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# Training in modern statistical methodologies and software tools for the definition and analysis of (stochastic) quantitative microbial risk assessment models with relevant food products for the Italian and Spanish food supply chains

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## Abstract

The fellowship, entitled 'Training in modern statistical methodologies and software tools for the definition and analysis of (stochastic) quantitative microbial risk assessment models with relevant food products for the Italian and Spanish food supply chains', was implemented at the Universidad Politécnica de Cartagena (UPCT), Spain. Supervised by Dr. Alberto Garre and Prof. Pablo S. Fernandez and coordinated by Dr. Virginia Filipello of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Italy, the fellowship aimed to provide hands-on training in quantitative microbial risk assessment (***QMRA***). The fellow benefited from UPCT's expertise in microbiological risk assessment, gaining knowledge of methodologies, terminologies and software tools essential for ***QMRA***. The focus of the fellowship was on the risks associated with plant-based milk products, which are increasingly popular as sustainable alternatives to dairy milk. Despite the heat treatments these beverages undergo to ensure safety, risks persist, such as cross-contamination during post-processing or the survival of heat-resistant spores like Bacillus cereus. A recent European outbreak linked to contaminated oat milk underscored the importance of assessing these risks. The project was conducted in two phases: first, at UPCT's Food Microbiology Laboratory, where the fellow handled and characterised the thermal resistance of various B. cereus strains using a thermoresistometer; and second, through remote analysis of experimental data using risk analysis software tools. The fellow developed skills in microbiological techniques, such as spore preparation and thermal resistance evaluation, and became proficient in data analysis using the R programming language and the biorisk package. The fellowship culminated in the development of a ***QMRA*** model to estimate the likelihood of B. cereus-related foodborne illness from plant-based milks, considering different heat treatments and bacterial strains. The fellow's training covered all stages of risk assessment, including hazard identification, exposure assessment, hazard characterisation and risk characterisation, providing a comprehensive foundation for a career in food safety and microbial risk assessment.

**KEYWORDS**

bacillus cereus, plant-based milk, quantitative microbiological risk assessment

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# 1 | INTRODUCTION

The fellowship entitled 'Training in modern statistical methodologies and software tools for the definition and analysis of (stochastic) quantitative microbial risk assessment models with relevant food products for the italian and spanish food supply chains' was conceived and executed by the Universidad Politecnica de Cartagena (UPCT, Spain) within the Department of Food Safety and Preservation (ETSIA), under the guidance of Dr. Alberto Garre (supervisor) and Prof. Pablo S. Fernandez (co-supervisor). The sending organisation, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Italy), coordinated the fellowship through Dr. Virginia Filipello.

The Food Safety and Preservation research group at UPCT has extensive experience in developing tools and mathematical models for microbiological risk assessment. This expertise provided the EU-FORA fellow with a valuable opportunity to gain in-depth knowledge of methodologies, terminologies and practical skills commonly applied in quantitative microbial risk assessment (***QMRA***). The fellow benefited from hands-on experience with modern methodologies and software tools for constructing ***QMRA*** models.

Plant-based products are a significant advancement in the food industry, driven by the increasing consumer demand for sustainable and healthy alternatives to animal-based products. This shift towards plant-derived foods aims to reduce the environmental impact of food production and improve the overall sustainability of the agricultural sector (Tachie et al., 2023). Examples of these alternatives are plant-based milk, such as soy, almond, rice and oats, which are becoming increasingly popular as alternatives to dairy milk. Recent studies estimate that the global milk alternatives market could potentially exceed revenues of $38 billion by 2024 (Giugliano et al., 2023).

Although commercially available plant-based beverages undergo heat treatment to ensure they are microbiologically safe, there remains a risk of cross-contamination of the finished products after processing or the possibility that bacterial spores might survive the UHT process (Bartula et al., 2023). A recent outbreak in Europe (April 2022) involving UHT plant-based oat milk contaminated with *Bacillus cereus* resulted in illness for at least two people (Whitworth, 2022). Plant-based drinks are also easy to prepare at home, which increases the risk of cross-contamination. This is due to the high likelihood that beverages made at home may not receive adequate heat treatment in the home environment (Bartula et al., 2023).

