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EU-FORA SERIES 8

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EU-FORA SERIES 8

efsa JOURNAL

Foreword to the 8th Special Issue of *The EFSA Journal*

I'm pleased to introduce the 8th Special Issue of *The EFSA Journal* dedicated to the EU-FORA Programme – continuing a tradition starting in 2018 of highlighting scientific collaboration and capacity building across Europe. EU-FORA will soon turn 10, and since its inception, it has supported the development of future generations of Europe's experts in food safety and risk assessment.

This issue, as its predecessors, brings together a rich collection of contributions covering a wide range of topics, all closely aligned with EFSA's work and of great interest to the organisations involved in the Programme. With these publications, we recognise the work and dedication of the fellows, the cooperation of all the organisations involved in the Programme and the support provided by EFSA's colleagues across different departments. Partnership and cooperation are core values for EFSA, and EU-FORA is built around the idea of partnership – fostering collaboration among competent organisations across Member States on issues central to EFSA's work.

EFSA is committed to the creation of a European risk assessment community and will continue working towards that objective.

I would like to finish by extending our sincere thanks to all contributors to the Programme – fellows, supervisors but also the training consortium – for the successful cycle. We applaud our newly graduated fellows and hope they continue inspiring dialogue, innovation and cooperation in the years to come. EFSA is committed to the creation of a European risk assessment community and will continue working towards that objective.

Barbara Gallani

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EU-FORASERIES 8

efsa JOURNAL

Introduction to the 8th special issue – EU-FORA Programme

We are happy to introduce this Special Issue of the *EFSA Journal*, dedicated to the eighth cohort of the EU-FORA Fellowship Programme. It provides an insight of the work programmes and activities performed by our fellows in relevant areas of food risk assessment. Each report tells a story of professional growth, collaboration, networking and scientific curiousness.

To the fellows: Congratulations on your accomplishments. Together with my team, we hope you expand your networks, share the knowledge, build upon the experience gained during this year and continue to invest in the EU-FORA Community as Alumni.

Finally, we would like to thank everyone who was involved and contributed to yet another cycle of this well-established and welcome initiative, extending our gratitude to EFSA's Management and Staff, Member States and competent organisations, and our training consortium for shaping it.

On behalf of the EU-FORA Programme,

Victoria Villamar

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EU-FORA SERIES 8

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In Memoriam: Prof. Konstantinos (Kostas) Koutsoumanis (1973–2025)

Before we delve into the scientific articles in this special issue of the EFSA Journal, it is only fitting that we pay tribute to our late esteemed colleague and friend Dr. Konstantinos (Kostas) Koutsoumanis who acted as mentor and tutor for many of the fellows who have participated in EFSA's EU-FORA programme over the years. His untimely passing earlier this year has greatly saddened his many colleagues in EFSA and the wider European food safety community. A prominent researcher in food microbiology, teacher and global advocate for food safety, Kostas passed away on 25th of February at the age of just 52. His passing leaves an irreplaceable void in the field of food microbiology and a profound sense of loss among those whose lives he touched through his commitment to food safety research and policy.

In addition to his academic career at Aristotle University, he found time to collaborate widely across Europe and well beyond, encompassing not just EFSA but also the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). Over the course of his distinguished career, he authored more than 250 peer-reviewed articles, 15 book chapters and 150 conference proceedings, making him one of the most cited academics on food safety of his generation.

He first began to contribute to EFSA in 2007 and over the years held a number of key positions: as a member and Chair of EFSA's Panel on Biological Hazards and more recently as Vice-Chair of its Scientific Committee. His expertise was greatly appreciated on the Editorial Advisory Board of this Journal. Those of us who had the good fortune to work with Kostas remember him for his dedication and collaborative nature. He always found time to support colleagues and nowhere was this more evident than in his collaboration with the EU-FORA Programme, a vital platform for preparing the next generation of European food risk assessors.

Colleagues at EFSA remember Kostas not only for what he achieved but for how he did it, with compassion and an unwavering commitment to science. While he received numerous awards throughout his distinguished career, perhaps the most meaningful testament to his work lies in the improved safety of food systems around the world and in the generations of professionals who continue to build on his legacy.

All involved in the EU-FORA programme offer their sincere condolences to Kostas' family and his wide circle of friends.

May he rest in eternal peace.

Arthur Healy

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Cumulative exposure of xenobiotics of emerging concern from agrifood under the One Health approach (XENOBAC4OH)

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The declarations of interest of all scientific experts active in EFSA'S work are available at https://open.efsa.europa.eu/experts.

Abstract

Anthropogenic activities, such as industrial processes, urban development, intensive agriculture and waste disposal, have significantly contributed to the continuous introduction and accumulation of a wide array of xenobiotic compounds into natural ecosystems. Among them, emerging contaminants (ECs) such as pharmaceuticals, endocrine-disrupting chemicals (EDCs), and per- and polyfluoroalkyl substances (PFAS) are of increasing concern due to their persistence, bioactivity and limited regulation. ECs enter ecosystems through diverse pathways including wastewater discharge, agricultural runoff and atmospheric deposition. Once released, many of these xenobiotics can bioaccumulate in organisms and enter the food chain, posing serious risks to food safety and public health. Traditional physico-chemical remediation methods are often insufficient or environmentally taxing, prompting a shift toward bio-based alternatives like bioremediation. These approaches, which rely on the activity of microbial communities to degrade pollutants, offer more sustainable solutions but require further interdisciplinary research to optimise their use. The One Health framework provides an effective model for addressing the complex risks posed by xenobiotics. This research programme aims to harmonise methodologies for cumulative dietary risk assessment across Europe and explore microbial strategies for xenobiotic degradation. By integrating microbiomics, toxicology, environmental science and food safety, this approach supports the development of safer food systems and more effective pollution management in line with the 'farm to fork' and One Health principles.

KEYWORDS

cumulative risk assessment, emerging contaminants, 'farm to fork', microbial remediation, one health, xenobiotics

CONTENTS

1 | INTRODUCTION

Pollution resulting from anthropogenic activities, such as industrial practices, urbanisation, waste management, livestock farming and agriculture, introduces a wide range of chemical compounds into ecosystems (Strokal et al., 2021; Wen et al., 2017). A key characteristic of many of these substances is that they are foreign to biological systems and may cause unexpected and potentially severe adverse effects on both environmental and human health. These substances, known as xenobiotics, represent a growing concern due to their persistence, bioactivity and uncertain long-term impacts (Chen, 2020). Within this broad category, a subset known as emerging contaminants (ECs) has attracted growing scientific attention (Puri et al., 2023). ECs encompass a wide and expanding group of anthropogenic and naturally occurring substances increasingly detected in the environment that are not yet well-regulated or understood in terms of their potential health or ecological risks (Miglani et al., 2022).

Traditionally, risk assessment frameworks have focused primarily on human health. However, growing awareness of the interconnectedness between human, animal and environmental health has led to the adoption of the One Health approach, an integrated, unifying perspective that aims to sustainably balance and optimise the health of people, animals and ecosystems (Adisasmito et al., 2022; Buschhardt et al., 2021). This approach is especially critical for ensuring food safety, nutrition, controlling zoonotic diseases, managing environmental contamination and addressing the global challenge of antimicrobial resistance (WHO, 2023). Agricultural activities, veterinary drug use and industrial emissions can lead to the presence of ECs in food products. Once these substances accumulate in crops, livestock or aquatic organisms, they may be transferred through the food chain, thereby serving as a significant pathway of human exposure (Colopi et al., 2024; Onyeaka et al., 2024). Adopting a One Health perspective is therefore essential for understanding and managing the transfer of emerging contaminants through the food chain, helping to protect human health while preserving the integrity of environmental systems.

1.1 | Classification and sources of emerging contaminants

ECs are a heterogeneous group of pollutants that include pharmaceuticals and personal care products (PPCPs), endocrine-disrupting chemicals (EDCs), plasticizers, per- and polyfluorinated substances (PFAS), metals, nanomaterials, pesticides and other exogenous substances whose ecological and human health impacts remain largely unknown (Hassan et al., 2022; Li et al., 2024)). The widespread application of chemicals in sectors like industry and agriculture has led to the unavoidable release of ECs into ecosystems (Feng et al., 2023).

These compounds enter the environment through various routes, including domestic, industrial and hospital wastewater, thereby forming a complex xenobiotic mixture (Carraro et al., 2016; Su et al., 2020). Although wastewater treatment plants (WWTPs) are designed to remove conventional pollutants, they often have limited capacity to fully eliminate many ECs due to their persistence and chemical complexity (Archer et al., 2017). Agricultural runoff is another major source, especially for pesticides and metals, which can leach from treated soils or be carried by rainwater into nearby streams, rivers and groundwater (Casado et al., 2019; Rai et al., 2019). Additionally, atmospheric deposition can transport volatile or particulate-bound contaminants over long distances. Chemicals released into the air from manufacturing, combustion or application processes eventually settle onto land or water surfaces, introducing ECs into ecosystems far from their original source (Wollenweber et al., 2025; Zhu et al., 2024).

1.2 | Health risks and mitigation strategies of ECs

ECs pose multiple threats to human health, both from short-term and long-term exposure. These risks include altered immune function (Bansal et al., 2017; Fenton et al., 2021), carcinogenesis (Tokar et al., 2010; Yasasve et al., 2022), neurological and developmental disorders (Tran & Miyake, 2017), dysregulation of reproductive function (Bhattacharya & Keating, 2012; Bonde et al., 2017), metabolic dysfunction (La Merrill et al., 2020) and damage to major organs (Liu & Meng, 2005). Furthermore, the widespread presence of antibiotics as emerging pollutants can affect microbiome composition or structure and promote the development of antibiotic resistance in certain pathogenic microorganisms, thereby complicating their treatment (Alidoosti et al., 2024). Considering their harmful impacts on human health and ecological systems, the need for effective remediation strategies to eliminate xenobiotics is increasingly urgent.

Conventional remediation of contaminated environments often involves physico–chemical methods. However, these traditional treatments often face significant challenges, including the need for complex machinery and the risk of generating secondary pollutants (Ghorbannezhad et al., 2021). Recently, there has been a growing shift toward bio-based remediation techniques like bioremediation and biodegradation (Alidoosti et al., 2024). These bio-based remedial techniques, particularly those involving microbial communities, offer a more energy-efficient and environmentally sustainable alternative by harnessing the synergistic activity of microorganisms to degrade pollutants. However, these communities are highly complex and challenging to study (Kumar et al., 2021). Understanding their structure, dynamics and responses to environmental contamination is essential for developing effective bioremediation strategies. Achieving this requires a multidisciplinary approach that integrates microbiology and environmental science to fully unlock the potential of microbial communities in restoring polluted ecosystems.

1.3 | Implementation of the One Health approach

Given the complex and interconnected risks posed by xenobiotics, there is an urgent need to adopt the One Health approach, which recognises the intrinsic links between human, animal and environmental health (Buschhardt et al., 2021). Xenobiotics, including many emerging contaminants, are increasingly entering ecosystems. Once released, many of these xenobiotics can bioaccumulate in organisms and enter the food chain, raising serious concerns about food safety and public health (Mahmood et al., 2017). Addressing these challenges requires coordinated and multidisciplinary teams for establishing collaboration across microbiology, environmental science, nutrition, food safety and public health sectors. The integration of advanced technologies, such as omics-based tools and microbiota analysis can enhance our understanding of microbial roles in xenobiotic degradation and support the development of sustainable bioremediation strategies (Ampatzoglou et al., 2022; Gruszecka-Kosowska et al., 2022; Kostka et al., 2024). By bridging knowledge across disciplines and sectors, the One Health framework offers a holistic and effective approach to ensuring food safety, protecting ecosystems and enhancing human health (Garcia et al., 2020).

2 | DESCRIPTION OF THE WORK PROGRAMME

2.1 | Aims

Cumulative exposure to xenobiotics and their combined effects have significant implications for health research. Addressing these complex challenges requires the integration of diverse scientific disciplines, including microbiology, nutrition, toxicology, environmental science and personalised medicine. In addition, collaboration across technological platforms, research communities and professional sectors is essential to generate meaningful and actionable insights. Advances in risk assessment, particularly those incorporating microbiome analysis, metabolomics and other omics technologies, are paving the way for a deeper understanding of how various factors and substances influence human health. Consequently, there is a growing need to strengthen the connection between environmental and microbiological research on xenobiotics throughout the entire food chain and food safety.

The programme aims to harmonise and exchange methodologies to advance the practice of food risk assessment in Europe, specifically in the area of cumulative risk assessment of multiple xenobiotics that may be present in food. The work programme also included the microbiological expertise of the consortium to explore the potential of bacteria to degrade xenobiotics in the environment. This dual approach supports safer food production in alignment with the 'from farm to fork' strategy and the principles of the One Health approach.

2.2 | Main activities

2.2.1 | Systematic literature review of bacterial degradation of ECs

In the work programme, the process began with grouping ECs into two categories: inorganic contaminants, represented by heavy metals and organic contaminants, specifically bisphenols, PFAS and pesticide residues. A systematic literature review was carried out using major scientific databases, including PubMed, Web of Science and Scopus and employing a carefully selected set of keywords related to these chemical groups as well as relevant biological processes.

A total of 3114 articles related to heavy metals and 3944 articles on organic contaminants were initially retrieved. Next steps following e.g. PRISMA approach will involve applying specific research questions and well-defined inclusion and exclusion criteria, followed by a critical appraisal to ensure conclusions are based on robust and scientifically sound evidence. Results will be published as a systematic review in a peer-reviewed journal.

2.2.2 | ECs in water and soil

To achieve this objective, a field campaign was conducted at the beginning of the Fellow's stay at the hosting site in the Bukowno region of Poland, specifically targeting the vicinity of a lead–zinc (Pb–Zn) smelting and mining complex known for its historical and ongoing industrial activity. The primary objective of this campaign was to collect soil and water samples around the site in order to evaluate levels of investigated ECs for potential bioremediation and mitigation strategies planned as the continuation of the research in the next work programme. Chemical analysis of the collected samples is currently ongoing, and the detailed results and findings are intended to be published in peer-reviewed scientific journals.

2.2.3 | ECs in freshwater sediments

Regarding this activity, sediment samples for the Fellow were obtained from various inland water reservoirs in Southern Poland, each influenced by various anthropogenic activities, as well as from a reference site – a drinking water reservoir

serving the inhabitants of Krakow, under the AGH University of Krakow grant no. 6794, under the programme Excellence initiative – research university, entitled 'Investigation of UV sunscreen filter occurrence in the aquatic environment and assessment of associated environmental risks'. During the Fellow's stay at the hosting site, organic ECs, specifically bisphenols (BPs), per- and poly-fluoroalkyl substances (PFAS), pharmaceuticals and personal care products (PPCPs), were determined using ultra-performance liquid chromatography–mass spectrometry (UPLC–MS). Inorganic ECs, meaning heavy metals, were measured using inductively coupled plasma–mass spectrometry (ICP–MS) system and inductively coupled plasma–optical emission spectrometry (ICP–OES) system. Environmental risk associated with the analysed ECs concentrations was evaluated using the risk quotient (RQ) methodology, as developed by the US Environmental Protection Agency (Khare et al., 2023). RQ values were determined by calculating the ratio of measured environmental concentrations (MECs) to predicted no-effect concentrations (PNECs) for each of the investigated ECs. The average concentrations of the investigated ECs were used as MECs for environmental risk calculations. Additionally, the worst-case scenario was evaluated by using the highest measured EC concentrations as MECs. PNEC values were obtained directly from the ECHA Chemical Database. Human Health Risk Assessment followed USEPA (1989) methodology, with Monte Carlo simulation performed in @RISK software (Lumivero).

Primary results were submitted to the 14th International SedNet Conference 2025, Madrid, Spain, 8–10 October 2025 and were invited to be presented as oral presentation entitled 'Emerging contaminants (ECs) in inland water sediments of the anthropogenically affected areas in Poland. A One Health perspective'.

2.2.4 | Effect of copper on edible plants

This activity was based on the experiment performed to evaluate the impact of varying copper concentrations in the soil on germination and seedling growth of four edible plant species (wheat, spinach, broccoli and radish) as well as the copper accumulation in their tissues. The experiment utilised garden soil with six different Cu concentrations: control sample (Cu0) with natural copper content and five contaminated samples (in mg/kg dry mass): Cu0–24.9, Cu1–756, Cu2–1619, Cu3–2637, Cu4–6741 and Cu5–16,126. For each plant species, 100 seeds were sown in each soil portion. After 1 week of cultivation, the number of germinated seeds was recorded, root and shoot lengths were measured. Concentrations of Cu in each soil portion, and in plant tissues, were determined using atomic absorption spectrometry (AAS).

Results of this research were presented in the IAFP's European Symposium on Food Safety 2025 under the title of 'The Effect of Copper on the Germination and Growth of Selected Edible Plant Species: Pot Experiment Study'. Ortiz, P., Aguilera, M., Załączkowska, A., Gruszecka-Kosowska, A., Kostka, A. Oral communication. 6–8 May 2025, Madrid.

2.2.5 | Impact of fertiliser on tomatoes fruits

This activity was based on the experiment conducted to (I) evaluate the impact of various forms of fertilisation on the growth of seedlings, (II) determine the potassium content in fruits, (III) assess the taste of fruits and (IV) examine the fruits' resistance to spoilage. The experiment utilised an 'Oxheart tomato' variety. Seedlings were cultivated in a home plantation and divided into groups; each watered with either chemical fertiliser (EC certified), homemade nettle slurry or water only (control group). The potassium content in the fruits, soil and fertilisers was measured using AAS. The taste of tomatoes was evaluated through sensory analysis, and spoilage resistance was assessed by measuring the time until the first signs of spoilage appeared on harvested fruits.

Results of this research were presented in the IAFP's European Symposium on Food Safety 2025 under the title of 'The Impact of Fertilizer Components on the Growth, Development, and Flavor of Tomatoes'. Szatko K., Ortiz, P., Aguilera, M., Gruszecka-Kosowska, A., Kostka, A. Oral communication. 6–8 May 2025, Madrid.

2.2.6 | Impact of extraction methods on risk assessment values in food safety

During her stay at the hosting site, the Fellow was involved in the AGH University of Krakow grant no. 4046, under the programme 'Excellence initiative – research university', entitled 'Experimental evaluation of the influence of chemical speciation methodologies for potentially harmful elements on health risk assessment outcomes in the context of remedial decision-making'. The effect of the participation in the project was co-authorship of the manuscript on examination the presence of essential trace elements in edible plants, focusing on their dual role in providing necessary nutrition while potentially posing health risks if present in excessive or unsafe levels. The manuscript was submitted to the peer-reviewed journal.

