Temperature effect on bacterial growth rate: quantitative microbiology approach including cardinal values and variability estimates to perform growth simulations on/in food

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Abstract

Temperature effect on growth rates of Listeria monocytogenes, Salmonella, Escherichia coli, Clostridium perfringens and Bacillus cereus, was studied. Growth rates were obtained in laboratory medium by using a binary dilutions method in which 15 optical density curves were generated to determine one μ value. The temperature was in the range from 2 to 48 °C, depending on the bacterial species. Data were analysed after a square root transformation. No large difference between the strains of a same species was observed, and therefore all the strains of a same species were analysed together with the same secondary model. The variability of the residual error, including both measurements errors and biological strain difference, was homogenous for suboptimal temperature values. To represent this variability in bacterial kinetic simulation, the 95% confidence interval based on an asymptotic Normal distribution, around the growth rate value was determined. With this modelling approach, the behaviour of bacterial species on food, irrespective of the strain or the laboratory, was described. This growth simulation with confidence limits has several applications, such as to facilitate comparisons between a challenge-test and simulation results, and, to appreciate if the temperature change has or has not a significant effect on a bacterial growth profile, with regard to the uncontrolled factors. The integration of this piece of work in the Sym’Previus software is now in process. Results obtained in five French laboratories will be extended by working on new food and new microbial species and improved by further work on variability estimation.

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1. Introduction

Predictive modelling has been widely developed since the 1980s (Van Impe et al., 1992; McMeekin et al., 1993; Baranyi and Roberts, 1994; Rosso et al., 1995). This field combines the knowledge of bacterial growth responses over a range of conditions with the power of mathematical modelling to enable predictions of growth. The main objective is to simulate bacterial growth as function of temperature, pH and water activity.

Several French laboratories (among which industrial ones), working in collaboration in the national Sym’Previus project, have collected data concerning bacterial strains from the species *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* (including O157:H7), *Clostridium perfringens* and *Bacillus cereus*, to enable simulation of bacterial behaviour in foods in the event of contamination.

The first objective of this paper is to quantify the variability due to both measurement errors and biological difference between various bacterial strains, and therefore, to suggest a modelling approach including this variability. The second objective is to illustrate how such a modelling tool is able to predict the bacterial behaviour on food at different temperatures (Membre’ et al., 2003; Leporq et al., 2004).

2. Materials and methods

2.1. Microorganism, culture and sub-culture conditions

The microorganisms used were bacterial strains isolated from several foods: 21 strains of *L. monocytogenes* from pork meat (5 strains), seafood (2), dairy products (7), poultry (3) and food plants (4); 10 strains of *E. coli* from a meat product (1), bovine faeces (3), dairy products (3) and human isolates (3); 10 strains of *B. cereus* from seafood (1), dairy products (6), egg or egg based products (2) and pasta (1); 5 strains of *C. perfringens* from pork (1), dairy products (3), poultry (1); 9 strains of *Salmonella* from sausage and pork meat (2), dairy products (1), poultry (1), dairy plants (3), bakery products (2).

For all the species, except *C. perfringens*, the growth medium was brain heart infusion (BHI) supplemented with glucose (0.2%) and yeast extract (0.3%). From frozen strains, two sub-cultures were carried out at 30 °C. Concerning *C. perfringens*, the growth medium was thioglycolate prepared under N2, the two sub-cultures were carried out at 37 °C.

Whatever the species, the second sub-culture was diluted and then inoculated in the culture medium to obtain an initial concentration of about $10^5$ cfu/ml into the first dilution tube, D1. Then, a minimum of four consecutive binary dilutions were performed to obtain different inoculum levels (D2 up to D5). A Bioscreen C (Labsystems, Helsinki, Finland) was used to measure the turbidity at 600 nm. The microplates containing 350 μl were incubated at the target temperature level and agitated 30 seconds per minute. Three replicates of the same dilution for each temperature level were generated. A minimum of seven temperature levels were studied for each bacterial strain, in a range within 2–48 °C according to the species.

2.2. Microbial and statistical methodologies to obtain growth rates

To obtain one growth rate value for each bacterial strain studied at a given temperature level, 15 optical density curves were generated (Fig. 1). The statistical analysis of Bioscreen curves was done according to a method adapted from Cuppers and Smelt (1993) and described previously in Membre’ et al. (2002). By computing a regression in the linear phase of turbidimetry curves (Fig. 1), 15 detection times were estimated. Then, these detection times were plotted versus the initial dilution values (D1–D5). The slope of linear regression corresponded to $-\mu$.