*B. cereus* is a Gram-positive, spore-forming, facultative anaerobic, rod-shaped bacteria, which is ubiquitously found in the environment (Jessberger et al., 2020). *B. cereus* is capable of enduring and surviving in adverse environmental conditions through the production of endospores and the formation of biofilms. The spores of *B. cereus* are elongated and consist of a core that is enveloped by an inner membrane, a peptidoglycan cortex, an inner coat and an outer coat (Bressuire-Isoard et al., 2018). These spores are extremely resistant to environmental assaults that would normally kill vegetative bacteria, thereby facilitating persistence in the environment until more favourable conditions return (Hornstra et al., 2009). *B. cereus* produces a variety of virulence factors, including toxins, cereulide, hemolysins, enterotoxins, proteases and phospholipases. These factors contribute to its ability to cause foodborne illnesses and infections (Tuipulotu et al., 2021). In 2022, *B. cereus* toxins ranked first for the number of reported foodborne outbreaks, with an increase of 251.7% compared to 2021 (EFSA and ECDC, 2023). Given the considerations, plant-based products, such as plant-based milks, can provide a favourable environment for the survival of *B. cereus* spores and may lead to foodborne toxin infections. This work aimed to select various strains of *B. cereus* and phenotypically characterise them in terms of their heat resistance by evaluating their thermal inactivation dynamics and using this information to construct a ***QMRA*** model. Therefore, this work programme aimed to provide the fellow essential knowledge in ***QMRA*** through a hands-on learning approach. This included experimental methods, statistical analysis, mathematical modelling and stochastic simulations in plant-based milk. The programme encompassed all stages of risk assessment: hazard identification, exposure assessment, hazard characterisation and risk characterisation. The specific training objectives were: (1) establishing a strong understanding of methodologies and software related to microbiological risk assessment (MRA); (2) providing practical experience in obtaining experimental data necessary for kinetic models for ***QMRA***; (3) developing and validating predictive models based on experimental data; and (4) implementing a ***QMRA*** model to evaluate the behaviour of *B. cereus* in plant-based milk.

# 2 | DATA AND METHODOLOGIES

## 2.1 | Study setting

The project was carried out in two distinct phases. The first phase took place at the Food Microbiology Laboratory of the Polytechnic University of Cartagena (UPCT) in Spain. During this phase, various strains of *B. cereus* were manipulated and phenotypically characterised using a thermoresistometer. The second phase was conducted remotely and focused on learning various risk analysis software tools. These tools were subsequently used to analyse all experimental data obtained during the period in Cartagena. In the first phase, the thermoresistometer was utilised to evaluate the thermal resistance of *B. cereus* strains. This involved subjecting the strains to different temperature treatments to determine their survival rates and resistance characteristics. This data is crucial for understanding the potential risks of *B. cereus* in food products, particularly those subjected to thermal processing.

In the second phase, the risk analysis software provided a comprehensive platform for modelling and simulating the behaviour of *B. cereus* under various conditions. The integration of experimental data into these models allowed for the

prediction of contamination risks in different food supply chains, emphasising the importance of thermal resistance data in microbial risk assessment.

## 2.2 | Methodologies

During the first phase of the programme, the fellow was part of the working group in the microbiology laboratory at UPCT, through which he gained new knowledge in microbiology, food safety and risk assessment. Initially, the fellow was supervised through the various laboratory stages and was trained in the use of the different equipment available in the laboratory. This training continued until he acquired the skills necessary to work independently. Concurrently with laboratory activities, the fellow was also instructed in the use of various software tools, including R, to equip him with the necessary tools to develop a ***QMRA*** model.

### 2.2.1 | Spore preparation

Two *B. cereus* strains, INRA AV Z421 and TZ415, provided by the Station de Technologie des Produits Vëgëtaux (Institut National de la Recherche Agronomique, Avignon, France) were used throughout this study. The INRA AV Z421 strains is highly resistant to heat and the INRA AV TZ415 strain is psychrotrophic. Both strains were isolated from cooked chilled foods containing vegetables (Fernández et al., 1999). The initial task performed in the laboratory involved learning how to manage *B. cereus* bacterial strains, starting from thawing the strains to preparing the spores, which were subsequently used in various thermoresistance experiments. In more detail, following the initial thawing of the frozen stock, a loopful of the culture was aseptically transferred onto tryptic soy agar (TSA) plates to obtain isolated colonies. These colonies were subsequently inoculated into BHI broth and incubated at 37°C with shaking to encourage robust growth. After reaching the stationary phase, the cells were transferred to a sporulation medium and incubation was continued at 30°C to promote spore formation. Sporulation was monitored using microscopy, and the process was allowed to continue until a high percentage of cells had formed spores (Li et al., 2022).

Finally, the spores were collected by centrifugation, washed multiple times with distilled water and stored at 4°C. These prepared spores were then utilised for experiments assessing the thermoresistance of *B. cereus*.

### 2.2.2 | Thermal treatments

Thermal treatments were carried out using a Mastia thermoresistometer (Conesa et al., 2009). This device allows to perform thermal treatments in liquid media with a temperature profile that can be programmed within the maximum heating and cooling rates of the equipment (40°C/min). The vessel of the thermoresistometer is constantly stirred during the treatment, ensuring a homogeneous temperature distribution. Before starting the treatment, the vessel was filled with 400 mL of the medium. Sterile TSB and soya milk were used as heating media. Isothermal experiments were conducted at temperatures of 85, 90, 92.5 and 95°C. Dynamic experiments were performed from 60°C to 95°C with heating times of 5, 15 and 35 min. For each specific treatment, the thermoresistometer was set to maintain a constant temperature. After the temperature in the chamber stabilised, a 0.2 mL aliquot of the spores was introduced. For experiments under non-isothermal conditions, 3 distinct temperature profiles were evaluated for both strains. Viable counts were determined following the same procedure for both isothermal and dynamic profiles. Samples were taken at preset times and collected in Eppendorf tubes, subsequently, 0.1 mL aliquots were plated and mass homogenised in TSA. Plates were incubated at 37°C for 24 h and then counted. All the experiments were performed in triplicate for each temperature profile (isothermal and non-isothermal), each liquid media (TSB and soya milk) and each strain (Z421 and Z415) for a compressive number of 96 different experiments.

