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Training in tools to develop quantitative microbial risk assessment of ready-to-eat food with a comparison between the Romanian and Spanish food supply chains

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Abstract

The prevention and control of bacterial contamination on ready-to-eat (RTE) fresh produce is an essential task to ensure food safety. Therefore, the development of novel and effective decontamination technologies to ensure microbiological safety of fruits and vegetables has gained considerable attention and new sanitisation methods are needed. The antimicrobial activity of essential oils (EOs) is well documented, but their application in fresh produce remains a challenge due to their hydrophobic nature. Thus, nanoemulsions efficiently contribute to support the use of EOs in foods by enhancing their dispersibility, their contact area and facilitating the introduction into bacterial cells. The combination of these factors ultimately increases their antimicrobial activity. Quantitative microbial risk assessment (QMRA) is gaining more attention as an effective tool to assess and prevent potential risks associated with food-borne pathogens. In this context, the current project aims to study the effectiveness of different washing methods based on nanoemulsified EOs, comparing them against traditional methods, using a QMRA model for *Escherichia coli* O157:H7 on cherry tomatoes. Different simulations within a stochastic risk assessment model were implemented using the biorisk package for R, aiming to describe microbial behaviour and biological risk along the Romanian and Spanish food supply chains of RTE fresh produce. Nanoemulsions were prepared using oregano and rosemary EOs, each from Romania and Spain. The four nanoemulsions were evaluated as decontamination treatments to control the growth of *E. coli* O157:H7 on artificially contaminated cherry tomatoes. The decontamination treatments showed encouraging results, comparable to commonly used chlorine solutions. Therefore, oregano and rosemary nanoemulsions are promising and could be a feasible alternative for chlorine solutions in the reduction of microbiological contaminants.

Keywords: food safety, produce safety, risk assessment, *Escherichia coli*, oregano, rosemary, nanoemulsions

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Table of contents

1. Introduction

The European Food Risk Assessment Fellowship (EU-FORA), is a practical 'training by doing' programme that aims to improve the pool of food safety risk assessment experts available in Europe and to stimulate the involvement of Member States in risk assessment work (Bronzwaer et al., 2016). The fellowship programme '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 Romanian and Spanish food supply chains' was developed and implemented by the Universidad Politécnica de Cartagena (Polytechnic University of Cartagena [UPCT], Spain), Food Safety and Preservation in the Agronomic Engineering Department (ETSIA), as hosting site, under the supervision of Drs. Alberto Garre (supervisor) and Pablo S. Fernández (co-supervisor), together with the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (USAMV CN), Romania, as sending organisation, under the coordination of dr. Giorgiana Cătunescu.

The research group of Food Safety and Preservation at UPCT has extensive experience in the development of tools and mathematical models for biological risk assessment, thereby they were able to provide the EU-FORA fellow the opportunity to acquire knowledge in methodologies, terminologies and practical skills commonly used in quantitative microbial risk assessment (QMRA). Even though the fellow had a different background – veterinarian – due to the experience and involvement of the UPCT supervisors, the fellow had the opportunity to gain first-hand experience in modern methodologies and software tools for building QMRA models.

There is a global increase in consumers' demand for fresh produce because of the health benefits provided by its high content of vitamins, minerals and fibres (Dávila-Rodríguez et al., 2019). This increase in demand has also brought additional challenges for food safety. The main food-borne pathogens associated with fresh produce include *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Shigella* spp., *Yersinia* spp., *Clostridium* spp. and *Campylobacter* spp. (CDC, 2017; WHO, 2023). Recent studies have revealed an increase in the number of outbreaks linked to fresh produce (CDC, 2019). Food-borne pathogens are responsible for several diseases, many seriously affecting human health and causing an impact to the economy (Pizzo et al., 2023). Of the 9.4 million food-borne illnesses reported each year in the US, 60% are associated with fresh vegetable consumption (CDC, 2017).