2.3. Statistical analysis

The linear and non-linear regressions were computed with Splus (AT&T Bell Laboratories, Murray Hill, New Jersey, USA), with SAS (SAS Institute, Cary, NC, USA) or Excel (Microsoft Excel 1997) according the software available in the different
laboratories (results were similar with the three programs).

3. Results and discussion

3.1. Effect of temperature on growth rates

The growth rates, $\mu$, were plotted versus temperature (Figs. 2–4). No large difference of behaviour at suboptimal temperature values between the strains of a same species was observed, although the strains were selected to be representative of different food contexts. Therefore all the strains of a same species were analysed together with the same statistical model.

The residual error variance was analysed graphically (data not shown). This variance was composed of uncertainty (lack of accuracy due to the measurements errors, the discrepancy between the laborato-

Fig. 1. Illustration of optical density curves generated using the Bioscreen C. Data obtained on Salmonella at 25 °C, three replicates of five binary dilutions, D1–D5.

Fig. 2. Illustration of temperature effect on growth rates including variability. Data collected with E. coli (symbols), fitted by a secondary model (solid line) and given with a 95% asymptotic confidence interval of the response prediction (dotted lines).
ries, etc.) and of biological strain variability. However, our study did not lead to a characterisation of each compound of this variability: our purpose was to suggest a modelling approach to simulate the temperature effect on the behaviour of bacterial species on food, whatever the strain or the labo-

Fig. 3. Illustration of temperature effect on growth rates including variability. Data collected with *B. cereus* (symbols), fitted by a secondary model (solid line) and given with a 95% asymptotic confidence interval of the response prediction (dotted lines).

Fig. 4. Illustration of temperature effect on growth rates including variability. Data collected with *L. monocytogenes* (symbols), fitted by a secondary model (solid line) and given with a 95% asymptotic confidence interval of the response prediction (dotted lines).
ratory. Particularly with *E. coli* (Fig. 2) and *B. cereus* (Fig. 3), the residual error was bigger when temperature values were above the optimal temperature than for other temperature levels. This phenomenon could be explained by biological considerations such as high sensibility of the strain to these mild temperature treatments. Consequently, to calculate the interval limit (see below), the supraoptimal temperature levels were not taken into account.

3.2. Temperature-dependent growth model, including variability

The temperature-dependent term $f(T_i)$ for *B. cereus, C. perfringens, Salmonella, L. monocytogenes* and *E. coli*, was the secondary model suggested by Rosso et al. (1993), including the cardinal values $T_{\min}, T_{opt}$ and $T_{\max}$ close to the minimal, optimal and maximal temperature for growth. Concerning *L. monocytogenes*, growth rates were also described by an adapted secondary predictive model (Le Marc et al., 2002) including the particular behaviour of *L. monocytogenes* at low temperatures (Bajard et al., 1996). This model was selected because it gave accurate predictions in foodstuffs at refrigerated temperatures (Membre et al., 2004).

To take the temperature effect on growth rates into account, a statistical model was employed (Eq. (1)). To stabilise the variance of the error, the square root of the growth rate was considered as the response. At each temperature value, $T_i$, the growth rate of $j$th strain, was written $\mu_{ijk}$, with $i$ as reference to the temperature level, $j$ to the strain and $k$ to the repetition of the experiment with the same strain at the same temperature value.

$$
\begin{align*}
\eta_{ijk} &= \sqrt{\mu_{ijk}} \\
\hat{\eta}_{ij} &= \left( \eta_{ijk} - \bar{\eta}_i \right) + \bar{\eta}_i \\
\bar{\eta}_i &= f(T_i)
\end{align*}
$$

(1)

$\eta_{ijk}$ represents the square root value of the growth rate, $\mu_{ijk}, \bar{\eta}_i$ the mean value observed for all the strains at a given temperature value, $i$, and $\bar{\eta}_i$ the predicted value obtained with a temperature-dependent secondary model.