### 2.2.3 | Microbial inactivation models

The data obtained from the isothermal treatments was analysed using the Bigelow, Mafart and Peleg models. The Bigelow model, as shown in Equation (1), assumes that the log-microbial concentration ($N$) varies linearly with time during the treatment, with respect to the initial concentration ($N_0$). In this model, the inactivation kinetics are characterised by the $D$-value ($D$).

$$\log N = \log N_0 - t / D. \tag{1}$$

Regarding secondary models, the Bigelow model assumes a log-linear relationship between the $D$-value and the treatment temperature ($T$). As shown in Equation (2), the sensitivity of the microorganism to temperature changes is characterised by the $z$-value ($z$). This model introduces a reference temperature ($T_{ref}$) to improve parameter identifiability, with $D_{ref}$ being the $D$-value at $T_{ref}$.

$$\log D = \log D_{\text{ref}} - \frac{T - T_{\text{ref}}}{z}. \tag{2}$$

The Mafart model is an extension of the Bigelow model that enables curves to have curvature. It is defined by Equation (3), where the curvature is defined by parameter $p$ ($p < 1$ indicates upwards curvature and viceversa). The inactivation rate is also defined by the delta-value (δ) that corresponds to the time for the first log-reduction.

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p. \tag{3}$$

The Mafart model uses a similar secondary model as Bigelow (Equation 4), where the relationship between the delta-value and temperature is assumed log-linear.

$$\log \delta = \log \delta_{ref} - \frac{T - T_{\text{ref}}}{z}. \tag{4}$$

The primary model of the Peleg is equivalent to the one of the Mafart model, although it uses a different parameterisation (Equation 5). In this model, parameter $n$ is equivalent to $p$ while parameter $b$ is related to the inactivation rate.

$$\log N = \log N_0 - b \cdot t^n. \tag{5}$$

The secondary model of the Peleg model is defined in Equation (6). It assumes that, below a critical temperature ($T_{\text{crit}}$) the inactivation rate is practically zero and that for temperatures much larger than $T_{\text{crit}}$ the relationship between $b$ and $T$ is practically linear with slope $k$.

$$b = \ln\left(1 + e^{k(T - T_{\text{crit}})}\right).$$

The models were fitted to the data using the online version of bioinactivation (Garre et al., 2017, 2018), available at (https://foodlab-upct.shinyapps.io/bioinactivation4/). The models were fitted using a one-step approach, where both the primary and secondary models are fitted to the data. Bioinactivation was also used for making predictions based on the inactivation models fitted. This enabled validating the models under dynamic conditions, as well as on the food product.

### 2.2.4 | Definition of the risk assessment model

An academic risk assessment was performed as part of the training programme for ***QMRA***. The model was developed as a Modular Process Risk Model (Nauta, 2008) with five elements: initial contamination, inactivation treatment, growth during storage and consumer storage.

Due to the lack of data on the prevalence and initial concentration of *B. cereus* in vegetable-based milk, it was assumed that every item was contaminated with 1 CFU/mL.

For the inactivation treatment, four different scenarios were considered (Tachie et al., 2023): sterilisation, HST, pasteurisation and blanching. The time/temperature combinations were described using uniform distributions with the limits described in Table 1. The inactivation kinetics were described using the Peleg model fitted and validated using the data obtained within the fellowship (Table 2).

**TABLE 1** Thermal inactivation scenarios considered for the ***QMRA*** model.

| Scenario | Min. Temperature | Max. Temperature | Min. Time | Max. Time |
|---|---|---|---|---|
| UHT | 110°C | 121°C | 15 min | 30 min |
| HTST | 70°C | 72°C | 25 s | 30 s |
| Pasteurisation | 135°C | 150°C | 5 s | 5s |
| Blanching | 85°C | 95°C | 2 min | 5 min |

The growth during storage was described using the kinetic model by Carlin et al. (2013). It was considered that the product is stored refrigerated with temperatures and time according to Bodea et al. (2023).

The probability of illness was described using a discrete model. Considering that *B. cereus* outbreaks are often related to toxin production and that these are produced during the late exponential/early stationary growth phases, it was assumed that portions with a bacterial concentration of at least 5 log CFU/mL would cause illness.

