EU-FORA SERIES 6

University of Veterinary Medicine Budapest

efsa JOURNAL

APPROVED: 15 September 2023

doi: 10.2903/j.efsa.2023.e211014

Training in modern statistical methodologies and software tools for the definition and analysis of (stochastic) quantitative microbial risk assessment models with a comparison between the Hungarian and Spanish food supply chains

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Abstract

Human pathogenic *Salmonella enterica* strains have been infecting people since historical times. The original human pathogens, typhoid *Salmonella* strains (e.g. *S.* Typhi) played a huge role in the previous centuries but nowadays in the developed world the number of cases or outbreaks caused by these serotypes deceased due to the development of personal and public hygiene. Nowadays in these regions the animal-borne zoonotic serotypes (e.g. *S*. Enteritidis) became more important because of their high prevalence in intensive animal husbandry. But these bacteria can also appear in fruits and vegetables. The fellow joined the scientific work of the Polytechnic University of Cartagena, Spain about the safety of plant-based products, where he could gain experience in microbiological laboratory exercises and theoretical calculations of statistics and modelling. The activities in the laboratory were part of the research lines already established at the host institution, being based on the protocols they have already implemented. Nonetheless, the fellow had the opportunity to design his own experiment, do the experimental work required and analysed the data within the context of a qualitative microbiological risk assessment. The main focus was on the heat resistance of two strains of zoonotic *Salmonella* spp. at different temperatures. Experiments were done using a reference strain and an extremely resistant variant to evaluate this rare phenotype. The experiments were executed using a Mastia thermoresistometer, a device patented by the host institution that provides more control when studying thermal treatments than traditional methods. The data was analysed using the principles of predictive microbiology, using the D-value as an estimate of heat resistance that provides insight into the bacterial behaviour. For this, the fellow used the *bioinactivation* software, developed within the host group. Through the work and results the fellow learned the principles of quantitative microbiological risk assessment (QMRA) and predictive microbiology, which was the aim for the EU-FORA programme.

Keywords: QMRA, R programme, Salmonella, heat elimination

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Acknowledgements: This report and the whole programme could not have come into being without the two fantastic supervisors from the hosting site (UPCT), Pablo Fernández and Alberto Garre. Beside them there were several researchers who helped my work and education at UPCT. I also would like to thank my colleagues from my university, as well, to let me accomplish this programme. I also would like to thank Cristina Alonso Andicoberry and the whole EFSA for helping us during this year immediately, when we had a problem.

Declaration 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.

Suggested citation: EFSA (European Food Safety Authority), Pleva, D., Garre, A., & Fernández Escámez, P. S., 2023. Training in modern statistical methodologies and software tools for the definition and analysis of (stochastic) quantitative microbial risk assessment models with a comparison between the Hungarian and Spanish food supply chains. *EFSA Journal*, *21*(S1), 1–11. https://doi.org/10.2903/j.efsa.2023.e211014

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

Summary

The European Food Risk Assessment Fellowship Programme is an innovative project of the European Food Safety Authority to educate the food risk assessors of the future in Europe. In the cohort of 2022/2023 the fellow had the opportunity to join the programme and besides the lectures organised by the European Food Safety Authority, the fellow could visit the Polytechnic University of Cartagena, Spain, as a hosting site due to the consortium. There were already several ongoing research projects in several topics at the hosting site, and the fellow also could do his own research in the laboratory and at the computer, as well. He joined an experiment about the heat resistance of *Bacillus subtilis*, and an experiment about the microbiology of kale products. The fellow's own topic was the investigation of heat resistance of two zoonotic *Salmonella enterica* serotypes, *S*. Enteritidis and *S*. Senftenberg. For the treatment a Mastia thermoresistometer was used that was able to provide samples at different times. These samples were poured into agar plates and after the proper method the colonies were counted. These data let the fellow and the supervisors calculate the D-value of the strains to execute further calculations for qualitative microbiological risk assessment of *Salmonella* in various foodstuffs, especially fruits. In addition to the laboratory work the fellow got introduced to the background of statistics and modelling by the application of several packages of R programme. He also took part at a workshop about quantitative microbial risk assessment tools and a 2-day-long visit to the Spanish Agency for Food Safety and Nutrition to participate at theoretical and practical lectures about the operation of the food safety authorities and risk assessment at a state level.

