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Training in quantitative microbial risk assessment of *Listeria monocytogenes* in processing chains: Quantification of biofilm-cells transfer integrating virulence and persistence factors

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Abstract

Food safety is a global challenge, with nearly 1 in 10 people worldwide falling ill each year from consuming contaminated food. The risk is particularly high in ready-to-eat (RTE) products, which are consumed without further cooking to eliminate harmful microorganisms. To address this, the University of Cordoba and the University of Bologna, in the framework of the EU-FORA programme, developed a training programme focused on quantitative microbial risk assessment (QMRA) for *Listeria monocytogenes* in RTE food processing chains, a significant public health concern due to its association with severe foodborne illnesses. The programme aimed to train the fellow in advanced food microbiology techniques, predictive modelling and comprehensive QMRA methodologies. The fellow gained hands-on experience with predictive microbiology models applied to real-world scenarios, particularly RTE meat and fish products. Activities included developing predictive models for microbial growth and conducting challenge tests to evaluate *Listeria* behaviour in various foods. Emphasising data collection and statistical analysis, the fellowship explores the dynamics of Listeria within the food supply chain. A case study on sliced cooked ham demonstrates QMRA's application, using Monte Carlo simulations to estimate Listeria concentrations at consumption, ultimately informing risk management strategies. This initiative aimed to increase the number of food safety risk assessment experts in Europe, thereby enhancing public health outcomes related to foodborne diseases.

KEYWORDS

Listeria monocytogenes, QMRA, ready-to-eat, risk assessment, RTE-fish, RTE-meat

CONTENTS

1 | INTRODUCTION

1.1 | Background

The European Food Risk Assessment Fellowship (EU-FORA) is a practical training program aimed at increasing the number of food safety risk assessment experts in Europe and promoting Member States' participation in risk assessment activities (Bronzwaer et al., 2016). The fellowship project, titled 'Training in Quantitative Microbial Risk Assessment of *Listeria monocytogenes* in processing chains. Quantification of biofilm-cells transfer integrating virulence and persistence factors,' was developed through a partnership between the University of Cordoba (Spain), specifically the Department of Food Science and Technology, UIC Zoonosis y Enfermedades Emergentes (ENZOEM), under the guidance of Prof. Antonio Valero Diaz (supervisor), and the University of Bologna (Italy) as the sending organisation of the fellow Dr. Federico Tomasello.

1.2 | Introduction

Listeria monocytogenes is a Gram-positive, non-spore-forming, facultatively anaerobic rod-shaped bacterium. It poses a health risk to both humans and animals. Although foodborne listeriosis is infrequent, it is a serious illness with high hospitalisation and mortality rates, making it a notable public health issue. In 2022, the European Union (EU) reported listeriosis as the fifth most common zoonotic disease, with 2738 confirmed invasive cases in humans, leading to 1330 hospitalizations and 286 deaths (EFSA and ECDC, 2023). The low detection rate of outbreaks is due to most invasive listeriosis cases being sporadic, which contributes to uncertainty about the true extent of listeriosis among different population groups. Underreporting is estimated to be at around a factor of two in the UK and North America (EFSA BIOHAZ Panel, 2018).

In the past decades, numerous quantitative risk assessment (QRA) models have been created worldwide to assist in food safety decision-making and risk management for *L. monocytogenes*. These models, varying in complexity, aim to systematically evaluate potential contamination routes of *L. monocytogenes* at different stages of the food production chain (Gonzales-Barron et al., 2023). While soil and water are primary sources of *L. monocytogenes* contamination for plants, feed, animals and the food chain, the bacterium can also withstand harsh environmental conditions and persist by forming biofilms (Gray et al., 2018) in processing environments.

QRA models, regardless of their focus on the entire farm-to-table process, processing stages, retail or consumption, aim to quantify the public health risks associated with consuming specific food products and to evaluate potential risk reduction strategies (Gonzales-Barron et al., 2024).