The ***QMRA*** model was solved by forward uncertainty propagation using Monte Carlo simulations. Calculations were done in R version 4.2.3 using the *biorisk* package for R (available at https://github.com/albgarre/biorisk). Considering the four treatment scenarios and two different bacterial strains, a total of eight different scenario were observed.

# 3 | RESULTS

## 3.1 | Characterisation of the thermal resistance of *B. cereus*

Figures 1 and 2 illustrate the fit of the Bigelow, Mafart or Peleg model to the isothermal inactivation data obtained for *B. cereus* strains 415 and 421, respectively. The data showed a slight curvature, so the Bigelow model would be not be appropriate for describing the response of these microorganisms. This is further confirmed in Table 2, which reports the parameter estimates. For both bacterial strains, parameter *p* was significantly different from one, so there would be enough evidence to conclude that the microbial response is non-linear.

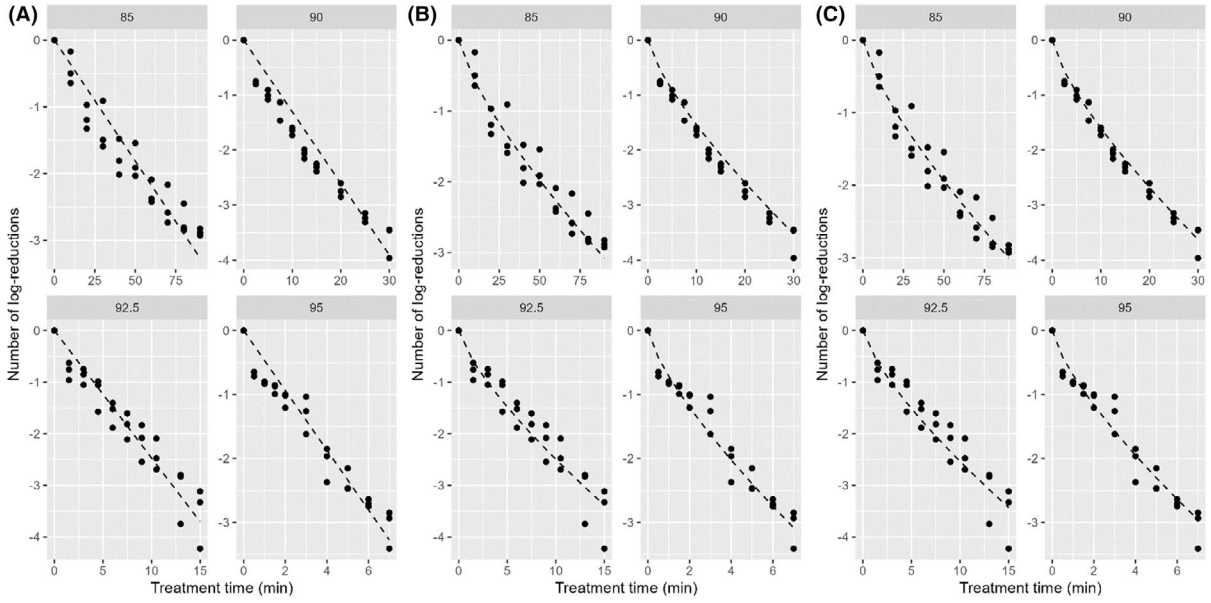


**FIGURE 1** Fit of the Bigelow (A), Mafart (B) and Peleg (C) models to the data on the isothermal inactivation of *B. cereus* 415.