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1. Introduction

1.1. Fellow's personal background

During his scientific studies and work, food toxicology and microbiology took a huge part of the fellow's interest. The topic of his PhD dissertation, 'Impact of home frying and cooking methods on chemical and microbiological safety and quality characteristics of poultry meat' included both food toxicological and microbiological aspects and during this work, he realised that modern food safety cannot exist without risk assessment. That is why he also assessed the risk of potentially carcinogenic heterocyclic amines (IARC, 2020) in heat treated chicken meat and tried to find the balance between the controversial demands of temperature–time parameters of toxicological and microbiological safety during cooking (Pleva et al., 2020).

The microbiological leg of his experiments was based on Salmonella Enteritidis elimination, so these bacteria were in the focus. At the same time the biggest Hungary-based food scandal in the EU of the past years was connected to smoothies: in Western Europe nine deaths were caused by an infectious *Listeria monocytogenes* strain from a Hungarian frozen vegetable plant because the consumers used the vegetables without heat treatment (EFSA and ECDC, 2018). These were connection points with the research of the hosting site: examination of *Salmonella* strains is smoothies.

1.2. Heat elimination of different *Salmonella* strains

Salmonella is one of the most frequently detected foodborne pathogens. Although the prevalence of some human pathogenic serotypes (*S*. Typhi, *S*. Paratyphi) has declined in developed countries, some serovars still circulate among farm animals and can cause diseases in humans (Locht et al., 2002; EFSA, 2019). Therefore, the European Union launched a programme to reduce the occurrence of the most important *Salmonella* serotypes from a public health point of view: *S*. Typhimurium and *S*. Enteritidis (EC, 2006). According to EU law, poultry meat must be free from these strains (EC, 2005). But not only these serotypes can cause human infections, e.g. *S*. Dublin, *S*. Infantis or *S*. Senftenberg can also be a source of human contagion (Aparecida Fernandes et al., 2022; ECDC, 2023).

1.3. Food safety of 'smoothies'

When it comes to the safety of vegetables, the focus is often put on *L. monocytogenes* contamination. However, it is not the only microbe that can be dangerous to the consumers of products of plant origin. Due to imperfect agricultural or processing technologies, they can be contaminated with human pathogenic and zoonotic *Salmonella* spp., as well as other pathogenic species (Laczay, 2008). Root vegetables and ground fruits can be contaminated from the soil, being particularly relevant if the irrigation takes place with contaminated water (Truchado et al., 2021; Garre et al., 2022). Poor hygienic conditions at the level of the processing plant can also cause contamination, either from the surfaces or the personnel. The risk of Salmonella in smoothies is worsened by the fact that these products cannot be treated at high temperatures, due to the impact of these treatments in the product quality (Viswanathan and Kaur, 2001; Quiroz-Santiago et al., 2009; González-Tejedor et al., 2017).

Some smoothies may contain milk because of its protein and calcium content that makes it a fine ingredient for a healthy diet. However, milk, as an animal-borne product may have an even higher hazard level than vegetables, as it can be contaminated by zoonotic pathogens including *Salmonella* (Van Kessel et al., 2011; Singh et al., 2018). Milk-based products are mostly pasteurised due to these hazards but there are many parameters that can affect the efficacy of the pasteurisation of such complex products, e.g. milk containing smoothies. That is why this field needs more multiparametric risk analysis and quantitative microbiological risk assessment (QMRA) is a perfect way to do so.

1.4. Quantitative microbiological risk assessment

QMRA is nowadays a keystone of food safety control systems. It is a part of the modern Risk Analysis concept besides Risk Management and Risk Communication (WHO, 2021). It contains four major elements that constitute a logical and sequential pathway: hazard identification, hazard characterisation, exposure assessment and hazard characterisation (Giaccone and Ferri, 2005). The European Union and EFSA developed methods for several product (Snary et al., 2016) and there are also investigations for fruits as well, that mentions *Salmonella* spp. in the top 14 microbiological hazards of fruit products due to their pH resistance (Bassett & McClure, 2006).

A QMRA needs mathematical models that provide quantitative estimates of the microbial response within the food chain (Allende et al., 2022). In this sense, the field of predictive microbiology is a well-established methodology that defines the experimental and numerical protocols to define such models (Perez-Rodriguez & Valero, 2013), that has served to define growth and inactivation models for most food pathogens (Guillén et al., 2021a,b; Alvarenga et al., 2022; Georgalis et al., 2022). One of the main limitations of this approach is the complexity in the implementation of these mathematical models. However, this hurdle is currently being circumvented through the implementation of user-friendly software applications (Possas et al., 2022).

