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Applying advanced predictive microbiology techniques to static and dynamic growth studies of *Listeria monocytogenes*

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Abstract

This project, titled 'Listeria Control,' aimed to advance expertise across Europe in applying predictive microbiology to shelf-life studies of *Listeria monocytogenes* in ready-to-eat (RTE) products. By increasing the capacity of the two participating organisations in predictive microbiology, this initiative strengthens Europe's overall ability to manage and mitigate the risk of *L. monocytogenes* in RTE foods. The project's first experimental phase involved experimental trials that examined the growth of *L. monocytogenes* under both constant and dynamic temperature conditions. Subsequent analysis fitted existing primary growth models to the constant temperature growth data. The resulting models were then employed to predict *L. monocytogenes* growth under fluctuating temperature scenarios. Given the limited reported research on modelling *L. monocytogenes* growth in dynamic environments, this work represents a significant contribution to this emerging field. Furthermore, this fellowship facilitated collaboration between IZS-Teramo and UCD, leading to enhanced and harmonised expertise in experimental and predictive techniques for *L. monocytogenes* shelf-life studies – a partnership that both organisations are committed to continuing beyond the fellowship's duration.

KEYWORDS

inactivation studies, *Listeria monocytogenes*, predictive microbiology, shelf-life studies

CONTENTS

SUMMARY

The 'Listeria Control' project was initiated with the primary objective of developing and disseminating advanced expertise across Europe in the application of predictive microbiology techniques to shelf-life studies of *Listeria monocytogenes* in food products. *L. monocytogenes* is a serious food-borne pathogen that poses significant health risks, especially in ready-to-eat (RTE) foods requiring refrigeration, as it can thrive at low temperatures. The urgency of enhancing expertise in this area has been underscored by recent events, such as the *Listeria monocytogenes* ST155 outbreak in Italy (the host nation of one of the participating organisations) between July and December 2022, which originated from a contaminated cooked meat product. This outbreak led to 109 cases and six fatalities, highlighting the critical need for better predictive modelling of *L. monocytogenes* growth in RTE meat products. Such advancements are essential for enabling rapid and effective risk management and mitigation strategies.

The project was structured into two key phases. The first phase involved conducting trials to measure the growth of *L. monocytogenes* under both constant and dynamic temperature conditions. In the second phase, the growth data obtained from these trials were fitted using existing classic predictive models, such as the Huang model (Huang, 2013). The primary objective of this analysis was to use the models to fit the experimental data and then allow the prediction of the dynamic growth behaviour of *L. monocytogenes* in fluctuating temperature environments. This aspect of the project is particularly important because there are few studies that focus on modelling *L. monocytogenes* growth under dynamic conditions. Therefore, this work represents a valuable contribution to the field of predictive microbiology. Predictive microbiology, in general, holds significant potential for improving food safety and quality. By leveraging advanced modelling techniques and data analysis, predictive models can enhance the accuracy of exposure assessments, leading to better risk assessment in food safety protocols (Baranyi et al., 2024a, 2024b). Additionally, these techniques are crucial for determining the shelf-life of RTE foods, particularly in understanding the growth behaviour of pathogens like *L. monocytogenes* at refrigeration temperatures (Tarlak, 2023). From a commercial perspective, predictive models empower food producers to make informed decisions about product safety and quality, ultimately reducing food waste and boosting consumer safety and confidence. The fellowship component of this project facilitated increased collaboration between the participating organisations, resulting in enhanced and harmonised expertise in experimental and predictive techniques for *L. monocytogenes* shelf-life studies. This collaboration not only addressed crucial food safety issues but also made significant contributions to the European Food Safety Authority's (EFSA) broader food safety program. The knowledge and techniques developed through this initiative are expected to have a lasting impact, strengthening the overall food safety framework across Europe.

1 | INTRODUCTION

L. monocytogenes, a cold-tolerant pathogen, is a significant food-borne hazard, being capable of growing in refrigeration conditions commonly used in food storage and transport (Myintzaw et al., 2022). With global food chains becoming increasingly complex, food products can experience dynamic temperature fluctuations which can impact the growth of *L. monocytogenes* and therefore the associated risk of food-borne illness. Consequently, a robust understanding of Listeria's growth behaviour under dynamic temperature conditions is crucial for the development of effective food safety measures. Predictive microbiology has offered a scientifically grounded approach to estimating microbial behaviour, shelf-life and safety of food products (Taiwo et al., 2024), underpinning the design of control measures and management of risk.