Ready-to-eat (RTE) foods that are not heat-treated or do not undergo listericidal treatments before consumption are crucial in the transmission of foodborne listeriosis (EFSA BIOHAZ Panel, 2018). Foods commonly linked to human listeriosis include meat and meat products, fish and fish products, milk and milk products, plant-based foods and frozen foods. According to the 2018 EFSA scientific opinion on listeriosis, meat and fish products were the most consumed RTE food subcategories per person per year in the EU/EEA. These subcategories were also the primary food sources implicated in listeriosis outbreaks in 2022 and previous years (EFSA and ECDC, 2023).

The primary aim of this programme was to train the fellow in the main practices of risk assessment through real-case scenarios. Specifically, the application of QMRA models to RTE meat and fish products to assess the risk of listeriosis in subgroups of the Italian population was the main scenario developed as a case study.

2 | DESCRIPTION OF WORK PROGRAMME

2.1 | Aims

The primary aim of the work programme was to provide the fellow with a comprehensive understanding of the complex dynamics involved in microbial risk assessment. To do so, the pathways associated with *L. monocytogenes* contamination in RTE foods was used as a case study. This goal encompassed a detailed focus on quantifying the transfer of planktonic and biofilm cells and integrating factors related to the pathogen's virulence and persistence.

The programme covered all critical stages of the risk analysis process, including hazard identification, exposure assessment, hazard characterisation and risk characterisation (FAO and WHO, 2006). A robust statistical approach was applied throughout, with careful consideration given to uncertainty and variability.

Additionally, the fellow was trained in three key areas to support these objectives; acquisition of advanced techniques in food microbiology; gaining expertise in the application of predictive models for microbial growth, inactivation, and cross-contamination; and in-depth training in advanced QMRA models, tailored to food safety contexts.

These aims were designed to build a comprehensive skill set for effective microbial risk assessment and management in food safety.

2.2 | Activities/methods

The main training activities during the fellowship were related to the development of predictive microbiology models and their incorporation into a risk assessment scheme for *L. monocytogenes* in RTE foods. Four main tasks were carried out: (1) Training of the fellow on repositories, software tools and methodologies related to predictive microbiology and microbial risk assessment; (2) Development of dedicated predictive models for their incorporation in the exposure assessment; (3) Fine-tuning of existing dose–response (DR) models for *L. monocytogenes*; (4) Building process risk models into a probabilistic QMRA framework.

2.2.1 | Laboratory experience

As part of the training during the fellowship, two main activities were performed in the wet lab. Firstly, in the premises of the sending institution, the fellow performed a set of challenge tests to evaluate the fate of *L. monocytogenes* in artisanal dry-cured fish products during refrigerated shelf-life. The results of this experimental design, under the guidance of the supervisor from the hosting site, where used to validate an existing predictive microbiology model from Mejlholm et al. (2015). The results of this activity are part of a scientific paper under preparation and were also presented to the latest 28th FoodMicro conference held in Burgos (Spain). The second wet-lab activity was performed during the secondment at the hosting site. The aim of this activity was to assess the anti-listerial activity of two plant extracts (i.e. date seed and carob extracts). An in vitro experimental design was conducted, evaluating the effect of the extracts in laboratory liquid media through the turbidimetric assay. The collected data were used to calculate the minimum inhibitory concentration (MIC) and the microbial kinetic parameters. Detailed results of this work will be included in the master thesis of an exchange student from Tunisia hosted at the University of Cordoba at the same time of the fellow.

2.2.2 | Training on repositories and software

During the first period of the fellowship, the fellow was introduced to and trained on a variety of software, repositories and tools highly valuable for predictive microbiology and QMRA. Regarding repositories, the fellow was trained on some of the most common ones, namely Combase Browser, to extract data on microbial behaviour in food and lab media, RAKIP-Web Model Repository, Open FSMR and EFSA Comprehensive European Food Consumption Database. The first is a web-based model repository developed to provide risk assessment data and models, the second is a search engine for predictive microbial models and the third a source of information on food consumption across the EU. Furthermore, the fellow gained firsthand experience with a variety of valuable tools for predictive microbiology and QMRA, including:

Combase DMFit: A web-based application for fitting primary models (https://browser.combase.cc/DMFit.aspx).