2. Description of work programme

2.1. Practical work in the laboratory

The Polytechnic University of Cartagena (UPCT) is recognised for their work in thermal resistance of microorganisms, especially under dynamic conditions thanks to the availability of the Mastia thermoresistometer (Conesa et al., 2009).

The main advantage of this device is that it is very easy to model heat treatment methods of liquids: the temperature and the duration can be set, even changes in the temperature level during the process; and it is easy to take samples with a resolution of up to 3 s to measure the bacterial concentration during the experiment. Because of its flexibility in temperature settings, it is very useful in the study of bacterial inactivation under dynamic conditions, allowing the analysis of how faster or slower heating rates will affect the microbial response (Garre et al., 2018a; Clemente-Carazo et al., 2020).

2.1.1. Participation in ongoing projects at the host institution

During the autumn of 2022 there were two empirical works the fellow could join as an assistant before the onset of his own tasks for the EU-FORA programme. These activities served as a training-by-doing of the laboratory skills required to carry out the research project.

One of these experiments was an investigation of the heat resistance of *Bacillus subtilis*, a spore-former, spoilage bacteria, which is a common model microorganism for heat resistance (Berendsen et al., 2015). This work provided the fellow with the know-how needed to work with spoilage microorganisms, especially when it comes to sample preparation, highlighting the importance of separating vegetative cells from spores.

Another topic where he could be involved was an experiment of the examination of microbiological safety of a kale-based semi-liquid product. In this situation the main challenge of the research was the high viscosity of the product, making it hard to analyse with the thermoresistometer. Hence, the fellow participated in the testing and application of some modifications of the device (e.g. changes in the stirrer configuration) to be able to perform thermal treatments in this product without clogging the system.

2.1.2. Own research

My main focus during the stay in Cartagena was on the thermal resistance of two human pathogenic *Salmonella* serotypes: *S*. Enteritidis (higher virulence) and *S*. Senftenberg (higher heat resistance). *Salmonella enterica* serovar Enteritidis CECT 4300 and *Salmonella enterica* serovar Senftenberg CECT 4565 strains were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). The comparison of the heat resistance of the two strains was performed in the Mastia thermoresistometer, during the several pre- and main experiment isothermic and gradient temperature combinations were set with the duration of the heat treatment. The results were detected by pouring agar plates and plate counting.

The final results were obtained on 12–15 December, 2022, the results are displayed on Figures 1–4. The treatment conditions were 55 and 60°C for the less heat resistant *S*. Enteritidis, 60 and 65°C for *S*. Senftenberg. The samples were taken at pre-defined time points and 10-fold diluted (the desired dilution level was also specified by the pre-experiments) in peptone water. The diluted samples were then transposed by pouring-plate method into Petri dishes with trypticase soy agar and after 24 h of incubation at 37°C the Salmonella-like colonies were counted manually. Two independent replicates were taken from each combination.