Pathogenic *E. coli* O157:H7 is linked to multiple reported outbreaks, which account for ~ 20% of the total cases associated to fresh produce-related issues (CDC, 2019). Multiple cases associated with Shiga toxin-producing *E. coli* (STEC) outbreaks have been reported throughout Europe, including Austria, the Czech Republic, Denmark, France, the Netherlands, Norway, Poland, Spain and Sweden, as well as the UK (EFSA, 2011). In Germany, on 22 May 2011, a significant increase number of patients with severe diarrhoea and uremic syndrome (HUS) caused by STEC were reported. Over 1,000 STEC cases and over 400 HUS cases were announced (EFSA, 2011).

Therefore, novel and effective decontamination technologies should be developed and implemented to mitigate the risk of microbial contamination and ensure the safety of fresh produce. Preventing outbreaks is challenging because during the production chain, the bacteria can be transferred to the produce from various sources: during production, harvest, postharvest, storage or transportation (Pizzo et al., 2023). This is specially concerning because it is not possible to apply a harsh decontamination method (e.g. thermal pasteurisation) to fresh produce without compromising the quality of the product (Buchanan et al., 2018). Instead, mild treatments are applied, such as washing the produce with water often containing chemical sanitisers (Pizzo et al., 2023). The most widely used synthetic sanitisers during the washing of fresh vegetables are sodium hypochlorite, hydrogen peroxide and peroxyacetic acid (PPA). They have been widely used on fresh vegetables to reduce the load of pathogens (Dávila-Rodríguez et al., 2019). However, these sanitisers can be corrosive to surfaces, are potentially harmful to consumers because of chlorine by-products and pose a health risk to workers and consumers (Pizzo et al., 2023). Thus, there is a crucial need to find alternative antimicrobials, particularly of natural origin, for the fresh produce industry (Bhargava et al., 2015).

Plant-based antimicrobial agents, especially EOs and their bioactive compounds, have become a promising alternative to chemical sanitisers as antimicrobials in postharvest washing systems. EOs provide a novel, eco-friendly method to wash fruits and vegetables with minimal environmental impact (Pizzo et al., 2023). Several studies have reported that different EOs showed an antimicrobial effect that inhibited or inactivated bacteria (Bhargava et al., 2015; Dávila-Rodríguez et al., 2019; He et al., 2021). In addition, the US Food and Drug Administration (FDA), states in its Code of Federal Regulation (CFR) that EOs are categorised as 'generally recognized as safe' (GRAS) (FDA, 2016;

de Souza et al., 2021). However, the direct applications of EOs are limited because: (1) they contain lipophilic compounds with low solubility in water (Dávila-Rodríguez et al., 2019); (2) they possess strong flavour and odour, which can exceed the sensory rejection threshold (de Souza et al., 2021); (3) large amounts are needed to induce their antibacterial effects (Dávila-Rodríguez et al., 2019); (4) the antimicrobial efficiency against Gram-negative bacteria is often reduced due to the impermeable outer membrane of these bacteria (He et al., 2021). To resolve these limitations, EOs can be encapsulated in nanoemulsions to exhibit characteristics such as: superior physical stability of active compounds; enhanced antimicrobial activity over equivalent Eos; limiting their oxidation and degradation; and mask the undesirable flavouring properties (Ros-Chumillas et al., 2017; Garre et al., 2020; He et al., 2021; Huertas et al., 2021).

2. Description of work programme

2.1. Aims

The aim of the EU-FORA working programme was to provide the fellow with basic knowledge on QMRA through a training-by-doing approach that covered experimental methods, statistical analysis, mathematical modelling and stochastic simulations in RTE fresh food produce, with a comparison between the Romanian and Spanish food supply chains. It covered every step of the risk assessment: hazard identification, exposure assessment, hazard characterisation and risk characterisation. To achieve this purpose, the following training objectives were formulated: (1) to set up a solid knowledge on specific methodologies and software related to Microbiological Risk Assessment (MRA); (2) to provide the fellow hands-on experience in obtaining experimental data needed for kinetic models for QMRA; (3) to develop and validate predictive models based on experimental data; (4) to implement a QMRA model with a comparison between the Romanian and Spanish food supply chains in RTE, highlighting not only similarities and differences, but also their relevance for consumer health.