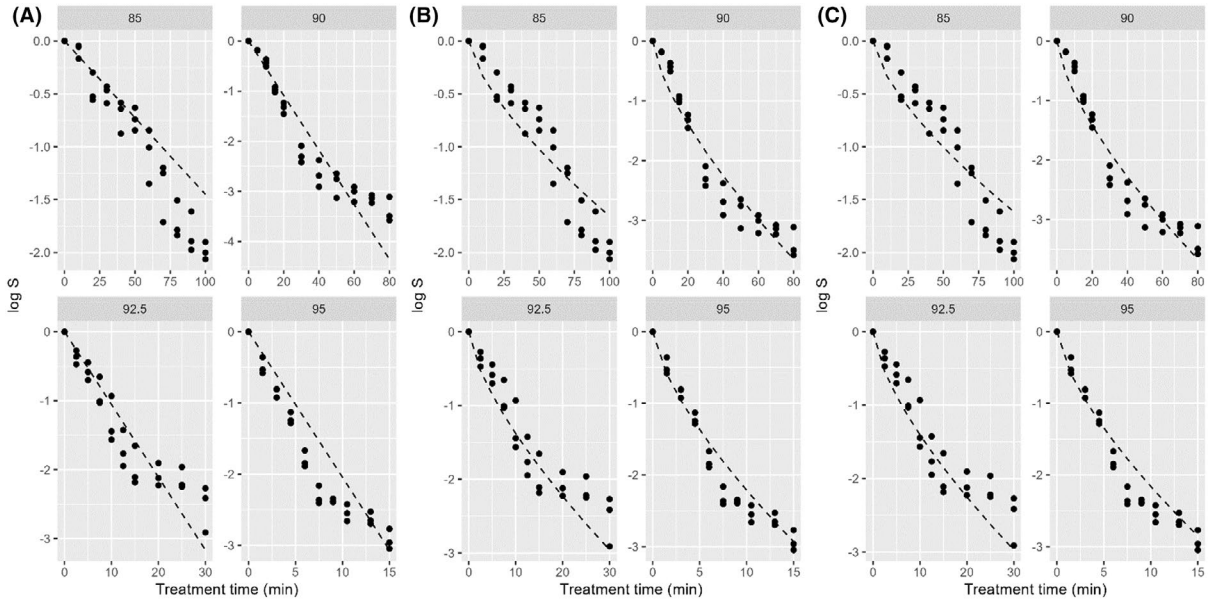


**FIGURE 2** Fit of the Bigelow (A), Mafart (B) and Peleg (C) models to the data on the isothermal inactivation of *B. cereus* 421.

**TABLE 2** Parameters of the inactivation models fitted to the isothermal inactivation data on *B. cereus* 415 and *B. cereus* 421.

| Model | Parameter | *B. Cereus* 415 | *B. Cereus* 421 |
|---|---|---|---|
| Bigelow model | $D_{90}$ (min) | 7.67 ± 0.11 | 18.37 ± 0.43 |
| | $z$ (°C) | 9.01 ± 0.14 | 8.70 ± 0.26 |
| Mafart model | $\delta_{90}$ (min) | 5.63 ± 0.24 | 12.42 ± 0.59 |
| | $z$ (°C) | 9.01 ± 0.14 | 8.47 ± 0.22 |
| | $p$ | 0.75 ± 0.03 | 0.69 ± 0.03 |
| Peleg model | $k_b$ | 0.22 ± 0.01 | 0.21 ± 0.01 |
| | $T_{crit}$ | 94.98 ± 0.27 | 97.77 ± 0.39 |
| | $n$ | 0.74 ± 0.02 | 0.68 ± 0.03 |

The models fitted to the isothermal inactivation data were validated under dynamic conditions using an independent dataset. Figures 3, 4 compare the predictions of the Bigelow, Mafart and Peleg model against the dynamic data for strains 415 and 421, respectively. In every case, the Peleg model is able to describe the overall trend of the changes in the microbial concentration. Therefore, this model would be validated for describing the inactivation of the spores of both strains under dynamic conditions.

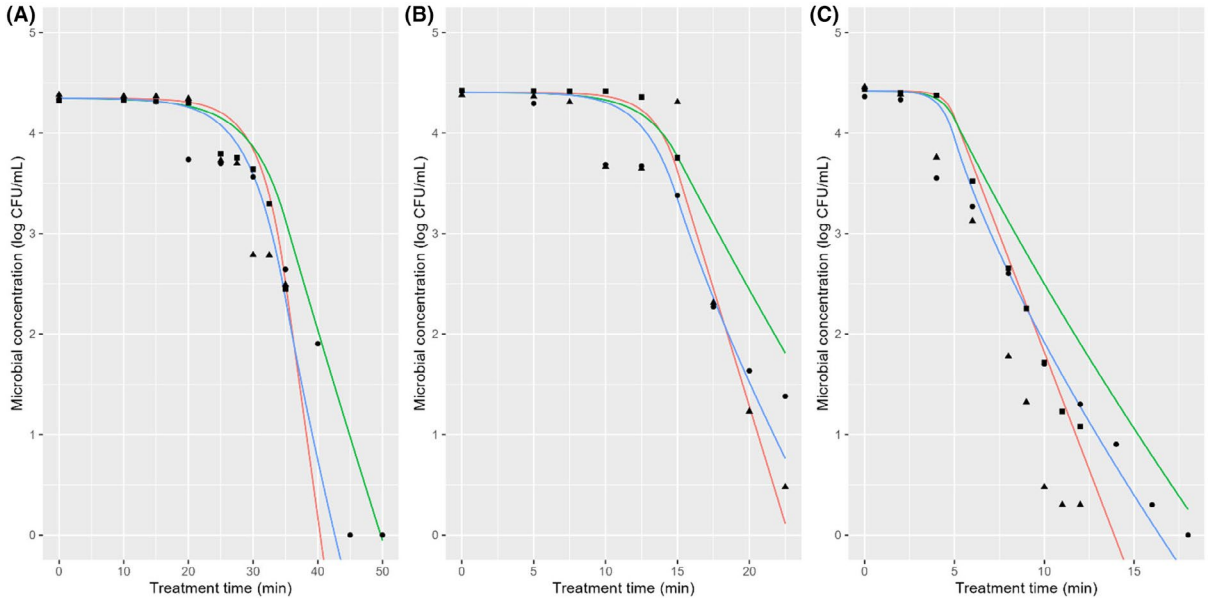


**FIGURE 3** Comparison between the predictions of the Bigelow (red), Mafart (green) and Peleg (blue) models against the microbial concentrations observed for strain 415 in dynamic treatments with a heating time of 35 (A), 15 (B) or 5 (C) minutes.