2.3 | Additional activities

During the fellowship period, Pilar Ortiz, as a fellowship holder, participated in five EU-FORA training modules, organised by the European Food Safety Authority (EFSA), in cooperation with other European institutions, including the Austrian Agency

for Health and Food Safety (AGES), the German Federal Institute for Risk Assessment (BfR), the Hellenic Food Authority (EFET) and SAFOSO (Swiss consultancy, research and capacity-building company). Additionally, the fellow attended several conferences, workshops, training sessions and Institutional visits:

- State Congress of Biosciences Students (CEEBI). Collaboration in the micro-course: 'HANDS-ON! Learn Different Techniques for Analyzing the Gut Microbiota Through Practical Examples'. 15–18 Jul 2025, Granada.
- FEMS MICRO 2025. The potential correlation between metals and metalloids in faecal samples and obesity in children. Kostka, A., Ortiz, P., Luque, G., López-Moreno, A., Ruiz-Rodríguez, A., Gruszecka-Kosowska, A., Aguilera, M. Poster communication. 14–17 Jul 2025, Milan.
- 3rd Annual MISTRAL Project Meeting convened at AGH University of Science and Technology. 22–23 May 2025, Kraków.
- EU-FORA training module on ONE HEALTH organised by the European Centre for Disease Prevention and Control (ECDC), 12–15 May, online.
- IAFP's European Symposium. The Effect of Copper on the Germination and Growth of Selected Edible Plant Species: Pot Experiment Study. Ortiz, P., Aguilera, M., Załączkowska, A., Gruszecka-Kosowska, A., Kostka, A. Oral communication. 6–8 May 2025, Madrid.
- EU-FORA training visit to the Spanish National Centre for Food (CNA), covering Food Contact Materials, Food Processing Contaminants, Biotechnology, Microbiology and Antimicrobial Resistance, 19 Feb 2025, Madrid.
- EU-FORA training visit to the Spanish Agency for Food Safety and Nutrition (AESAN), covering Risk Assessment and the AESAN Scientific Committee, Risk Communication and Risk Management of Biological and Chemical Hazards, Nutritional Safety, Food Official Control and Alerts, 18 Feb 2025, Madrid.

3 | OUTCOMES

The activities carried out under the EU-FORA fellowship programme generated significant scientific and collaborative outcomes that strengthen both the research field and the network of partners involved.

- **Foundation of a shared data pool**. The fellowship laid the groundwork for a structured pool of experimental and field data on ECs. This resource establishes a basis for future joint analyses and methodological harmonisation between consortium partners, thereby promoting sustained collaboration and strengthening research capacity at the European level.
- **Advancing the One Health approach**. By linking environmental and human health risk assessment of contaminants, the fellowship contributed to advancing the application of the One Health approach. The integration of human exposure assessment with ecological risk evaluation provided new insights into the cumulative impact of xenobiotics and reinforced the holistic perspective needed for policy-making and food safety.
- **Scientific dissemination**. Results were disseminated through several international scientific events, including one conference poster and two oral presentations at the IAFP's European Symposium and one invited oral presentation at the 14th International SedNet Conference 2025. These opportunities enhanced the visibility of the project within the scientific community and promoted engagement with specialists in environmental science, food safety and risk assessment.
- **Advancing risk assessment skills**. The fellow had the opportunity to be comprehensively trained in cumulative risk assessment through the EU-FORA training programme organised by EFSA and partner institutions and continuing during her stay at the hosting site through both theoretical and hands-on practical activities. This training encompassed a wide range of interconnected fields, including microbiology, environmental science, food safety and risk assessment, and was further reinforced by active participation in conferences, workshops and institutional visits, thereby ensuring a multidisciplinary and applied learning experience.

ABBREVIATIONS

AAS atomic absorption spectrometry
AGES Austrian Agency for Health and Food Safety
BfR German Federal Institute for Risk Assessment
BPs bisphenols
ECs emerging contaminants
EDCs endocrine-disrupting chemicals
EFET Hellenic Food Authority
ICP–OES inductively coupled plasma–optical emission spectrometry
MECs measured environmental concentrations
PFAS per- and polyfluoroalkyl substances
PNECs predicted no-effect concentrations
PPCPs pharmaceuticals and personal care products
RQ risk quotient
SAFOSO Swiss consultancy, research and capacity-building company
UPLC–MS ultra-performance liquid chromatography–mass spectrometry
WWTPs wastewater treatment plants

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DISCLAIMER

Detailed scientific results of the fellow's research are not fully included in this report to avoid copyright claims, as parts of the analyses are yet ongoing and are intended to be published in peer-reviewed scientific journals. Continued collaboration between the fellow's sending institution and hosting sites is planned beyond the duration of the programme. The EU-FORA fellowship and funding will be acknowledged in later manuscripts submitted for peer-reviewed scientific journals.

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EU-FORA SERIES 8

Probabilistic MIC modelling for AMR risk assessment

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Abstract

Understanding the minimum inhibitory concentration (MIC) of antibiotics is crucial for developing targeted strategies to combat antimicrobial resistance (AMR) in the food chain. Traditional MIC testing methods provide a single and deterministic value without taking into account the inherent inter-individual variability in the response of foodborne pathogens to antimicrobials. A more accurate representation of the complex interactions influencing AMR in foodborne pathogens could be probabilistic MIC modelling. The incorporation of probabilistic MIC modelling into a classic quantitative microbiological risk assessment (QMRA) of AMR for food pathogens could provide a more realistic risk estimate. The EU-FORA fellowship's objective was to develop a probabilistic QMRA for an antimicrobial-resistant foodborne pathogen in a food product, incorporating the variability of the MIC of individual bacterial cells. The project involved the collection of data on *Listeria monocytogenes* in ready-to-eat (RTE) cooked ham, the selection of an appropriate growth model, the conducting of MIC assessment laboratory experiments for ampicillin based on the plate-count agar method and Monte Carlo analysis. The QMRA model was constructed using the R programming language. The final outputs obtained were a total of 1000 simulated doses of *L. monocytogenes* in servings of cooked ham at the time of consumption, as well as the maximum and 95th percentile of single-cells MIC values of ampicillin for each dose. Moreover, a sensitivity analysis was conducted.

KEYWORDS

AMR, cooked ham, *Listeria monocytogenes*, QMRA, RTE, scMIC

[†]Deceased.

This work is dedicated to the memory of Professor Konstantinos ('Kostas') Koutsoumanis, whose passing represents a great loss to the EU-FORA community. He was an enthusiastic supporter of the EU-FORA programme, recognising its value in nurturing the next generation of food risk assessment experts. His generosity, guidance and example have left an enduring mark on the field and on all who had the privilege to be taught by and work alongside him. His legacy will continue to inspire scientists within EU-FORA and far beyond.

CONTENTS

1 | INTRODUCTION

In recent years, antimicrobial resistance (AMR) has emerged as a new concern in the field of food safety. AMR is defined as the capacity of bacteria to resist the effects of antimicrobials. This can lead to consequences such as persistent infections, prolonged hospital stays, increased costs and, most importantly, an increased mortality rate (Bennani et al., 2020; WHO, 2015). To avoid the increase of AMR and ensure effective and optimal treatments, it is crucial to know the minimum inhibitory concentration (MIC), defined as the lowest concentration of an antibiotic that can inhibit the growth of a pathogen. A variety of methods have been employed to determine the MIC, such as the dilution of the antibiotic in agar or liquid medium (microdilution and macrodilution), as well as gradient methods employing strips impregnated with a concentration range of the antibiotic (Kowalska-Krochmal & Dudek-Wicher, 2021). These conventional testing methods provide a single and deterministic value, however they do not take into account the inter-individual variability in the response of foodborne pathogens to antimicrobials. Factors such as the initial pre- and post-processing contamination, storage conditions, food matrices (pH, a_w, etc.) and biological variability contribute to the different microbial responses (Aspridou & Koutsoumanis, 2015). By considering the distribution of MIC values, and not just a single value, variability and uncertainty could be incorporated and therefore increase the probability of identifying resistant strains within foodborne pathogen populations. In fact, integrating probabilistic MIC modelling into food safety risk assessments improves the precision of AMR predictions in foodborne pathogens.

Listeria monocytogenes, a foodborne pathogen that causes listeriosis, was the fifth most commonly reported zoonosis in humans during 2023 in the EU (EFSA and ECDC, 2024). Despite having a low incidence rate, it can cause severe symptoms (fever, meningitis and septicaemia) in susceptible groups including pregnant women, elderly, newborns and immunosuppressed people (EFSA and ECDC, 2024). Moreover, treatment of listeriosis can be complicated by antimicrobial resistance, which may affect the effectiveness of standard antibiotic dosing regiments. This pathogen can be present in a variety of foods products, nevertheless, the main source of infection in the USA and Europe, according several risk assessment studies, is the consumption of RTE foods and in particular deli meats (EFSA BIOHAZ Panel, 2018; Pérez-Rodríguez et al., 2017; Pradhan et al., 2010; Tsaloumi et al., 2021). In addition, its ability to grow at low temperatures, acidic conditions and high salt concentration poses a significant risk due to it has the capacity to grow during refrigerated (EFSA BIOHAZ Panel, 2018; Matle et al., 2020).

The EU-FORA project was focused on improving the risk assessment of AMR in foodborne pathogens, such as *L. monocytogenes*, by modelling and simulating variability in the single-cell minimum inhibitory concentration (scMIC) values.

2 | DESCRIPTION OF THE WORK PROGRAMME

2.1 | Aims

The objective of the work programme was to develop a probabilistic QMRA for a foodborne pathogen in a food product incorporating the MIC variability on the cellular level. This was achieved by using R and Monte Carlo simulations, and incorporating the variability of scMIC values for the selected hazard-antimicrobial combination, in order to provide a more realistic risk estimate. The fellow received training in all the necessary steps for QMRA (hazard identification, hazard characterisation, exposure assessment and risk characterisation), predictive microbiology tools, use of R programming in risk assessment (probability distributions, fitting and visualisation of distributions, etc.), growth modelling and data collection.

2.2 | Activities/methods

2.2.1 | Selection of food-hazard-antimicrobial combination

The first activity conducted by the fellow was the selection of an appropriate food-hazard-antimicrobial combination for analysis. As described above, *L. monocytogenes* poses a significant virulence in susceptible populations and it is often transmitted via RTE products, such as cooked ham. Moreover, ampicillin is the antibiotic of choice for treating listeriosis in pregnant women and cases overall (American College of Obstetricians and Gynecologists, 2014; Ramos-Ospina & Moncada-Vallejo, 2024; Temple & Nahata, 2000). Therefore, a QMRA for *L. monocytogenes* in cooked ham sliced at retail was developed.

2.2.2 | scMIC estimation experiments

A total of six experiments were performed in order to determine the MIC of ampicillin for a strain of *L. monocytogenes* FSL R2-764, responsible for an epidemic and isolated from sliced deli meat in 2002, in order to incorporate this information in the risk assessment model. These experiments were carried out during the on-site visit in the hosting site. Several ampicillin concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7 and 8 μg/mL) were tested using the plate-count agar method. Briefly, these ampicillin concentrations were incorporated into Mueller-Hinton II Agar (MHA, Biolab) medium before it

solidified. Then, 100 µL of three inoculums at concentrations of 10^2, 10^3 and 10^4 CFU/mL were inoculated onto the agar plates surface, with two replicate plates per ampicillin concentration. The inoculated plates were then incubated for 72 h at 37°C. Following this time, the colonies were counted to calculate the probability of growth (P_g) of individual cells at each ampicillin concentration according to:

$$P_g = \frac{N}{N_0},$$

where N = the colonies counted at a given ampicillin concentration and N_0 = the inoculum.

2.2.3 | Development of a probabilistic QMRA in R

2.2.3.1 | *Literature search*

An extensive search and analysis of scientific literature about *L. monocytogenes* and the selected product from different scientific bibliographical databases such as Scopus, Google Scholar, PubMed and EFSA, was carried out to collect all the necessary data for the model's input variables. These included the level of contamination of *L. monocytogenes* and lactic acid bacteria (LAB) in cooked ham sliced at retail, the time and temperature during the transportation from retail to home, the time of storage, the temperature of domestic refrigerators and the physicochemical characteristics of cooked ham (pH, water activity (a_w) and nitrite concentration). The studies focused on Spain were selected wherever possible, including the data about the concentration of LAB, temperature and duration of domestic storage, pH and a_w of cooked ham.

2.2.3.2 | *Construction of QMRA model in R*

The model was developed as a Modular Process Risk Model (Nauta, 2007) starting from retail and covering the possible growth of *L. monocytogenes* during transportation from retail to home and domestic storage. The QMRA model was built using the R programming language, for which the fellow received online training lessons during the fellowship. Data collected from published studies were used to simulate ingested doses of *L. monocytogenes* at the time of consumption. All the probability distributions, parameters and calculations used as inputs are shown in Table A1 in Appendix A. The initial concentration of *L. monocytogenes* in cooked ham (N_0) was described as a beta-general distribution by EFSA BIOHAZ Panel (EFSA BIOHAZ Panel, 2018). For the concentration of LAB at retail (N_{LAB}), a normal distribution was selected (Pérez-Rodríguez et al., 2010). In addition, a normal distribution was used to described the variability of pH and a_w values of the cooked ham (Bover-Cid et al., 2024). However, the pert distribution was used for the nitrite concentration (Tsaloumi & Koutsoumanis, 2024). Concerning the transport module, the temperature variability was calculated according Serra-Castelló et al. (2025) and Mataragas et al. (2010), and the transport time was described using a cumulative distribution (Mataragas et al., 2010). The temperature of Spanish domestic refrigerators was described with a normal distribution (Jofré et al., 2019), and a cumulative distribution was applied for the domestic storage time (Bover-Cid et al., 2015). Furthermore, the maximum population densities (N_{max}) of *L. monocytogenes* and LAB from the FSSP software (8.5 log CFU/g) were utilised, and it was assumed that the serving size was a single slice of cooked ham of 10 g (Maciejewska et al., 2024) and that the Lag phase was 0.

2.2.3.3 | *Exposure assessment*

Microbial growth of *L. monocytogenes* in cooked ham under transport and domestic storage conditions was modelled using the Mejlholm and Dalgaard model (Mejlholm & Dalgaard, 2007). This growth model considers the effects of interactions between different parameters (pH, a_w, nitrite, temperature, etc.) and the Jameson effect approach in the growth of *L. monocytogenes* (Mejlholm & Dalgaard, 2007). The Jameson effect, describes the influence of LAB inhibiting the growth of *L. monocytogenes* (Mejlholm & Dalgaard, 2007). The growth rates of *L. monocytogenes* and LAB during transport and storage were estimated using the cardinal parameter growth and growth boundary models, and the differential form of the simple logistic model (Mejlholm & Dalgaard, 2007). A total of 1000 Monte Carlo simulations were run in R for the complete model in order to calculate the final concentration of *L. monocytogenes* at the time of consumption. Finally, to estimate the doses ingested by consumers per serving, this result was multiplied with the serving size (10 g).

2.2.3.4 | *Sensitivity analysis*

A sensitivity analysis, a tornado graph based on Spearman's rank correlation, was performed to assess the effect of the different input factors of the developed QMRA model on the simulated doses of *L. monocytogenes* at the time of consumption.

2.2.3.5 | *Integration of the scMIC variability into the QMRA model*

Based on the P_g values of the six experiments performed, the probability of inhibition ($1 - P_g$) by ampicillin at each antibiotic concentration tested was calculated. A cumulative normal distribution was then fitted to the inhibition probabilities across concentrations, describing the scMIC distribution. Using the 'nls' function in R we estimated the nonlinear (weighted) least-squares estimates of the parameters (mean μ and standard deviation σ) of the fitted distribution. This normal distribution was used in the developed QMRA model to simulate doses of ingested number of *L. monocytogenes* cells per slice (1000 iterations). For each simulated dose:

- Dose sizes were rounded to integer values.
- Fractional doses (< 1 CFU/slice) were conservatively rounded up to 1 CFU/slice to ensure meaningful sampling.
- A Monte Carlo procedure was applied to draw *n* scMIC values (from the fitted normal distribution truncated at 0 µg/mL), where *n* corresponded to the size of the simulated dose. This ensured that each individual cell in each dose was assigned its own scMIC.

For each simulated infection dose, the maximum scMIC and the 95th percentile of the scMIC values were obtained.

3 | RESULTS

The collected data from previous studies on the different inputs of the QMRA model was used to obtain 1000 *L. monocytogenes* simulated doses (log CFU) per slice of cooked meat (10 g) at the time of consumption (Figure 1). The concentration of *L. monocytogenes* in a single slice ranged from 0 to 9.14 log CFU, with a mean and median of 1.40 and 0.85 log CFU, respectively. The 50% of the simulated doses showed a concentration of 0 to 2.17 log CFU/slice (Figure 1). These concentrations are lower than the limit proposed by the Commission Regulation (EC) No 2073/2005 which establishes a maximum of 100 CFU/g (i.e. 2 log CFU/g or 3 log CFU in 10 g), in RTE foods to ensure product safety during shelf life. However, it should be noted that approximately 25% of the simulated doses exceed this limit, with some doses reaching 9.14 log CFU/10 g, which is well above the limit (Figure 1).

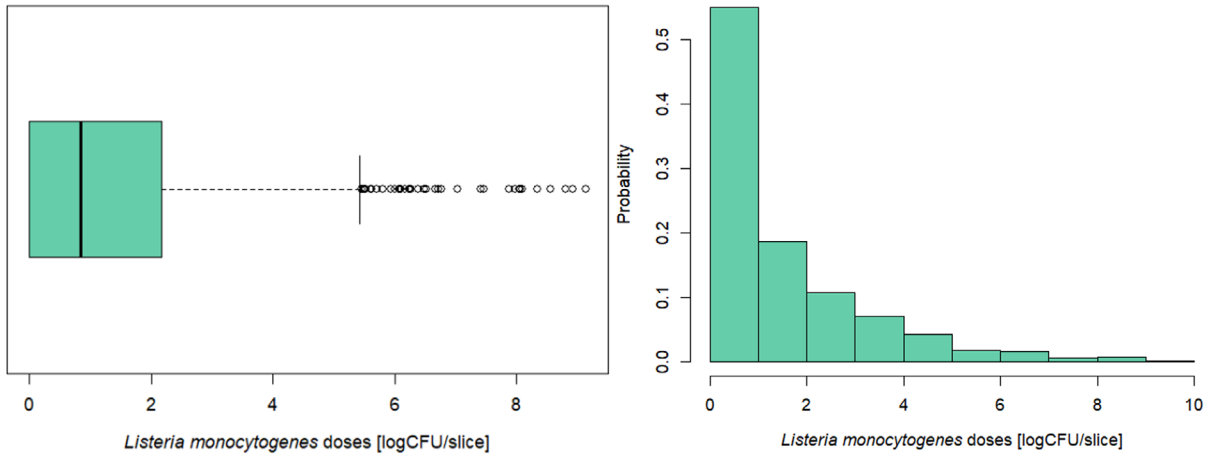

FIGURE 1 Boxplot (left) and histogram (right) representing the 1000 simulated infection doses [log CFU] of *Listeria monocytogenes* per single slice of cooked ham at the time of consumption.

The results of the sensitivity analysis are presented in a Tornado graph (Figure 2). The input with the greatest impact on the output obtained in our QMRA (*L. monocytogenes* doses in one slice of cooked ham at the time of consumption) was the *L. monocytogenes* initial contamination (N_0). The time of storage at home and the temperature of the domestic refrigerators, can have a certain influence on the final output. It is therefore important that consumers take into account the best-before dates and to control the temperature of refrigerators.

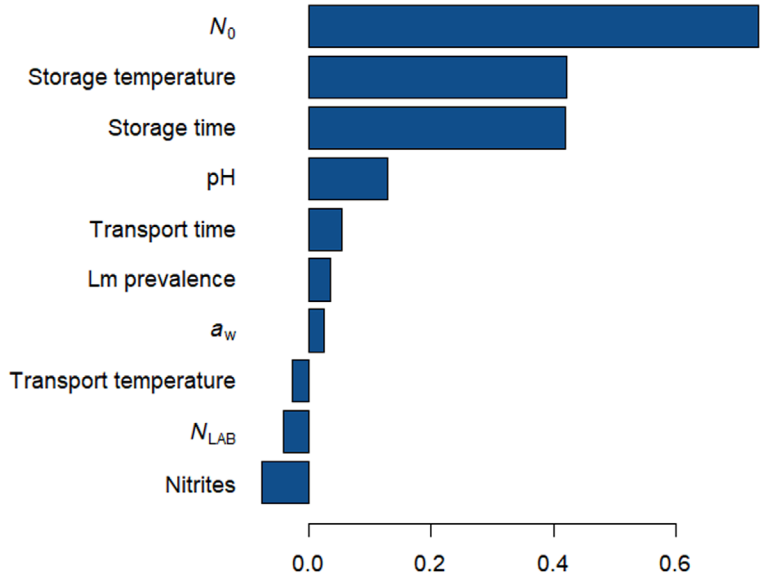

FIGURE 2 Sensitivity analysis for the effect of the model input values on the *Listeria monocytogenes* doses in one slice of cooked ham at the time of consumption.

