, and 42 ºC

Lactobacillus delbrueckii strain	Maximal growth rates (μ_m) at different temperatures				
	25 ºC	30 ºC	33 ºC	37 ºC	42 ºC
B5	*0,198*	*0,297*	*0,372*	*0,481*	*0,659*
B7	*0,176*	*0,363*	*0,467*	*0,605*	*0,874*
B8	*0,214*	*0,307*	*0,448*	*0,510*	*0,630*
K2	*0,185*	*0,315*	*0,377*	*0,472*	*0,724*
K3	*0,205*	*0,424*	*0,508*	*0,608*	*0,976*
K5	*0,205*	*0,356*	*0,358*	*0,519*	*0,778*
K6	*0,168*	*0,351*	*0,434*	*0,569*	*0,753*
K14	*0,186*	*0,315*	*0,386*	*0,490*	*0,811*
K15	*0,209*	*0,454*	*0,577*	*0,790*	*0,979*
K16	*0,207*	*0,379*	*0,411*	*0,683*	*0,977*
K20	*0,208*	*0,392*	*0,505*	*0,674*	*0,949*
K21	*0,220*	*0,395*	*0,488*	*0,702*	*0,773*
K22	*0,197*	*0,362*	*0,453*	*0,626*	*0,756*
K24	*0,194*	*0,332*	*0,400*	*0,574*	*0,671*
K26	*0,159*	*0,306*	*0,396*	*0,510*	*0,675*
K27	*0,153*	*0,329*	*0,416*	*0,538*	*0,768*
O42	*0,156*	*0,291*	*0,385*	*0,563*	*0,811*
O43	*0,176*	*0,341*	*0,396*	*0,458*	*0,916*
O45	*0,212*	*0,437*	*0,577*	*0,747*	*0,957*
O47	*0,245*	*0,435*	*0,436*	*0,764*	*1,045*

The logistic model gave no difficulties in finding the least-square parameters. The strain *Lactobacillus delbrueckii* O45 was arbitrary chosen as an example of the fitting of the observed data and those predicted by the model (**Fig. 3**). The statistic parameters such as the correlation coefficient (R), the coefficient of determination (R^2), and adjusted R^2 are represented in **Table 3**. These parameters show how well the logistic model describes the data observed. The calculated values close to 1 show that good fittings with the experimental growth data were obtained. The model did show no difficulties for predicting the values in the log phase, the only deviations were found in beginning of the lag phase.

TABLE 3

Statistic parameters

Temperature of incubation	R	R^2	Adjusted R^2
25 ºC	*0,9982*	*0,9964*	*0,9960*
30 ºC	*0,9998*	*0,9996*	*0,9995*
33 ºC	*0,9998*	*0,9996*	*0,9995*
37 ºC	*0,9989*	*0,9978*	*0,9973*
42 ºC	*0,9992*	*0,9985*	*0,9981*

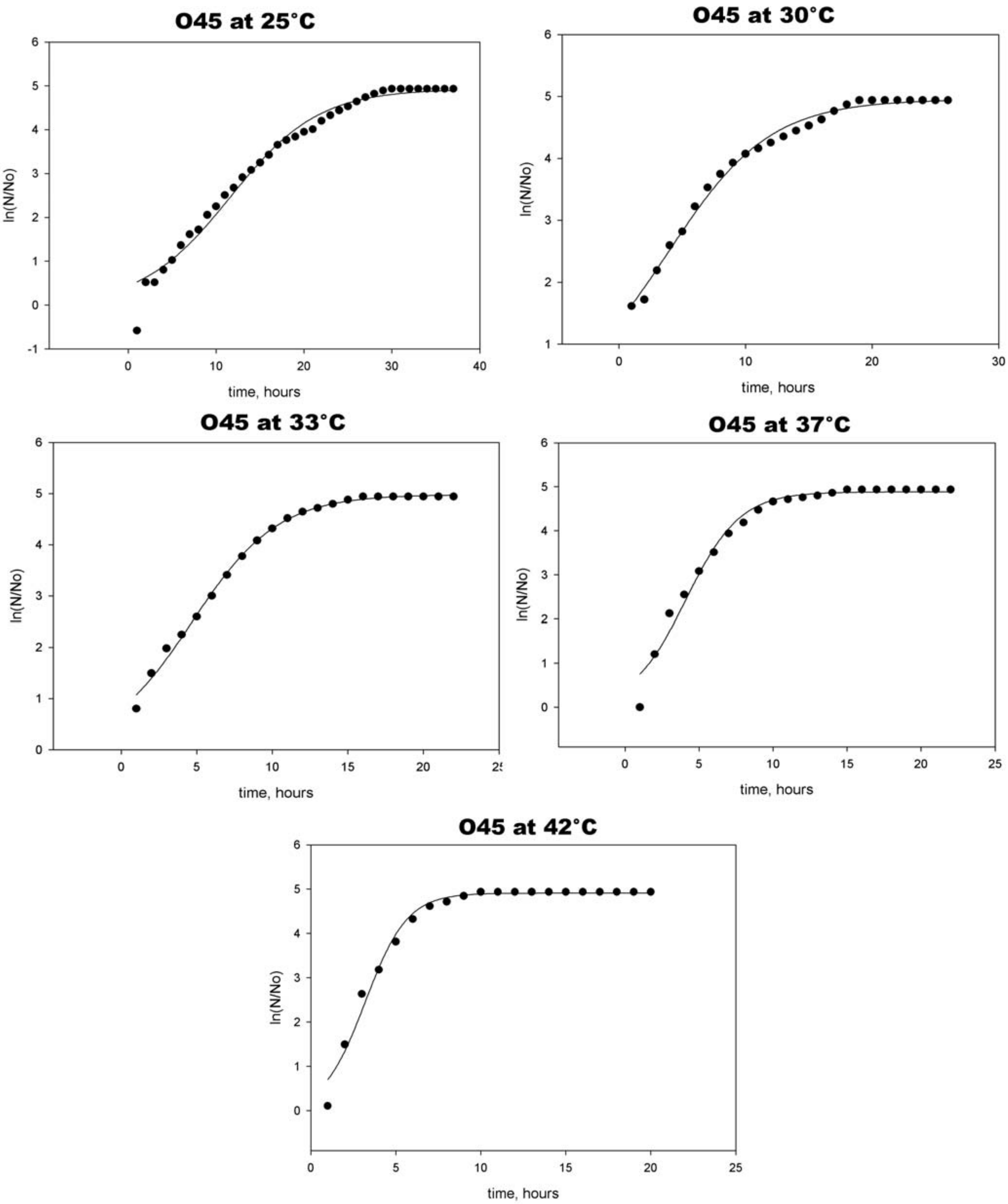

Fig. 3. Fitting of the observed data and those predicted by the model for the strain *Lactobacillus delbrueckii* O45 at five different temperatures: 25 ºC, 30 ºC, 33 ºC, 37ºC, and 42 ºC

Conclusions

Growth rates for all studied strains indicated that temperatures 37 ºC and 42 ºC are optimal for the cultivation, which is in correlation with the artisan mode of production. However some differences existed between the different strains independently of the origin their isolation (cow, buffalo or sheep yoghourts).

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