Biogrowth: A Shiny app for fitting primary and secondary models and making predictions (https://foodmicrowur.shinyapps.io/biogrowth/).

Biorisk: An R package for quantitative microbial risk assessment.

Growth Predictor: A Shiny app for fitting primary models, estimating cardinal values of microorganisms and developing modular process risk assessments (https://skandamis.shinyapps.io/Microbial-Growth-Predictor-Dashboard/).

MicroHibro: A web application that serves both as a repository for predictive microbiology models and as a tool for developing modular process risk assessments (https://microhibro.com).

BIKE: A Shiny app for exposure assessment of microbiological and chemical hazards that uses occurrence and consumption data to estimate both chronic and acute exposures for consumers through a Bayesian approach (https://avoinkoulutus.ruokavirasto.fi/).

Additionally, the fellowship provided an opportunity to enhance the fellow's programming skills using the R programming language. Alongside R, the fellow also utilised Excel with the @Risk Advanced Risk Analysis add-in, which employs Monte Carlo simulation for risk analysis.

2.2.3 | Data collection and development of a predictive model

The second task conducted during the training was the development of dedicated predictive models for their incorporation in the exposure assessment. In detail, a cardinal model for the growth of *L. monocytogenes* in RTE meat and fish was developed. To accomplish the task, an extensive literature search for growth data of *L. monocytogenes* in RTE products was conducted through ComBase browser and published papers retrieved from the main scientific bibliographical databases (Web of Science, PubMed, Scopus). RTE-fish products were categorised into smoked and salted products, and fish pâté, while RTE-meat products comprised cooked ham, cooked meat, non-fermented cooked sausages and meat pâté. The obtained records were examined, with maximum growth rates (μ_{max}) either extracted directly or calculated from microbial counts and standardised at 5°C. Type of product, products' formulation and characteristics (pH, a_w, organic acids, NaCl%, packaging conditions, other antimicrobials) and strain details were recorded. Results were incorporated into growth database previously generated in a funded EFSA Tender of QMRA of *L. monocytogenes* in RTE foods to the hosting

institution. Finally, a descriptive analysis was carried out to summarise the collected data. Subsequently, the data were analysed to identify factors affecting the growth behaviour of the pathogen in these products, and, after the statistical analysis, probability distributions were fitted to the collected μ_{max} standardised at 5°C to describe the variability in the ability of *L. monocytogenes* to grow in different products of the same category (e.g. different formulations of cooked ham). The goodness of fit of the probability distributions for the collected data was statistically tested using various methods, chi-square test, Kolmogorov–Smirnov test, Cramer–Von Mises test, Anderson–Darling test, Akaike Information Criterion and Bayesian Information Criterion. As an output from this task, a database of *L. monocytogenes* growth rates and food characteristics was produced as well as probability distributions summarising and describing the variability of the μ_{max} values at 5°C.

These probability distributions were then applied to estimate the μ_{max} at a specific temperature (T) using this simplified secondary model (Equation 1), with $T_{min} = -2.83$°C as derived from Mejlholm et al. (2015).

$$\mu_{max}(T) = \mu_{max}(5^{\circ}C) \times \left(\frac{T - T_{min}}{5 - T_{min}}\right)^2 \text{ if } T < T_{min} \rightarrow \mu_{max}(T) = 0. \quad (1)$$

Using this approach allowed to estimate the μ_{max} at specific temperatures using as only parameter of the model temperature whilst considering the variability due to a variety of product characteristics (e.g. pH, a_w).

2.2.4 | Training in risk assessment

The working programme aimed to familiarise the fellow with all aspects of QMRA by providing hands-on experience in conducting a risk assessment of RTE meat and fish products. The fellow engaged in activities ranging from relevant data collection to mathematical modelling using both R and Excel with the @Risk add-in. Emphasis was placed on describing the variability and uncertainty of the model inputs by applying a stochastic approach.

The first activities conducted were the selection of a RTE meat product to use as case study, the definition of the conceptual model (Figure 1) and the collection of relevant data to build the QMRA model.