Different responses to ampicillin were observed in the experiments performed by the fellow. As shows in Figure 3, a cumulative normal distribution with mean = 3.88 and SD = 0.702 was fitted to the results from the six experiments.

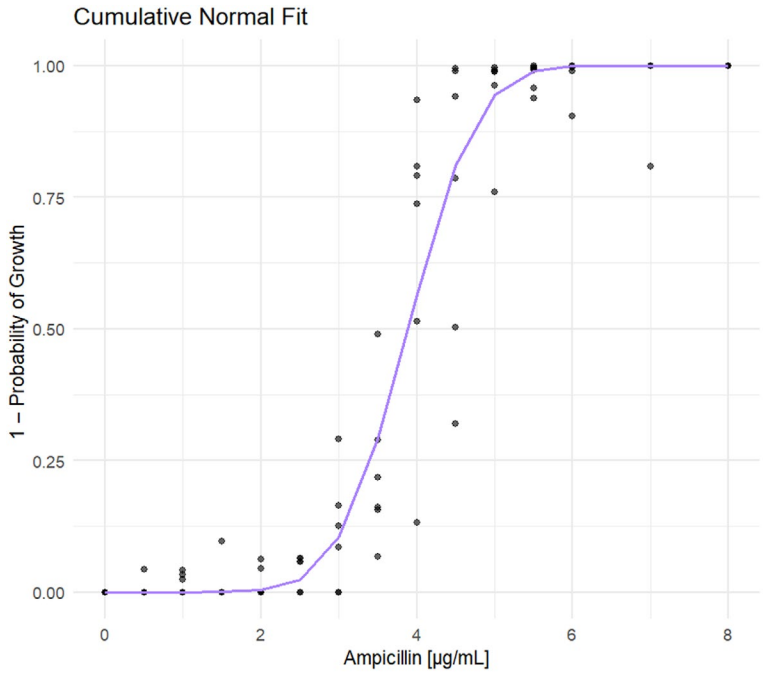

FIGURE 3 Cumulative normal distribution of the probability of inhibition ($1 - P_g$) of *Listeria monocytogenes* at different ampicillin concentrations [µg/mL].

Furthermore, the maximum scMIC values of each simulated *L. monocytogenes* dose ranged from 1.95 to 8.16 µg/mL of ampicillin, with a mean and median of 4.95 and 4.97 µg/mL, respectively (Figure 4). This means that among the 1000 *L. monocytogenes* simulated doses, the most resistant cells were inhibited at concentrations of ampicillin ranging from 1.95 to 8.16 µg/mL.

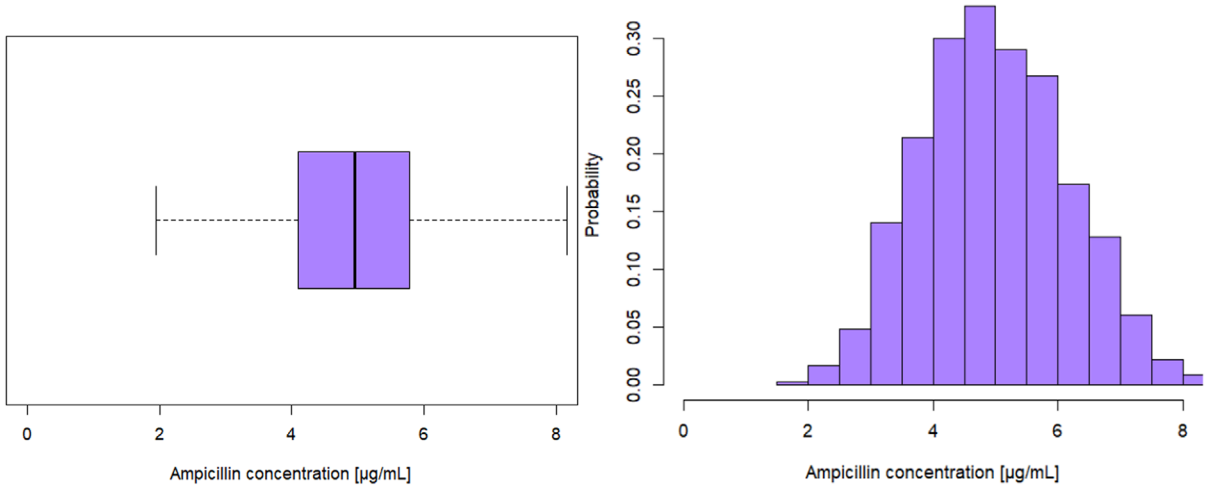

FIGURE 4 Boxplot (left) and histogram (right) representing the maximum scMIC values of ampicillin [µg/mL] of each simulated infection doses of *Listeria monocytogenes*.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) establish that *L. monocytogenes* is resistant to ampicillin with a MIC >1 µg/mL (Mejlholm & Dalgaard, 2007), therefore our modelling suggests that, every simulated dose includes at least one cell with an estimated scMIC above the EUCAST breakpoint. Moreover, we observed that at higher doses of *L. monocytogenes* in a single slice, higher concentrations of ampicillin are required to inhibit this pathogen (Figure 5). In addition, the 95th percentile of the scMIC values of ampicillin of each simulated doses of *L. monocytogenes* was calculated (Figure 5). At low doses (approximately 0–1 log CFU/slice), the variability of the 95th percentile of the scMIC is higher, however, at higher doses, it is more or less constant. Specifically, the calculation of the 95th percentile indicated that the 95% of the scMIC values were below 5 µg/mL (Figure 5). This suggests that the concentration of ampicillin required to inhibit the majority of the *L. monocytogenes* cells is 5 µg/mL.

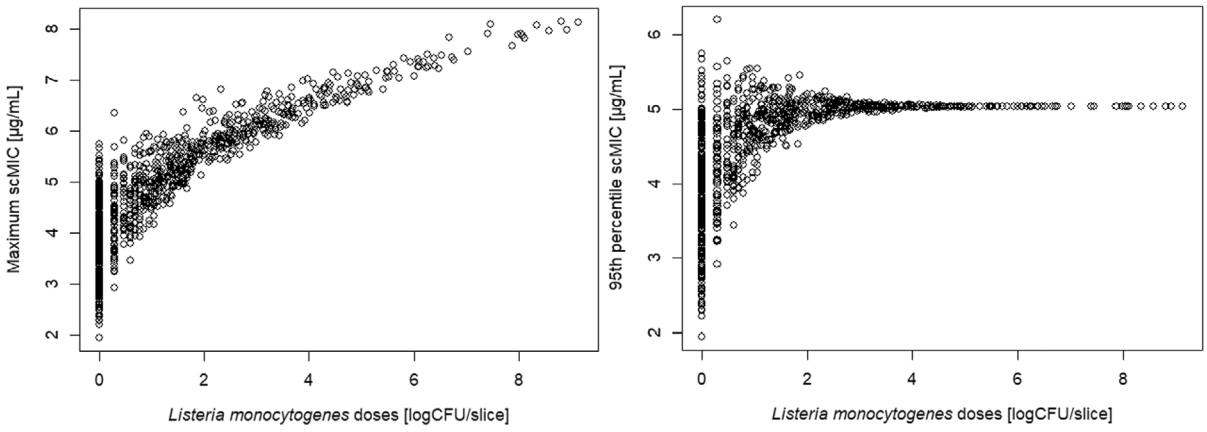

FIGURE 5 Relation between the maximum scMIC (left) and the 95th percentile of the scMIC (right) values of ampicillin [µg/mL] for each simulated infection doses [log CFU/slice].

4 | CONCLUSION

The objective of the project was to enhance the risk assessment of AMR in foodborne pathogens by employing probabilistic modelling to simulate variability in the MIC. The project work programme enabled the fellow to acquire expertise in risk assessment, guiding them through every step required to build a complete QMRA model in R using growth models, predictive microbiology tools, Monte Carlo analysis, and the handling of variability and uncertainty in a stochastic risk assessment. The developed QMRA model considering the inherent variability in MIC values, provided a more realistic representation of the spectrum of MIC values present in a population, due to the MIC of the most resistant cells within each simulated dose was determined. The results of the study showed that in the 1000 simulated doses, the maximum scMIC was higher than the clinical breakpoint for ampicillin, therefore the presence of resistant cells among the *L. monocytogenes* population. With the integration of probabilistic MIC modelling into food safety risk assessments the potential phenotypic variability can be detected.

5 | ADDITIONAL ACTIVITIES

During the EU-FORA fellowship, the fellow had the opportunity to attend the workshop 'Making sense of data in food science' held by Dr. József Baranyi, a leading figure in predictive food microbiology, held in Thessaloniki (Greece) from 5 to 7 November 2024. The fellow also visited the Spanish Agency for Food Safety and Nutrition (AESAN) in Madrid and the National Center for Food Laboratory in Majadahonda (Spain) on 18–19 February 2025, and completed the online training module on One Health organised by the European Centre for Disease Prevention and Control (ECDC) from 12 to 15 May 2025. Moreover, the fellow received training lessons on the use of R in risk assessment by the research team, and participated in working group meetings, seminars and social activities organised by the hosting site. The fellow presented her project work at the lab team's joint meeting held in June and a presentation to share the results of this project with the Department of Food Hygiene and Technology staff of the University of León (Spain) is scheduled for September–October. Furthermore, the establishment of a new collaboration between the research group of the hosting site and the sending was facilitated by this fellowship. The objective of this collaboration is to develop a risk assessment study for *Acinetobacter* in the meat production chain, as part of the fellow's PhD thesis.

ABBREVIATIONS

AMR	antimicrobial resistance
CFU	colony forming units
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EU-FORA	European Food Risk Assessment
MIC	minimum inhibitory concentration
P_g	probability of growth
QMRA	quantitative microbiological risk assessment
RTE	ready-to-eat
scMIC	single-cell minimum inhibitory concentration

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APPENDIX A

TABLE A1 Parameters, distributions and calculations of the risk assessment model.

Model parameters [units]	Description	Distribution/calculation	Ref.
N_0 [logCFU/g]	Concentration of *L. monocytogenes* at retail	Beta-general ($\alpha = 0.502$, $\beta = 2.908$, min = −1.69, max = 6)	EFSA BIOHAZ Panel (2018)
N_{LAB} [logCFU/g]	Concentration of LAB at retail	Normal (mean = 5.24, SD = 1.19). Truncated (min = 5.24 − 3 × 1.19, max = 8.5)	Adapted from Pérez-Rodríguez et al. (2010)
pH	pH of the product	Normal (mean = 5.97, SD = 0.44)	Bover-Cid et al. (2024)
a_w	Water activity of the product	Normal (mean = 0.973, SD = 0.004)	Bover-Cid et al. (2024)
Nitrites [ppm]	Concentration of nitrites in the product	Pert (min = 2.80, mode = 40.50, max = 68.42)	Tsaloumi and Koutsoumanis (2024)
T_T [°C]	Temperature during transport from retail to home	T_1: Temperature at retail Normal (mean = 5.44, SD = 2.32). Truncated (min = 0, max = 10) T_2: potential temperature change during transport Normal (mean = 3.72, SD = 2.82). Truncated (min = mean − 3 × SD, max = mean + 3 × SD) $T_T = T_1 + T_2$	Serra-Castelló et al. (2025) Adapted from Mataragas et al. (2010) Mataragas et al. (2010)
t_T [h]	Transport time from retail to home	Cumulative (min = 0, max = 225)/60[a]	Mataragas et al. (2010)
T_H [°C]	Temperature of domestic refrigerators	Normal (mean = 5.4, SD = 2.3). Truncated (min = −0.03, max = 12.7)	Jofré et al. (2019)
t_H [h]	Storage time at home	Cumulative (min = 0, max = 21) × 24[b]	Bover-Cid et al. (2015)
S [g]	Serving size	10 g slice of cooked ham	Maciejewska et al. (2024)
$N_{storage}$ [logCFU/g]	Concentration of *L. monocytogenes* at the end of domestic storage (time of consumption)	$N_{storage} = \begin{cases} N_{transport}, \text{ if } t_H \leq \text{Lag}, \mu_{max} = 0, \text{Lag} = \infty \\ \min\left(N_{max}, \log\left(10^{N_{transport}} e^{(\mu_{max}(t_H - \text{Lag}))}\right)\right) \end{cases}$	Mejlholm and Dalgaard (2007)
D [CFU/slice]	Dose	$S \times 10^{N_{storage}}$	–

[a]Transport time in minutes converted to hours.
[b]Storage time in days converted to hours.

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EU-FORA SERIES 8

Assessing allergenic potential, exposure and risk of *Anisakis* spp.

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

Parasites in seafood are a recognised food safety concern worldwide. Anisakiasis is a human disease caused by ingestion of larvae of parasitic nematodes of the genus ***Anisakis***, typically present in raw or undercooked seafood. It is considered a significant emerging foodborne disease and was recently ranked among the top 10 of 24 parasitic foodborne infections in Europe, with a rapidly increasing potential to cause illness. Many clinical cases are often misdiagnosed as other gastrointestinal disorders, leading to misinterpretation of data and likely a substantial underestimation of their true prevalence. The issue is further complicated by a rising number of asymptomatic individuals who develop sensitivity to thermostable ***Anisakis*** antigens. These otherwise healthy individuals often show elevated anti-***Anisakis*** IgE antibody levels and may have recovered from a mild or undiagnosed gastric anisakiasis without showing allergic symptoms. Because these cases lack obvious clinical signs, this 'silent' form of anisakiasis could affect more than 12% of certain populations and is typically identified only through sero-epidemiological research. Earlier detection approaches, including DNA-based methods such as real-time PCR, demonstrated the potential to identify *Anisakis* as an allergy-causing agens in marketed seafood. However, these methods had not been validated, and the results, therefore, required cautious interpretation. Here, we present the first validated real-time PCR method designed to improve the detection of *Anisakis* as an allergy-causing agens and to support more accurate monitoring within the EU seafood market. Additional work to express two known allergic proteins (Ani s 1 and Ani s 7) for immunological testing of hypersensitised patients can open venues for a future complete risk assessment of anisakiasis.

KEYWORDS

allergy, *Anisakis*, gene expression, seafood, validation

CONTENTS

1 | INTRODUCTION

Anisakidosis is a zoonotic disease caused by nematodes belonging to the family Anisakidae, while anisakiasis (also referred to as anisakiosis) specifically results from infection by species of the genus *Anisakis* (Adams et al., 1997; Oshima, 1987; Vidal-Martinez et al., 1994). Recognised as one of the most important emerging foodborne diseases globally, its rising prevalence has been attributed to stricter conservation policies protecting marine mammals, the definitive hosts of *Anisakis*, alongside evolving culinary habits across Europe that favour raw or lightly processed seafood. These environmental and gastronomic shifts have led to an increased infection rate in paratenic fish hosts and, consequently, in humans. Growing medical awareness and improved diagnostic practices have contributed to a rise in reported human cases in recent years. However, anisakiasis remains significantly underdiagnosed, particularly in the Mediterranean region. Infection occurs through ingestion of third-stage *Anisakis* larvae, typically via thermally unprocessed or lightly treated seafood. Known high-risk dishes include sushi and sashimi in Japan; tuna or sparid carpaccio and marinated, pickled, or salted anchovies in the Mediterranean (Rello et al., 2009); smoked or fermented herring (maatjes) in the Netherlands (Levsen & Lunestad, 2010); dry-cured salmon (gravlax) in Norway; raw salmon (lomi-lomi) in Hawaii; and ceviche in South America (Dias et al., 2011).

Emerging evidence links *Anisakis* infections not only with allergic reactions but also with more severe complications, including an increased risk of upper gastrointestinal bleeding in patients using nonsteroidal anti-inflammatory drugs (NSAIDs) (Chai et al., 2005; Dorny et al., 2009; Hochberg et al., 2010) and potential associations with neoplastic or carcinogenic changes in the gastrointestinal tract (Petithory et al., 1990; Yoo et al., 2008). The allergic dimensions of *Anisakis* infections have been extensively researched, particularly in Spain, where hundreds of allergy-related cases have been documented since 1995 (Mattiucci et al., 2002; Rello et al., 2009; Tejada et al., 2006). These findings underscore the importance of conducting serological surveys in other Mediterranean populations, including both healthy individuals and those with a history of food allergies, to better understand the regional burden of *Anisakis* infections.

Despite this, the European Food Safety Authority (EFSA) database on zoonoses and foodborne outbreaks recorded only 11 human outbreaks linked to unspecified *Anisakis* species across the EU from 2010 to 2022. In contrast, a recent quantitative risk assessment model estimates between 7700 and 8320 cases per year in Spain alone (Bao et al., 2017). Countries with high fish consumption, such as Spain, have incorporated routine testing for *Anisakis* species, but comprehensive assessment of species presence in fresh and processed seafood for other high fish consuming countries such as Portugal, France or Scandinavian countries remains limited. Ensuring safe seafood products requires mandatory testing for these allergens.

Hence, the aim of this EFSA EU-FORA work programme entitled 'Assessing allergenic potential, exposure and risk of *Anisakis* spp.' was to comprehensively assess the prevalence of *Anisakis* in commercially available seafood, with the following approach: development of a validated real-time PCR method for detecting *Anisakis pegreffii* and *A. simplex* DNA in raw and processed seafood products. We applied the validated method to analyse seafood and seafood-containing samples collected from both the host and partner institutions. Moreover, the goal of the study was to evaluate the allergenic potential of *Anisakis* in vitro to generate both qualitative and quantitative data so that these findings can be used in future detailed risk assessment (RA).

The project included an early-stage fellow (BCAS), two mid-stage professionals (BfR) and a high-stage professional from BCAS with complementary expertise who collaborated synergistically. The team was composed of professionals with well-developed backgrounds in parasitology, immunology, molecular biology and proteomics, and the project contributed to strengthening their experience in RA. By applying different approaches to conduct a RA of *Anisakis* allergens in marketed seafood: immunology, molecular biology and more, across two highly specialised research institutions involved in food safety and parasitology, the project helped establish a new scientific collaboration between BfR and BCAS. It also consolidated future directions for exchanging practices and methodologies. Based on knowledge transfer between the partners, the project intensified exchange and cooperation among German and Czech food safety agencies and EFSA, thereby stimulating the involvement of Member States in regulatory risk assessment work.

2 | DATA AND METHODOLOGIES

2.1 | Data

Various seafood/seafood matrix samples from the supermarket, nematodes from Max-Rubner Institute and BfR as well as fish, plant and mammal samples from the collection of the 'National Reference Laboratory for Animal Proteins' at the BfR have been collected for DNA analysis. Altogether more than 70 samples were tested in the project schedule, while additional samples arrive at a later stage and will be additionally analysed by the hosting site. Furthermore, the EU-FORA fellow has produced a model food (fish-burger patties) with incurred *Anisakis* larvae (two larvae/209 g fish) that is in the lower ppm range. DNA was isolated from both raw and cooked fish patties and successfully analysed. The model food will be used as reference material for planned interlaboratory comparison of the validated method.