2.2. Activities/methods

Throughout the 1-year fellowship programme, the fellow obtained general information on quantitative risk assessment activities, both remotely and on site. Initially, the fellow was trained online. During this period, the priority of the hosting site was to provide the fellow fundamental understanding and hands-on experience on all the steps and tools required to perform a QMRA. Thus, at the beginning of the programme, the fellow had weekly online meetings with the supervisors, which provided guided learning on specific topics, such as: (1) handling of available databases (EFSA, FAO, ComBase) for hazard identification; (2) defining models based on published data using online databases; (3) using statistical concepts on experimental design to study microbial growth and inactivation kinetics as a first step to build QMRA models; (4) learning R programming language using the *biogrowth, bioinactivation* and *biorisk* packages (developed by the UPCT group); (5) interpreting the results of QMRA simulations. This provided the fellow a strong theoretical background required to perform a QMRA, complementing her previous education and training, as well as the one provided by EFSA during the 3-week induction training in microbiological and chemical risk assessment.

2.2.1. Laboratory experience

Afterwards, the fellow joined the working team at UPCT, which has proven expertise in risk assessment. She gained hands-on experience in obtaining experimental data, which were further used in the QMRA development. During this period, the fellow gained in-depth experience and know-how on laboratory techniques used for the characterisation of the microbial response within the scope of a microbial risk assessment of foods.

The training was designed to integrate the new knowledge on fundamental aspects of QMRA together with the fellow's scientific background on bioactivity of plant extracts. Thus, during the training, the fellow assessed the decontamination potential of nanoemulsified oregano and rosemary EOs against *E. coli* O157:H7, as a potential washing solution for cherry tomatoes.

Preparation of nanoemulsions

First, the fellow was trained in the preparation of nanoemulsions from EOs, following a 'learning-by-doing' approach. Thus, oregano and rosemary EOs, purchased from both Romania and Spain, were used for the preparation of the nanoemulsions. Two individual protocols were used to prepare the

rosemary and oregano nanoemulsions. Both Romanian and Spanish rosemary nanoemulsion were prepared following a modified method described by Zhang et al. (2014), by mixing distilled water, propylene glycol, Tween 80 and rosemary EO. The second protocol was used for both Romanian and Spanish oregano EOs, by using the method previously described by Sow et al. (2017). Tween 80, sunflower oil, oregano EO and distilled water were mixed to obtain the oregano nanoemulsions. The obtained nanoemulsions were subjected to continuous sonication using a Hielscher UP400St sonicator (Hielscher Ultrasonics, Germany) to produce disruptive forces which decreased the droplet size. Afterwards, the droplet size was determined by the laser light scattering method using the Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) (Maté et al., 2017; Ros-Chumillas et al., 2017).

Decontamination treatments of cherry tomatoes

Cherry tomatoes with uniform shape, colour and size, purchased from a local market, were selected for the study. The tomatoes were kept at 4°C until the treatment. They were rinsed with water to eliminate impurities, allowed to dry and afterwards they were artificially contaminated by immersion into an *E. coli* O157:H7 (CECT 5947) (Spanish Type Culture Collection) suspension.

The *E. coli* O157:H7 strain was cultured in 500 mL Luria–Bertani broth, incubated at 37°C overnight under agitation to yield an initial population of approximately 10^7 CFU/mL. Subsequently, the cherry tomatoes were dipped in the overnight culture, then removed and left to dry on a sterile metal rack in the laminar flow cabinet, to artificially contaminate them.

Inoculated cherry tomatoes were then treated by different decontamination processes. Washing solutions of varying concentrations of oregano and rosemary EOs nanoemulsions were prepared. Experiments were also performed with distilled water and a commercial chlorine solution for fresh produce, as controls. At the same time, a positive control consisting of contaminated tomatoes without washing treatment was used to evaluate the initial contamination. The effect of the different treatments was evaluated by immersing three contaminated cherry tomatoes in 300 mL treatment solution for 15 min, without agitation.

The antimicrobial efficacy of different washing solutions against *E. coli* O157:H7 was investigated by the plate colony counting method. After application of the sanitisation procedures, the contaminated tomatoes were homogenised in peptone water and mixed in a sterile stomacher bag using a masticator. Bacterial counts were performed by serial dilution in peptone water of the homogenised samples and enumerated on Luria–Bertani agar media. Plates were incubated at 37°C for 24 h.

