

Estimation of temperature dependent growth rate and lag time of *Listeria monocytogenes* by optical density measurements

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Abstract

An automated turbidimetric system, Bioscreen C, was used to monitor growth of ten strains of *Listeria monocytogenes* at different temperatures. Several methods for estimation of maximum specific growth rate (μ_{\max}) and lag time (lag) from turbidimetric data were compared to values estimated from viable count data. By using a calibration factor, reliable estimations of μ_{\max} could be obtained from turbidimetric measurements. On the other hand, accurate estimations of lag required some viable count data. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Development of models in predictive microbiology requires large quantities of data. These data are generally obtained from viable count (VC) growth curves. Accurate estimations of growth parameters (lag time and maximum specific growth rate) need a large number of viable count determinations (Bratchell et al., 1989) which are labour and time-consuming. The measurement of optical density (OD) with automated turbidimetric systems provides for liquid and translucent media a rapid and inexpensive method to monitor bacterial growth. Turbidimetry is then an attractive method which has recently been used to acquire growth parameters (McClure et al., 1993; Young and Foegeding, 1993; Barbosa et al.,

1994; Jones et al., 1995; Bégot et al., 1997; Bréand et al., 1997; Stephens et al., 1997; Arino et al., 1998; Cheroute-Vialette et al., 1998).

There are, however, some problems with this technique and different methods have been proposed to derive growth parameters from OD measurements.

For estimation of growth rates, authors frequently recommend the use of classical growth models on OD data from a knowledge of the relationship between OD and VC. It is generally assumed that there is a proportional relation between OD and VC (Corman et al., 1986; Young and Foegeding, 1993; Barbosa et al., 1994; Dalgaard et al., 1994; Bégot et al., 1996; Bégot et al., 1997; Bréand et al., 1997; Arino et al., 1998; Cheroute-Vialette et al., 1998) but quadratic (McClure et al., 1993) and cubic (Stephens et al., 1997) calibration equations has been proposed as well. Growth models (e.g. logistic, Gompertz) are used on the whole growth curves

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(Corman et al., 1986; McClure et al., 1993; Barbosa et al., 1994; Dalgaard et al., 1994; Bégot et al., 1996; Bégot et al., 1997; Stephens et al., 1997; Arino et al., 1998; Cheroutre-Vialette et al., 1998) or only in the exponential growth phases (Young and Foegeding, 1993; Bréand et al., 1997; Stephens et al., 1997; Arino et al., 1998).

Lag times has been estimated by Bégot et al. (1996, 1997), Cheroutre-Vialette et al. (1998) and Hudson and Mott (1994) by applying the Gompertz equation to OD growth curves for which the inoculum level was sufficiently high to produce an initial OD above the detection threshold of the turbidimeters used. When inoculum size is such that the detection threshold is not reached, the initial cell count and the calibration equation must be known. McClure et al. (1993) used the logistic function constrained at the initial cell counts to fit OD growth curves while other authors (Bréand et al., 1997; Stephens et al., 1997; Arino et al., 1998) estimated lag times from the intersection between the extrapolated straight line, the slope of which is the estimated growth rate, at a point of visible growth and the log of the initial cell count horizontal.

Whatever the method used, it seems that the growth parameters estimated from OD and those estimated from VC are unequal. Thus, Dalgaard et al. (1994) showed that growth rates estimated from OD data were smaller than those obtained from VC data. Hudson and Mott (1994) showed with *Pseudomonas fragi* that lag times calculated from OD data were shorter than those derived from VC data. Similarly, McClure et al. (1993) supposed that their lag times and/or doubling times estimates were shorter in the system they had used.

In the present work, lag times and growth rates of ten strains of *Listeria monocytogenes* were estimated at different temperatures using both OD measurements and VC data, a novel method involving OD data and a few VC data is proposed to accurately estimate growth parameters.

2. Materials and methods

2.1. Strains

Ten strains of *Listeria monocytogenes* were used, Table 1 indicates their reference and their origin. The

Table 1

References, serotypes and origins of tested strains of *L. monocytogenes*

Strain	Serotype	Origin
Scott A ^a	4b	Human
CLIP 22485C ^b	4b	Food
CLIP 22573 ^b	4b	Human
CLIP 19884 ^c	1/2c	Meat products
27 795 ^c	1/2a	Minced meat
925 318 ^c	1/2b	Guinea-fowl
IG 1 ^d	1/2a	Industrial sites
IG 114 ^d	3a	Industrial sites
IG 74 ^d	1/2c	Industrial sites
IG 65 ^d	1/2a	Meat products

^a Strains were donated by: Dr. Agnès Brouillaud (Centre National d'Etudes Vétérinaires et Alimentaires, Paris, France).