Synthetic DNA gene constructs encoding the target allergens AniS1 and AniS7 were obtained from a commercial source and re-cloned into the pET-22b(+) expression vector, a widely utilised plasmid system for recombinant protein production in *Escherichia coli*. All produced constructs of expressed proteins are stored at −20°C with the addition of proteinase inhibitors.

2.2 | Methodology

2.2.1 | DNA-based methods

A reliable real-time PCR method was developed to detect Anisakis DNA in food. Since existing primers and probes for the ITS and COX2 regions lacks specificity, new ones were designed in silico from the COX2 gene of *A. simplex* (GenBank: KF972438.1). The assay achieved 95% primer efficiency and 98% probe efficiency, confirmed using serial DNA dilutions (10 ng–10^{-6} ng) of *A. simplex* and *A. pegreffii*. Specificity was validated by BLAST and experimental testing with DNA from ~50 plant and animal species, while sensitivity (LOD_{12}, ≥ 95%) was defined as the lowest concentration detected in 12/12 replicates, even in the presence of salmon DNA (Figure 1). The method reliably detected *A. simplex* and *A. pegreffii* down to 0.01 pg., with near-perfect efficiencies ($R^2 = 0.999$). Tests in spiked salmon fillets, including cooked samples, showed successful detection across concentrations from 100,000 ppm to 1 ppm. Robustness was confirmed using different PCR mixes, machines and annealing temperatures, with only minor variation (≤ 2 Ct) (Figure 1). Finally, to test for real-world application, more than 70 commercial seafood samples were collected (2023–2025). Only spiked positive controls showed amplification, while all commercial samples and negative controls were *Anisakis*-negative, confirming the high specificity and suitability of the assay for routine screening.

2.2.2 | Immunological methods

Synthetic gene constructs encoding the target allergens were obtained from BioCat, cloned into the pET-22b(+) expression vector, a widely used plasmid system for recombinant protein production in *Escherichia coli*. Protein expression was initiated following the standard protocol previously established at the BfR. However, initial attempts failed to yield detectable levels of the recombinant proteins.

Following troubleshooting and consulting with internal collaborators and external experts, we proceeded with a conventional cloning strategy using plasmid systems that had been effective in related expression studies. Following restriction enzyme digestion of the constructs and the expression vector, the constructs were ligated into the cloning vector pUC57 and introduced into *E. coli* Top10 cells via heat-shock transformation, a well-established method for efficient plasmid propagation.

After propagation, plasmid DNA was extracted using a commercial miniprep kit (Macherey-Nagel, Düren, Germany) and analysed by restriction enzyme digestion. The digested products were separated by agarose gel electrophoresis, and bands corresponding to the expected gene insert sizes were excised and purified for subsequent cloning. Finally, the gene constructs were ligated into the in-house pET-22b(+) expression vector using T4 ligase, and protein expression was re-initiated according to the standard BfR protocol.

3 | ASSESSMENT

Nematodes of the genus Anisakis are recognised as a relevant biological hazard in seafood, capable of causing both infectious disease and allergic reactions in humans. Ingestion of viable third-stage larvae may result in anisakiasis, a zoonosis associated with gastrointestinal pain, nausea, vomiting and, in severe cases, intestinal obstruction or perforation. In addition to acute infections, increasing concern is directed towards allergic responses caused by thermostable proteins such as Ani s 1 and Ani s 7, which remain active after standard freezing or cooking. These proteins can induce IgE-mediated hypersensitivity ranging from urticaria and angioedema to severe anaphylaxis, and sensitisation has also been observed in asymptomatic individuals, complicating risk characterisation. The probability of exposure depends strongly on fish species and preparation methods, with raw or lightly processed products such as sushi, sashimi, ceviche and anchovy marinades representing the highest risk. While freezing at −20°C for at least 24 h or adequate cooking effectively inactivates larvae, allergenic proteins persist and may retain biological activity in processed matrices. In the present study, application of a validated real-time PCR assay to over 70 retail seafood samples in German markets revealed no detectable Anisakis DNA, suggesting low prevalence under these conditions; however, epidemiological data indicate higher incidence in Mediterranean regions, where thousands of cases are reported annually. For the general EU population, infection risk from retail seafood is currently considered low when regulatory freezing requirements are respected, although sporadic infections cannot be excluded. Allergic risk, by contrast, remains relevant even in the absence of viable larvae, particularly for previously sensitised individuals, for whom trace amounts may elicit severe reactions. Uncertainties persist due to underreporting, diagnostic limitations, undefined dose–response relationships and limited prevalence data for specific fishing grounds, but overall, the evidence supports continued monitoring of both infection and allergenicity risks. Research into standardised allergy-causing species detection methods, harmonised surveillance across Member States and clarification of dose–response thresholds is needed to improve consumer protection and support risk management decisions.

4 | CONCLUSIONS

This study successfully established and validated a real-time PCR method targeting the *cox2* gene for the detection of Anisakis spp. DNA in both raw and processed seafood. The newly designed primers and probes demonstrated high sensitivity and specificity, with detection limits down to 0.01 pg. of *A. simplex* DNA and of *A. pegreffii* DNA, even in commercial food matrices. Furthermore, screening of over 70 commercially available seafood products revealed no detectable Anisakis DNA in the tested samples, indicating the absence of the parasite in the sampled German retail market products during the study period. This suggests that current handling and processing practices may be effective in minimising Anisakis contamination in consumer-ready seafood.

For the immunological part, the project faced initial setbacks in protein expression of recombinant Anisakis allergens, but subsequent optimisation using conventional cloning methods into *E. coli* expression systems resulted in successful gene insertion and protein induction. These recombinant allergens will be instrumental in future allergenicity testing and might contribute to the risk assessment process (RA).

The study demonstrated the value of combining molecular and immunological approaches for assessing the allergenic potential and prevalence of Anisakis spp. in seafood. Additionally, the project fostered international collaboration, knowledge exchange and capacity-building among European food safety institutions.

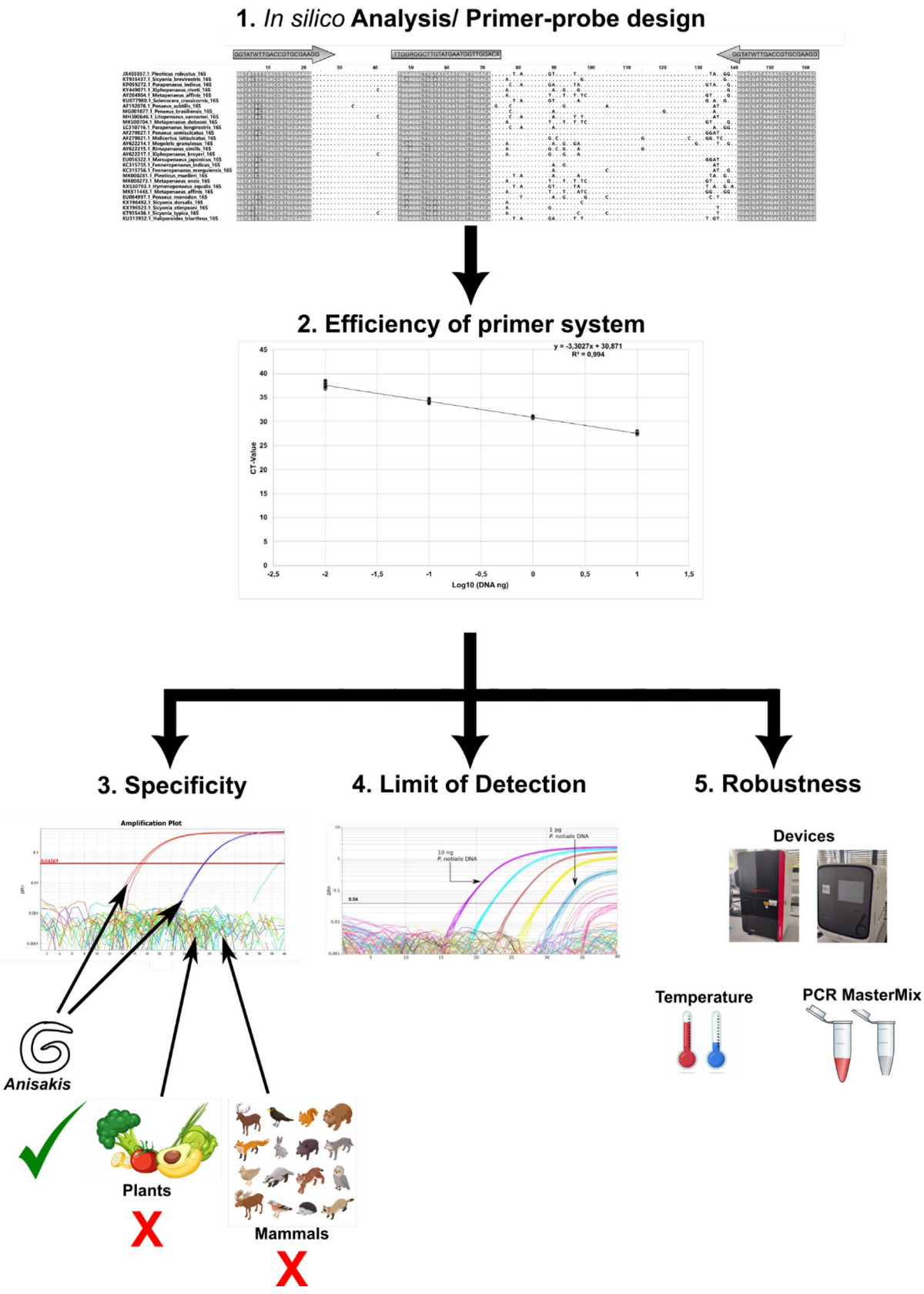

FIGURE 1 Overview of real-time PCR method validation scheme.

5 | RECOMMENDATIONS

EFSA should encourage the implementation of standardised, validated qPCR protocols for detecting Anisakis DNA in seafood products across member states. This would improve the comparability of data and help monitor prevalence consistently.

Moreover, given the allergenic potential and the occurrence of asymptomatic sensitisation, EFSA should recommend including Anisakis spp. detection (in addition to currently standardised detection methods of whole larvae) in routine food safety monitoring – especially for products consumed raw or undercooked. Awareness campaigns aimed at consumers, medical professionals and seafood producers on the risks of *Anisakis* infection and allergy would help reduce the public health burden.

ABBREVIATIONS

AP	*Anisakis pegreffii*
AS	*Anisakis simplex*
COX2/COII	cytochrome oxidase subunit II
Ct value	cycle threshold value
DNA	deoxyribonucleic acid
H_2O	molecular water
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	lysis Buffer
LOD_{12}	limit of detection
MM	mastermix
MW	molecular weight
N/A	not applicable
ND	NanoDrop
NSAIDs	nonsteroidal anti-inflammatory drugs
NTC	non template control
QC	quality control sample
RA	risk assessment
RT F	forward primer
RT P	probe
RT R	reverse primer
RT-PCR	real-time PCR
Undt	undetermined

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APPENDIX A

Additional Activities

	Title/description	Date
Trainings	Trainings on method description and method validation provided by fellow's department Training on allergy assessment provided my allergology unit of BfR	**25–27.3.2025**
Meetings with departments	Meetings, presentations and discussions with departments in hosting Institute: Toxogenomic Unit, Allergology Unit, Risk Assessment Group meetings between BC CAS and BfR	**Every Tuesday Biweekly**
Department Seminars	Interactive seminars where master and PhD students would present their work and have open discussions with the rest. The topics were related to allergology risk assessment in insect food.	**Every 2nd Monday**

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EU-FORA SERIES 8

Development of a qualitative risk assessment for meat from wild boar from a One-Health perspective

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

The focus on this EU-FORA fellowship was to develop a qualitative risk assessment with flowchart as way of communicating the risk assessment transparently at each stage including the indication of data uncertainty and variability. Even though qualitative risk assessments are widely used, they do not always include uncertainty and variability and are not always presented in scenarios, as way of transparent communication. As case studies a microbial pathogen and a contaminant were chosen because they exemplify different types of risk. During this fellowship, a qualitative risk assessment flowchart was developed for *Trichinella* spp. and per- and polyfluoroalkyl (PFAS) for consumers of Latvia and Germany, from consuming wild boar meat. For each potential hazard, a literature search was done. For the flowchart, prevalence and concentration for each hazard were estimated, based on available literature. Furthermore, the flowchart was supplemented with additional nodes, that might increase or decrease the risk for the consumer. In the end, two different qualitative risk assessment flowcharts were developed, out of which, the *Trichinella* spp. risk assessment has been presented in two conferences.

KEYWORDS

exposure, Latvia, Germany, consumers, contamination, PFAS, trichinella

CONTENTS

1 | INTRODUCTION

1.1 | Background and introduction to the work programme

The European Food Risk Assessment Fellowship Programme (EU-FORA), a collaboration between the Institute of Food Safety, Animal Health and Environment "BIOR" (BIOR) of Latvia and the German Federal Institute of Risk Assessment (BfR) of Germany, was established to jointly work on the "Development of a qualitative risk assessment for meat from wild boar from a one-health perspective" project, with the focus on creating a qualitative risk assessment flowchart for *Trichinella* spp. and Per- and polyfluoroalkyl (PFAS) substances.

1.2 | Introduction for the qualitative risk assessment and hazards

Even though qualitative risk assessments are widely used they do not always include uncertainty and variability and are not always presented graphically with a detailed scenario as way of transparent communication. As case studies a microbial pathogen and a contaminant were chosen because they exemplify different types of risk. During this fellowship, a qualitative risk assessment flowchart was developed for *Trichinella* spp. and Per- and polyfluoroalkyl (PFAS) for consumers of Latvia and Germany, from consuming wild boar meat.

With the increase of wild game, especially wild boar, meat consumption and handling, there are also increased concerns regarding food safety (Gomes-Neves et al., 2021). Wild boars can harbour several pathogens and chemical contaminants, which can enter the food chain and cause health problems for the consumers (Gomes-Neves et al., 2021; Mertens et al., 2025). As these concerns increase, a qualitative risk assessment (QRA) can be a useful tool for the initial evaluation and characterisation of hazards, particularly when data availability is limited or the hazard is new. The flowcharts in QRA are also a good vehicle for communication which can indicate well how the risk may change along the pathway and where control may be useful.

One of the major pathogens that causes a zoonotic risk, which can enter the food chain via consuming undercooked or raw wild boar meat, is *Trichinella* spp. *Trichinella* spp. is a nematode with a direct life cycle that, upon initial larva ingestion, can cause the disease trichinellosis, which manifests as gastrointestinal symptoms, such as diarrhoea, stomach ache and vomiting (Murrell & Pozio, 2011). After 2 weeks post-infection, myalgia, ocular and facial oedema, fever can develop and in 0.1% of cases, even death (Murrell & Pozio, 2011). Although *Trichinella* spp. is a parasite, where for which control measures exist in the European Union (EU), there still have been human infection cases in both Latvia and Germany (EFSA & ECDC, 2024).

In the last two decades, chemical contaminants such as per- and polyfluoroalkyl substances (PFAS), especially perfluorooctanoic acid (PFOA), erfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA) and perfluorohexanesulfonic acid (PFHxS) have been increasingly recognised as a risk to human health and are often referred to as 'forever chemicals' (Bell et al., 2021). PFAS are synthetic chemical compounds with several carbon-fluorine bonds, they are resistant to biodegradation, hydrolysis, degradation at high temperatures and repel water, oil and dirt (Bell et al., 2021; Leung et al., 2023). PFAS have been widely used in coating agents, lubricants, adhesives, paints, textile and leather finishing agents, fire extinguishing foams, aviation fluids and historically were used in paper production (Leung et al., 2023) Because PFAS do not biodegrade in the environment, they accumulate not only in the soil, but also in plants, water and animals with concentrations often increasing along the food chain (Wang et al., 2023). Wild boars have been suggested to be bioindicators of environmental PFAS contamination, as PFAS tend to bioaccumulate primarily in their livers and, to an extent, skeletal muscle. Since 2020, EFSA has set threshold for the main four (PFOA, PFOS, PFNA, PFHxS) substances, with a tolerable weekly intake (TWI) of 4.4 ng/kg of bodyweight (bw) per week (EFSA, 2020). Additionally, Commission Regulation (EU) 2023/915 of 25 April 2023 establishes maximum levels for certain contaminants, including PFOS, PFOA, PFNA, PFHxS and the sum of these four PFAS, in various foodstuffs such as meat and offal from game animals (EU 2023/915).

The aim of the research was to conduct a qualitative risk assessment model with a pathway and decision tree, accounting for data uncertainty, variability and estimated input impact on the risk itself, for *Trichinella* spp. and ∑4PFAS (PFOA, PFOS, PFNA, PFHxS) from the consumption of wild boar meat in consumers of Latvia and Germany, and to compare the risk between the countries.

2 | DESCRIPTION OF THE WORK PROGRAMME

2.1 | Aims

The goal of this work programme was to introduce the fellow to both microbiological and chemical qualitative risk assessment of wild boar meat from a One Health perspective. The qualitative risk assessment focused on one microbiological hazard – *Trichinella* spp., and one chemical contaminant group – PFAS (the four most common compounds – PFOA, PFOS, PFNA and PFHxS, hereafter referred as ∑4PFAS). The aims were to develop a pathway for each hazard, providing a detailed description of the risks at each point of the pathway to ensure transparency and to account for variability and uncertainty in the risk assessment.

2.2 | Activities and methods

2.2.1 | Literature research and pathway development for *Trichinella* spp. risk assessment

First step was the development of the QRA flowchart for *Trichinella* spp. and a previously published flowchart about microbiological risk to consumers from small game birds, was taken as a basis (Horigan et al., 2014).

To estimate the combined probability of infection for an individual consumer, eight potential sources of the risk (hazards) were identified: 'Prevalence of *Trichinella* spp. in wild boar meat', 'Concentration of *Trichinella* spp. in wild boar meat', 'Legislation regarding testing wild boar meat for the parasite', 'Human errors', '*Trichinella* larvae survival while cooking and freezing', 'Test sensitivity for *Trichinella*' and 'Dose response'. However, to estimate the overall risk of the population with *Trichinella* spp. larvae from consuming wild boar meat, two additional risk inputs were added – 'Wild boar meat consumption' and 'Severity of trichinellosis in humans'.

Available data about the prevalence and concentration of *Trichinella* spp. in wild boar in Latvia and Germany were gathered by searching the «SCOPUS» database with keywords '*Trichinella*' AND '*Sus scrofa*' OR 'wild boar' AND 'prevalence.' The search years were from 1976 to 2024, with the language set to English. Afterwards, available articles were used for the QRA ($n = 3$). Furthermore, additional information about *Trichinella* spp. prevalence, intensity and species in wild boar from the German (Department of Biological Hazards, BfR) and Latvian (Parasitology group, BIOR) national reference laboratories for *Trichinella* spp. were gathered to supplement the information.

To improve the flowchart, we added additional pathways that might affect the probability of illness of infection in human populations. The pathway 'Legislation regarding testing wild boar meat for the parasite' was added, due to the possible impact of testing or not of all hunted wild boar meat intended for human consumption. The pathway 'Human errors', includes sample mix-up, an inexperienced laboratory technician, not removing the infected animals from the market and testing muscles that are not the primary target for the parasite. The pathway 'Test sensitivity' was added as a possible risk enhancer or minimiser, if different diagnostic tests are used, however, both countries oblige by the EC regulation 1375/2015, in which, the methods that can officially be used for *Trichinella* spp. detection are highly specific and sensitive (Riehn et al., 2013).