In predictive microbiology, the integration of variability and/or uncertainty in predictions has already been suggested (Cassin et al., 1998; Nauta, 2000; Poschet et al., 2003; Pouillot et al., 2003; Shorten et al., 2004) by using simulation techniques. Our purpose in this study was to integrate mainly the variability due to the bacterial strains around the growth rate value, and then, the 95% confidence interval was determined. Based upon Huet et al. (1996) approach, the variance of an individual predicted response, $\eta_{ijk}$ as the square root of $\mu_{ijk}$, was calculated at each temperature value, $T_i$ by using Eq. (2).

$$
\begin{align*}
\text{Var}(\eta_{ijk}) &= \text{Var}(\eta_{ijk} - \bar{\eta}_i) + \text{Var}(\bar{\eta}_i - \hat{\eta}_i) \\
S_t(\eta_{ijk}) &= \sqrt{\text{Var}(\eta_{ijk})} \\
I_{95\%} &= [\hat{\eta}_i - \delta_{2.5\%}S_t(\eta_{ijk}); \hat{\eta}_i + \delta_{2.5\%}S_t(\eta_{ijk})]
\end{align*}
$$

(2)

$S_t$ is the standard deviation, the term $\text{Var}(\eta_{ijk} - \bar{\eta}_i)$ represents the variability of the square root of growth rates, $\mu_{ijk}$, observed at $T_i$, about its mean (“average” response of the species), including both uncertainty and strain variability, the term $\text{Var}(\bar{\eta}_i - \hat{\eta}_i)$ is the lack of fit due to the model. Of course, when an appropriate secondary model is employed, the second term is reduced and then becomes more negligible compared to the first one.

The term $\delta$ was calculated by using two methods (Huet et al., 1996). First, the Bootstrap method, a resampling technique appropriate to determine a confidence interval for an individual predicted value, was used to approximate the $\eta_{ijk}$ distribution without assuming Normality hypothesis. The main disadvantage of this method, especially when the number of experimental data is small, was to obtain interval limits fluctuating with the experimental data and then with the temperature values. On the other hand, the interval limit was also calculated by assuming Normality hypothesis of $\eta_{ijk}$ (in this case, $\delta$ corresponds to the Normal percentiles). The main advantage of this method was to obtain symmetric and identical intervals around the estimated value, $\hat{\eta}_i$, whatever the temperature value.
Since the distribution of individual errors, \( \eta_{ijk} \), was not too far from the Normal distribution (data not shown), for a practical use in simulation of bacterial behaviour on food whatever the temperature, the interval limit based on asymptotic level was preferred. Results obtained with Normal percentiles in Eq. (2) are presented in Figs. 2–4, with \( E. coli \), \( B. cereus \), and \( L. monocytogenes \) species, respectively. Concerning \( L. monocytogenes \) species, both secondary temperature-dependent models with three cardinal values (Rosso et al., 1993) and five cardinal values (Bajard et al., 1996; Le Marc et al., 2002) were employed. Comparison between predictions made by these two models and challenge-tests obtained in/on foods was also investigated. More details are given in Pinon et al. (2003) and Membre´ et al. (2004). Basically results were acceptable with the two models. The five cardinal values-model seemed more appropriate to describe \( L. monocytogenes \) behaviour in cooked pork products at low temperatures but on the other hand, the three cardinal-values-model present the advantage of parsimony and consistency with the other bacterial species studied in Sym’Previus. In Table 1, the cardinal values estimate obtained with \( B. cereus \), \( L. monocytogenes \), \( E. coli \) and Salmonella when the three cardinal-values-model developed by Rosso et al. (1993) was applied, are given. The estimated values of the minimal temperature where the growth is observed varied across a range of 6.6 °C as function of the species. Concerning \( C. perfringens \) species, so far only five strains were studied and we considered that more data were necessary before introducing results in the Sym’Previus software.

3.3. Bacterial kinetics on food at different temperatures

Growth rates (predicted and interval limits) were introduced in a primary model (Eq. (3)) to predict bacterial kinetics on food, at different temperatures.

\[
\begin{align*}
\ln N & = \ln N_0 + \ln \left[ 1 + \exp(-\hat{\mu}_g(t-L)) \right] \left( \frac{N_{\max}}{N_0} - 1 \right) & \text{if } t < L \\
\ln N & = \ln N_{\max} - \ln 1 + \exp(-\hat{\mu}_g(t-L)) & \text{if } t \geq L
\end{align*}
\]

(3)

In Eq. (3), \( t \) is the time (h), \( N_{\max} \) (CFU g\(^{-1}\)) the maximal biomass quantity and \( N_0 \) (CFU g\(^{-1}\)) the initial biomass quantity. The term \( \hat{\mu}_g \) corresponds to the predicted value at the temperature \( T_i \) (\( \hat{\mu}_g = \hat{\eta}_i^T \) from Eq. (1)). To focus the variability influence study only on the growth rate, the value of Lag time (L) was chosen arbitrarily as 0.