^b Strains were donated by: Dr. Jocelyne Rocourt (Institut Pasteur Paris, France).

^c Strains were donated by: Drs. Isabelle and André Lebert (Institut National de la Recherche Agronomique, Theix, France).

^d Strains were donated by: Inès Giovanacci (Centre Technique de la Salaison, de la Charcuterie et des Conserves de Viande, Maisons-Alfort, France).

strains were maintained by monthly transfers on tryptone soya agar (Oxoid, Unipath, Ltd., Basingstoke, Hampshire, UK) slopes stored at 4°C.

2.2. Growth experiments

L. monocytogenes strains were subcultured onto tryptone soya (Oxoid) plus 0.6% yeast extract (AES, Combourg, France) agar slopes at 37°C for 24 h and then into tryptone soya (Oxoid) plus 0.6% yeast extract (AES) (TSYE) broth at 30°C for 24 h. Inocula were then diluted in TSYE broth to obtain concentrations of approximately 10^8 and 10^6 cfu \times ml⁻¹, and a series of five half-dilutions was prepared from this last dilution. Aliquots of 400 μ l of each dilution and non-inoculated medium were placed in the wells of honeycomb sterile plates. The inoculated honeycomb plates were placed in the reading chamber of a Bioscreen C (LabSystem, LabSystem France SA, Les Ulis, France) where they were incubated at constant temperature in the range of 4.0°C–35.4°C and shaken at medium intensity for 30 s/min. Bioscreen C was placed in a room at 3.5°C and the incubator was pre-heated at the set-point temperature for 2 h before starting growth experiments.

The automated turbidimeter, Bioscreen C, was

used to monitor the growth of *L. monocytogenes* by reading OD at a wavelength of 600 nm at regular time intervals. OD growth kinetics were constructed by plotting the OD of suspensions minus the OD of the non-inoculated medium vs. the time of incubation. In between readings, wells containing the dilution of about 10^6 cfu \times ml $^{-1}$ were sacrificed to perform VC of the suspensions. VC growth curves were generated with 13–19 points.

2.3. Variance stabilizing transformations for VC and OD data

Stabilization of the variance of the VC data was done by using the usual logarithmic transformation (Dalggaard et al., 1994). The Box–Cox method (Box et al., 1978) was used on two sets of 90 replicates at 4.0 and 28.7°C to find the variance stabilising transformation, s , for the OD data. The log of the standard deviation of replicates was plotted against the log of the average OD and the slopes (α) of the regression lines gave an indication of the variance stabilizing transformation to use. Values of α of 0.0, 0.5, 1.0, 1.5, and 2.0 correspond to no transformation, square root transformation, log transformation, reciprocal square root, and reciprocal transformation, respectively.

2.4. Estimation of growth parameters from VC growth curves

Growth curves were fitted using the logistic growth model with delay, i.e., with a breakpoint at the transition between the lag and the exponential phase (Kono, 1968; Baranyi et al., 1993; Rosso et al., 1996):

$$x(t) = \begin{cases} x_0 & , \quad t \leq \text{lag} \\ \frac{x_{\max}}{1 + \left(\frac{x_{\max}}{x_0} - 1\right) \times \exp(-\mu_{\max} \times (t - \text{lag}))} & , \quad t > \text{lag} \end{cases} \quad (1)$$

where $x(t)$ is the bacterial concentration (cfu \times ml $^{-1}$) at time t (h), x_0 is the initial bacterial concentration (cfu \times ml $^{-1}$), x_{\max} is the maximum bacterial con-

centration (cfu \times ml $^{-1}$), lag is the lag time (h) and μ_{\max} is the maximum specific growth rate (h $^{-1}$).

This function, called f , was fitted to the logarithm of VC. VC maximum specific growth rates and lag times were estimated at 4.0, 7.8, 11.6, 15.4, 24.0 and 35.4°C.

2.5. Calibration of optical densities against cell concentration

OD was calibrated against VC in a given range of OD values using equation:

$$\text{OD} = p_n(x) \quad (2)$$

where p_n is a polynomial of order n . In order to know what model between cubic, quadratic or linear was the most appropriate to calibrate OD data, F tests were used to compare nested models of degree n and $n-1$ (Bates and Watts, 1988). Calibrations were done with OD and VC data obtained from growth of suspensions with initial cell number of approximately 10^6 cfu \times ml $^{-1}$.