A case study was chosen to evaluate the risk of listeriosis connected with consumption of sliced cooked ham in the Italian population. It was decided to model from the end of the production process, through post-processing operations (i.e. slicing), distribution (including the storage a various points of the distribution chain), up to domestic storage and consumption. This approach assumed that the production process of this product effectively inactivates *L. monocytogenes* and that the risk

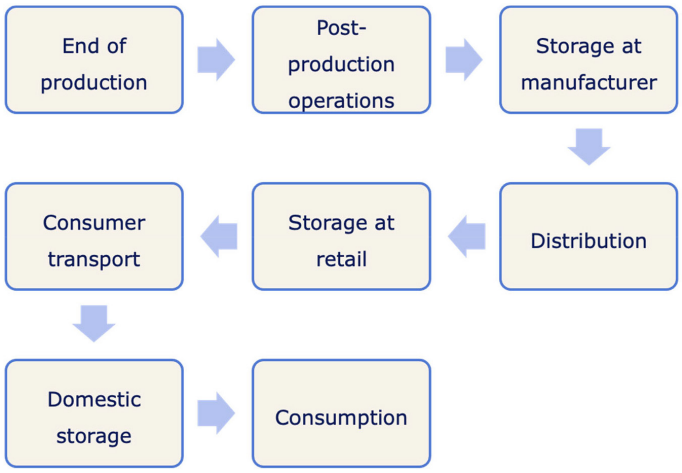

FIGURE 1 Conceptual model for the QMRA case study on sliced cooked ham.

for consumer is posed mainly by recontamination during post-processing operations followed by storage.

Once defined the pathway to model, the necessary data were identified and collected. Appropriate distributions were chosen to describe the model inputs. For the prevalence of *L. monocytogenes* in the processing environment (i.e. slicing operations) a beta distribution was chosen to incorporate the uncertainty resulting from the limited data, similarly for initial concentration (N_0) was assumed a beta-PERT distribution. The next step was to identify the data needed to model the post-production operations module, for that a transfer model describing the transfer ratio of *L. monocytogenes* from slicer to cooked ham and vice versa was selected from literature (Chaitiemwong et al., 2014) and used to model the cross-contamination due to the slicing operation. Concerning the storage and distribution modules data were retrieved from scientific literature when possible or probability distributions were used to model experts' opinion in case where no published data were available. The data collection focused mostly on time and temperatures profiles of the different modules.

Furthermore, the simplified cardinal model described above (Equation 1) was coupled with an exponential primary model (Equation 2) to estimate the *L. monocytogenes* concentration at the end of each step (N_t).

$$\mathrm{Log}(N_t) = \mathrm{Log}\left[N_0 \times \exp(\mu_{\max} \times \mathrm{time})\right]. \quad (2)$$

This primary growth model excludes lag time, based on the general assumption that the lag phase ends before RTE foods are purchased (EFSA BIOHAZ Panel, 2018). This conservative assumption was based on previous studies showing that *L. monocytogenes* could grow in different RTE food commodities at refrigeration temperatures without lag phase (Hussein et al., 2023).

The output of this exposure assessment module was an estimate of the concentration of *L. monocytogenes* at the moment of consumption. This was achieved using a stochastic approach. A total of 100,000 simulations were run for the complete model, sampling from the input distributions using Monte Carlo simulation. This produced a probability distribution for the final concentration of *L. monocytogenes* at consumption. These results were then combined, using a Poisson distribution (Condoleo et al., 2017), with a probability distribution that modelled the uncertainty around the single serving size (SS), as obtained from the EFSA Comprehensive European Food Consumption Database, to estimate the dose (λ) ingested by consumers (Equation 3).

$$\lambda = N_t \times \mathrm{SS}. \quad (3)$$

The ingested dose was estimated for three subpopulation groups: those older than 65, those between 18 and 65, and those younger than 18 years. The parameter lambda (λ) was then used to calculate the probability of illness using the exponential dose–response (DR) model (Equation 4) by FAO/WHO. This DR model was further enhanced by integrating the work of Pouillot et al. (2024), who developed a new set of *r* parameters. *r* is the parameter that expresses the probability of illness after the consumption of a single cell for the population group being considered. They incorporated the EFSA DR model (EFSA BIOHAZ Panel, 2018) for 14 different age-sex subgroups and categorised strain virulence into three classes ('less virulent', 'virulent', and 'more virulent') using epidemiological data.