The obtained results were implemented in the development and application of stochastic mathematical modelling for QMRA using the *bioinactivation* and *biorisk* R packages (Garre et al., 2017, 2018, 2023; Possas et al., 2022), by assessing a comparison between the Romanian and Spanish food supply chain of RTE fresh produce, and how their similarities and differences affect the risk to the consumer.

Training on bacterial inactivation

During her stay at the hosting site, the fellow also benefited from trainings on multiple laboratory equipment, among which, she gained experience on the use of a thermoresistometer Mastia (Conesa et al., 2009), patented by the UPCT group. The Mastia thermoresistometer was used to study the inactivation kinetic behaviour of a *Listeria* spp. In particular, the fellow focused on the inactivation of *L. monocytogenes* under dynamic heating conditions, as there is evidence that different heating rates may induce an increased resistance in this species (Garre et al., 2019; Clemente-Carazo et al., 2020).

2.2.2. Training in risk assessment

Together with the expertise gained during the four modules and induction training, the fellow was also trained at the hosting site on QMRA. Thus, after obtaining the experimental data, the last step of the training programme was the implementation of a QMRA model as an application of the skills gained during the previous tasks. This activity provided knowledge on methodologies for data collection to characterise *E. coli* O157:H7 kinetic behaviour along the food chain. The pathway of *E. coli* behaviour, incorporated in the QMRA model, started from the initial concentration of *E. coli* on cherry tomatoes, followed by processing and storage, and ending with the risk of illness after consumption. The risk assessment model was developed based on both data collected from literature and experimental results. The model was developed in R software, by using the *biorisk*, *bioinactivation* and *biogrowth* packages, developed by the UPCT group (Garre et al., 2017, 2018, 2023; Possas et al., 2022).

The prevalence and initial concentration of *E. coli* were calculated based on data collected from literature. The processing step was based on experimental data and describes the potential of different washing treatments on tomato decontamination. The last step – the storage - was based on data gathered from ComBase datasets. Several studies have investigated the prevalence of *E coli O157:H7* on cherry tomatoes, and a prevalence ranging from 1.6% (Pagadala et al., 2015) to 65% (Gomez-Aldapa et al., 2013) was reported. The initial bacterial concentration was calculated based on the number of samples, the sample size of the positive and negative samples, data extracted from scientific literature.

Additionally, experimental growth curves, under different conditions, of *E. coli* in broth media were collected from ComBase. Data describing and predicting the survival and growth of *E. coli* under varying environmental conditions was selected according to the pH of the tomatoes (4.5 to 5 pH). Additionally, data describing all ComBase available temperatures (from 10°C to 42°C) were collected. The collected data were used to describe the storage temperature for cherry tomatoes in this QMRA study.

The data gathered from literature and available datasets, together with the experimental results, were implemented to build the QMRA model. The model was implemented by using probability distributions for the model parameters and environmental factors, accounting for variability and uncertainty (Nauta, 2000). The risk was calculated by implementing a stochastic approach, by simulating and analysing QMRA models based on Monte Carlo simulations. Different scenarios and different modelling approaches were defined for the characterisation of the microbial response in the food chain. Finally, the fellow was able to interpret the results of QMRA simulations, including the evaluation of variability and uncertainty. The models were implemented to show the similarities and differences between Romanian and Spanish food supply chains of RTE fresh produce, but mainly to assess the biological risk of these produce.

2.3. Secondary scientific activities during fellowship

Together with the scheduled tasks, additional training and other scientific activities were provided by the hosting (UPCT) and sending (USAMV CN) organisations, both in person and remotely. This improved the fellow's general knowledge on risk assessment and communication. Thus, the fellow was encouraged and supported to attend and gain experience from the following activities:

- The fellow attended a hybrid Workshop held by 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'.
- The fellow visited the headquarters of the Spanish Agency for Food Safety and Nutrition (AESAN) in Madrid and the National Centre for Food laboratory, in Majadahonda on 17 and 18 January. During the visit, the fellow benefited from the presentation of AESAN's work on risk assessment, management and communication. She also had the opportunity to present her work and activities to the AESAN Scientific Committee.
- The fellow was invited as a guest lecturer and delivered a seminar for Food Quality Management Master students enrolled in the biological hazard assessment and control in food quality management course at USAMV CN. She presented her research activities under the EU-FORA programme.
- The fellow also attended the presentations of two Final MsC Reports related to decontamination with nanoemulsions of RTE fresh produce at the hosting organisation (UPCT).