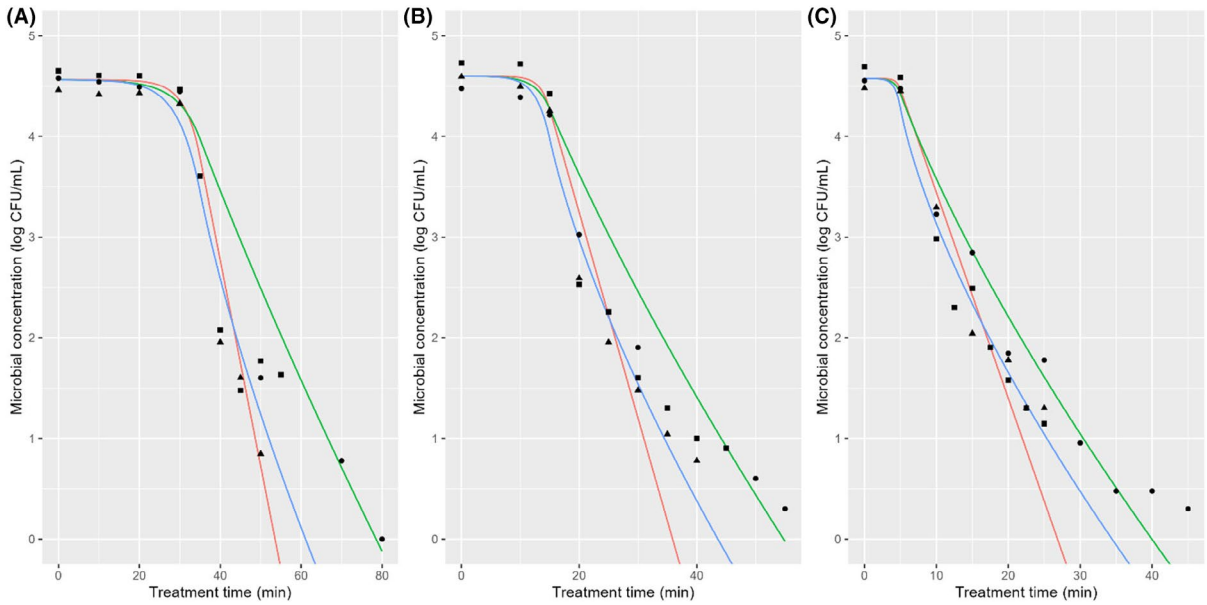


**FIGURE 4** Comparison between the predictions of the Bigelow (red), Mafart (green) and Peleg (blue) models against the microbial concentrations observed for strain 421 in dynamic treatments with a heating time of 35 (A), 15 (B) or 5 (C) minutes.

The predictive power of the models was also validated on vegetable-based milk. Figures 5, 6 compares the predictions against the microbial concentrations observed under isothermal treatments. In every case, the Peleg and Mafart models successfully described the overall trend of the data. Therefore, considering also the results obtained under dynamic conditions, the Peleg model would be validated for the description of the inactivation kinetics of the spores of both *B. cereus* strains.