Predictive microbiology generally is based on isothermal growth experiments and many models exist to characterise the growth of bacterial cells. Predictive models are commonly divided in primary and secondary models (Baranyi et al., 2024a, 2024b). The primary predictive mathematical models are those that describe changes in the concentration of bacteria in foods as a function of time. The secondary predictive mathematical models describe the parameters of the primary predictive models as a function of environmental conditions such as temperature, pH and aw. The methods for producing predictive models were standardised in the 1980s and 1990s and since then the fundamentals of this discipline have substantially remained unchanged. Gibson et al. (1988) identified the basis for reporting the phases of bacterial growth in mathematical formulas, proposing the use of the Gompertz sigmoid function for the primary models and the polynomial quadratic function for the secondary models. Not having been originally developed to model bacterial growth, the Gompertz model had important limitations. This basic approach was progressively improved to reduce the margin of error related to the fact that bacterial cells are living organisms, and as such do not always adhere precisely to simple mathematical rules. Consequently, a model was specifically developed by Baranyi et al. (1993) and Baranyi and Roberts (1994, 1995), capable of reflecting the natural environmental changes that can occur during the shelf-life of foods: The 'history' of bacterial cells prior to contamination was identified as a determining factor, which influences the ability to adapt to the new environment and consequently the duration of the transitional phase (lag phase) which precedes the start of exponential growth (exponential phase). More recently, Huang (2013) developed an alternative approach to describe the growth of bacteria under constant temperature conditions that is increasingly being used in modelling applications (Huang, 2016; Li et al., 2013). As regards secondary models, despite some alternative proposals in the 1990s (Rosso et al., 1995), the use of polynomial multivariate empirical models remains the normally preferred approach.

During the following decades, several dynamic modelling experiments for different pathogens have been published (Huang, 2017); however, full data sets of isothermal and non-isothermal are not always included, which can be a challenge for comparative analysis and model validation. Discrepancies between model predictions and observed growth in dynamic conditions suggest the need for further research and model refinement. This study aims to contribute to this critical area by investigating the growth of *L. monocytogenes* in broth under dynamic temperature conditions.

In recent years, there has been substantial activity within the European Union to provide both detailed and practical information on how to conduct shelf-life studies on *L. monocytogenes* in ready-to-eat foods to ensure compliance to the European Commission microbiological criteria. Predictive microbiology has a central role in interpreting the results of challenge tests and extending their application. Modelling can predict when concentration of *L. monocytogenes* reaches an unacceptable level in different environmental conditions and can explore combinations of environmental and product formulation conditions that would otherwise result in an inordinate amount of experimental characterisation. Predictive microbiology is useful to predict bacterial growth in various environmental conditions, investigate potential variability between batches and optimise formulation (additives, pH, salt) to assure product safety. There is a pressing need to compliment recent developments in information on how to conduct shelf-life studies on *L. monocytogenes* in ready-to-eat foods to ensure food safety with advanced predictive microbiology techniques to best leverage the amount of information gathered from experimental shelf-life studies. The objective of this study was to conduct parallel static and dynamic challenge studies of the growth of *L. monocytogenes* at low temperatures and to use the data generated by the static challenge tests to predict the dynamic temperature growth outcomes.

2 | DATA AND METHODOLOGIES

2.1 | Experimental methodologies

The focus of this work was on modelling the growth of *L. monocytogenes* as it is a significant food-borne hazard, and capable of growing in refrigeration conditions commonly used in food storage and transport. The initial laboratory work carried out a series of constant temperature growth experiments at low temperatures to underpin the development of primary growth models for the organism. In parallel, a series of dynamic non-isothermal growth experiments were carried out. The objective was to use the growth models derived from the constant temperature experiments to predict the dynamic non-isothermal growth and compare the predicted values with the experimental data. Through this work and other training activities, the fellow obtained a high-level training in the application of predictive modelling techniques using R and other online predictive modelling packages.

Briefly, the experimental protocol was as follows: The strain of *L. monocytogenes* used was from the EURL collection (EURL *Lm* 2013), strain 12MOB118LM isolated from cheese. The strain was first incubated at 37°C overnight in brain heart infusion broth, and then, aliquots were transferred to fresh BHI broth and further incubated at 10°C for 3 days, until the exponential phase was reached. The suspension of *L. monocytogenes* was diluted to produce a concentration of approximately $1 \times 10^{\wedge 3}$

CFU/mL. The suspension was divided into six volumes of 20 mL in sterile tubes. The first four volumes were incubated at four different isothermal conditions –4°C up to a maximum of 552 h, 8°C up to a maximum of 264 h, 10°C up to a maximum of 192 h and 12°C up to a maximum of 168 h. The last two 20 mL volumes were incubated at two different dynamic non isothermal conditions – one at 4°C for 264 h and then at 12°C until 336 h and the other at 12°C for 48 h and then shift at 4°C until 336 h. Samples for enumeration were taken at the start ($t=0$) and at approximately 24-h intervals thereafter up to the end of the trial. All experiments were performed for four times and each time repeated on two different occasions.

2.2 | Data modelling

A no-lag reduced growth primary model was chosen to model the experimental constant temperature growth data as the results indicated no lag phase in the growth curves. A three parameter logistic model was used to describe bacterial growth curves (Fang et al., 2012).

$$Y(t) = Y_0 + Y_{\max} - \ln(e^{Y0} + (e^{Y\max} - e^{Y0})\, e^{-\mu t}),$$

where $Y(t)$ is the natural logarithm of the bacterial counts (N) at time t; Y_0 and $Y_{\max}$ are the natural logarithms of the initial and the stationary phase counts; μ is the maximum growth rate. The growth curves were fitted to the logistic model using the nls () function in *R*.