2.6. Estimation of growth rates from OD growth curves

OD growth rates were estimated at 4.0, 6.0, 7.8, 9.7, 11.6, 13.5, 15.4, 19.2, 24.0, 28.7 and 35.4°C.

2.6.1. From suspensions with initial OD above the bioscreen C 's detection threshold

The model used to fit these growth curves, called model GP , was the modified Gompertz equation, g (Zwietering et al., 1990), which gives with calibration equation and variance stabilizing transformation:

$$s[\text{OD}_a(t)] = s(p_n(x_0 \times \exp[g(t)])) \quad (3)$$

where

$$g(t) = A \times \exp\left(-\exp\left(\frac{\mu_{\max} \times e}{A} \times (\text{lag} - t) + 1\right)\right),$$

OD_a is the OD from suspensions with initial OD above the detection threshold, and A is the logarithmic increase of bacterial population ($\ln(\text{cfu} \times \text{ml}^{-1})$).

2.6.2. From suspensions with initial OD below the bioscreen C's detection threshold

From the suspension with an initial cell count of approximately $10^6 \text{ cfu} \times \text{ml}^{-1}$ and the five half-dilutions series, generation time (T_g) was defined as the time separating two successive curves when OD was in the range of 0.010–0.600. This technique is valid assuming there is no effect of inoculum size on growth parameters. Maximum specific growth rate was derived from the average generation time achieved with the six growth curves using:

$$\mu_{\max} = \frac{\ln(2)}{T_g} \quad (4)$$

In the second method, the whole OD growth curve was fitted using the second term of the f model (Eq. (1)) with calibration equation and by constraining the initial OD as being $p_n(x_0)$, with x_0 known. This model, called W , is described by the equation:

$$s(\text{OD}_b(t)) = s(p_n[f(t)]) \quad (5)$$

where OD_b is the OD from suspensions with initial OD below the detection threshold.

In the third technique an exponential model with delay, called E , was used only on the exponential growth phase. Its equation is:

$$s(\text{OD}_b(t)) = s(p_n(x_0 \times \exp[\mu_{\max} \times (t - \text{lag})])) \quad (6)$$

2.7. Estimation of lag times from OD growth curves

Lag times were estimated from suspensions with an initial OD above the Bioscreen C's detection threshold with model GP (Eq. (3)), and from suspensions with an initial OD below the detection threshold with models W and E (Eqs. (6) and (7)).

A new method consisted in calculating lag times by using the f model (Eq. (1)), knowing μ_{\max} , x_0 , x_{\max} , and $x(t)$ at time t (Rosso et al., 1996):

$$\text{lag} = t + \frac{1}{\mu_{\max}} \times \ln \left(\frac{x_0 \times (x_{\max} - x(t))}{x(t) \times (x_{\max} - x_0)} \right) \quad (7)$$

This model was called M .

2.8. Variance stabilising transformations for growth parameters

To compare maximum specific growth rates and

lag times achieved with the different techniques, we had to stabilize the variances of these parameters. We chose for maximum specific growth rates the square root transformation which seems the most appropriate (Ratkowsky et al., 1991; Alber and Schaffner, 1992; Schaffner, 1994; Ratkowsky et al., 1996) and the logarithmic transformation for lag times (Ratkowsky et al., 1991; Alber and Schaffner, 1992; Zwietering et al., 1994; Delignette-Muller, 1998).

2.9. Validation of the method selected for the acquisition of growth parameters from OD data

Validation of the best method was done by predicting bacterial growth from estimated growth parameters and by comparing these predictions with observed VC growth curves of *L. monocytogenes* Scott A incubated in TSYE broth at 6°C. The validation was done with eight growth curves achieved from different pre-incubation conditions applied after the culture prepared in TSYE broth at 30°C for 24 h: pre-incubations at 12°C for 10, 22 and 32 h; pre-incubations at 37°C for 1, 3 and 5 h and pre-incubations at 4°C for 52 days and 10 months.

2.10. Model fit

Fits were performed by linear or non-linear regression using the least squares criterion (Box et al., 1978). Estimation of parameters was carried out by minimizing the sum of the squared residuals (SSR) where SSR is defined as follows:

$$\text{SSR} = \sum_{i=1}^n (\text{value}(i)_{\text{observed}} - \text{value}(i)_{\text{fitted}})^2$$

where n is the number of data points.