Bacterial species kinetics could be generated at any temperature value, \( T_i \), included in a \( T_{\min} - T_{\opt} \) range (Fig. 5), with a growth curve corresponding to the “average” behaviour of all the strains characterised in this study, and with an interval limit taking variability (particularly the strain variability and experimental error) of the specific growth rate into account. The limits were computed as the square of the confidence interval built by using Eq. (2), and thus, the interval limit around the growth rate value is not symmetric anymore (due to the non-linear square transformation).

With this modelling approach, the behaviour of bacterial species on food, whatever the strain or the laboratory, was described. It allows comparisons between experimental data (challenge-test) and simulation. In fact, before using a predictive model as a food safety tool, it is now admitted that predicted kinetics should be compared to data collected independently on food (Dalgaard and Jorgensen, 1998). This validation step is generally based on discrepancies between observed and predicted growth rates (or generation times) by using bias factors (Ross, 1996; Mellefont et al., 2003) but for a practical use in a food industrial context, analysis should be carried out on kinetics as well. Indeed, the time when a maximal authorised biomass value is achieved could be the value of interest. Likewise, if the exposition of food at a given temperature for a determined period is analysed in a risk assessment procedure, the validation should be performed on bacterial kinetics.

<table>
<thead>
<tr>
<th>Bacterial species (number of strains)</th>
<th>Minimal temperature estimate ( (T_{\min}) ), °C</th>
<th>Optimal temperature estimate ( (T_{\opt}) ), °C</th>
<th>Maximal temperature estimate ( (T_{\max}) ), °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus (10)</td>
<td>3.9</td>
<td>39.9</td>
<td>49.8</td>
</tr>
<tr>
<td>Listeria monocytogenes (21)</td>
<td>-1.7</td>
<td>38.1</td>
<td>45.1</td>
</tr>
<tr>
<td>Escherichia coli (10)</td>
<td>4.9</td>
<td>41.1</td>
<td>45.8</td>
</tr>
<tr>
<td>Salmonella (9)</td>
<td>4.6</td>
<td>39.5</td>
<td>45.6</td>
</tr>
</tbody>
</table>
However, to improve the bacterial kinetic confidence interval as illustrated in Fig. 5, further studies should be carried out. Firstly, variability was studied through the temperature factor while factors such as pH, organic acid, water activity or the food itself should be integrated as well. Secondly, the confidence interval built depended on experimental conditions obtained in our five laboratories and with the 55 bacterial strains; it depended also on the methodology to obtain the growth rate values and of course on the secondary model employed as statistical model.

Finally, in this paper, the variability taken into account referred to the growth rate variations whilst lag time is also a source of variability (McKellar and Knight, 2000; Koutsoumanis, 2001; Coleman et al., 2003). Therefore, the confidence interval limits could be improved in the future by collecting new data and by using extended models.

However, for the present, as the temperature is the main factor involving in pathogenic bacteria proliferation when fresh food are stored, this methodology could be considered as a helpful tool. For instance, to evaluate if the temperature change has or has not a significant effect on a bacterial growth profile, with in regard to the uncontrolled factors, a graphical comparison could be easily made (Fig. 5). In fact, if the interval limits built for two different temperatures are superposed, the bacterial kinetic is not significantly modified by the change in temperature. On the other hand, if the two interval limits are separated, temperature effect is preponderant.

4. Conclusion

The integration of this piece of work in the Sym’Previus software is now in process (Leporq et al., 2004). Thus, results obtained in five French laboratories to obtain model parameters and generate growth kinetics on food will be extended to the information collected on other bacterial species and strains, and other foodstuffs. Indeed, data provided from literature or industrial partners, stored in the Sym’previus database, could be used (i) to improve the variability estimation by adding new strains, (ii) to modify the predicted model if it is necessary (for instance to introduce lag time estimation) and (iii) to extend the application domain by working on new food and new microbial species.

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References