$$P_{\mathrm{ill}} = 1 - \mathrm{e}^{(-r \times \lambda)}. \quad (4)$$

As part of the risk characterisation step, the probability of illness (P_{ill}) was utilised to calculate the number of listeriosis per 100,000 servings in the Italian population. Additionally, a sensitivity analysis was performed to assess which of the model parameters had the greatest impact on the final risk for the consumers. The findings from the analysis subsequently informed the selection of mitigation strategies, which were evaluated through scenario analysis. This process involved re-running the QMRA model under an alternative set of input conditions. Detailed results of this work will be included in a scientific publication that is currently in progress.

2.2.5 | Secondary scientific activities during fellowship

Conferences:

- IAFP annual meeting. 14–17 July, 2024 Long Beach, California – Long Beach Convention Center. Oral presentation: 'Comprehensive Analysis Of *Listeria Monocytogenes* Growth In Ready-To-Eat Fish And Meat Products: Understanding Variability To Assess Public Health Risks'.
- 28th International ICFMH Conference FOOD MICRO 2024. Burgos, 8–11 July, 2024. Poster: 'Ensuring Safety in Dry-Cured Fish Products: describing the behaviour of Listeria monocytogenes in Artisanal RTE Salmon, Swordfish and Tuna'.
- Jornada sobre *Listeria monocytogenes*: un reto para la seguridad alimentaria. Ministerio de Derechos Sociales, Consumo y Agenda 2030. Madrid, 4-6-2024.

Training course:

- Modelling Food Safety and Animal Health Risks Using R (postponed to 4/2025).

Lectures:

- Lectured for 16 h in the workshop 'Data Extraction and Analysis to Assess Biological Risks within Circular Agrofood Systems' held at the University of Bologna as part of the 'Workshop in Animal Metabolism and Management'. of the Master course in 'Food animal metabolism and management in the circular economy'. The lectures focused on the application of data analysis in the field of risk assessment. (16 students)
- Lectured for 2 h in the course of 'Food Hygiene' of the degree of 'Food Science and Technology' held at the University of Cordoba. The lecture focused on the main bacterial foodborne pathogens. (three students)

3 | CONCLUSIONS

The primary goal of the work programme was to equip the fellow with a thorough understanding of microbial risk assessment by developing and applying a stochastic QMRA model to estimate the health risk of listeriosis from consuming RTE meat products. Through this programme, the fellow gained in-depth expertise in the various components of QMRA by focusing on the specific food-hazard combination of RTE meat and fish products and *L. monocytogenes*.

The training programme successfully integrated laboratory experience, selecting appropriate data from scientific literature, identifying data gaps, designing risk assessment pathways, mathematically modelling bacterial growth and developing a comprehensive QMRA model that accounted for variability and uncertainty in inputs and outputs. A particularly valuable aspect of this process was the enhancement of the fellow's skills in R programming and Excel with the @Risk add-in, enabling the probabilistic design and conception of the model using Monte Carlo simulations, along with training on several other relevant tools.

The fellowship programme provided an invaluable opportunity for the fellow to acquire new scientific knowledge and skills and to join the vibrant and expanding network of EU-FORA fellows and alumni, thereby promoting the dissemination of risk assessment expertise across Europe.

ABBREVIATIONS

DR	dose–response
ECDC	European Centre for Disease Prevention and Control
EU-FORA	European Food Risk Assessment Fellowship
FAO	Food and Agricultiure Organization
IAFP	International Association for Food Protection
ICFMH	International Committee
MIC	minimum inhibitory concentration
QMRA	quantitative microbial risk assessment
QRA	quantitative risk assessment
RTE	ready-to-eat
SS	serving size
WHO	World Health Organization

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