Available data for pathways '*Trichinella* spp. larvae survival while cooking' and '*Trichinella* spp. larvae survival while freezing' were obtained by searching the «SCOPUS» database with keywords '*Trichinella*' AND '*Sus scrofa*' OR 'Wild boar' AND 'Survival' or 'Freezing' or 'Cooking.' These two pathways were added due to their effect on the presence of the parasite in the meat, if proper handling techniques were used. Heat treatment is a good way to minimise the larvae load in the meat if proper cooking times are met, such as when the internal temperature reaches between 63°C and 71°C (Noeckler et al., 2019). Freezing the meat, however, is not a reliable method for minimising the larval load, mainly due to intra-species differences (Noeckler et al., 2019).

Dose response in the context of *Trichinella* spp. is, how many larvae need to be ingested by a person to become infected and develop trichinellosis. Available data for this pathway was obtained by searching the keywords '*Trichinella*' OR 'Trichinellosis' AND 'Humans' OR 'Outbreak.' This search revealed eight articles, that contained trichinellosis outbreak investigations and possible dose of which the humans became infected from consuming wild boar meat or its meat products in Europe.

To estimate the overall risk for the population of getting infected with *Trichinella* spp. larvae from consuming wild boar meat, two additional inputs were added: 'Severity of trichinellosis in humans' and 'Consumption of wild boar meat'.

Trichinellosis severity in humans was obtained by searching not only original research but also review articles that consolidated available information about trichinellosis and case reports, by using the keywords '*Trichinella*' OR 'Trichinellosis' AND 'Humans' AND 'Clinic' OR 'Impact' OR 'Epidemiology.' This input was added to estimate, how severe the disease is in humans.

The final and one of the most important inputs for a risk assessment was the consumption data of wild boar meat. For this, the EFSA 'Food consumption statistics' in FoodEx2 were used, selecting 'Latvia' and 'Germany' as the countries and 'Wild boar fresh meat' under the 'Exposure hierarchy (L6)' option. In Germany, four surveys were conducted 2001 and 2006, and in Latvia three surveys were conducted between 2008 and 2012, collecting information on wild boar meat consumption.

The final outline of the flowchart for 'Overall risk estimate for the population of getting ill with *Trichinella* spp. larvae from consuming wild boar meat' is represented in Figure 1 and was created in Lucidchart (www.lucidchart.com).

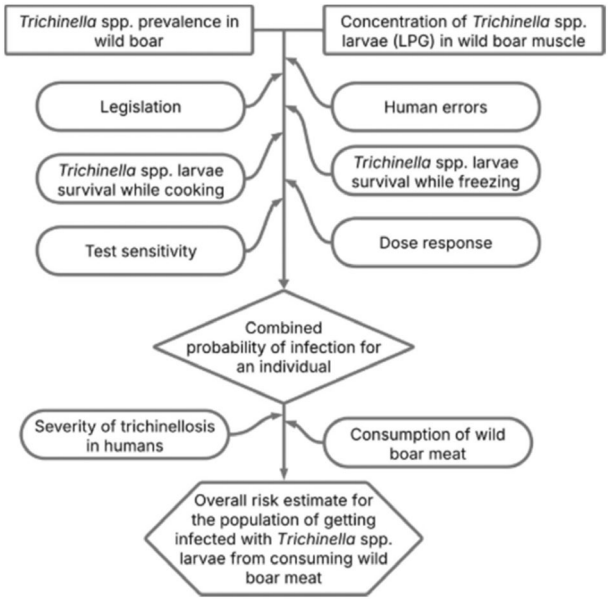

FIGURE 1 Example of a flowchart for the risk estimate for the population of getting infected with *Trichinella* spp.

2.2.2 | Literature research and pathway development for per- and polyfluoroalkyl substances (∑4PFAS)

To outline the flowchart for the overall risk estimate for the population from ∑4PFAS, a similar approach to that used for *Trichinella* spp. was applied, however, some pathways were adapted for chemical exposure. Unlike microbiological agents, chemical contaminants do not grow or multiply within the body. Instead, their risk lies in bioaccumulation and in the potential to metabolise into other harmful compounds.

Similar to the *Trichinella* spp. flowchart, the first step was to estimate the PFAS exposure in the wild boar. The flowchart starts with the 'Environmental contamination of ∑4PFAS', 'Occurrence of ∑4PFAS in wild boar meat' and 'Concentration of the ∑4PFAS in wild boar meat'. The chemical risk assessment also began with a literature search, using several combinations of keywords in the databases 'Scopus', 'Web of Science' and 'ScienceDirect'. In all four search engines, the literature was searched by article title, abstract and keywords. The keywords used for the literature search were: 'PFOS' OR 'PFAS' OR 'PFOA' OR 'PFNA' OR 'PFHxS' AND 'Wild boar' OR 'Sus scrofa,' which resulted in a total of 16 results in 'Scopus', however, an overlap of 11 articles from 'Web of Science' and 5 from 'ScienceDirect' were also found. Of these, seven articles were from Germany and none from Latvia. Out of the seven articles from Germany, only four focused on PFAS in wild boar tissue (liver or meat).

The overall risk estimate for the population from PFAS exposure through wild boar meat consumption was based on two additional risk inputs: 'Adverse health effects of PFAS in humans' and 'Tolerable weekly intake of the combination of four PFAS'.

The tolerable weekly intake (TWI) threshold for the main PFAS substances is set to 4.4 nanograms/kilograms/body weight per week (ng/kg/bw/week) (EFSA CONTAM Panel, 2020). It was set by EFSA in 2020. For the risk assessment, the TWI was taken into account together with wild boar meat consumption and the average consumer body weight, which in Germany for men was 85.8 kg, but for women – 69.2 kg). In Latvia, no official data are available, however, the obesity prevalence among adults in Latvia is approximately 26.8% for women, and 23.9% for men (Eurostat, 2024), while in Germany, it is estimated at approximately 9.0% (Schienkiewitz et al., 2022). Based on the obesity prevalence, it was assumed that the average adult body weight in Latvia is broadly comparable to that in Germany, although this assumption introduces some uncertainty.

Chronic, long-term exposure to PFAS has been shown to have adverse health effects on the human body (Fenton et al., 2021). Literature search in the 'Scopus' database was performed with the keywords 'PFOS' OR 'PFAS' OR 'PFOA' OR 'PFNA' OR 'PFHxS' AND 'Toxicity' OR 'Adverse health effects' AND 'Human,' obtaining 1950 articles, out of which, articles with long-term studies were selected.

The final outline of the flowchart for 'Overall risk estimate for the population from PFAS exposure through wild boar meat consumption' is represented in Figure 2 and was created in Lucidchart (www.lucidchart.com).

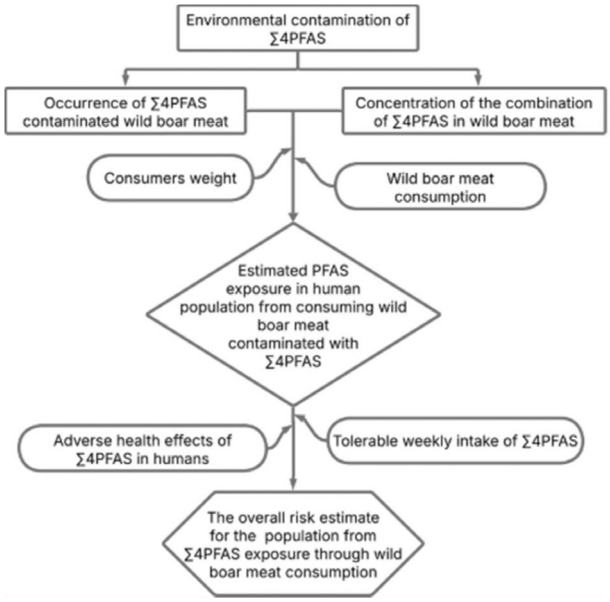

FIGURE 2 Example of a flowchart for the qualitative risk assessment for the risk estimate for the hunter and the general population.

2.2.3 | Uncertainty and variability

For each flowchart pathway, the uncertainty and variability were determined and assessed, based on the available literature and the quality of the available data for each of the risk input. For data uncertainty, we established the following definitions:

- Low – the available data is sufficient for data interpretation and to determine the associated risk. Data comes from research articles, expert data.
- Medium – while there is some data available, there is lack of sample size, animal species or other factors, that can complicate the risk determination.
- High – there is lack of data in any animal species.

For data variability, we also established our own interpretation for the possible variable. The variability the interpretations were:

- Low – Values or information do not vary between articles, individuals or other findings. There is an overlap between the concentration ranges of the studies, but no substantial differences are observed. The data agrees well.
- Medium – There is some variability of available data between articles, individuals or other findings.
- High – There is a large variability of available data between articles, individuals or other findings.

For each risk input, the likelihood of risk was estimated, and the interpretation was taken from EFSA guidelines. To estimate likelihood of risk for each input, we took into consideration also variability and uncertainty (Table 1).

TABLE 1 Likelihood of risk and their interpretations.

Likelihood of risk	Interpretation	Representing colour in the flowchart
Very low	The event is very unlikely to occur	
Low	The event is unlikely to occur	
Medium	The event is equally likely to occur or not to occur	
High	The event would be very likely to occur	

3 | QUALITATIVE RISK ASSESSMENT

3.1 | Flowchart for the *Trichinella* spp. qualitative risk assessment

During this EU-FORA project, it was decided between the fellow and the supervisor to do one publication on the QRA for *Trichinella* spp. in humans in both Germany and Latvia.

The fellow has reported the results in two conferences:

1. Mateusa, M., Ozoliņa, Z., Müller-Graf, C., and Deksne, G. '*Trichinella* spp. and *Alaria alata* – a Risk of Wild Boar Consumption for Human Health in Latvia?', 1st SafeGameMeat Conference, 26.–28.5.2025., Porto, Portugal.
2. Mateusa, M., Johne, A., Müller-Graf, C. '*Trichinella* spp. - a risk from consuming wild boar meat? A comparison between Germany and Latvia using a qualitative risk model', DACH-Epidemiologietagung, 03.–5.9.2025., Germany.

An example of a pathway that will be presented in the DACH-Epidemiologietagung conference is represented in Figure 3.

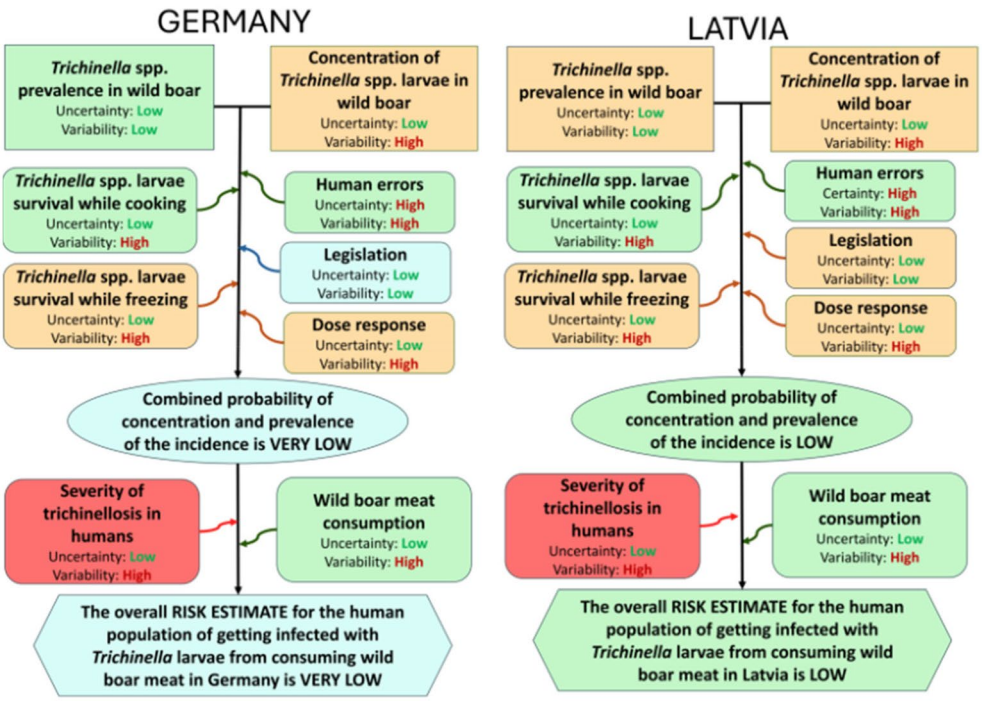

FIGURE 3 Qualitative risk assessment flowchart of the overall risk estimate for the human population of getting infected with *Trichinella* spp. larvae from consuming wild boar meat in Germany and Latvia.

The flowchart indicates that the overall risk for consumers in Germany is very low, whereas in Latvia it is low. The biggest difference between the two countries that affects the consumer risk is the prevalence of *Trichinella* spp. in wild boars, which was 0.3% in Latvia (communication with NRL of *Trichinella* in Latvia) and 0.004% in Germany (Johne et al., 2025). In addition, the prevalence of *Trichinella* in other wild animals is different: in Latvia, 50.6% of red foxes were infected (Deksne et al., 2016), while in Germany, only 1.9% of the raccoon dogs carried the parasite (Mayer-Scholl et al., 2016). Therefore, it is essential to test all wild boars and other *Trichinella*-susceptible animals for *Trichinella* larvae, if their meat is intended for human consumption. Additionally, Germany has strict mandatory testing at both national and EU levels. Comparatively higher risk in Latvia was estimated, as not all wild boar meat for personal consumption may be tested.

The qualitative risk assessment as flowchart is valuable, as it visualises each potential step of the risk pathway, making uncertainties and critical control points more transparent, which, for *Trichinella* is the mandatory testing of all wild boars before consumption, which has been highly effective in keeping consumer risk very low, due to the removal of *Trichinella*-positive carcases from the food chain. However, if testing rates are reduced or legislation is not implemented, the risk increases substantially, particularly in cases, where undercooked products (such as raw minced meat) are consumed. It is important to not, that, if an infection does occur, the consequences can be severe – from gastrointestinal symptoms and facial edema to long-term myalgia and even death (Murrell & Pozio, 2011). Flowchart for the ∑4PFAS qualitative risk assessment.

3.2 | Flowchart for the ∑4PFAS qualitative risk assessment

To estimate the ∑4PFAS exposure in the human population in Germany from consuming wild boar meat, occurrence and contamination of ∑4PFAS in wild boar meat was obtained from the available data on ∑4PFAS occurrence in wild boars; in total, four research studies and one scientific opinion were evaluated, out of which, two studies focused on wild boar meat (BfR, 2021; BfR, 2024; Felder et al., 2023; Kowalczyk et al., 2018; Mertens et al., 2025; Stahl et al., 2012). The most representative occurrence data of wild boars being contaminated with PFAS was reported in the BfR opinion from 2024. Three out of four wild boars were contaminated with PFAS (73.5%) with the mean concentration of PFOS, PFOA, PFNA and PFHxS

being 33.77 μg/kg and the high 95th percentile being 236.93 μg/kg (BfR, 2024). Regarding occurrence and concentration, we estimated the data uncertainty as medium, as only three research papers reported PFAS levels in wild boar meat (BfR opinion, 2024; Mertens et al., 2025; Stahl et al., 2012), focusing on particular regions in Germany. Therefore, there is lack of information about other wild boar populations. As for data variability, contamination with PFAS is more likely to be location-based, rather than being a background contamination. Therefore, depending on where the wild boar is hunted, the PFAS concentration in the muscle can vary drastically (BfR, 2021), hence the variability is high.

According to the EFSA consumption database, the general population, on average, consumes 0.17 g of wild boar meat per day, ranging between 0.01 and 0.17 grams. However, on consumption days, the general population consumes 155.48 grams of wild boar meat per day (reference years: 2001–2006) (FoodEx2, 2024). Among the hunter population, there is higher wild boar meat consumption. According to a smaller-scale study, hunters consume up to 158 grams of wild boar meat per week, which increases the risk of the hunter population being exposed to ∑4PFAS (Meisenberg & Gerstmann, 2021). Considering both the data age and the higher exposure to ∑4PFAS in hunter populations, the data uncertainty was assessed as medium, but variability as high. To fully estimate the possible PFAS exposure for individual consumers, the weight of the person should be added as a possible risk node for the pathway.

For the overall risk estimate for the population from PFAS exposure through wild boar meat consumption, adverse health effects and TWI were added. In the human body, PFAS bind to serum albumin and fatty acid binding proteins, resulting in excessive PFAS storage in the liver, kidneys and blood (BfR, 2024). Adverse health effects of PFAS in humans have been associated with thyroid disease, increased cholesterol levels (especially with PFOS, PFOA and PFNA), liver damage and increased probability of testicular and kidney cancers (Fenton et al., 2021). There have been reports of reduced response to several vaccines, such as tetanus, diphtheria and rubella if during pregnancy, there was an increased exposure to PFOS and PFOA (Fenton et al., 2021). Uncertainty for the adverse health effects was assessed as medium, but there was a high degree of variability. As for tolerable weekly intake, EFSA has set a weekly limit for the ∑4PFAS (4.4 ng/kg/bw/week) therefore, the uncertainty and variability was assessed as low. In the end, the final risk estimate for the German population from PFAS exposure through consuming wild boar meat was assessed as low (Figure 4). However, there are a lot of different routes for ∑4PFAS intake, so that for the overall picture the intake through the consumption through wild boar meat has to be combined with all other intakes.

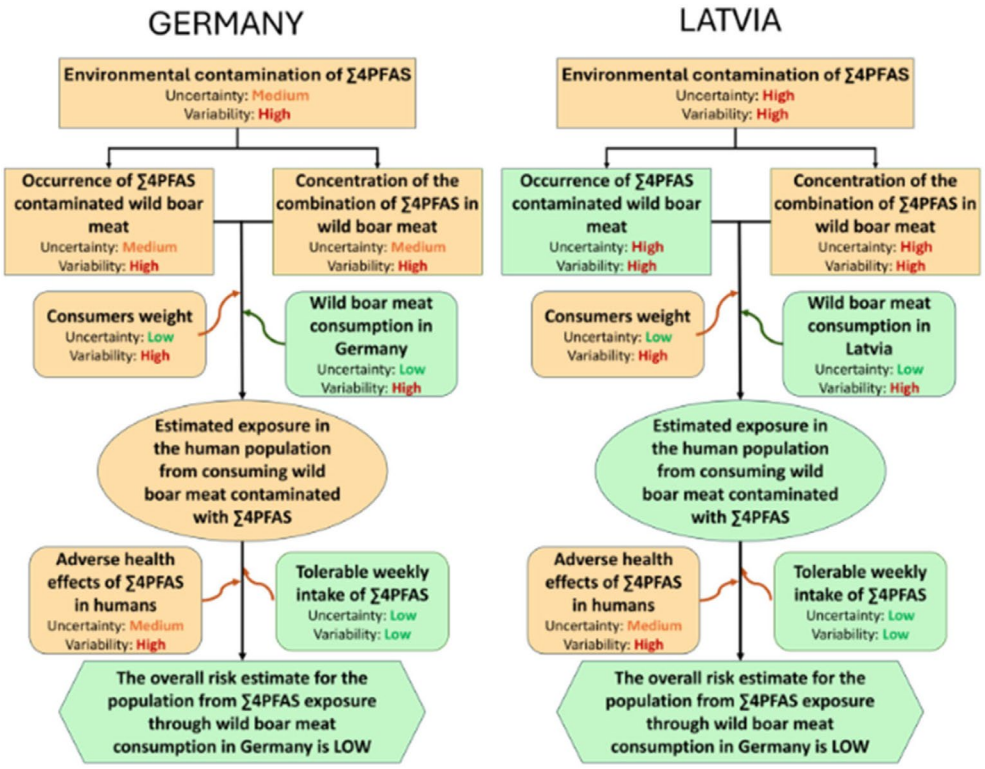

FIGURE 4 Qualitative risk assessment flowchart for Germany and Latvia, for the overall risk estimate for the population from ∑4PFAS exposure through wild boar meat consumption.

As for Latvia, it was more complicated to estimate the ∑4PFAS exposure in the human population from consuming wild boar meat contaminated with PFAS, as there is no publicly available data about PFAS in wild boar meat. However, there is information that PFAS have been detected in wastewater treatment plant influent and effluent (Zacs et al., 2022), European perch from inland waters (Zacs et al., 2025), and some foodstuffs, such as eggs, meat and offal, and fish products (Pasecnaja & Zacs, 2024), as well as around waste landfills in Latvia (Blumfelde et al., 2023). The 'Forever Pollution' project has published a map, which contains information about PFAS levels in various wastewater treatment plants, surface and

groundwaters, and is freely available (The Forever Pollution Project, 2024). Based on the lack of available information about PFAS levels in wild boars, the data uncertainty and variability were assessed as high.

Data about wild boar meat consumption in Latvia is available in the EFSA food consumption database from surveys done from 2008 to 2012, in which, one adult, on average, consumed 0.16 grams of wild boar meat per day, with 212.5 grams of meat being consumed on consumption days (FoodEx2, 2024). However, – as in Germany –there is only a certain percentage of the population which consume venison, whereas the others do not. There is the particular group of hunter households where the consumption is the highest. In these household, venison including wild boar meat, may be consumed almost daily. This group is most at risk and particular children may be a vulnerable group. Because the data is not up to date, the uncertainty was assessed as medium and the variability as high, as there are possible differences from household to household, especially in hunting communities. In analogy with the QRA of ∑4PFAS in Germany, the country-specific average weight of the consumer was added (average of 85.8 kg for men and 69.2 kg for women), with medium uncertainty and high variability. The additional inputs and their uncertainty and variability, to estimate the overall risk assessment for the population from ∑4PFAS exposure through wild boar meat was done the same way as for Germany. The QRA flowchart adapted for Latvia is represented in Figure 4.

Although the overall risk was estimated low for both countries (Figure 4), it may be higher in vulnerable groups, such as hunters' families, children and pregnant women, who either consume more wild boar meat or are more vulnerable to ∑4PFAS exposure (Peterson et al., 2023). The flowchart approach is useful also for chemical risk assessment, as it highlights the main exposure pathway, highlights uncertainties and illustrates the points, where proper interventions could reduce the risk. For PFAS, the potential control points include targeted monitoring of game meat from contaminated regions and environmental surveillance of known hotspots. Furthermore, if wild boars are exposed to elevated PFAS levels in their environment (soil, water or vegetation contaminated by industrial waste or firefighting foams), consumers who frequently eat their meat may face an increased risk of chronic exposure (Peritore et al., 2022). The main difference between the two countries lies in the availability of data on PFAS contamination in the environment and wild boars, as several studies have been conducted in Germany (Stahl et al., 2012; Kowalczyk et al., 2018; Felder et al., 2023; BfR, 2024; Mertens et al., 2025). For Latvia, data are available on PFAS contamination in water bodies, wastewater treatment plants and in pork meat, liver and poultry meat samples, where the ∑4PFAS concentrations ranged from < 0.04 to 0.43 μg/kg (Pasecnaja & Zacs, 2024; Zacs et al., 2022; Zacs et al., 2025). However, these levels are considerably lower than in Germany, where the mean ∑4PFAS concentration in wild boar meat was 33.44 μg/kg with the 95th percentile concentrations being 236.93 μg/kg (BfR, 2021). These findings show that continuous monitoring and good data availability are essential for reliable qualitative risk assessment, which then for PFAS has to include the intake of all other sources.

4 | CONCLUSIONS

The qualitative risk assessment for both *Trichinella* spp. and ∑4PFAS gave a deep insight and highlighted the differences in the way that QRA flowcharts are developed for microbiological and chemical risk assessments. While *Trichinella* represents an acute zoonotic microbiological hazard, PFAS illustrate chronic chemical exposure risk, underlining the importance of tailored approaches to QRA models and flowcharts. This work highlighted how important data availability is for risk assessment. Overall, the EU-FORA fellowship provided the fellow with an excellent opportunity to collaborate with experienced risk assessors at the BfR and to strengthen expertise in qualitative risk assessment.

ABBREVIATIONS

∑4PFAS	sum of perfluorooctanoic acid, Perfluorooctanesulfonic acid, Perfluorononanoic acid and Perfluorohexanesulfonic acid
BfR	The German Federal institute of risk assessment
BIOR	Institute of Food Safety, Animal Health and Environment
bw	body weight
EU-FORA	European Food Risk Assessment Fellowship Programme
kg	kilogram
ng	nanogram
PFAS	Per- and polyfluoroalkyl substances
PFHxS	perfluorohexanesulfonic acid
PFNA	perfluorononanoic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctanesulfonic acid
QRA	qualitative risk assessment
Spp	species
TWI	tolerable weekly intake

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EU-FORA SERIES 8

Risk prioritisation of (emerging) contaminants in aquaculture production

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Abstract

The European Union (EU) food safety legislative framework is designed to guarantee the safety of the entire food production chain through a comprehensive 'from farm to fork' approach. Chemical safety of the food production chain, encompassing contaminants, veterinary drugs and pesticide residues, food/feed additives, is further regulated by a specific legislative framework. Regulation (EU) 2017/625 requires each Member State to ensure that official controls are carried out to verify compliance with food, feed and animal health laws. These controls are to be performed in a risk-based manner through specifically designed multi-annual national control plans. In Norway, where aquaculture represents a highly developed sector, systematic monitoring of chemical contaminants throughout the aquaculture production chain is essential to safeguard food safety and identify potential risks to both public and animal health. Therefore, the present work programme was focused on the development and subsequent application of a ranking methodology for the prioritisation of chemical contaminants to be included in the Norwegian national monitoring programmes for the aquaculture production chain of Atlantic salmon (*Salmo salar*) fillet and complete feed for salmonids. The fellow was involved in the identification of the assessment criteria to be included in the semi-quantitative methodology, the selection of appropriate metrics for each criterion and testing the methodology on the selected contaminants relevant for Norwegian farmed fish production chain. The developed methodology systematically incorporates the assessment of contaminants present in feed materials, fish feed and fish fillet and is intended to provide documented basis for risk-based prioritisation of contaminants for monitoring plans.

KEYWORDS

fish feed, fish fillet, semi-quantitative risk ranking, undesirable substances

CONTENTS

1 | INTRODUCTION

The fellowship programme was performed through the partnership between Institute of Marine Research (IMR, Bergen, Norway) and Istituto Zooprofilattico Sperimentale della Lombardia ed. Emilia-Romagna 'Bruno Ubertini' (IZSLER, Brescia, Italy). The work was conducted at the hosting site (IMR), under the supervision of Dr. Annette Bernhard and the research group of Marine Toxicology.

1.1 | Background

The European Union (EU) food safety legislative framework is designed to guarantee the safety of the entire food production chain through a comprehensive "from farm to fork" approach. The basic EU rules with regard to food and feed law are laid down in Regulation (EC) No 178/2002 (European Commission, 2002) of the European Parliament and of the Council and its application is ensured by official controls implemented through Regulation (EU) 2017/625 (European Commission, 2017). Chemical safety of food production chains, encompassing contaminants, veterinary drugs and pesticide residues, food/feed additives, is further regulated by a specific legislative framework. However, Regulation (EU) 2017/625 states that each Member State should ensure the official controls are performed in a risk-based manner through specifically designed multi-annual national control plans (MANCPs). As a member of the European Economic Area (EEA), Norway must comply with EU food legislation.

In Norway, aquaculture is an important food production industry, which produced about 1.65 million tonnes of aquatic animals in 2022 with Atlantic salmon (*Salmo salar*) being the major farmed fish species (FAO, 2024). Monitoring of chemical contaminants through the whole aquaculture production chain is essential to ensure food safety and identify potential risks for public and animal health. The prioritisation of contaminants to include in the monitoring programmes requires a science-based systematic approach. To this end, multiple risk ranking methodologies have been developed in recent years, and a comprehensive review by Van der Fels-Klerx et al. (2018) provides an excellent overview of the currently available approaches. These approaches are broadly classified in quantitative, semi-quantitative and qualitative methodologies (FAO, 2020). Quantitative risk-ranking methodologies are based on the comparative quantitative risk assessment framework, are sustained by mathematical models and provide numerical outcomes with specific units, e.g. disability-adjusted life years (DALYs). However, the applicability of these methodologies prevalently pertains to the situation where different foodstuffs are ranked for a single contaminant (or a group of structurally similar contaminants) (FAO, 2020). Furthermore, the applicability of quantitative methodology highly depends on the quality of data used as input (e.g. occurrence data, consumption data, health-based guidance values (HBGVs)) in the model. Although these methods are considered "gold standard," the practical application of quantitative methods to rank a set of contaminants for prioritisation across the entire aquaculture production chain remains limited due to restricted data availability, particularly for emerging contaminants. Risk ratio methodologies have been commonly applied for the risk ranking of chemical contaminants in food (Hobé et al., 2023; van Asselt et al., 2024). Risk ratio methodology involves the ranking of the contaminants based on the calculated hazard quotient (HQ) or hazard index (HI) which is the ratio between the estimated daily intake (EDI) and established HBGV, e.g. tolerable daily intake (TDI), tolerable weekly intake (TWI). For genotoxic and carcinogenic compounds, where HBGVs cannot be established, the reference points for potential health concerns (RPHC) can be used, considering benchmark dose levels (BMDL) and taking the appropriate margin of exposure (MOE) into account (EFSA, 2017). However, in the context of prioritising chemical contaminants for inclusion in monitoring programmes, it is necessary to consider not only exposure and health impact endpoints, but also additional criteria such as legal monitoring requirements established by European regulations. Although risk ratio methodologies offer a quantitative framework for ranking contaminants in food matrices based on exposure and toxicological endpoints, their applicability is limited when evaluation criteria cannot be expressed as continuous numerical variables. Qualitative or categorical factors, such as evaluation of input data quality, knowledge gaps, regulatory mandates, are inherently incompatible with purely quantitative ranking approaches, necessitating the integration of complementary methods or multicriteria decision analysis frameworks. For this purpose, scoring systems and decision tree/flow chart approaches are suitable and have been extensively applied (EFSA, 2011, 2012). Decision trees and flow charts are structured approaches developed to classify contaminants in prioritisation categories based on the selected criteria/assessment questions. These methods imply categorization, rely on expert judgement and usually provide ranking of contaminants in predefined "priority groups" (van Asselt et al., 2018). Scoring-based ranking systems represent a semi-quantitative approach to prioritise chemical contaminants by converting qualitative or semi-quantitative information into numerical scores across multiple evaluation criteria (Mathisen et al., 2020). These systems are particularly useful in contexts where complete toxicological or exposure data are lacking, for instance for emerging contaminants, but a structured, transparent method is needed to support decision-making in risk management or monitoring programmes design. Another key advantage of these methods is their flexibility to incorporate non-numerical data, e.g. legal monitoring requirements, which are difficult to integrate in quantitative models furthermore allowing the application of weighting of the criteria for the contribution to the total evaluation.

2 | OBJECTIVES OF THE WORKING PROGRAMME

The overall aim of the present work programme was to acquire knowledge and hands-on experience in the development and application of a ranking methodology for the prioritisation of chemical contaminants to be included in the national monitoring programmes for the aquaculture production chain through the following objectives:

Objective 1: Identification, refinement and definition of the relevant criteria to include in the ranking methodology applicable to the entire aquaculture production chain from feed materials/complete feed to fish fillet of Atlantic salmon.

Objective 2: Application of the developed methodology to the selected contaminants relevant to Norwegian aquaculture of Atlantic salmon with particular focus on the emerging chemical contaminants/groups of contaminants.

Objective 3: Comprehensive analyses of the relevant occurrence data for selected chemical contaminants obtained by IMR from the monitoring programmes for fish feed materials, complete feed and fish fillet in Norway over the past 5 years (2019–2023), with the particular focus on the identification of potential knowledge gaps.

3 | METHODOLOGIES

3.1 | Development of the prioritisation methodology

The fellow screened the available literature regarding different approaches applied in the chemical risk ranking to select the appropriate model to be used for the prioritisation of the contaminants in the risk-based monitoring programmes for aquaculture. For the methodological approach, the following aspects were considered: (i) applicability to the whole production chain starting from feed materials/complete fish feed to the final product of fish fillet; (ii) inclusion of the selected main criteria: (1) public and animal health concerns, (2) monitoring requirements with respect to the legal limits, (3) occurrence data from the previous 5 years of the monitoring programmes and (4) identification of the knowledge gaps with respect to available occurrence database; (iii) applicability to the emerging contaminants with limited data availability. A semi-quantitative approach supported by a scoring system was found applicable to further develop the method. In the subsequent step, the systematic definition of the predefined criteria was included in the assessment and scores were assigned to each criterion. The fellow elaborated questions/criteria with three predefined categorisation answers assigning the relative score for each category ranging from the minimum of zero to the maximum of six depending on the question/criterion. The maximum score to be assigned for each criterion reflected the weight of the criterion to the means of the contribution to the total score. The health concern criterion (evaluation of dietary exposure to the contaminant and fish contribution to the exposure for fish fillet, fish health concerns and potential transfer from feed to fish fillet) was considered as the major contributor with the maximum of six points, followed by the occurrence data outcomes evaluation balanced with the knowledge gaps in the occurrence data with the maximum score of three for each criterion. Additional maximum score of two was assigned for the legal status of the contaminant (established maximum levels). The scores assigned for each criterion are summed to obtain the total assessment score in the range of 2–14. The method was applied separately for the evaluation of fish feed and fish fillet in order to ensure the prioritisation for the monitoring programmes performed at both steps (feed and food) and the coverage of the whole production chain.

3.2 | Application of the developed methodology

To test and validate the applicability of the developed methodology in different scenarios the fellow applied it to a series of contaminants relevant to Norwegian aquaculture.

3.2.1 | Selection of the contaminants

Following contaminants/groups of contaminants were selected:

(i) regulated contaminants with monitoring history: dioxins and PCBs – polychlorinated-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (dl-PCBs), non-dioxin-like polychlorinated biphenyls (ndl-PCBs), regulated per-and polyfluoalkyl substances (PFAS) including perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and perfluorohexanesulfonic acid (PFHxS), potentially toxic elements – cadmium (Cd), mercury (Hg), inorganic arsenic species (iAs) (regulated in feeds), mycotoxins – deoxynivalenol (DON);

(ii) non-regulated contaminants with monitoring history: polybrominated diphenyl ethers (PBDEs), potentially toxic elements – organic arsenic species (oAs), methylmercury (MeHg), nickel (Ni), mycotoxins – enniatins (ENNs);

(iii) non-regulated contaminants with no/limited monitoring history (emerging contaminants): polychlorinated naphthalenes (PCNs), novel brominated flame retardants – dibromoneopentyl glycol (DBNPG), dechloranes.

3.2.2 | Evaluation of the occurrence data

Occurrence data for contaminants in farmed fish production are collected through monitoring programmes for feed materials, complete fish feed and fish fillet, which are annually performed by IMR on behalf of the Norwegian Food Safety Authority (NFSA). The fellow retrieved the occurrence data for the selected contaminants covering a 5-years period (2019–2023) from the laboratory information management system (LIMS).

For each selected contaminant/group of contaminants, the following information was compiled: (i) number of samples analysed for different species of fish fillet (e.g. Atlantic salmon, rainbow trout, Atlantic cod, Atlantic halibut), complete fish feed and feed materials; (ii) geographical distribution of the collected and analysed fish fillet samples; (iii) descriptive statistical analysis of the contaminant concentrations for all the types of samples (fish fillet, complete fish feed and feed materials). The resulting analysis was used for the evaluation of the occurrence data. Specifically, for regulated contaminants the compliance/non-compliance with the established legal limit values in the EU, the mean detected percentage of the established legal limit (exceedance of 20% of the legal limit was considered higher risk) and the decreasing/increasing trends in the detected concentrations through the years were considered. For non-regulated contaminants, the detection of levels above the limit of quantification (LOQ) of the method, increasing trends in the detected concentrations through the years and exceedance of the concentrations reported in the risk assessments performed by EFSA or retrieved through the available literature were evaluated.

3.2.3 | Evaluation of knowledge gaps

For the evaluation of the completeness of the available occurrence database and identification of the knowledge gaps, the number of samples analysed per year, per species and per type of complete feed/feed material, the availability of continuous data through the 5-year period and geographical coverage were considered.

3.2.4 | Evaluation of consumer and animal health concerns criteria

To evaluate consumer health concerns, the fellow conducted a comprehensive screening of the available literature and existing risk assessments published by the European Food Safety Authority (EFSA) for each contaminant or group of contaminants under consideration. For those contaminants with established toxicological reference values, such as HBGVs or MOE, and available risk characterisations, the fellow assessed total dietary exposure and specific contribution of fish to the overall exposure. In cases where no toxicological reference values were defined, the fellow evaluated the available evidence of toxicity and the expected contribution of fish to overall exposure based on findings from the literature search. For the assessment of fish health concerns, the fellow performed several literature searches to identify reported toxicological effects in fish. Additionally, evidence of contaminant transfer from feed to edible fish tissues was taken into account.

4 | OUTCOMES

The primary outcome of the fellowship programme was the development of a semi-quantitative methodology for the prioritisation of contaminants to monitor within the aquaculture production chain. This methodology systematically incorporates the assessment of contaminants present in fish fillets, complete fish feed and feed ingredients, based on the following criteria: (i) established legal limits, (ii) evaluation of occurrence data collected during the monitoring period 2019–2023, (iii) concerns related to consumer and animal health and (iv) identified knowledge gaps in the available occurrence data. Subsequently, contaminants were evaluated against predefined prioritisation criteria. This evaluation yielded ranking lists of contaminants, to provide risk-based prioritisation strategies to design national monitoring programmes (Figure 1).

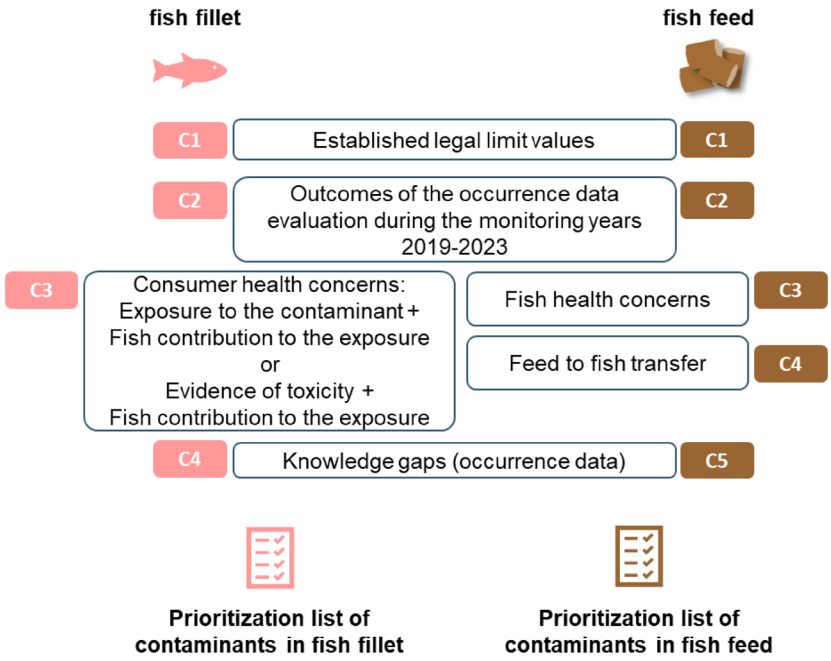

FIGURE 1 Schematic representation of the semi-quantitative methodology developed for the prioritisation of the contaminants in aquaculture production chain (fish fillet and fish feed).