Additionally, a project proposal for GP/EFSA/BIOHAW/2023/01 – Support to EFSA in the risk assessment of alternative methods for the use and disposal of animal by-products and derived products was submitted during the fellowship by the hosting organisation (UPCT) as coordinator and the sending organisation (USAMV CN) as one of the partners. This proposal was awarded, and it includes all the parties of the EU-FORA grant in the team (one of the supervisors at UPCT, the fellow and fellow's supervisor from sending organisation, USMAV CN) which ensures the continuation of their collaborations in risk assessment.

At the same time, during the fellowship, an Erasmus International Agreement was signed by the two organisations. The fellow's supervisor from the sending organisation (USAMV CN) was able to carry out an Erasmus teaching mobility at the hosting organisation (UPCT) under this agreement. Thus, a close collaboration between USAMV CN and UPCT was enabled.

The fellow submitted an abstract on the data obtained during the EU-FORA fellowship, which has been accepted for a poster presentation at the 37th International European Federation of Food Science and Technology (EFFoST) Conference 2023, which will take place between 6 and 8 November in Valencia, Spain.

Another outcome of the working programme will be a scientific manuscript which is under preparation. The manuscript is based on the results obtained during the EU-FORA fellowship.

3. Conclusion

The overall focus of the EU-FORA programme offered the fellow an opportunity to familiarise herself and gain experience in food risk assessment in general, and specifically in QMRA. This was also a valuable opportunity to expand her knowledge and acquire many new skills related to food safety, by working in a professional environment among a team with proven expertise in risk assessment. Additionally, the fellow gained an overview on different topics related to food safety risk assessment by attending the EU-FORA dedicated training modules. The activities were designed to provide the fellow with basic knowledge on QMRA through a training-by-doing approach that covered experimental methods, statistical analysis, mathematical modelling and stochastic simulations. The results of the risk assessment will be the basis of a stochastic model that will evaluate the minimum EO concentration that provides an acceptable level of protection.

In addition, the fellow was successfully integrated in the daily routine which created a great environment for professional and social interactions. Thanks to the EU-FORA programme, the fellow had the opportunity to learn by practice and apply the knowledge acquired, to the field of RTE food decontamination, such as tools for the prediction of bacterial behaviour and inactivation models, to build a QMRA. Also, the professional and pleasant working environment at the hosting site has ensured the success of the EU-FORA fellowship programme. The EU-FORA programme has set the stage for future collaborations not only between the fellow and the team at UPCT, but also between the two universities, the sending and the hosting organisations, such as the awarded project proposal accessed in consortium, and research manuscripts under preparation. Thus, this fellowship not only was an excellent opportunity for professional and personal development, having a significant impact on the fellow's future career, but also it provided a first instance in the collaboration and partnership of the two organisations: UPCT and USAMV CN.

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Abbreviations

AESAN	Spanish Agency for Food Safety and Nutrition
CFR	Code of Federal Regulation
CFU	colony forming units
EFFoST	European Federation of Food Science and Technology
EOs	Essential oils
ETSIA	Food Safety and Preservation in the Agronomic Engineering Department
EU-FORA	The European Food Risk Assessment Fellowship Programme
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GRAS	generally recognized as safe
HUS	haemolytic uraemic syndrome

MRA	Microbiological Risk Assessment
PPA	peroxyacetic acid
QMRA	Quantitative Microbial Risk Assessment
RTE	ready-to-eat
STEC	Shiga toxin-producing *E. coli*
UPCT	Universidad Politécnica de Cartagena; Polytechnic University of Cartagena
USAMV CN	University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca