Estimation of vaginal probiotic lactobacilli growth parameters with the application of the Gompertz model

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Abstract: Lactobacilli are widely described as probiotic microorganisms used to restore the ecological balance of different animal or human tracts. For their use as probiotics, bacteria must show certain characteristics or properties related to the ability of adherence to mucosae or epithelia or show inhibition against pathogenic microorganisms. It is of primary interest to obtain the highest biomass and viability of the selected microorganisms. In this report, the growth of seven vaginal lactobacilli strains in four different growth media and at several inoculum percentages was compared, and the values of growth parameters (lag phase time, maximum growth rate, maximum optical density) were obtained by applying the Gompertz model to the experimental data. The application and estimation of this model is discussed, and the evaluation of the growth parameters is analyzed to compare the growth conditions of lactobacilli. Thus, these results in lab experiments provide a basis for testing different culture conditions to determine the best conditions in which to grow the probiotic lactobacilli for technological applications.

Key words: Gompertz model, lactobacilli, growth parameters, vaginal probiotic.

Résumé : Les lactobacilles sont généralement décrits comme étant des micro-organismes probiotiques utilisés pour rétablir l'équilibre écologique de diverses canalisations animales ou humaines. Pour être considérées comme probiotiques, les bactéries doivent présenter certaines caractéristiques ou propriétés liées à la capacité à adhérer aux muqueuses ou aux épithéliums, ou à inhiber certains micro-organismes pathogènes. Il est donc du plus grand intérêt d'obtenir un importante biomasse et un viabilité élevée des micro-organismes sélectionnés. Dans cet rapport, nous avons comparé la croissance de sept souches de lactobacilles vaginaux dans quatre différents milieux de culture et à divers pourcentages d'inoculation, et les valeurs des paramètres de croissance (temps de phase de latence, taux de croissance maximum, densité optique maximale) ont été obtenus en appliquant aux données expérimentales le modèle de Gompertz. L'application et l'évaluation de ce modèle est discuté, et les paramètres de croissance sont estimés et analysés afin de comparer les conditions de croissance des lactobacilles. Ainsi, les résultats des expériences en laboratoire fournissent une base pour l'analyse de différentes conditions de culture dans le but de déterminer les meilleures conditions sous lesquelles devraient être cultivées les lactobacilles probiotiques pour des procédés technologiques.

Mots clés : modèle de Gompertz, lactobacilles, paramètres de croissance, probiotiques vaginaux.

[Traduit par la Rédaction]

Introduction

In bacterial replacement therapy, lactobacilli function as probiotic microorganisms (Redondo-López et al. 1990; Reid 1999). The beneficial and technological properties of lactobacilli must, therefore, be studied. The revisions of Fuller (Havenaar et al. 1992) and recently of Salminen et al. (1998) clearly state the importance of both the technological and probiotic characteristics, for they must be considered when selecting strains for probiotic purposes. Until now, the growth conditions of probiotic lactobacilli isolated from the human vagina have not been studied in depth. Reid et al. (1998) studied the effect of nutrient composition on the growth of urogenital lactobacilli and uropathogens, describing the microbial growth using mathematical symbols. The authors put the main emphasis on the stimulation of lactobacilli growth, to the detriment of the pathogens, rather than on the kinetic parameters of the growth.

As previously reported in other papers, the lactobacilli showing some adherence properties on their surface (as a way to predict their adhesion capabilities) and the ability to produce antagonistic substances, such as H_2O_2 and bacteriocin-like substances, have already been selected (Ocaña et al. 1999*a*, 1999*b*, 1999*c*, 1999*d*). To make decisions about the optimal culture media and inoculum percentage for each of the aforementioned lactobacilli, a comparison of different growth conditions was carried out.

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With the application of the classical methodology described in the literature (Beal et al. 1994), although rather tedious and time-consuming, the growth parameters are able to be evaluated.

The application of statistical models to analyze the growth of microorganisms is currently being used and is largely applied in the field of predictive microbiology to deal with contamination and storage of foods. The Gompertz model has been applied much to the data obtained on the growth (measured both as optical density (OD) or as log of the colony-forming units (CFU)) of different lactobacilli and pathogenic bacteria (Zwietering 1993; McClure et al. 1994*a*, 1994*b*; Zaika et al. 1994; Dengremon and Membré 1995; Neumeyer et al. 1997; Dufossé et al. 2001).

The objective of the present research was to compare the growth of seven potentially probiotic vaginal lactobacilli in two commonly used culture media for lactobacilli (LAPTg and MRS) and two enriched media (BHI and M17–glucose) inoculated at different inoculum levels to find the most favorable growth conditions required to obtain the highest biomass in the shortest possible time. The Gompertz model was applied to the changes of OD (as a measure of growth) to evaluate the growth parameters of vaginal probiotic lactobacilli. Nonlineal regression statistical methods were applied to estimate the growth curves and to calculate the confidence intervals of the estimated parameters.

Materials and methods

Microorganisms

Lactobacilli isolated from vaginal swabs of women from Tucumán, Argentina were used throughout this study (Ocaña et al. 1999*d*). The following strains were selected for the presence of good surface properties and the characteristics mentioned: (*i*) *Lactobacillus crispatus* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1266, *Lactobacillus delbrueckii* subsp. *delbrueckii* CRL 1333, *Lactobacillus paracasei* subsp. *paracasei* CRL 1251 and CRL 1289, for production of H_2O_2 (Ocaña et al. 1999*a*, 1999*b*); (*ii*) *Lactobacillus acidophilus* CRL 1294, for autoaggregating abilities; (*iii*) *Lactobacillus salivarius* subsp. *salivarius* CRL 1328, for production of a bacteriocin-like substance (Ocaña et al. 1999*c*); and (*iv*) *L. acidophilus* CRL 1259, for its acid production. Although all lactobacilli produce lactic acid, only a few vaginal strains were able to inhibit the growth of urogenital pathogens, tested by the plate diffusion technique. *Lactobacillus acidophilus* CRL 1259 was selected because of the bigger size of the inhibition halos obtained on pathogen plates (data not published).

Culture media

The following four different culture media, pH adjusted to 6.5, were used: LAPTg (1.5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, and 0.1% Tween 80) (Raibaud et al. 1963); MRS (Biokar Diagnostics, Beauvais, France) (De Man et al. 1960); BHI (Brain Heart Infusion, Laboratorios Britania, Argentina); and M17–glucose (M17 supplemented with 1.0% glucose; Biokar Diagnostics). LAPTg and MRS are two commonly used culture media for lactobacilli. M17 was included in the present study because it is a medium commonly used for the growth of other lactic acid bacteria, showing excellent growth rates. The energy sources in M17 are lower (0.25% tryptone, 0.25% peptone, 0.25% yeast extract, 0.5% meat extract, 0.5% lactose, with 1% additional glucose). BHI was included because is a highly enriched media used for very fastidious bacteria, with very complex ingredients (such as brain and heart extracts), but very low amounts of peptone (0.074%) and glucose (0.0148%).

Inoculum build-up

Before the experiments, each strain, stored in milk–yeast extract at –70°C, was propagated at 37°C for 24 h and subcultured twice at 37°C for 12 h. The last preculture was washed with saline solution (0.85% NaCl) to remove the spent media and resuspended in the same solution to give a final OD of 1.4 at 540 nm. This bacterial cell suspension was used as the inoculum for the growth experiments.

Growth conditions

One hundred millilitres of each medium (250-mL Erlenmeyer flasks) was inoculated with precultures, prepared as described above, and after each one was mixed equally, they were distributed over tubes for growth determinations. The tubes were incubated in a water bath in aerobic conditions, without agitation, at the constant temperature of 37°C. At each time point, a tube was removed from the incubator. Growth was followed by measuring absorbance at 540 nm by using a Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.) with glass cuvettes and a 10-mm light path. The initial absorbance values varied with the initial inoculum size; the lower initial OD_{540} corresponding to 0.1% inoculum was around 0.010 (detection threshold of the spectrophotometer used). A significant change of OD was observed when the measured values exceeded this level, which allowed estimation of the duration of the lag phase.

Experimental design

During the experimentation, randomization was carried out with certain restrictions presented by the difficulties of working simultaneously with many *Lactobacillus* strains under many different growth conditions. The randomization was completed for each of the microorganisms, taking the media and the inoculum as factors. The experiments were performed each day with one microorganism, and the growth curves were repeated two or three times on different days. The experiments were performed in different stages. The exploratory phase allowed us to encounter more precise information on (*i*) the behavior of the main variables (OD, culture media, and initial inocula, referring to their interactions) and (*ii*) the variations of the noncontrollable sources of our experimental conditions. With *L. crispatus* CRL 1266, the growth curves were performed using the complete factorial experiment (2×5): two culture media were studied, LAPTg and MRS, with 0.1, 0.5, 2, 5, and 10% inocula. The experiments with *L. salivarius* CRL 1328 and *L. acidophilus* CRL 1294 were done with the same culture media as mentioned above, but using only four inoculum levels (complete factorial designs 2×4 for each one of these microorganisms). From the information obtained in this stage, the experiments carried out with the other four strains presented in this study were performed using a complete factorial design of 4×2

with four different culture media (LAPTg, MRS, M17, and BHI) and two different inoculum levels (2 and 5%). In the previous strains, experiments were also performed with M17 and BHI broth. A total of 157 growth curves were analyzed.

Description of the bacterial growth curves

Gompertz model

The experimental data of the growth curves were adjusted with the reparameterized Gompertz model (Zwietering et al. 1990). Each one of the four parameters included (see function [1]) has a particular meaning within the bacterial growth dynamic because it reflects the behavior of each particular strain in each one of the assayed conditions. This application allows one to find initial values for each of the four parameters under study and takes into account the convergence of the estimation methods and the visual validation of the obtained estimations. In the present paper, an independent term is included, D_0, which represents the initial OD.

At time t, the reparameterized Gompertz model is expressed by the following function:

$$[1] \qquad D_t = D_0 + A \exp\{-\exp[(\mu e/A)(\lambda - t) + 1]\}$$

where D_t is OD at time t; t is time of growth in hours; D_0 is OD at $t = 0$; A is increase of OD between D_0 and OD_{max}; μ is maximum growth rate (h^{-1}); λ is duration time of lag phase in hours; e is base of neperian logarithm (2.718281828).

Growth curves estimation

To estimate the growth curves, the Nonlinear Estimation module included in the statistical software Statistica package (Release 5.5) was applied. The estimators of D_0, $D_0 + A$, μ, and λ were approximated by the application of the optimized algorithm Rosembrock – quasi-Newton (*STATISTICA for Windows* (computer program manual)) (StatSoft Inc. 1995), with some restrictions. The asymptotic standard deviation of the estimators was obtained by the approximation of finite differences.

As neither heterogeneity nor residual correlation were present in the growth of microbial populations, the Ordinary Least Square method for the estimation of parameters was applied.

Each one of the estimators, named with a hat over the variables, D_0, A, μ, λ, has an approximately normal distribution. Then, if one generically calls the true value of one parameter β_i, the distribution of its estimator $\hat{\beta}_i$ is given by the following expression:

$$[2] \qquad \hat{\beta}_i \approx N(\beta_i;\ S_i)$$

where β_i is, asymptotically, the expected value of the parameter estimator; S_i is the standard deviation of the estimator (often referred to as standard error of the estimator); and N is the Normal distribution.

By knowing the distribution of the estimators, it is possible to calculate approximately the confidence interval (I_{β_i}) given by the following expression:

$$[3] \qquad I_{\beta_i} = \left[\hat{\beta}_i - \sqrt{\frac{n}{n-4}}\, t_{1-a/2}\, \hat{S}_i;\ \hat{\beta}_i + \sqrt{\frac{n}{n-4}}\, t_{1-a/2}\, \hat{S}_i\right]$$

where n is the sample size, $t_{1-\alpha/2}$ is a percentile of the distribution of Student's t test with $n - 4$ freedom grades, α is the level of confidence of the interval ($\alpha = 0.05$), and $\hat{S}_i$ is the S_i estimators (Huet et al. 1996). The numeral 4 corresponds to the number of parameters that were estimated in the model. The freedom grades were different according to the sample size and varied according to each of the experimental conditions tested (data not shown).

Estimation of the exponential phase time

From A and μ, the exponential phase time in hours, ε_g, was calculated (Zwietering 1993). This parameter represents the time that the microorganism is under the exponential phase and is given by the following expression:

$$[4] \qquad \varepsilon_g = A/\mu$$

Because this last parameter is a ratio of the other two parameters, the expected mathematical value of ε_g estimator, $E(\hat{\varepsilon}_g)$, is not equal to $E(\hat{A})/E(\hat{\mu})$. Therefore, this ratio can not be estimated by the ratio of $\hat{A}$ and $\hat{\mu}$. Therefore, $E(\hat{A}/\hat{\mu})$ must be approximated by using a serial development of Taylor (Meyer 1973), around $E(\hat{A})/E(\hat{\mu})$, where E indicates the mathematical expectation of a random variable. The Taylor series approximation is in the second order, taking into consideration that the quadratic term correspondent to the second derivative. The following expression results:

$$[5] \qquad E(\hat{\varepsilon}_g) \approx E(\hat{A})/E(\hat{\mu}) - [1/E^2(\hat{\mu})]\mathrm{Cov}(\hat{A}, \hat{\mu}) + [E(\hat{A})/E^3(\hat{\mu})]\mathrm{Var}(\hat{\mu})$$

where Var is the variance and Cov is the covariance.

In this same manner, the approximated expression for the variance of $\hat{\varepsilon}_g$ is given by the following:

$$[6] \qquad \mathrm{Var}(\hat{\varepsilon}_g) \approx E^2(\hat{A})/E^2(\hat{\mu}) + \mathrm{Var}(\hat{A})/E^2(\hat{\mu}) - 4[E(\hat{A})/E^3(\hat{\mu})]\mathrm{Cov}(\hat{A};\ \hat{\mu}) + 3[E^2(\hat{A})/E^4(\hat{\mu})]\mathrm{Var}(\hat{\mu}) - E^2(\hat{\varepsilon}_g)$$

To obtain the estimation of $E(\hat{\varepsilon}_g)$ and $\mathrm{Var}(\hat{\varepsilon}_g)$, the parameters $E(\hat{A})$, $E(\hat{\mu})$, $\mathrm{Var}(\hat{\mu})$, and $\mathrm{Cov}(\hat{A}, \hat{\mu})$ are replaced by the estimated values obtained from the experimental data, respectively.

R^2, proportion of variance explained

The adjustment of the growth curves was evaluated with the proportion of the variance explained, R^2. The R^2 value represents the fraction of variation of the response explained by the model (Bates and Watts 1988; Nerbrink et al. 1999). Afterwards, with the same objective, the graph analysis of the residual values was performed to control the achievement of the following hypothesis: normality, independence and homogeneity of their variances (Bates and Watts 1988). The results are not presented in this paper.

Analysis of variance

The statistical quantification (ANOVA) of differences between the estimated values of growth parameters ($\hat{D}_0 + \hat{A}$, $\hat{\lambda}$, and $\hat{\varepsilon}_g$), under four different growth conditions (combination of two culture media, LAPTg and MRS, and two inoculum sizes, 2% and 5%), was calculated. The application of this method allowed us to know if the effects produced by the

different inoculum sizes and culture media used were statistically significant on the magnitude of the growth parameters obtained.

Results

Application of the model of Gompertz

During the exploratory phase of the experiments with *L. crispatus*, we observed that the 3.5% inoculum did not produce growth curves that varied greatly in comparison with those that were obtained with the 2% or the 5% inoculum. For this reason, the experiments involving *L. crispatus* CRL 1266 in MRS broth were not performed using the 3.5% inoculum. The growth curves obtained with the 0.5% inoculum did not differ from those obtained with the 0.1% inoculum in *L. crispatus* experiments; the reason by which the 0.1% and 0.5% inocula were eliminated in the remaining strains. Figure 1 summarizes (*i*) the experimental data obtained for the growth of *L. crispatus* CRL 1266 in LAPTg and MRS using different percentages of inoculum, *(ii)* the experimental data obtained from replicated growth curves under the same conditions, and *(iii*) the adjustment of those data through the application of the Gompertz model. These figures were obtained by drawing the graphs of the curves performed in duplicate or triplicate. In Fig.1, we observe that the systematic variation of the experimental data of the growth of one strain of lactobacilli, under different conditions, always fit with a very good adjustment to the Gompertz model. An identical behavior was observed with the estimated growth curves of the other lactobacilli (figures not shown).

The values of the growth parameters obtained varied with each species, culture media, and initial inoculum used. This variation has been statistically quantified (ANOVA) for the growth curves obtained from experiments performed in LAPTg and MRS, because the cellular mass obtained during the growth in M17–glucose and BHI was too low compared with that obtained in LAPTg and MRS. On the other hand, initial inoculum different than 2% and 5% was only taken into account when four or five different inoculum sizes were used (experiments performed only with *L. crispatus* CRL 1266, *L. salivarius* CRL 1328, and *L. acidophilus* CRL 1294). Both maximal OD and exponential phase time showed small variations. The lag phase was much longer for those values lower than 2% inoculum. For values higher than 5% inoculum, there was a decrease of λ. These values were not acceptable from the practical point of view because the final OD did not increase, and the time needed to reach that value was not significantly shorter.

Effect of the culture media (LAPTg and MRS) and initial inoculum on the values of the parameters

$D_0 + A$

The culture media used produced significant changes in the maximal OD, but the significance of these changes depended on the species of lactobacilli tested, as shown in Tables 1, 2, and 3. There were statistically significant differences among the maximum OD reached by *L. salivarius* CRL 1328, *L. acidophilus* CRL 1294, *L. delbrueckii* CRL 1333, and *L. paracasei* CRL 1289 in the two culture media assayed.

In the experiments performed with *L. salivarius* CRL 1328 and *L. acidophilus* CRL 1294, a higher final OD was obtained when MRS was used as culture media, as shown in Tables 1 and 2. For *L. delbrueckii* CRL 1333 and *L. paracasei* CRL 1289, the highest OD was obtained in LAPTg. The maximum OD reached by *L. crispatus* CRL 1266, *L. acidophilus* CRL 1259, and *L. paracasei* 1251 while growing in the two culture media did not show statistically significant differences (Table 3).

In reference to the effect of the initial inoculum on the maximum obtained OD, the variation from 2% to 5% did not produce significant differences in the final OD in any of the microorganisms studied (Table 3; Fig. 2).

If the particular behavior of each microorganism is analyzed while comparing the estimated values of $D_0 + A$, it is clearly observed that *L. salivarius* CRL 1328 (growing in MRS) is the microorganism that reaches the highest final OD, with values between 2.15 and 2.49 (2.32 ± 0.17) with the use of a 2% inoculum and between 2.04 and 2.52 (2.28 ± 0.24) with a 5% inoculum. These are extremes of the corresponding intervals of 95% confidence, as observed in Fig. 2. In regards to the lower end of the spectrum, *L. salivarius* CRL 1328 is the microorganism that shows the lowest growth in LAPTg, with a maximum OD between 1.16 and 1.60 (1.38 ± 0.22) with the 2% inoculum and between 1.29 and 1.67 (1.48 ± 0.19) with the 5% inoculum. Both in LAPTg and MRS media, the other microorganisms' OD growth is approximately 2.

λ

While analyzing the lag phase duration (as shown in Tables 1 and 2), it is indicated that λ is lower in LAPTg than in MRS for *L. crispatus* CRL 1266, *L. salivarius* CRL 1328, and *L. paracasei* 1289. In the other four lactobacillus strains, the behavior was different: in MRS broth, λ was shorter than in the LAPTg broth. The inoculum size had a more homogeneous effect on the lag phase for all the strains studied: at higher inoculum, there was a decrease of λ, except for *L. acidophilus* CRL 1259 in LAPTg as shown in Tables 1 and 2 and in Fig. 2.

In the ANOVA application presented in Table 3, the inoculum significantly affected the λ in the experiments performed with *L. crispatus* CRL 1266, *L. acidophilus* CRL 1294, and *L. delbrueckii* CRL 1333. For *L. acidophilus* CRL 1259, the lag phase was modified significantly with the culture media used. Both the culture media and the inoculum level significantly affected the λ values of *L. paracasei* CRL 1251 and *L. paracasei* CRL 1289.

As shown in Table 3, the lag phase time of *L. salivarius* CRL 1328 promotes an statistically significant interaction effect on the culture media and the inoculum size used. These results state that the effect that is produced by the increased inoculum is different in the two culture media assayed. As observed in Tables 1 and 2 and in Fig. 2, when *L. salivarius* CRL 1328 grows in LAPTg with either a 2% or 5% inoculum the value of λ is practically zero in both cases. However, when it grows in MRS, the lag phase is notably reduced from 1.64 to 0.67 h with the inoculum's in-

Table 1. Estimation of growth parameters of the Gompertz model, and the 95% confidence intervals, for microorganisms grown in LAPTg broth.

Strain	*C*	D_0*±95%	$(D_0 + A)$*±95%	μ*±95%	λ*±95%	ε_g†±95%	R^2
Lactobacillus crispatus	0.1	0.03±0.04	1.99±0.08	0.39±0.06	5.77±0.42	5.09±0.86	99
CRL 1266	0.5	0.04±0.04	2.04±0.07	0.38±0.03	3.75±0.24	5.28±0.44	99
	2	0.06±0.06	2.03±0.09	0.35±0.03	1.82±0.32	5.73±0.54	98
	3.5	0.09±0.07	2.04±0.11	0.35±0.03	1.22±0.34	5.59±0.56	98
	5	0.13±0.05	1.97±0.09	0.28±0.01	0.96±0.31	6.70±0.46	99
	10	0.27±0.18	2.02±0.25	0.33±0.03	0.00±0.67	5.32±0.66	97
Lactobacillus salivarius	0.1	0.01±0.05	1.13±0.09	0.19±0.03	2.91±0.54	5.98±0.99	96
CRL 1328	2	0.00±0.14	1.38±0.22	0.24±0.04	0.12±0.82	5.82±1.23	89
	5	0.11±0.14	1.48±0.19	0.30±0.06	0.00±0.42	4.55±0.76	84
	10	0.39±0.23	1.45±0.33	0.24±0.07	0.00±1.07	4.56±1.55	97
Lactobacillus acidophilus	0.1	0.01±0.04	1.97±0.08	0.28±0.03	6.02±0.37	7.03±0.73	99
CRL 1294	2	0.11±0.10	1.99±0.16	0.24±0.03	2.70±0.76	7.97±1.31	97
	5	0.23±0.05	1.99±0.07	0.27±0.01	1.34±0.28	6.60±0.43	99
	10	0.34±0.07	1.99±0.10	0.24±0.01	0.52±0.39	6.88±0.51	99
Lactobacillus delbrueckii	2	0.08±0.08	2.18±0.16	0.45±0.07	2.02±0.43	4.69±0.80	98
CRL 1333	5	0.08±0.20	2.19±0.34	0.38±0.06	0.56±0.88	5.62±1.34	97
Lactobacillus acidophilus	2	0.00±0.06	2.13±0.12	0.24±0.02	0.69±0.37	9.03±1.23	99
CRL 1259	5	0.21±0.08	2.08±0.13	0.32±0.03	1.18±0.41	5.83±0.64	99
Lactobacillus paracasei	2	0.13±0.04	2.00±0.09	0.36±0.03	3.54±0.29	5.21±0.57	99
CRL 1251	5	0.23±0.06	1.96±0.11	0.27±0.02	1.78±0.41	6.45±0.74	99
Lactobacillus paracasei	2	0.14±0.07	1.83±0.12	0.33±0.04	1.89±0.44	5.10±0.77	98
CRL 1289	5	0.13±0.12	1.85±0.18	0.27±0.02	0.35±0.62	6.32±0.83	99

Note: *C*, inoculum size (percentage); R^2, proportion of variance explained.
*Parameters of the Gompertz model (see Materials and methods).
†Exponential phase time (h).

crease. In this case, the 95% confidence interval indicates that the possible values shall always be greater than zero.

ε_g

The estimations of the parameters of interest and their standard deviation are shown in Tables 1 and 2. The modifications of the initial inoculum did not produce systematic variations of the ε_g. This behavior is even more evident in the *L. crispatus* CRL 1266, *L. salivarius* CRL 1328, and *L. acidophilus* CRL 1294, for more than two concentrations were assayed for each. Only *L. paracasei* CRL 1289 showed an increased time of the exponential phase, which is statistically significant according to the inoculum used, and had a value of ε_g, which is also sensitive to the culture media assayed (Table 3). The estimated values show that when the growth of the microorganism begins with 2% inoculum, the mean value of the exponential phase is 6.45 h, but when the inoculum is 5%, that phase has a mean of 8.7 h.

Statistically significant differences in the ε_g values were obtained for the other two microorganisms, *L. crispatus* CRL 1266 and *L. acidophilus* CRL 1259, growing in LAPTg and MRS.

Neither the culture media nor the inoculum used produce significant variations in the duration of the exponential phase of the *L. salivarius* CRL 1328, *L. acidophilus* CRL 1294, *L. delbrueckii* CRL 1333, and *L. paracasei* 1251, as shown in Table 3.

The shortest ε_g time was calculated for *L. crispatus* CRL 1266 growing in the MRS broth. These values were greater, with a statistically significant difference of approximately 2 h, when this strain grew in LAPTg. *Lactobacillus acidophilus* CRL 1259 produced the longest time difference of all the microorganisms (a value between 10 and 11 h) for the two inoculum used in MRS and produced a shorter time in LAPTg at 5% inoculum. The 5% inoculum experiments made with the *L. paracasei* CRL 1289 strain in MRS yielded an increase of 11 h, one of the higher ε_g values obtained.

The *L. salivarius* CRL 1328, *L. crispatus* CRL 1266, *L. delbrueckii* CRL 1333, and *L. paracasei* CRL 1251 and CRL 1289 strains growing in LAPTg presented a mean ε_g of 5–7 h. The same values were obtained for *L. salivarius* CRL 1328, *L. delbrueckii* CRL 1333, and *L. paracasei* CRL 1251 growing in MRS.

Optimal growth conditions

When the growth parameters estimation was analyzed (Tables 1 and 2 for LAPTg and MRS growth, respectively) the following conclusions could be made, remembering that our interest was to select the conditions in which the highest biomass could be reached in the shortest time possible. (*i*) *Lactobacillus salivarius* CRL 1328 growing in MRS broth showed the best growth conditions, reaching a $D_0 + A$ value of 2.3 (the highest value obtained in the entire experimentation presented in this paper) in approximately 5 h (lag phase plus the time of the exponential phase). Because this behavior was independent of the initial inoculum, the value

Table 2. Estimation of growth parameters of the Gompertz model, and the 95% confidence intervals, for microorganisms grown in MRS broth.

Strain	*C*	D_0*±95%	(D_0 + *A*)*±95%	μ*±95%	λ*±95%	ε_g†±95%	R^2
Lactobacillus crispatus	0.1	0.11±0.03	2.14±0.06	0.45±0.03	6.22±0.21	4.52±0.39	99
CRL 1266	0.5	0.13±0.06	2.17±0.12	0.41±0.06	4.36±0.43	5.02±0.79	99
	2	0.19±0.04	2.12±0.07	0.44±0.03	2.58±0.22	4.40±0.38	99
	5	0.24±0.10	2.04±0.17	0.47±0.08	1.46±0.44	3.90±0.73	97
	10	0.34±0.12	2.08±0.19	0.42±0.06	0.65±0.51	4.19±0.76	98
Lactobacillus salivarius	0.1	0.00±0.02	2.26±0.05	0.46±0.03	4.29±0.15	4.92±0.34	99
CRL 1328	2	0.09±0.10	2.32±0.17	0.44±0.05	1.64±0.41	5.09±0.73	97
	5	0.13±0.15	2.28±0.24	0.39±0.04	0.67±0.60	5.59±0.89	97
	10	0.34±0.11	2.31±0.16	0.31±0.01	0.00±0.42	6.36±0.49	99
Lactobacillus acidophilus	0.1	0.05±0.02	2.05±0.05	0.33±0.02	5.10±0.20	6.07±0.40	99
CRL 1294	2	0.13±0.03	2.08±0.05	0.24±0.01	2.06±0.21	8.02±0.34	99
	5	0.16±0.10	2.05±0.15	0.19±0.01	0.00±0.60	10.04±0.73	99
	10	0.40±0.10	2.00±0.15	0.20±0.01	0.00±0.62	8.02±0.79	98
Lactobacillus delbrueckii	2	0.02±0.09	2.10±0.15	0.36±0.04	1.54±0.46	5.86±0.80	98
CRL 1333	5	0.05±0.15	2.06±0.24	0.37±0.04	0.54±0.65	5.53±0.96	98
Lactobacillus acidophilus	2	0.00±0.12	2.13±0.20	0.19±0.01	0.68±0.84	11.24±1.33	98
CRL 1259	5	0.08±0.15	2.08±0.23	0.19±0.01	0.00±0.92	10.43±1.15	98
Lactobacillus paracasei	2	0.06±0.09	1.94±0.16	0.26±0.03	2.11±0.63	7.46±1.10	98
CRL 1251	5	0.03±0.17	1.92±0.27	0.26±0.02	0.35±0.91	7.44±1.21	98
Lactobacillus paracasei	2	0.12±0.02	1.74±0.04	0.21±0.01	4.35±0.23	7.77±0.46	99
CRL 1289	5	0.19±0.03	1.76±0.06	0.14±0.01	2.42±0.40	11.10±0.70	99

Note: *C*, inoculum size (percentage); R^2, proportion of variance explained.
*Parameters of the Gompertz model (see Materials and methods).
†Exponential phase time (h).

of 2% inoculum was selected to optimize resources. (*ii*) The behavior of *L. crispatus* CRL 1266 in LAPTg and MRS was also very interesting. This microorganism reached a final OD of 2.0 in 7 or 7.5 h of incubation in MRS and LAPTg, respectively, with a 2% inoculum. (*iii*) The third microorganism with a good growth pattern was *L. delbrueckii* CRL 1333, which showed growth with similar characteristics in both LAPTg and MRS. A final OD of 2.1 was reached at 7 h with 2% inoculum and at 6 h with 5% initial inoculum. (*iv*) The other four lactobacilli strains studied did not show good growth behavior (in the different growth conditions assayed), according to the technological conditions required to be considered a good probiotic strain. Even though they eventually reached higher values of final OD, the time required to reach those OD levels was longer.

Discussion

For the successful probiotic application of lactobacilli to the urogenital tract, it is critical that a scientific basis be established for the selection of strains (Reid and Bruce 2001). During the design process of probiotic products, two of the main characteristics studied are the growth conditions and the technological performance of the selected microorganisms (Mäyra-Mäkinen and Bigret 1998). Because of budget allowances, the culture media that provides for the highest biomass or the highest number of viable microorganisms in the shortest time possible (short lag phase and high growth rate) must be considered.

To study the optimal growth conditions and the characteristics of these conditions in such probiotic microorganisms (with potential applications from the technological point of view), it is necessary to measure growth curves. Determining multiple conditions at the same time in the laboratory is always time-consuming. The evaluation of growth parameters and growth data is also a very slow process when classical methodology is used. The amount of data required to generate reliable models has convinced some researchers to use less time-consuming methods and often indirect methods of data collection, such as turbidimetry in laboratory media rather than traditional (viable count) methods (Dalgaard et al. 1994; Nerbrink et al. 1999). Determining bacterial growth rates in broth systems using turbidimetric methods provides a rapid and inexpensive means of obtaining the numerical values to get comparative conclusions and reliable modeling. The reproducibility of this method is high because the results are always superimposed, as observed in Fig. 1. In Fig. 1 many growth curves obtained under the same experimental conditions are presented. They provide a good fit of the plot to the experimental values, as the R^2 values presented in the last column of Tables 1 and 2 clearly show.

The predictive modeling method is a promising field in the microbiology area (Baranyi et al. 1993). Models are used to describe the behavior of microorganisms under different physical and chemical conditions, such as temperature, pH, and culture media. These models are widely applied to the foods microbiology fields to predict the microbial safety and shelf life of products and to detect critical parts of the pro-

Table 3. Statistical significance of the effects produced by the culture media and inoculum on each one of the parameters, for each strain.

Strain	Growth parameter	Culture media*	Inoculum†	MI
Lactobacillus crispatus	$D_0 + A$	NS	NS	NS
CRL 1266	λ	NS	$P < 0.005$	NS
	ε_g	$P < 0.01$	NS	NS
Lactobacillus salivarius	$D_0 + A$	$P < 0.001$	NS	NS
CRL 1328	λ	$P < 0.000005$	$P < 0.0005$	$P < 0.005$
	ε_g	NS	NS	NS
Lactobacillus acidophilus	$D_0 + A$	$P < 0.005$	NS	NS
CRL 1294	λ	NS	$P < 0.005$	NS
	ε_g	NS	NS	NS
Lactobacillus delbrueckii	$D_0 + A$	$P < 0.01$	NS	NS
CRL 1333	λ	NS	$P < 0.005$	NS
	ε_g	NS	NS	NS
Lactobacillus acidophilus	$D_0 + A$	NS	NS	NS
CRL 1259	λ	$P < 0.005$	NS	NS
	ε_g	$P < 0.005$	NS	NS
Lactobacillus paracasei	$D_0 + A$	NS	NS	NS
CRL 1251	λ	$P < 0.01$	$P < 0.005$	NS
	ε_g	NS	NS	NS
Lactobacillus paracasei	$D_0 + A$	$P < 0.005$	NS	NS
CRL 1289	λ	$P < 0.01$	$P < 0.01$	NS
	ε_g	$P < 0.005$	$P < 0.01$	NS

Note: NS, effect not statistically significant ($P > 0.01$); $P < 0.01$, effect statistically significant; $D_0 + A$ and λ, parameters of the Gompertz model (see Materials and methods); ε_g, exponential phase time (h); MI, interaction between culture media and inoculum.

*LAPTg and MRS.

†Initial inoculum (2 and 5%).

duction and distribution process (McClure et al. 1994*a*, 1994*b*; Zaika et al. 1994; Dengremon and Membré 1995; Neumeyer et al. 1997; Nerbrink et al. 1999).

The growth curve that results from plotting the graph has a sigmoid shape with a lag phase and an exponential phase, followed by a stationary phase. To describe the growth curves and to reduce the measured data to a limited number of parameters of interest, a number of models were found available in the literature, such as the models of Gompertz, Richards, and Schnute (Zwietering et al. 1990).

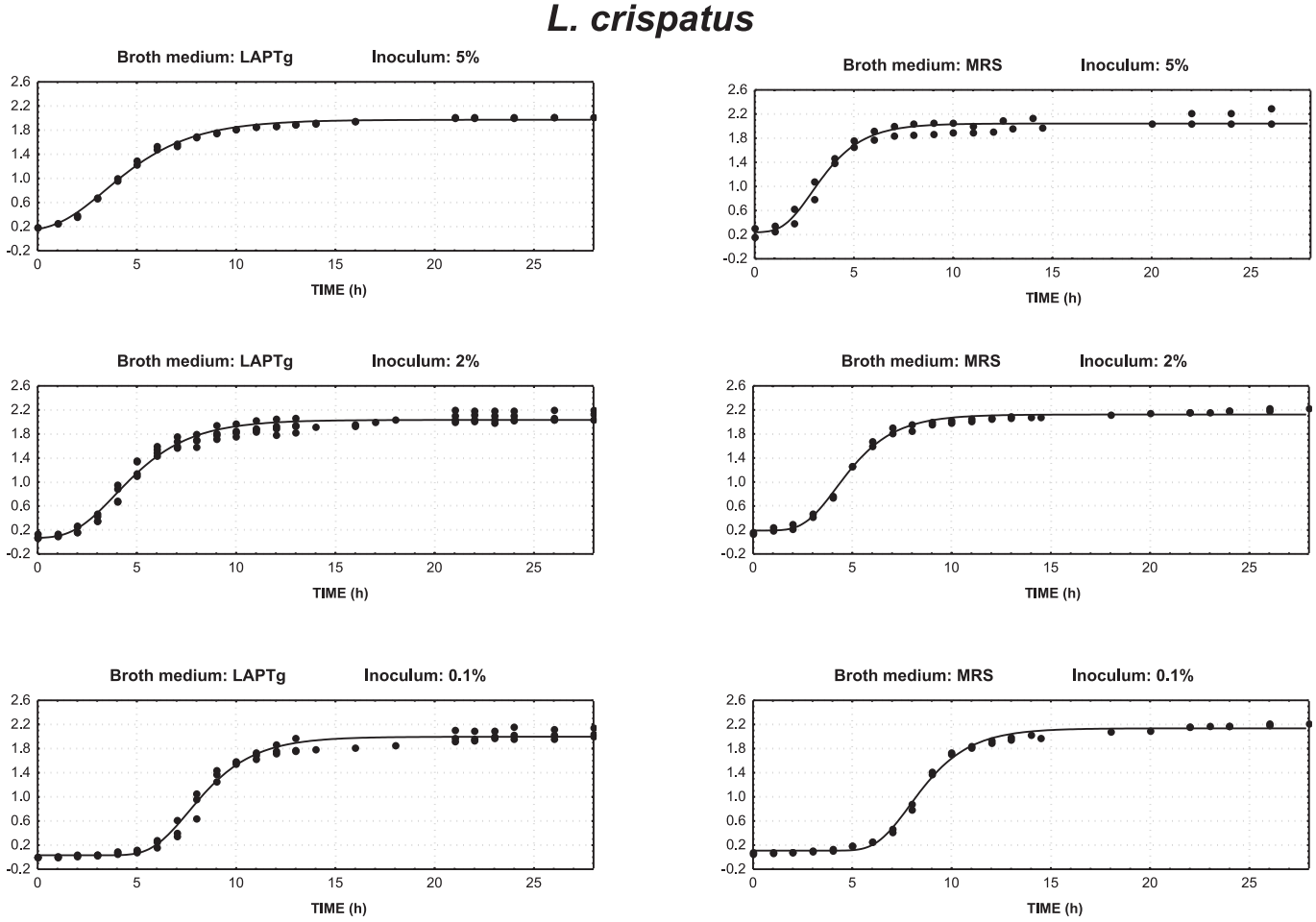
In this paper, the Gompertz model modified by Zwietering et al. (1990) was applied to the evaluation of the growth of seven probiotic lactobacilli. The growth parameters were obtained by the application of the model to the experimental data. The experimental data obtained in the laboratory from the spectrometric measures of lactobacilli growth fit perfectly into the mathematical formulation of the model (as shown in Fig. 1 under different culture media and inoculum percentage used).

The rapid calculation of the growth parameters allowed for an easy comparison of the data, and from that data, the preliminary conclusions (referring to the size of inoculum used) were able to be made. It was then concluded that the five different inocula that were originally to be used could be reduced to only two levels for the other strains of lactobacilli. The number of inocula used in this experiment was reduced mainly for the projection to industrial application, where the highest biomass in the shortest amount of time and at the lowest price is both desired and required.

Baranyi et al. (1993) and Dalgaard et al. (1994) have suggested that different numerical values of the generation times or growth rates may be obtained from the use of indirect methods applied to generate growth rates rather than those values determined by using viable counts. Some observations were made by different researchers (Dalgaard et al. 1994) concerning the fact that generation times calculated by the Gompertz function disagree with those obtained from the "traditional" steepest tangent. One of the possible differences in the values obtained is that the Gompertz model equation calculated the maximal growth rate using only one point of the curve, the point of maximum inflection. The "traditional", on the other hand, uses the tangent of the highest numbers of the points during the exponential growth. In this paper, the model of Gompertz was applied to obtain different parameters from the growth of vaginal probiotic lactobacilli. The growth rates obtained are different in value from those that were obtained from the application of the classical method, but the calculated growth rates show the same behavior and tendency in both cases (data not shown). The main reason for applying the parameters obtained from the Gompertz model is that it supplies the evaluation of the optimal conditions of the different microorganisms to be used later in technological applications.

As explained before, the growth rates obtained with the

Fig. 1. Growth curve of *Lactobacillus crispatus* CRL 1266 in LAPTg and MRS broth at different inoculum levels. The symbols represent the experimental data obtained, while the line was the result of the adjustment of the data through the Gompertz model.



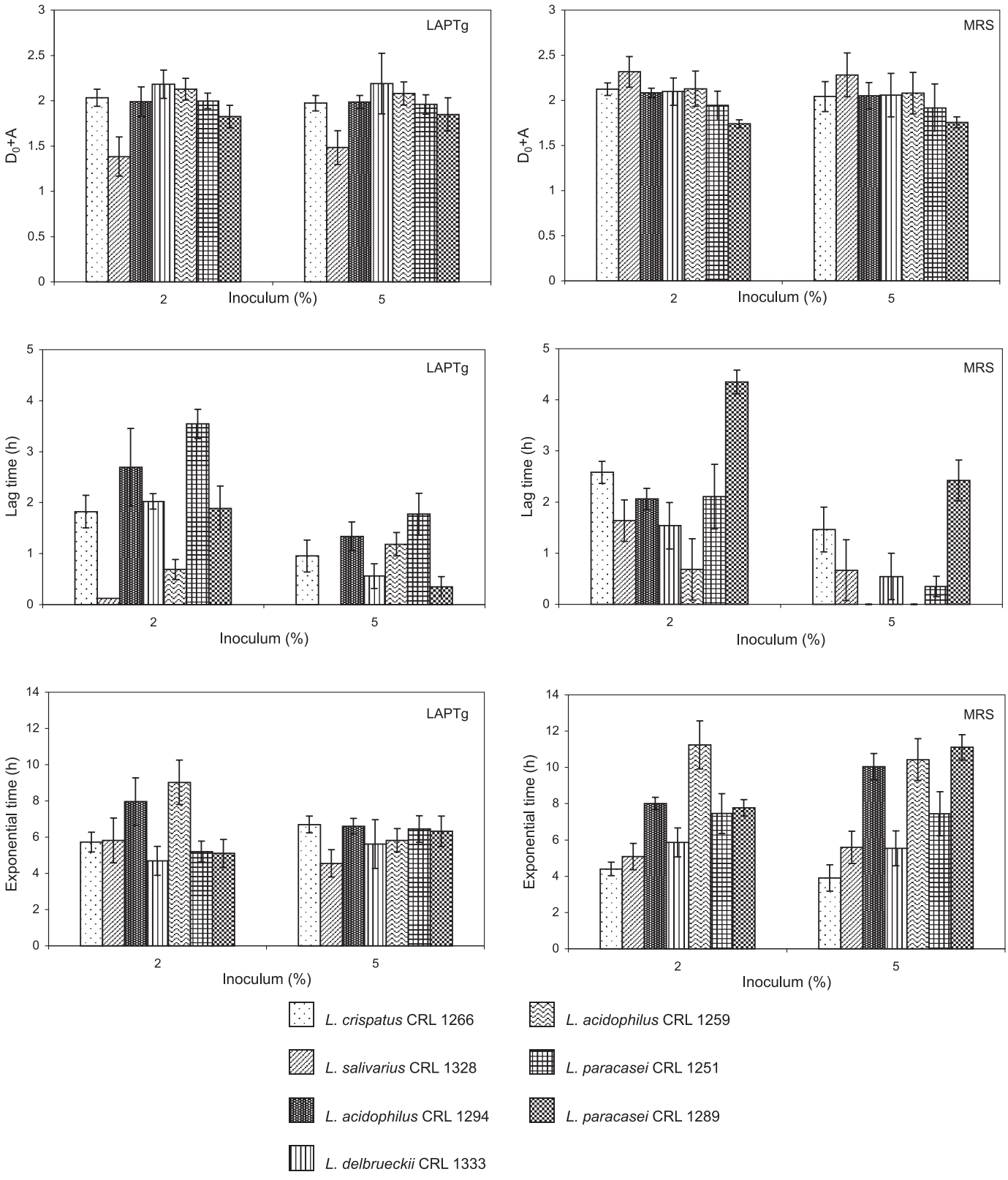
application of the model are quite different from those obtained with traditional methods. This finding agrees with the conclusions of Demetz and Dantigny (2000). They, however, state that no matter which method is used for growth rate calculation, the influence of the temperature on the growth rate of *Escherichia coli* can be determined. This means that the method of calculation of the growth rate does not affect the estimation of the optimum temperature for growth. These results highlight the interest of using a dimensionless approach that allows for the comparison of different ways to calculate the growth rate.

Through the application of the Gompertz model, the ANOVA analysis was used for the comparison and estimation of the parameters obtained. The determination of the factors that affect the different growth parameters (culture media, inoculum size, or both together) was thus able to be determined. From Table 2, we can conclude that each strain of microorganism must be evaluated individually, prohibiting the use of general conclusions. Even though some of the strains used are classified into the same species, because they show the same phenotypic characteristics, they present completely different behavior, as in the case of *L. paracasei* subsp. *paracasei* CRL 1251 and CRL 1289 and *L. acidophilus* CRL 1259 and CRL 1294. Nor can there be conclusions based on the metabolic group in which the different strains of lactobacilli are included because five of them are obligate homofermentative (classified as Thermobacterium-Orla Jenssen group), fitting *L. paracasei* into the facultative heterofermentative (Betabacterium-Orla Jenssen group). These results agree with the conclusions of different researchers, which state that each bacterial strain shows different characteristics, therefore, rendering it impossible to make general conclusions about their behavior before testing them at the experimental level (Sanders 1998; Jacobsen et al. 1999).

Growth substrate costs often represent the major part of the production cost of microbial cells from the industry. In general, only for *L. salivarius* CRL 1328 would the use of MRS as growth medium be justified; for the other strains, satisfactory yields are obtained in LAPTg (a cheaper medium than MRS) using an inoculum of 2–5%. Even though these are enriched laboratory media, they were used in the present study to allow good growth of the microorganisms to apply the statistical model. Other media used in the food or pharmaceutical industries, such as the whey-based media, to obtain the high amounts of microorganisms will be tested in a second stage.

Confidence intervals can also be obtained from the appli-

Fig. 2. Estimations of the growth parameters from the Gompertz model, with their respective confidence intervals for each microorganism, by using 2% and 5% inoculum in LAPTg and MRS broth.



cation of the Gompertz model to the growth parameters (resumed in Fig. 2). In this figure it is clearly observed that there is a very high tendency of the lactobacilli strains to obtain the maximum OD without being influenced by the inoculum used; this is a pattern characteristic of each strain. On the contrary, the lag phase, even though it is a character-

istic of each strain, showed modifications according to the inoculum level used, including changes due to the use of LAPTg or MRS. They were found to decrease when the inoculum level increased. The ε_g is sensitive to the different culture media used, LAPTg or MRS. Only *L. paracasei* CRL 1289 showed statistically significant variations when using a higher inoculum in MRS.

The results presented in the present paper allowed us to compare the growth conditions of several probiotic vaginal lactobacilli strains, in a very fast and computer-depending manner, as a way to suggest the better conditions to be applied in the technological uses of these lactobacilli.

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