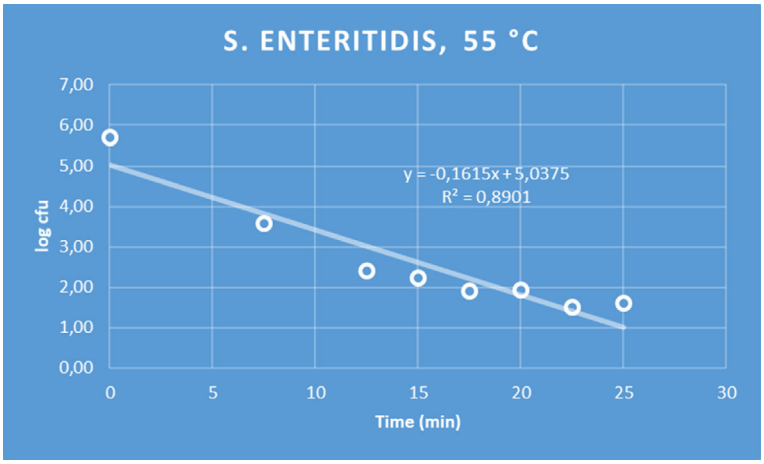

Figure 1: Heat inactivation of *S*. Enteritidis at 55°C

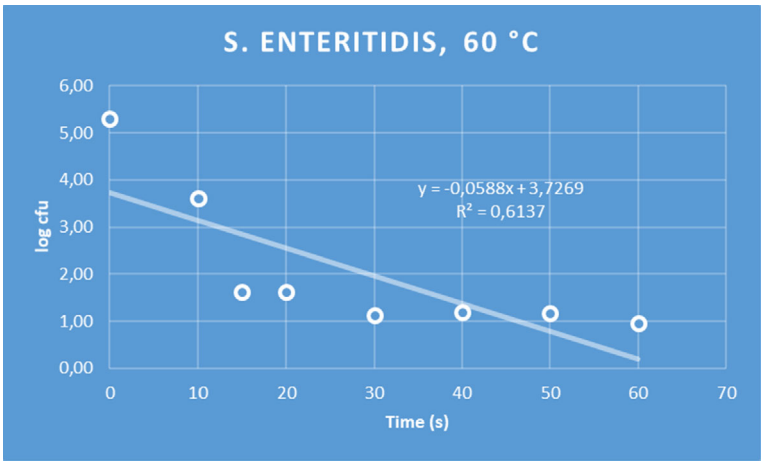

Figure 2: Heat inactivation of *S*. Enteritidis at 60°C

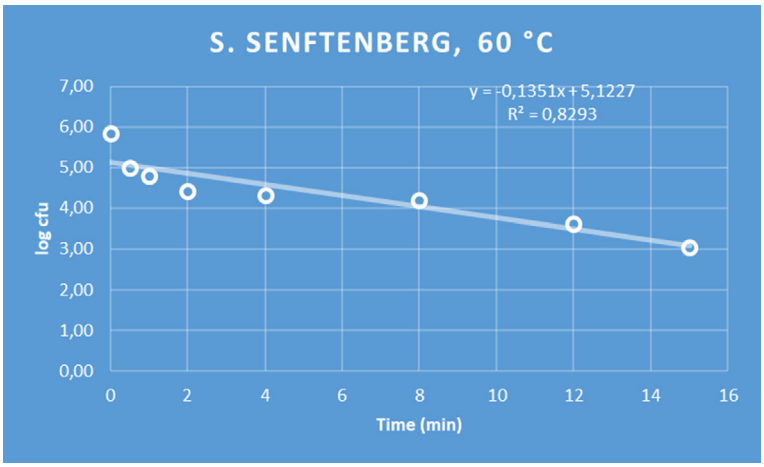

Figure 3: Heat inactivation of *S*. Senftenberg at 60°C

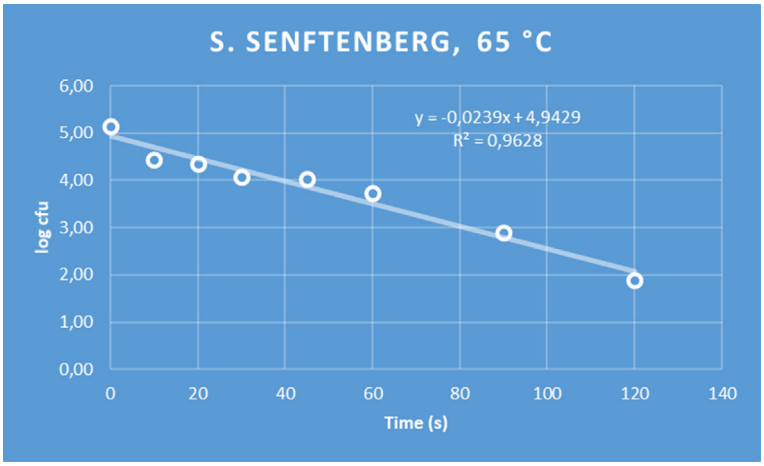

Figure 4: Heat inactivation of *S*. Senftenberg at 65°C

2.2. Theoretical work

2.2.1. Analysis of the inactivation data obtained

As expected, the research group's results showed that our *S*. Senftenberg strain had a higher heat resistance than the reference one. The heat resistance was quantified using the Bigelow model, commonly used within the field of predictive microbiology to analyse microbial inactivation. As shown in Equation (1), this model assumes a log-linear relationship between the microbial concentration (N) and the treatment time (t) with respect to the initial concentration (N_0).

$$\log N = \log N_0 - t/D. \quad (1)$$

The thermal resistance of the microorganisms was quantified using the D-value (D), which represents the opposite of the reciprocal of the slope of the survivor curve, and describes the time it takes to reduce a microbial population by 1 logarithm. The models were fitted using the software *bioinactivation*, developed within the host group (Garre et al., 2017; Garre et al., 2018b), using its web application currently available at: https://foodlab-upct.shinyapps.io/bioinactivation4/. Table 1 shows the D-values obtained for each condition tested.