The minimum SSR values were computed with the REGRESS and NLINFIT subroutines of MATLAB 5.2 software (The MathWorks Inc., Natick, MA, USA).

3. Results and discussion

3.1. Determination of the variance stabilizing transformation for OD data

The slopes of the regression lines of the log of the standard deviation of the 90 replicates against the log

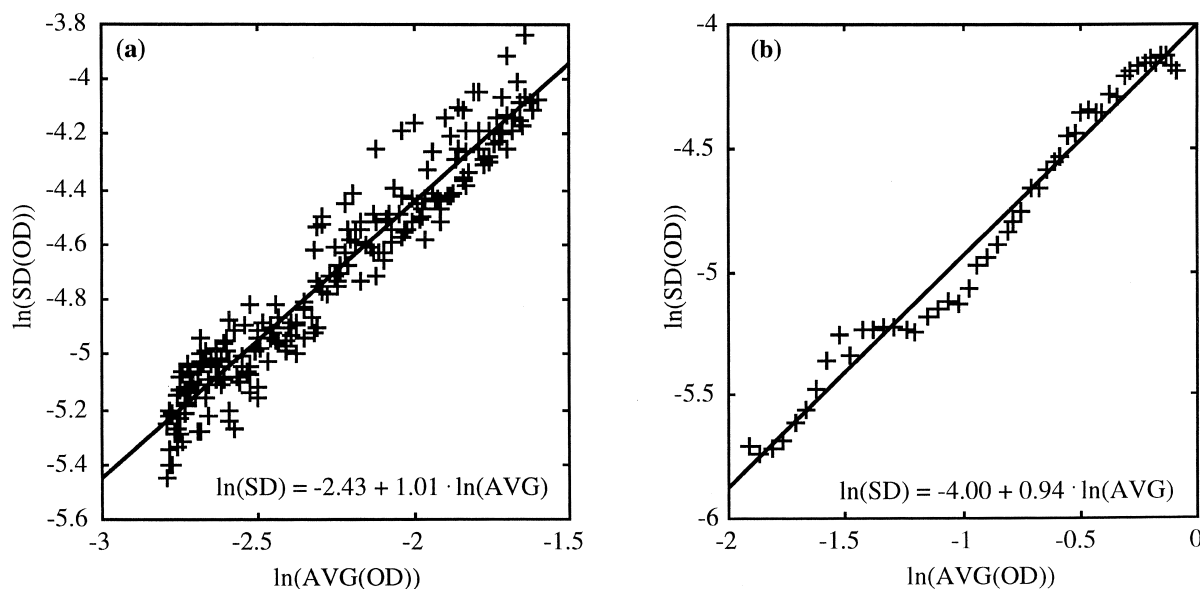


Fig. 1. Plots and regressions of the log of the standard deviation of OD against the log of the average obtained from 90 replicates of OD growth curves of *L. monocytogenes* Scott A at (a) 4.0°C and (b) 28.7°C.

of the average OD gave α -values of 1.01 ± 0.02 (SD) at 4.0°C (Fig. 1a) and 0.94 ± 0.02 (SD) at 28.7°C (Fig. 1b), indicating that the logarithmic transformation is suitable for OD data.

3.2. Calibration equation of OD against VC

Calibration equations were tested in the OD range of 0.0–0.6 for the ten strains because there were breakpoints at higher OD exhibiting an OD remaining at its maximum or decreasing slightly even though VC were increasing. The best fits of the data were achieved with polynomials of order 3 ($P < 0.001$). However, parameter values of calibration equations were significantly different from one temperature to another and these values were not linked to the temperature. Furthermore, by using the average values of parameter estimates at the different temperatures, the best fits of the whole data were obtained with polynomials of order 1. We then used, for calibration equations, polynomials of order 1 with constant term equal to 0 because confidence intervals of this parameter always included this

value. Eq. (2) can then be written in the OD range of 0.0–0.6:

$$OD = k \times x$$

where the k -values range from 2.85×10^{-10} to 4.46×10^{-10} for the ten strains.

3.3. Growth rates

3.3.1. Estimation of growth rates with the dilution series method

The hypothesis of no effect of inoculum size was checked by observing an almost constant time between successive OD growth curves for seven ten-fold dilutions (Fig. 2).