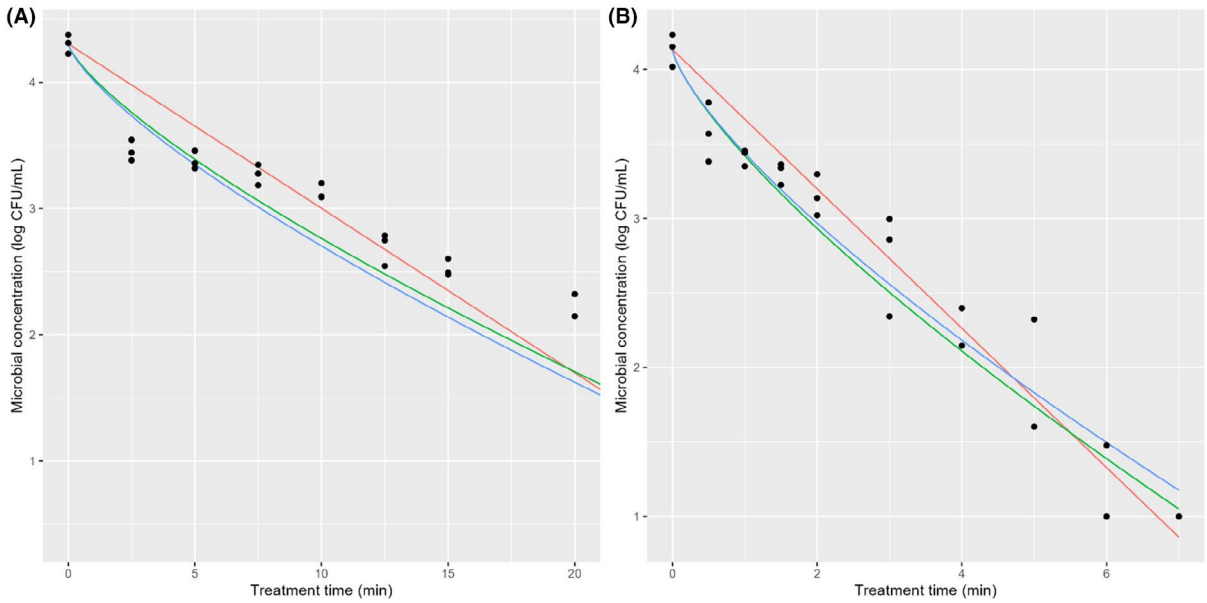


**FIGURE 5** Comparison between the predictions of the Bigelow (red), Mafart (green) and Peleg (blue) models against the microbial concentrations observed for strain 415 in vegetable milk during isothermal treatments at 90 (A) and 95°C (B).

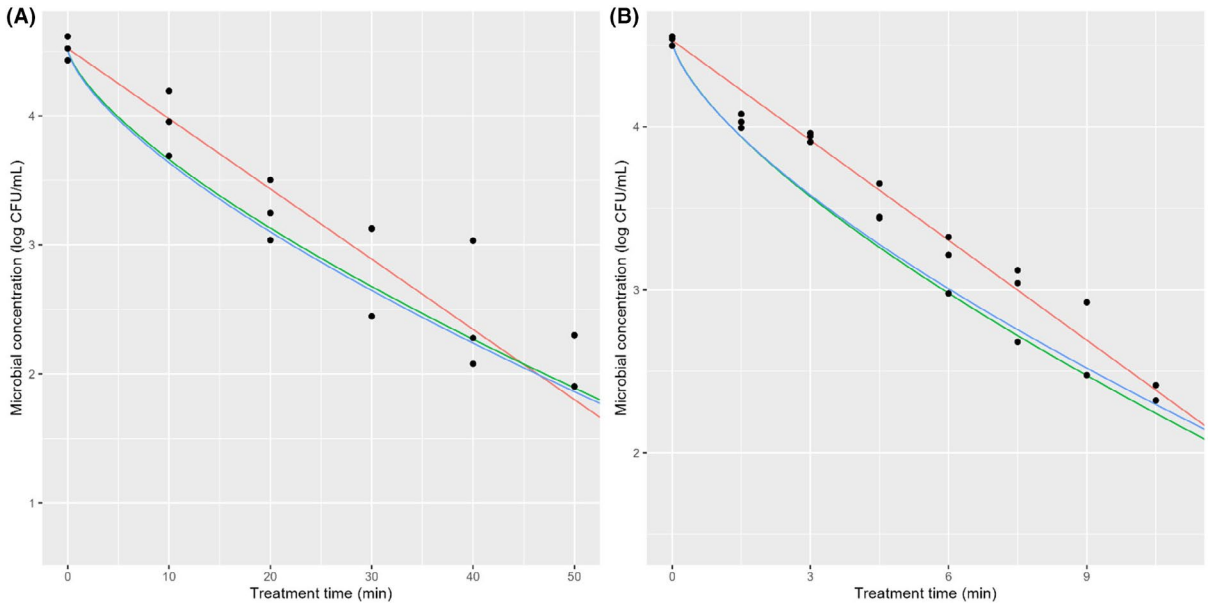


**FIGURE 6** Comparison between the predictions of the Bigelow (red), Mafart (green) and Peleg (blue) models against the microbial concentrations observed for strain 421 in vegetable milk during isothermal treatments at 90 (A) and 95°C (B).

## 3.2 | Risk assessment

The ***QMRA*** model was used to describe the variation in the *B. cereus* concentration through the food supply chain, as well as the probability of illness. Figure 7 illustrates the microbial concentration at the moment of consumer exposure for each of the scenarios considered. Strain 421 has overall higher concentrations for every scenario due to its higher thermal resistance (Table 2). Furthermore, there are large differences between scenarios. The UHT treatment results in extremely low concentrations, whereas the other treatments would result in a relevant exposure.