Table 1: D-values (estimate ± standard error) of *S*. Enteritidis and *S*. Senftenberg estimated at 55, 60 and 65°C

Temperature	*S*. Enteritidis	*S*. Senftenberg
55°C	6.19 ± 0.89 min	–
60°C	0.28 ± 0.09 min	7.40 ± 1.37 min
65°C	–	0.70 ± 0.06 min

2.3. Extra activities

- Participating at the 10-h hybrid Workshop held at the University of Cordoba (Spain) on 27–28 October 2022: 'Use of Quantitative Microbial Risk Assessment Tools. Case studies on foodborne pathogens in ready-to-eat foods'.
- Participation in a 5-day workshop on R programming provided by Alberto Garre within the Polytechnical University of Cartagena (Introduction, how to get started, overview of features and possibilities; Visualisation, gg plot; Importing data; Manipulating files, sorting, basic descriptive statistics; Statistical operations, transformation, optional bring your own files).
- Visiting AESAN (Spanish Agency for Food Safety and Nutrition) on 16–18 January 2023. The host, Vicente Calderon, head of the Department of Risk Evaluation of AESAN, organised an introduction of AESAN and EU-FORA fellows and several interesting theoretical and practical workshops with researchers of many fields of food science and risk assessment (AESAN, 2023). The programme is below:

17 January

- 8:30–9:00 Welcome & Presentation of AESAN
- 9:00–9:30 Presentation of EU-FORA fellows
- 9:30–10:30 Risk assessment/ Scientific Committee
- 10:30–10:45 EFSA Focal Point in Spain
- 10:45–11:15 Coffee break
- 11:15–11:45 Risk Management – Biological risks
- 11:45–12:15 Risk Management – Chemical risks
- 12:15–12:45 Risk Management – Nutritional safety
- 12:45–13:15 Food official control and alerts
- 13:15–14:45 Lunch
- 14:45–15:15 Spanish strategy for nutrition, physical activity and the prevention of obesity (NAOS)
- 15:15–15:45 Communication

18 January

- 8:30–9:30 Welcome & Presentation of CNA – National Centre for Food
- 9:30–10:15 Food contact materials
- 10:15–10:45 Toxins and food processing contaminants
- 10:45–11:15 Coffee break
- 11:15–11:45 Residues of veterinary drugs
- 11:45–12:15 Biotechnology
- 12:15–13:15 Microbiology and antimicrobial resistance

- Participated in the 30-h A1.2 Spanish language course led by Juana Mari Belchí Martínez at the Language Centre of UPCT, passing with excellent results (95%).

3. Conclusion

QMRA is an important part in modern food safety. It combines several sources of knowledge and data, and promotes trust in food processors and the controls of health authorities in the society. It enables us to use historical data to create models about the operation of microbiology and thanks to the development of computer science we can use these models for calculations in the future, which protects us from repeating the same mistakes and may prevent new ones.

Projecting this approach to the work plan of the programme, the integration of population, consumption and prevalence data, laboratory results and the skills of modelling softwares new opportunities became available. *Salmonella* strains were selected as representatives that are important due to their virulence or heat resistance and we performed a series of experiments to examine their heat resistance. These data can be used for further calculations, as it is expected in the future in smoothies and fruit products.

The whole EU-FORA programme helps to understand new points of view in food safety. In the cohort there are researchers with diverse backgrounds, different levels of knowledge in different topics and at the end all these people get to the same level by patching the missing information from the lectures and the practical work. In the fellow's case, by having a PhD in food hygiene from a university of veterinary medicine, he had a lack of mathematics, statistics and computer science background but during the programme he could acquire new knowledge in these fields, as well, and the application of it to the scientific work or to just simple everyday thinking about food safety opens up new horizons. It was tough to start this journey, it was a lot to learn, but according to the fellow thanks to the programme he started to move on the ascendant side of the Dunning–Kruger effect curve (Kruger & Dunning, 1999).

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Abbreviations

AESAN	Spanish Agency for Food Safety and Nutrition
CECT	Spanish Type Culture Collection
CNA	National Centre for Food at the Spanish Agency for Food Safety and Nutrition
D-value	decimal reduction time
EU-FORA	European Food Risk Assessment
IARC	International Agency for Research on Cancer
N	microbial concentration
N0	initial concentration
NAOS	Spanish strategy for nutrition, physical activity and the prevention of obesity
S.	*Salmonella*
t	treatment time
UPCT	Polytechnical University of Cartagena, Spain
WHO	World Health Organization