For all strains, the maximum specific growth rates obtained with the half-dilution series method were equal to those obtained with VC growth curves. However, this technique required several OD growth curves to obtain confident estimations of generation times. Indeed, the time separating the two successive growth curves was relatively variable (data not shown) and six successive dilutions were used to obtain average generation times. We moved then

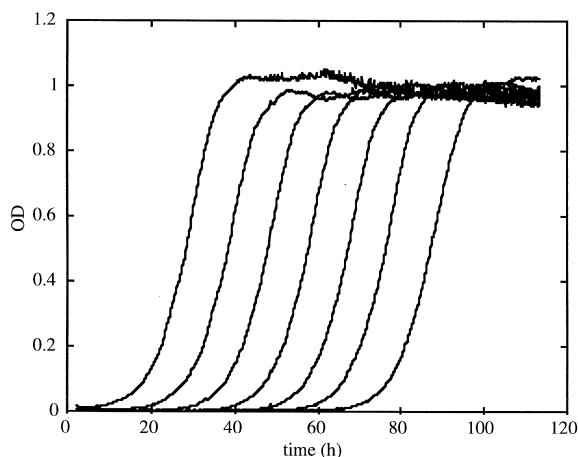


Fig. 2. OD growth curves of a series of ten-fold dilutions of *L. monocytogenes* Scott A at 14.5°C in TSYE broth (initial bacterial concentrations range from 10^7 to 10 cfu \times ml $^{-1}$).

towards methods requiring only one OD growth curve.

3.3.2. Estimation of growth rates with other methods on OD growth curves

As $s = \ln$ and $p_n(x) = k \times x$ in the OD range of 0.0–0.6, the model *GP* (Eq. (3)) became:

$$\ln\left(\frac{\text{OD}_a(t)}{\text{OD}_{a0}}\right) = A \times \exp\left(-\exp\left(\frac{\mu_{\max} \times e}{A} \times (\text{lag} - t) + 1\right)\right) \quad (8)$$

the model *W* (Eq. (5)) became:

$$\ln(\text{OD}_b(t) + 1) = \ln\left(\frac{k \times x_{\max}}{1 + \left(\frac{x_{\max}}{x_0} - 1\right) \times \exp(-\mu_{\max} \times (t - \text{lag}))} + 1\right) \quad (9)$$

and the model *E* (Eq. (6)) became:

$$\ln(\text{OD}_b(t) + 1) = \ln(k \times x_0 \times \exp(\mu_{\max} \times (t - \text{lag})) + 1) \quad (10)$$

The OD range corresponding to the exponential growth phase was estimated by plotting the observed growth rate $\frac{d\text{OD}}{dt}$ against OD (Fig. 3). $\frac{d\text{OD}}{dt}$ was estimated by $\frac{\Delta\text{OD}}{\Delta t}$ where ΔOD is the difference of OD between two successive readings and Δt is the time interval between two readings. We could consider that there were good linear correlations between $\frac{d\text{OD}}{dt}$ and OD for OD-values below 0.1. We then applied Eq. (10) in the OD range of 0.0–0.1.

In Eqs. (9) and (10), x_0 was the average of two initial VC and x_{\max} was the average of the maximum bacterial concentrations estimated with VC fits.

These three models gave growth rates strongly correlated with reference ones. The average square roots of ratios (Fig. 4) for the models *GP*, *W* and *E* were, respectively, 0.97 ± 0.06 (SD), 1.05 ± 0.08 (SD) and 0.97 ± 0.04 (SD). The best model for which the spread was the smallest was then the model *E*. As Dalggaard et al. (1994), we observed that this technique under-estimated the maximum specific growth rates and that the growth rates achieved must be divided by $0.97^2 = 0.94$. This is not surprising because OD is only detected when bacterial suspensions reached high cell concentrations, and at those high concentrations, the specific growth rate is significantly lower than μ_{\max} . If we consider that μ is continually broken as following a logistic function, we have $\mu = \mu_{\max} \times \left(1 - \frac{x}{x_{\max}}\right)$. By using the average factor of proportionality between OD and VC achieved with the ten *L. monocytogenes* strains (3.83×10^{-10}), we obtain a bacterial concentration of 1.3×10^8 cfu \times ml $^{-1}$ for an OD of 0.050. By taking for x_{\max} the average maximum bacterial concentration of the ten strains (2.3×10^9 cfu \times ml $^{-1}$), we can see that the specific growth rate, when the OD=0.050, is indeed equal to 94% of the maximum one.