The developed methodology is intended to be published in a peer-reviewed scientific journal (submitted for publication), therefore only a brief description of the main features was provided in the 'Methodologies' section. The same applies to the most of the tested contaminants. The results reported in the present technical report are focused on the application of the developed methodology to the emerging contaminants which are presented as 'case studies' and were not included in the aforementioned publication.

4.1 | Case study – Dechloranes

Dechlorane related compounds (DRCs) are a group of polychlorinated flame retardants used in the electronic, textile, plastic and other manufacturing chains. Dechlorane, also known as Mirex was banned in the USA in the 1970s due to its high toxicity and persistence (Ghelli et al., 2021). The commercial mixture 'Dechlorane Plus' (DP) contains syn- and anti-isomers in an approximate ratio of 1:3 and may also contain mono-adducts (e.g. 1,5-Dechlorane Plus Mono Adduct) and dechlorinated DP (Cl10-Dechlorane Plus, Cl11-Dechlorane Plus). DP was added to Annex A of the Stockholm Convention on Persistent Organic Pollutants in 2023. Dechlorane 601 (Dec-601), Dechlorane 602 (Dec-602), Dechlorane 603 (Dec-603), Dechlorane 604 (Dec-604) are also structurally related chlorinated flame retardants.

Tables 1 and 2 display the application of the developed methodology to the DRCs in fish fillet and fish feed, respectively. The rationale for each criterion is provided concurrently with the assigned score. Both assessments yielded a total score of 8, within the possible range of 2–14. This outcome is primarily driven by the maximum scores assigned to the criteria on health concerns and gaps in occurrence data.

TABLE 1 Application of the developed semi-quantitative risk prioritisation method for the ranking of dechlorane related compounds (DRCs) in fish fillet (Total score = 8/14)

Criteria	Criteria evaluation	Score
C1	No legal limits in fish fillet are currently established for this group of substances in the European legislation	0
C2	None of the DCRs were detected above the LOQ of the method, with the exception of anti-Dechlorane Plus in one fillet sample (17.3 pg/g). Data on the occurrence of DRCs in Atlantic salmon fillet samples available in the literature are limited. L'Homme et al. (2015) reported the LB (lower bound) mean concentration of 4.24 pg/g ww of syn-DP and 1.15 pg/g of anti-DP isomer in eight samples of Atlantic salmon from the Belgian market. Higher concentrations of DRCs were detected in wild salmon from the Baltic region, reporting mean levels of 85.6 pg/g ww for syn-isomer and 159.0 pg/g lw for anti-DP isomer (Rjabova et al., 2016).	0
C3	No toxicological reference values are available for this group of contaminants. Available studies in mammals suggest that DP isomers are not carcinogenic, genotoxic, mutagenic or toxic for reproduction, although the data are limited and carcinogenic potential is not excluded (ECHA, 2020). Acute toxicity is reported to be of low concern (ECHA, 2020). Data gaps are identified for long-term exposure studies. Oxidative stress, liver impairment and endocrine effects were reported in mammals (ECHA, 2020). 'Fish and seafood' is highly likely to be a food category with high contribution to the dietary exposure to the DRCs (ECHA, 2020; Ghelli et al., 2021). However, significant differences may occur between the concentrations detected in wild caught and farmed fish and only few specific data on the occurrence of DRCs in farmed salmon are available (L'Homme et al., 2015)	5 (2 + 3)
C4	The available database is considered not sufficient, only 17 Atlantic salmon fillet samples are available for the year 2024. Novel and emerging brominated flame retardants are part of the annual call for data issued by EFSA for the year 2024 with the Dechlorane 604 mentioned in the priority list.	2 (1 + 1)
Sum		**8**

TABLE 2 Application of the semi-quantitative risk prioritisation methodology to DRCs in fish feed (Total score = 8/14).

Criteria	Criteria evaluation	Score
C1	No legal limits in animal feed are established for this group of substances in the European legislation.	0
C2	Occurrence data from national monitoring programmes are not available.	0
C3	As reported in the drafted risk profile for DP, adverse effects in fish include oxidative stress, neurodevelopmental toxicity, effects on the sex and thyroid hormones pathways in zebrafish, as well as alterations in gene expression indicating immune modulation effects in carp (ECHA, 2020).	3
C4	Evidence of dietary uptake in fish has been reported, but limited studies are available. Tomy et al. (2008) conducted feeding trials with DP in juvenile rainbow trout, which suggested linear accumulation in several tissues for syn-isomer and higher biomagnification factor (5.2) for syn-isomer than anti-isomer (1.9), suggesting higher bioavailability for the syn-isomer. Dietary uptake laboratory experiments were also conducted in common carp suggesting a complex and multifactorial process (Zeng et al., 2014). The muscle was the main organ for contaminants deposition, with the DP and analogs percentages above 60% during the whole experiment, with the maximum of 95% reached at the day 75 (Zeng et al., 2014).	3
C5	Complete lack of data. Moreover, to our knowledge, data on the occurrence of DRCs in fish feed are very scarce in the current literature. Von Eyken et al. (2016) analysed four fish oil samples intended for animal consumption detecting the concentrations of DP isomers and Dec-602, −603, −604 up to 384.2 pg/g (for Dec-602) in crude salmon oil.	2 (2 + 0)
	No monitoring requirements in feed and feed materials are currently issued.	
Sum		**8**

5 | ADDITIONAL ACTIVITIES

5.1 | Additional laboratory activities

During the physical placement at IMR in Bergen, besides the planned activities, an additional learning opportunity was arranged for the fellow. Specifically, the fellow had the opportunity to explore the suspect and non-target screening analysis of contaminants in farmed Atlantic salmon fillet collected from the samples analysed for the monitoring programmes.

Current state-of-the-art analytical techniques for the identification and quantification of contaminants in food and feed, including those from aquaculture production, are primarily based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). These methods are widely employed in routine analysis of samples collected for official monitoring purposes. While these targeted methods offer high sensitivity and selectivity, they are typically limited to a predefined list of analytes and are often constrained by the availability of analytical reference materials, particularly for emerging, not well characterised contaminants. High-resolution mass spectrometry (HRMS) provides an alternative approach, enabling the detection of a potentially unlimited number of compounds (Čelić et al., 2021). Using suspect screening approaches,

HRMS and HRMS/MS data can be matched against databases containing compounds of interest, facilitating the identification of both known (already included in monitoring programmes) and emerging contaminants in fish fillet and fish feed (Ali et al., 2025; Carrizo et al., 2022). This approach offers the possibility to detect previously unrecognised chemical contaminants of potential concern specifically associated with the farmed fish production chain.

The fellow was introduced to different steps of untargeted analysis: (i) sample preparation, (ii) instrumental analysis, (iii) data evaluation. Specifically, five farmed Atlantic salmon muscles samples were extracted and purified through QuEChERS (quick, easy, cheap, effective, rugged, and safe) technique and analysed with ultra-high-performance liquid chromatography (UHPLC) (Vanquish Flex UHPLC system, Thermo Fisher Scientific, USA) coupled to HRMS platform (Orbitrap Exploris™ 120, Thermo Fisher Scientific, USA). Suspect screening analysis on acquired raw data was performed using Compound Discoverer 3.3.SP3 (Thermo Fisher Scientific, USA). Different workflows were applied to investigate the presence of environmental contaminants, including PFAS and pharmaceutically active compounds. The fellow received training on the application of the software to non-target screening analysis and performed part of the data analysis to gain experience and become familiar with the software. The data analysis has not been completed and the data are still under further investigation.

5.2 | Other relevant scientific activities

During the fellowship programme the fellow held several scientific presentations, listed below:

1. Norwegian Scientific Committee for Food and the Environment (VKM), 8 May, 2025, Oslo, Norway – Introduction to the work of VKM. During the meeting the fellow gave a presentation of the work performed during the EU-FORA programme, titled: 'Prioritisation method for risk-based monitoring of contaminants in the aquaculture production chain'.
2. Norwegian Food Safety Authority (Mattilsynet), 9 May, 2025, Oslo, Norway. Presentation: 'Prioritisation method for risk-based monitoring of contaminants in the aquaculture production chain'.
3. EUROTOX 2025, Athens, Greece (14–17 September, 2025). Poster presentation (accepted): 'Developing a ranking methodology for risk-based monitoring of contaminants along the aquaculture production chain'.
4. Submitted scientific paper: Butovskaya, E., Sele, V., Hannisdal, R., Ørnsrud, R., Berntssen, M.H.G., Lundebye, A.-K., van Asselt, E., and Bernhard, A. 'Ranking methodology for risk-based monitoring of contaminants in the Norwegian Atlantic Salmon production chain' (currently under review).

6 | CONCLUSION

During the 1-year working programme, the fellow gained a comprehensive understanding of various available methodologies for risk prioritisation of chemical contaminants, including their advantages and limitations. Furthermore, practical experience was acquired by developing a semi-quantitative ranking methodology for prioritising chemical contaminants to be monitored in the aquaculture production chain, building upon an initial framework provided by the supervising group. Criteria and a scoring system were established for evaluating the contaminant levels, data quality and identification of knowledge gaps. The developed method was then applied to selected contaminants, which enabled the fellow to investigate the complexity of occurrence data collected through the National monitoring programmes in Norway for farmed fish and fish feed. Additionally, the fellow gained practical experience in reading and critically assessing scientific opinions issued by EFSA and other risk assessment bodies. The practical experience was supported by five training modules (7 weeks distributed throughout the year) delivered as part of the EU-FORA Fellowship Programme, covering the full scope of food risk assessment. Overall, the fellow developed substantial expertise in chemical risk ranking, critically engaging with its inherent challenges and acquiring a more comprehensive understanding of the broader risk assessment framework.

ABBREVIATIONS

BMDL	benchmark dose levels
Cd	cadmium
DALYs	disability-adjusted life years
DBNPG	dibromoneopentyl glycol
dl-PCBs	dioxin-like polychlorinated biphenyls
DON	deoxynivalenol
DP	dechlorane plus
DRCs	dechlorane related compounds
EDI	estimated daily intake
EEA	European Economic Area
ENNs	enniatins
HBGVs	health-based guidance values
Hg	mercury

HI	hazard index
HQ	hazard quotient
HRMS	high-resolution mass spectrometry
iAs	inorganic arsenic species
LC–MS/MS	liquid chromatography coupled with tandem mass spectrometry
LIMS	laboratory information management system
LOQ	limit of quantification
MANCPs	multi-annual national control plans
MeHg	methylmercury
MOE	margin of exposure
ndl-PCBs	non-dioxin-like polychlorinated biphenyls
NFSA	Norwegian Food Safety Authority
Ni	nickel
oAs	organic arsenic species
PBDEs	polybrominated diphenyl ethers
PCDDs	polychlorinated-p-dioxins
PCDFs	polychlorinated dibenzofurans
PCNs	polychlorinated naphthalenes
PFAS	per-and polyfluoalkyl substances
PFHxS	perfluorohexanesulfonic acid
PFNA	perfluorononanoic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
QuEChERS	quick, easy, cheap, effective, rugged, and safe
RPHC	reference points for potential health concerns
TDI	tolerable daily intake
TWI	tolerable weekly intake
UHPLC	ultra-high-performance liquid chromatography

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EU-FORA SERIES 8

RBA4SDS – Risk–benefit assessment for sustainable dietary shifts

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

Diets are evolving to cope with the ecological transition and reduce the environmental impact of agri-food systems. The alternatives currently available will influence the nutritional content of diets and will also change consumers' exposure to microbiological and chemical hazards. Health impacts need to be considered to suggest safe, nutritious and sustainable alternatives. Quantifying this overall health impact is now possible using methods of food risk–benefit assessment (RBA), a recognised and well-established approach that integrates scientific knowledge on nutrition, toxicology, microbiology and human epidemiology. The Risk–Benefit Assessment for Sustainable Dietary Shift (RBA4SDS) project aimed to train a fellow to analyse the overall health impacts of dietary transitions towards more climate-friendly diets. This included performing RBA and applying the necessary quantitative tools to evaluate nutritional, microbiological and toxicological risks and benefits. The present project focused on a practical case considering the substitution of meat by insects and plant-based alternatives including lentil and red kidney bean for Croatia, France and Greece. As part of the programme, the fellow was involved in the following activities: (i) understanding key steps of food health RBA, with a focus on nutrition, microbiology and toxicology; (ii) defining and characterising various sustainable diet alternatives, including meat, insect-based and plant-based options; (iii) identifying available and missing data inputs needed for RBA of selected alternative scenarios; (iv) applying RBA methods to assess a chosen alternative scenarios; (v) identifying strategies for further development of RBA methodologies. Within the research group, multi-criteria decision analysis (MCDA) tool was used to rank sustainable options for alternative scenarios. The project enabled the fellow to define different sustainable food alternatives and to identify both available and missing inputs required to perform RBA and to suggest strategies to develop further RBA methodology.

KEYWORDS

alternative proteins, beef meat, food safety, lentil, public health impact, red kidney bean

CONTENTS

SUMMARY

RBA aims to assess beneficial and adverse health impacts associated with the consumption of foods, food ingredients or diets. It is now a recognised and well-established approach that integrates scientific knowledge on nutrition, toxicology, microbiology and human epidemiology. RBA is used to inform food safety policies or to provide dietary advice based on the available scientific knowledge and to prevent food-associated diseases. Significant progress has been made with more than 200 RBAs already performed, demonstrating its feasibility and added value. EFSA has established the basis of the risk–benefit assessment (RBA) method with a guidance published in 2006 and 2010 that was recently revised (2024). EU-FORA Programme has been hosted by the mixed unit INRAE-ONIRIS, named Secalim, located in Nantes, France under the supervision of Prof. Géraldine Boué, awarded by EFSA to the Croatian Agency for Agriculture and Food in Croatia as sending institution. The present work programme aimed to train a fellow to analyse the overall health impact related to dietary transition towards more climate-friendly diets by performing RBA of sustainable dietary shifts and the associated tools needed to assess the risks and the benefits in a quantitative way, related to nutrition, microbiology and toxicology.

Main objectives of this programme were: (i) understanding key steps in food health RBA, with a focus on nutrition, microbiology and toxicology; (ii) defining and characterising potential sustainable diet alternatives, including meat, insect-based and plant-based options; (iii) identifying available and missing data inputs needed for RBA of selected alternative scenarios; (iv) applying RBA methods to assess a chosen alternative scenarios; (v) proposing strategies for further development of RBA methodologies.

The RBA case study in this work extended the model developed through the EFSA-supported NovRBA project (Novel foods as red meat replacers – an insight using RBA methods)that was developed to estimate health impacts of substituting beef meat by insects powder in burgers, linked to seven nutrients, six microorganisms and one chemical. This new version includes two plant-based alternatives, lentil and red kidney bean, for Croatia, France and Greece. Methodologies employed in this research included extensive data search and management, development of ranking tool for sustainable plant-based alternatives, definition of the input variables and considering sources of variability and uncertainty. The fellow became autonomous in using databases to perform RBA on dietary transition towards more climate-friendly diets. This programme helped to better understand the challenges involved in expanding and adapting the existing model for application across additional countries, exposure scenarios and components within the RBA framework.

1 | INTRODUCTION

Concerns over carbon footprint and animal welfare have heightened awareness of the environmental impact of red meat production, intensifying debates in Western countries about reducing red meat consumption. Research has also highlighted public health risks associated with red meat consumption, particularly its processed forms, linked to an increased prevalence of chronic diseases such as type 2 diabetes, cardiovascular diseases and colorectal cancer. Consequently, reducing red meat intake, particularly processed varieties, is considered a key step in public health strategies to lower the incidence of these diseases. However, it remains essential to evaluate health implications of alternative protein sources in order to avoid merely shifting the problem elsewhere.

1.1 | Background and Terms of Reference as provided by the requestor

Secalim is recognised in the field of RBA at the international level and participated in 3 projects founded by EFSA over the past years: RISKBENEFIT4EU (Alvito et al., 2019), NovRBA (Naska et al., 2022) and ALTERNATIVA (Assunção et al., 2024). RBA and multicriteria analysis are highly connected to risk assessment but integrate other scientific domains. In this topic 2 PhD theses were supervised, one devoted to the risk–benefit evaluation associated with the consumption of infant milk in the first 6 months of children's life, and the second one, on the risk–benefit evaluation associated with consumption of red meat. The nominated supervisor has 9 years of experience in food public health RBA and has contributed to all three RBA-related projects at Secalim. Under the NovRBA project (Novel foods as red meat replacers – an insight using RBA methods) in 2019–2022, partnering grant funded by EFSA a RBA model was created associated with the substitution of red beef by Acheta domesticus insect-based powder in ground beef. Methodological framework for the harmonised identification, ranking and selection of nutritional, microbiological and toxicological components in RBA was developed. Calculations in NovRBA project were performed for adult population scenarios in Greece, France and Denmark.

2 | DATA AND METHODOLOGIES

2.1 | Data

Consumption data for minced beef patties were taken from national dietary surveys. The existing model already included French (ANSES, Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (France), 2017) and Greek surveys (Magriplis et al., 2019), while the Croatian data (Sokolić et al., 2024) were newly incorporated into the model. Environmental impact scores were obtained from the French Agribalyse LCI database (eng. Life Cycle Inventory) (Colomb et al., 2015) and Nutri-Score (Scientific Committee of the Nutri-Score, 2022) was calculated for food items listed in the Ciqual database (ANSES, 2024). For plant-based alternatives, toxicological data came from chemical monitoring, while no additional microbiological hazards were included due to absence of data. Data for *Acheta domesticus* were based on a systematic review (Ververis et al., 2022). Estimates of foodborne illness were sourced from the World Health Organization's Global Burden of Disease (GBD) data for *Toxoplasma gondii* and *Salmonella* spp., and from French national data for *Clostridium perfringens*. Dose–response data for nutrition, toxicology, microbiology were obtained from the NovRBA project (Naska et al., 2022; Ververis et al., 2024). Incidences and Disability Adjusted Life Years (DALY) per rate for diseases, as well as population numbers were retrieved from GBD Study for year 2021 (Institute for Health Metrics and Evaluation, 2021) using country-specific data when available.

2.2 | Methodologies

Food alternative scenarios to support sustainable dietary shifts were defined by ranking potential plant-based alternatives based on both nutritional quality (Nutri-Score) and environmental impact (Single Score EF 3.1) using Ciqual and Agribalyse databases. Multi-criteria decision analysis tool (MCDA) was used to calculate a composite Nutri-Eco ranking index.

The methodology for component selection, including nutrients, microbial and toxicological hazards, is described in (Boué et al., 2022). Dose–response models for nutritional components and inorganic arsenic (iAs) were included in NovRBA model, while cadmium (Cd) was assessed as additional toxicological component. For nutrient composition, Ciqual food composition database (ANSES, 2024) was used. For microbiological hazards in patties made from cricket powder a bottom-up approach was applied, using exposure assessment and microbial risk modelling with a threshold model for *Bacillus cereus* and an exponential dose–response for C. perfringens, described in (Ververis et al., 2024).