A suboptimal Ratkowsky square-root model (Ratkowsky et al., 1983) was used as the secondary model to characterise the effect of temperature on growth rate. The model is of the form:

$$\sqrt{\mu} = a(T - T_0),$$

where μ is the maximum growth rate, a is a coefficient, T is temperature and T_0 is the nominal minimum growth temperature. A suboptimal Ratkowsky square-root model was chosen as the temperature rang was only between 4°C and 12°C.

Finally, the dynamic temperature conditions were modelled using the deSolve package in R to solve numerically the logistic growth model expressed in its differential form:

$$\frac{\mathrm{dN}}{\mathrm{dt}} = \mu N\left(1 - \frac{N}{N\max}\right),$$

where N is the bacterial count at time, t; $N_{\max}$ is the maximum stationary phase count; μ is the maximum growth rate. The square root model was used to model the effect of temperature on growth rate.

3 | ASSESSMENT

The series of constant temperature growth experiments at low temperatures quantified the ability of this strain of *L. monocytogenes* to grow at low temperatures. Figure 1 indicates a typical experimental growth curve obtained at 4°C and the fitted logistic model. Generally, the growth curves exhibited no lag phase and achieved the stationary phase in the experimental time period. The logistic model was successfully applied to all of the constant temperature growth experiments and the resulting growth rates were obtained using a Ratkowsky square-root model as the secondary model to characterise the effect of temperature on growth rate. Subsequent modelling work used the deSolve package in R to model the dynamic temperature experiments with a high level of agreement between predicted and experimental values. Figure 2 is an example of the growth of *L. monocytogenes* under dynamic temperature conditions (namely 4°C for 264 h followed by 12°C for the remaining hours). Superimposed on the plot is the predicted dynamic growth which closely follows the experimental values. It is anticipated that ongoing analysis of the data generated by the project will result in a joint scientific publication between the two participating partners.

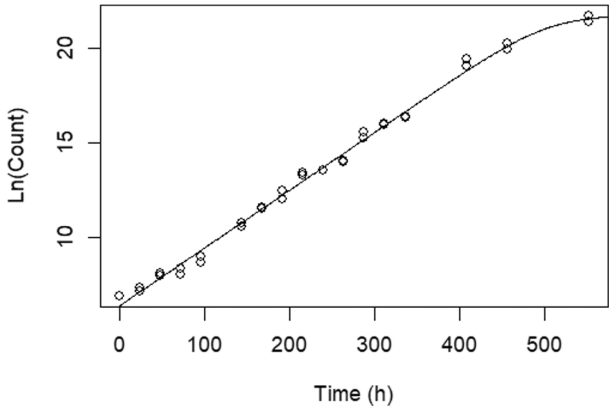

FIGURE 1 Typical growth curve of *Listeria monocytogenes* at a constant 4°C and fitted logistic model.

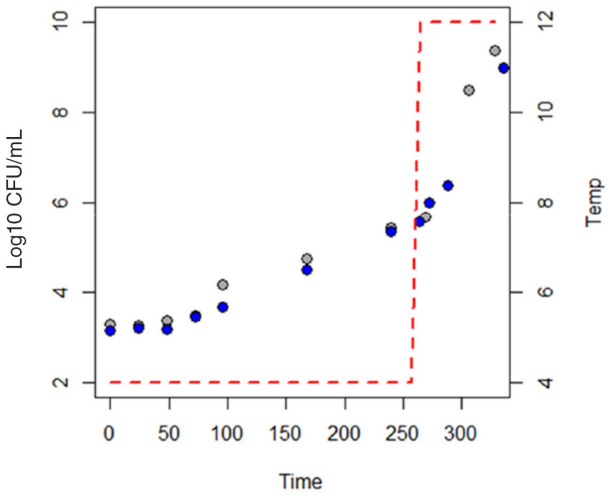

FIGURE 2 Predicted (blue) and experimental (grey) growth of *Listeria monocytogenes* under dynamic temperature conditions (4–12°C, red line).

4 | CONCLUSION

Developing a high level of predictive microbiology expertise and capacity across Europe as predictive microbiology has a critical role in interpreting the results of experimental challenge tests and extending their application under varying environmental conditions. This knowledge and the models derived through predictive microbiology underpin microbial risk assessment and are critical for the delivery of EFSA's mandate in this area. This Fellowship successfully applied predictive microbiology techniques to model the growth of *L. monocytogenes* in dynamic temperature situations which represent the actual conditions experienced by foods during the cold chain. There are only a few reported studies modelling the growth of *L. monocytogenes* in dynamic conditions, so this work will make a valuable contribution in the area. In addition, this fellowship facilitated the cooperation between staff at IZS-Teramo and UCD to develop an enhanced and harmonised expertise in experimental and predictive techniques for shelf-life studies for *L. monocytogenes* that will continue beyond the period of the fellowship.

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

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