3.4. Lag times

Lag times were estimated with models *GP*, *W*, *E* and *M*. With this last model, the maximum specific growth rates were the values obtained with model *E*

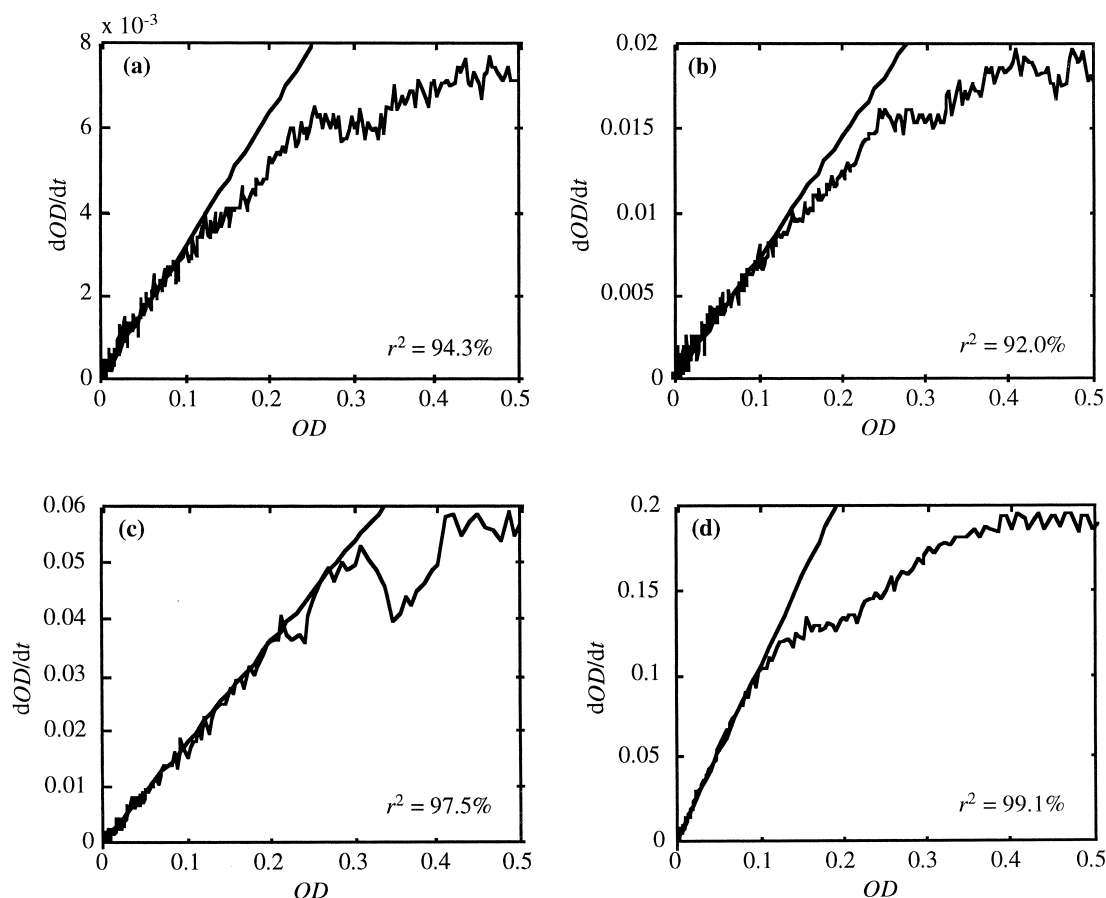


Fig. 3. Relation between OD growth rate and the OD of *L. monocytogenes* Scott A at (a) 4.0°C , (b) 7.8°C , (c) 13.5°C and (d) 35.4°C in TSYE broth. Regression lines and coefficients of determination of linear regressions between $\frac{dOD}{dt}$ and OD in the range of 0.0–0.1 are shown.

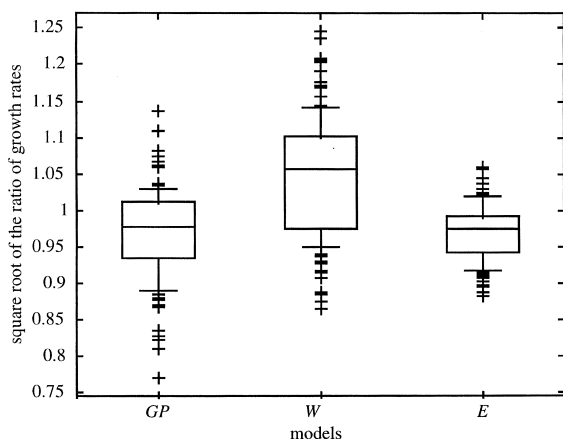


Fig. 4. Distribution of the square roots of the ratios of growth rates for the models GP, W and E (box plots displaying the 10th, 25th, 50th, 75th and 90th percentiles of the ratio).

divided by the calibration factor 0.94. Always for this model, lag times were the averages of two estimations obtained with two VC in exponential growth phase.