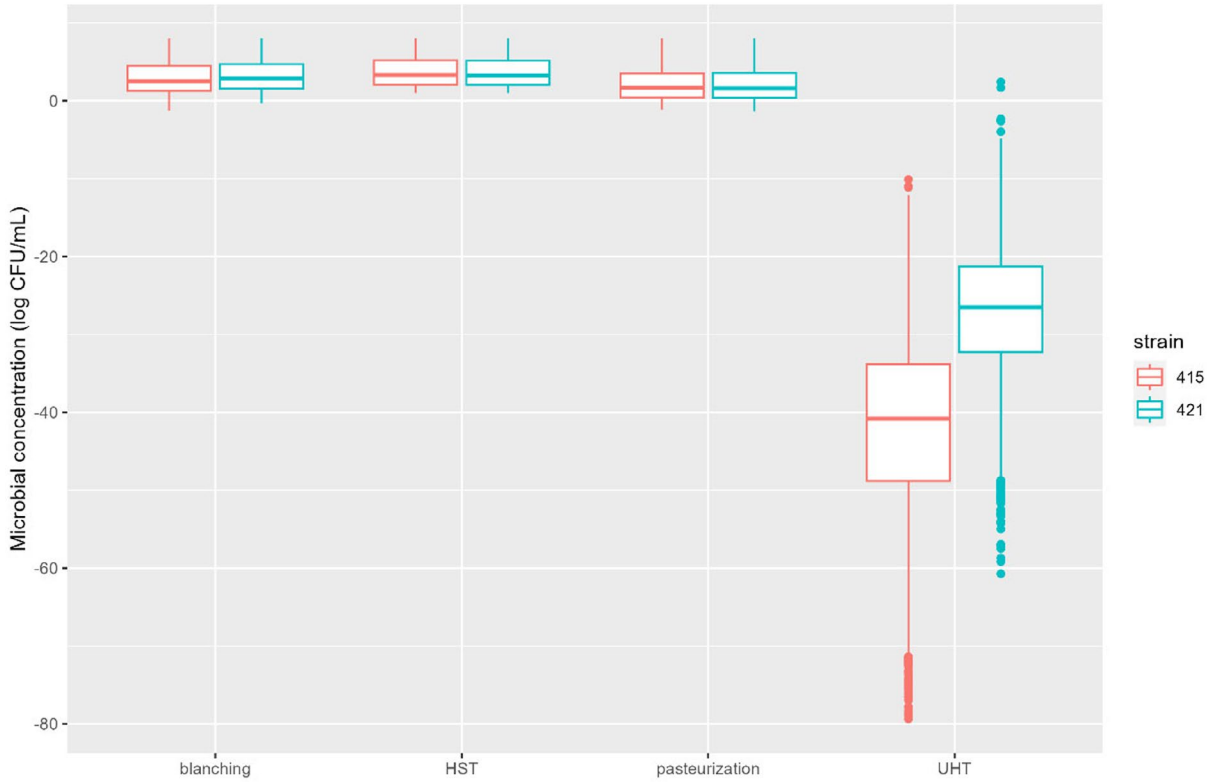


**FIGURE 7** Microbial concentration at the point of consumer exposure for each of the scenarios considered.

This is further evidenced in Table 3 that reports the proportion of servings that would cause disease. For the UHT treated products, the risk would be zero (from 100,000 MC simulations). On the other hand, the other three treatments would have

a proportion between 0.13 and 0.28. In particular, the HTST treatment would result in the higher risk, followed by blanching and pasteurisation.

**TABLE 3** Proportion of servings that would cause disease according to the academic ***QMRA*** model for each scenario considered considering a 100% prevalence.

| Type of treatment | *B. cereus* 415 | *B. cereus* 421 |
|---|---|---|
| UHT | 0 | 0 |
| HTST | 0.28 | 0.27 |
| Pasteurisation | 0.15 | 0.13 |
| Blanching | 0.27 | 0.24 |

## 4 | CONCLUSION

The fellowship provided the fellow with comprehensive training and hands-on experience in various aspects of food safety, microbiology and ***QMRA***. Throughout the programme, the fellow gained valuable knowledge and skills that are crucial for a career in food safety and risk analysis. The fellow acquired practical skills in handling and manipulating bacterial strains, specifically *B. cereus*. This included learning the techniques for spore preparation, culturing and identifying microbial strains. The fellow also gained proficiency in using specialised laboratory equipment, such as the thermoresistometer, to evaluate the thermal resistance of microorganisms under different conditions. The fellowship provided the fellow with extensive experience in data analysis using various software tools. The fellow was trained in using the R programming language and the biorisk package, which are integral for developing and analysing ***QMRA*** models. The fellow also became proficient in using online platforms such as Bioinactivation, which allows for the development of bacterial inactivation models based on experimental data. These skills enabled the fellow to conduct detailed statistical analysis, develop predictive models and interpret complex datasets, which are essential for quantitative risk assessments. The fellow gained a deep understanding of the methodologies and processes involved in ***QMRA***. This included learning about hazard identification, exposure assessment, hazard characterisation and risk characterisation. In addition, the fellow gained hands-on experience in constructing ***QMRA*** models to estimate the risk of foodborne illness from *B. cereus* in plant-based milks. This involved the use of Monte Carlo simulations for forward uncertainty propagation, providing the fellow with practical experience in stochastic simulations, which are crucial for risk assessment in the food industry. The fellowship also helped the fellow develop strong research skills, including the ability to design experiments, analyse results and draw meaningful conclusions. The fellow learned to critically evaluate scientific literature and integrate data from various sources to inform the development of risk assessment models. This ability to think critically and approach problems systematically is a key skill in scientific research and risk assessment.

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