Estimated lag times from OD growth curves with models GP, W and E were correlated with those obtained with VC data but numerous aberrant values were obtained with these models (Fig. 5). The average value of the \ln of the ratio of lag times for the model GP was -0.56 ± 0.35 (SD) indicating that lag times achieved with this method were almost the half of VC lag times. This phenomenon was observed by Hudson and Mott (1994) with *Pseudomonas fragi* who obtained an average \ln of the ratio of -0.54 ± 0.35 (SD). With models W and E, lag

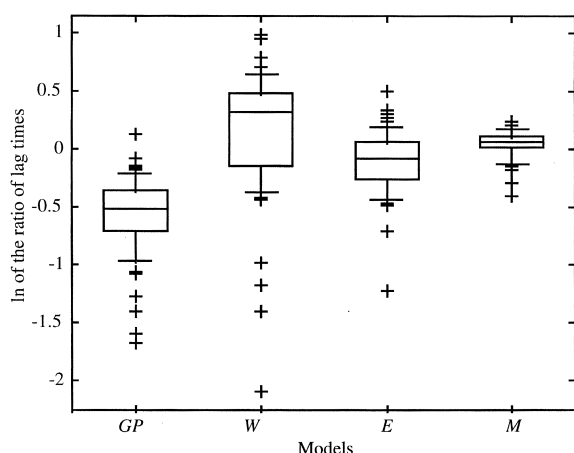


Fig. 5. Distribution of the \ln of the ratio of lag times for the models GP, W, E and M (box plots displaying the 10th, 25th, 50th, 75th and 90th percentiles of the ratio).

times achieved were on average almost the same that VC estimates but the ratios ranged from 0.1 to 2.7 (average \ln of the ratios of, respectively, 0.16 ± 0.56 and -0.10 ± 0.29). This can be explained in part by the variability of the factor of proportionality between OD and VC. It was then not possible to obtain reliable estimations of lag times with only the OD growth curves. We then preferred to use some VC in the exponential growth phase to estimate the lag time knowing the maximum specific growth from the OD data (model M). This method gave ratios of lag times less spread (Fig. 5) and lag times achieved with this method were almost equal to lag times estimated with VC growth curves (average \ln of the ratio of 0.05 ± 0.12).

3.5. Validation

Models E (Eq. (10)) and M (Eq. (7)) were chosen to determine growth parameters with OD growth curves. To use model M, the initial cell count, the maximum bacterial concentration and viable counts in the exponential growth phase must be known.

Maximum bacterial concentration was supposed to be equal to the average value achieved with the VC data regressions. Initial cell counts were estimated from two initial VC. Maximum specific growth rates were estimated by using model E on OD growth curves in the range of 0.0–0.1 and by dividing the

growth rates obtained by 0.94. Lag times were the averages of two estimates obtained by using model M on two VC in exponential growth phase. Knowing all these parameters, predicted growth curves were constructed with the growth model f (Eq. (1)).

Predicted growth curves were consistent with the observed VC for the eight experiments (Fig. 6).

4. Conclusion

Turbidimetric measurements are an effective way to accurately estimate growth rates of *Listeria monocytogenes* at different temperatures. It was shown that a reliable estimation of μ_{\max} can then be obtained with only the beginning ($OD=0.1$) of one OD growth curve when the initial OD is below the detection threshold by knowing the calibration factor to correct the growth rate observed. We saw that this calibration factor was constant for ten strains of *L. monocytogenes* and for the incubation temperature in the range of 4.0–35.4°C. Knowing μ_{\max} with high confidence, we can then estimate the lag time with only a few VC data. Consequently, the method proposed is highly appropriate for a rapid acquisition of dynamic growth parameters for a large number of curves obtained in different conditions. Nevertheless, this method has to be reconsidered for other organisms and environmental conditions (e.g. growth medium, pH, water activity, etc.) to demonstrate the validity of the model and to estimate the appropriate calibration factor. For example, this factor may vary with the factor of proportionality between OD and VC (e.g. the size of the organism used) and with the maximum population reached. But given the simplicity of the model proposed, this validation and new calibration may be easily performed.

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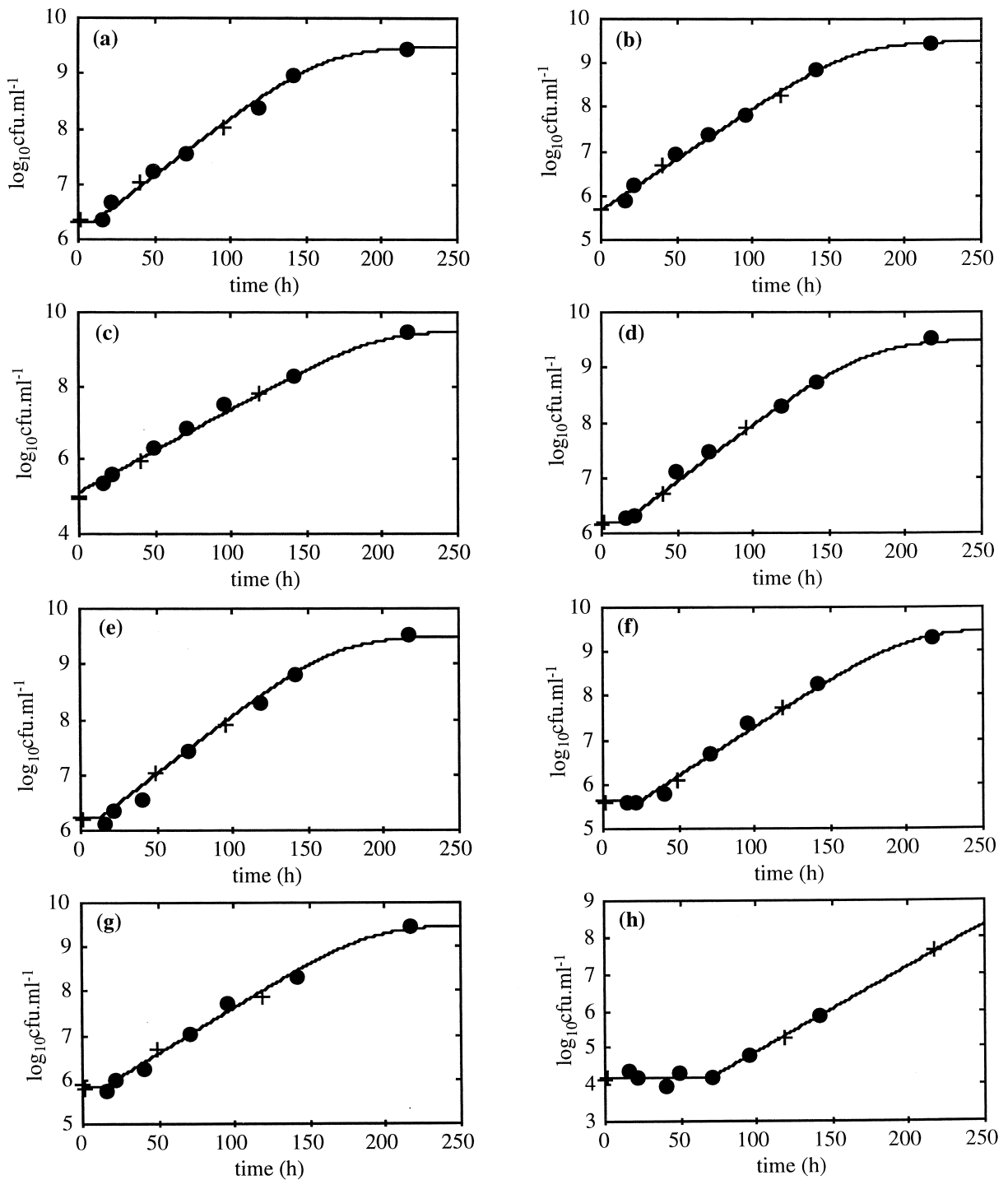


Fig. 6. Predictions of *L. monocytogenes* Scott A growth curves at 6°C in TSYE broth for different pre-incubation conditions: (a) 12°C for 10 h, (b) 12°C for 22 h, (c) 12°C for 32 h, (d) 37°C for 1 h, (e) 37°C for 3 h, (f) 37°C for 5 h, (g) 4°C for 52 days and (h) 4°C for 10 months. (●) are observed VC data, (+) are observed VC used in the model and the lines are the predicted growth curves.

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