The RBA methodology followed key steps illustrated in Figure 1 (EFSA Scientific Committee, 2010, 2024). The RBA model employed a probabilistic approach using @Risk® add-in software version 8.1 (Palisade Corporation, Ithaca, NY, USA) with Monte Carlo simulations considering variability and uncertainty. Health impacts of each alternative scenario were quantified using DALYs, a standard metric in food RBA representing years of healthy life lost.

The model chosen for training and further development was on the one created by the NovRBA project, which addressed net health impact of partially or totally substituting the beef in burger patties with cricket powder in the adults of Denmark, France and Greece.

2.2.1 | Computational activities

Different data processing strategies were applied throughout the implementation of the work programme. The first ap-

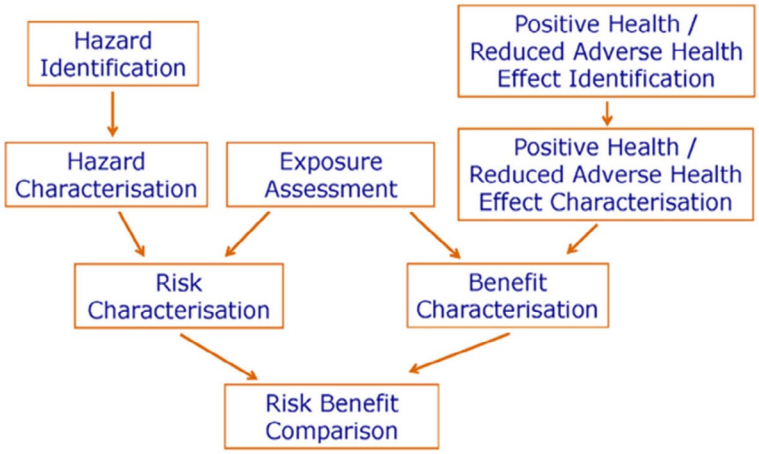

FIGURE 1 Risk–benefit assessment framework (EFSA Scientific Committee, 2024).

proach involved the use of Excel and @Risk, as developed in the NovRBA project, for conducting exposure assessments and DALY calculations. In parallel, a second approach was undertaken using R software, which included an additional toxicological component and code custom-written by Technical University of Denmark (Thomsen et al., 2022). This phase involved gaining familiarity with various computational models related to Cd exposure. Strategies were selected and applied based on the specific objectives and requirements of the research.

2.2.2 | Secondary scientific outcomes

Conferences:

- Alternative Proteins for Food and Feed International Conference, 03–05 December 2024, Berlin, BfR – online participation
- 1st International Congress on Sustainable Food, Green Chemistry and Human Nutrition, 6–9 April 2025, Dubrovnik – lecture *Evaluating the public health impact of plant based alternatives: a risk – benefit assessment to guide the protein transition*
- 14th International Food Data Conference (IFDC), 1–3 September 2025, FAO Headquarters, Rome, Italy – lecture *Integrating food composition and environmental impact data: identifying and ranking sustainable food choices for risk–benefit assessment*

Knowledge acquisition and exchange:

The fellow was involved in multiple activities at the hosting organisation, enrolling in modules of the Master 2 One Health Ermerge at Oniris and including attending presentations of PhD students and working group meetings. The fellow also held a presentation regarding EU-Fora project activities at Secalim Info Science meeting. At 17th Food Consumption Data Network Meeting fellow held a presentation with title *Risk–Benefit Assessment for sustainable dietary shift: Evaluating the public health impact of plant-based alternatives*.

Publications:

The fellow plans to submit two publications to international scientific journals to communicate results and methodologies developed.

3 | ASSESSMENT

3.1 | Definition of the RBA scenario and formulation of the question

Ranking of food items from Ciqual database showed promising substitutes for beef burger patty with priority given to red kidney bean-based or green lentil-based burger patty. To explore alternatives to red meat, four scenarios were proposed: a reference scenario (current beef consumption), an insect-based patty (from NovRBA) and two plant-based alternatives

(Table 1). Final RBA question was defined as 'What would be the net health impact of totally substituting the beef in burger patties with cricket powder or red kidney bean or green lentil in the adult populations of France, Greece and Croatia?'

TABLE 1 Patty ingredients for each scenario.

Ingredients	Amount (%)			
	Reference[a]	A[b]	B[c]	C[d]
Minced beef	90			
Other ingredients	10	10	10	10
Insect powder				18
Lentil, green, boiled/cooked in water		90		
Red kidney beans, boiled/cooked in water			90	
Water				72

[a]Beef.
[b]Lentil, green cooked.
[c]Red kidney beans, cooked.
[d]*Acheta Domesticus* insect powder.

3.2 | Selection of model components and related health outcomes

Methodological framework for the harmonised identification, ranking and selection of nutritional, microbiological and toxicological components in RBA developed in NovRBA was used to select model components for new scenarios (Boué et al., 2022). Each food component was ranked and selected based on its prevalence, the severity of related health outcomes, and the availability and reliability of supporting data. Final list of components included in current study is presented in Table 2.

TABLE 2 Final list of components included in RBA model.

Domain	Components	Minced beef	Lentil, green, cooked in water	Red kidney beans, cooked in water	Insect powder
Nutrition	*Calcium*	X	X	X	
	Fibre		X	X	X
	Iron	X	X	X	
	Magnesium	X	X	X	
	Selenium	X	X	X	
	Sodium	X	X	X	
	Vitamin B12	X			X
	Zinc	X	X	X	
Microbiology	*Toxoplasma gondii*	X			
	Salmonella spp.	X			
	Bacillus cereus				X
	Clostridium perfringens				X
Toxicology	*iAs*	X	X	X	X

3.3 | Exposure assessment

Exposure assessment was done with Monte Carlo simulations to account for variability in concentration of components as well as patty intake by different countries. Cumulative distribution of current intake of beef patties is depicted in Appendix A (Figure A1).

3.4 | Risks and benefits characterisation

Health risks and benefits of food substitution were assessed by combining exposure data with dose–response models across nutrition, toxicology and microbiology. Disease cases were estimated using relative risks, potential impact fractions and national consumption data. A top-down approach used existing disease data and attribution estimates (nutrition and toxicology domain), while a bottom-up approach relied on exposure levels and dose–response models (microbiology

domain). The impact of Cd as toxicological component could not be observed, as the threshold for an adverse outcome estimation was set at 1.0 µg/g creatinine, while substitution a single food source results in urinary cadmium levels below this threshold.

3.5 | Scenario comparison in DALYs

The net public health impact of replacing beef burger patty with low-environmental impact foods such as lentil, red kidney bean or insect powder patties was estimated in DALYs. Shifting from the reference scenario to any alternative one results in notable changes in the intake of the components present in the analysed scenarios. For instance, alternative scenarios led to increased fibre intake, which is associated with a reduced risk of various cancers and cardiovascular diseases (ΔDALY < 0). In contrast, a reduction of iron intake is expected across all alternative scenarios leading to increased risk of oesophageal cancer (ΔDALY > 0). The analysis showed that the most impactful domain in terms of shaping the overall health impact is the one of nutrition (Figure 2).

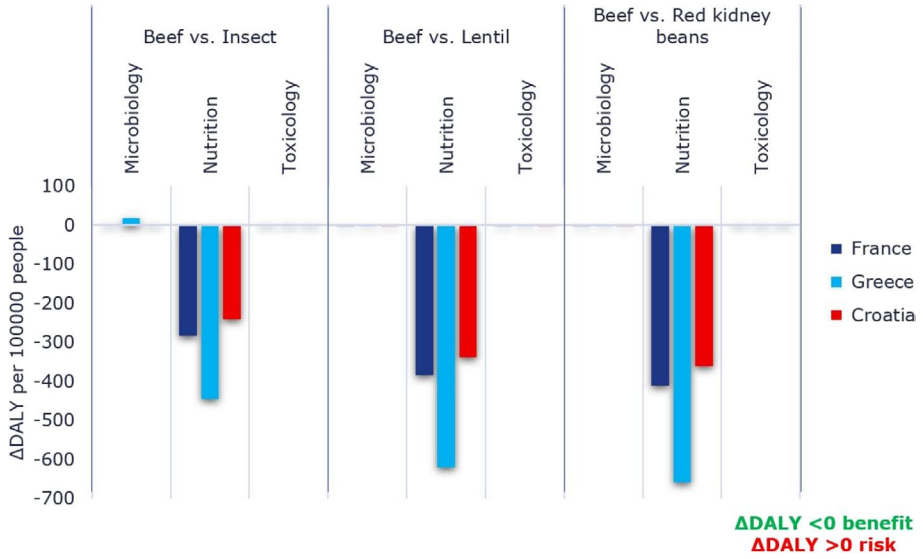

FIGURE 2 Change in disability adjusted life years (DALYs) across seven nutritional components when switching from the reference to alternative scenarios in three countries.

4 | CONCLUSION

Replacing beef burger patties with plant-based alternatives reflects a broader shift towards more sustainable diets. RBA supports this transition by evaluating nutritional and food safety trade-offs, providing evidence-based insights for consumers and policymakers. According to this research, if beef in burger patties was fully substituted with insect powder or plant-based alternatives (red kidney bean or lentil) DALYs will be saved in all countries under consideration.

This research involved structured data management, the development of a ranking tool for assessing plant-based alternatives and the definition of key input variables, incorporating sources of variability and uncertainty. The fellow gained practical skills in using databases to conduct RBAs related to dietary shift. The project also explored methodological challenges in adapting and scaling the existing RBA model to different countries, exposure scenarios and food components.

As part of the EU-FORA programme, the fellow received comprehensive training. Training consisted of an EFSA-led induction course, four modules and practical work in RBA at the host institution, including modelling incorporating Monte Carlo simulations and DALY calculations. This food substitution case study contributed to a broader RBA framework and supported continued collaboration among participating institutions, fellows and the EU-FORA network.

ABBREVIATIONS

Cd	cadmium
DALY	disability adjusted life years
GBD	Global Burden of Disease Study
iAs	inorganic arsenic
LCI	French Agribalyse database Life Cycle Inventory
MCDA	multi-criteria decision analysis
RBA	risk–benefit assessment
RBA4SDS	Risk–Benefit Assessment for Sustainable Dietary Shift

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APPENDIX A

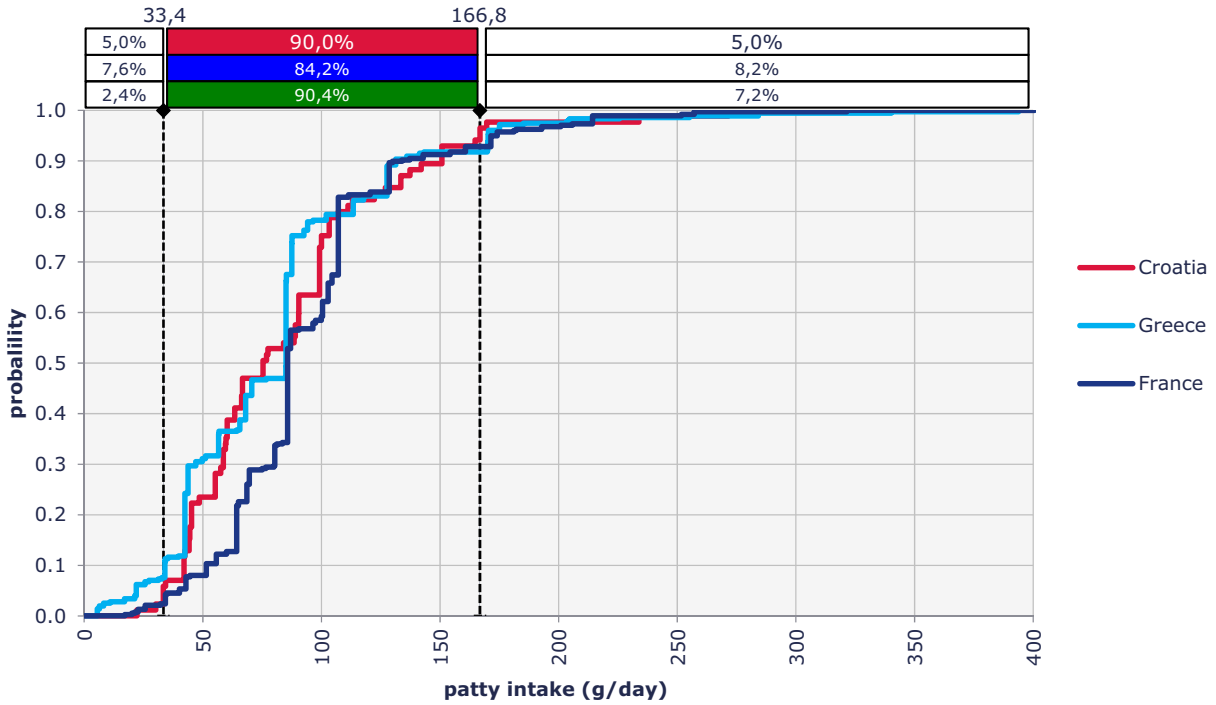

FIGURE A1 Cumulative distribution of current intake of minced beef patties.

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EU-FORA SERIES 8

In vitro metabolomics approaches to decipher the biochemical and cellular response to genotoxic compounds

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

New approach methodologies (NAMs) are gaining increasing attention within the scientific community as strategies to reduce the use of animal testing. Their integration into regulatory frameworks for chemical hazard and risk assessment in food and feed would directly support the goals of the European Food Safety Authority (EFSA). Within the framework of the EU-FORA fellowship programme, this study investigated the use of metabolomic approaches to evaluate cellular responses to genotoxic agents, serving as a proof of concept for the development, standardisation and future regulatory application of metabolomics in genotoxicity testing. Human liver-derived cells (HepRG) were exposed to two well-characterised genotoxicants, and both intracellular (endometabolome) and extracellular (exometabolome) metabolites were extracted and analysed using gas chromatography–mass spectrometry (GC–MS). Metabolites associated with energy metabolism, sugar metabolism and antioxidant capacity were identified, and both multivariate and univariate statistical analyses were applied to characterise metabolic profiles. Results obtained under the experimental conditions tested in this pilot study, do not highlight significant differences between exposed and control cultures. Nevertheless, the findings demonstrate the potential of metabolomics to capture biologically relevant pathways, supporting its future application in genotoxicity assessment under alternative experimental conditions and designs.

KEYWORDS

genotoxicity, in vitro, metabolomics, new approach methodologies (NAMs), risk assessment

CONTENTS

SUMMARY

This study evaluated the potential of metabolomics as a tool to investigate cellular responses to genotoxic substances. Human liver-derived cells were exposed to two genotoxic agents (i.e. methanesulfonate (MMS) and ethyl methanesulfonate (EMS)), and after 24 h of exposure, intracellular and extracellular metabolites were extracted using a liquid–liquid extraction protocol. Metabolite profiling was performed by gas chromatography–mass spectrometry (GC–MS) Data were processed and analysed with bioinformatics and statistical tools. Under the tested conditions, no significant metabolic alterations were detected, suggesting that metabolomics, as applied in this study, may not be sufficiently sensitive to detect the effects of these genotoxic agents. Nonetheless, further exploration of metabolomics under alternative experimental conditions is warranted. As part of the development of new approach methodologies (NAMs), such efforts could contribute to reducing animal testing while supporting reliable safety assessments in the food and feed sector.

1 | INTRODUCTION

A roadmap for action was recently proposed by EFSA identifying priorities before the incorporation of new approach methodologies (NAMs) into regulatory hazard and risk characterisations of chemicals in food and feed (Escher et al., 2022). In the context of genotoxicity assessment, new methods are needed to reduce uncertainties and to strengthen conclusions when applying a Weight of Evidence approach (EFSA, 2017). Generating supportive evidence for the use of metabolomics as a NAM is essential to enhance its acceptance and regulatory uptake, in line with EFSA's goals

2 | BACKGROUND

Metabolomics is the -omics discipline most closely related to classical knowledge of disturbed biochemical pathways. The analytes of interest are the small endogenous molecules produced and/or transformed by cells as a result of cellular metabolism (Lindon et al., 2004; Patti et al., 2012). These metabolites provide a direct snapshot of biochemical activity within a biological system, offering valuable insights into both the functioning of metabolic pathways and their potential disruption. Consequently, metabolomics facilitates the investigation of cellular responses to various stimuli, including xenobiotics (Fiehn, 2002). This approach enables researchers to link changes in the metabolome to specific biochemical processes, providing new insights into the toxicological effects and mechanisms of action of the compounds under study (Bouhifd et al., 2013). Furthermore, recent evidence has shown that DNA damage can be associated with alterations in cellular metabolism (Milanese et al., 2019).

Within the scope of the EU-FORA programme, this project proposed an in vitro metabolomics approach to investigating the biochemical and cellular responses to genotoxic compounds with the aim of improving hazard prediction. Two model genotoxicants, methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS), were selected and their effects studied using the human hepatic HepaRG cell line, a defined and controlled experimental system. This in vitro model was employed to ascertain whether exposure to these compounds induces distinguishable alterations in biochemical pathways compared to untreated controls. By applying metabolomics to evaluate the biological responses to genotoxic agents, the project aims to identify molecular markers of effect that support the biological relevance of the observed changes. This study could lay the foundation for the development, standardisation, and future regulatory application of metabolomics in genotoxicity assessment. This would support the EFSA goal of promoting the integration of NAMs into the regulatory framework for the hazard and risk assessment of chemicals in food and feed.

3 | DATA AND METHODOLOGIES

3.1 | Methodologies

The work programme was carried out within the framework of the European Food Risk Assessment (EU-FORA) Fellowship Programme, involving two institutions: the fellow's home institution, the Toxicology Laboratory at the Faculty of Pharmacy, University of Porto (Porto, Portugal) and the host institution, the Department of Environment and Health together with the Core Facilities at the Italian Institute of Health (ISS) in Rome, Italy. Dr. Francesca Marcon, dr Egidio Iorio and Dr. Paula Guedes de Pinho served as the main supervisors, providing guidance and support in experimental work focused on in vitro genotoxicity and metabolomics.

3.1.1 | Cell line and culture conditions

The human hepatoma HepaRG cell line was obtained from BIOPREDICT (France). Cells were cultured in basal hepatic cell medium (MIL700116, BIOPREDICT) supplemented with 10% fetal bovine serum (FBS) and antibiotics and maintained in a humidified incubator at 37 °C with 5% CO_2. Following an adaptation period, experiments were conducted over the subsequent 20 passages, during which cells were subcultured with fresh medium twice a week.

3.1.2 | Cell viability assay

To select concentrations for genotoxicity and metabolomics experiments, a cell viability assay was performed using the MTT reduction method, as previously described (Valente et al., 2016). Briefly, cells were seeded at a density of 2×10^5 cells/mL in 96-well culture plates. After 24 h of incubation, MMS at concentrations of 0.5, 1, 1.5, 2, 5, 10, 15 and 20 μg/mL, and EMS at 0.5, 1, 1.5, 2, 5, 10, 20 and 40 μg/mL, were added to the wells. Cells were then incubated for an additional 24 h before testing for viability.

3.1.3 | Genotoxicity experiments

The micronucleus test was applied to measure the genotoxic effects induced by MMS and EMS in HepaRG cells treated for 24 h with three concentrations of the model compounds. Cytochalasin B, a substance blocking cytoplasmic division at the end of mitosis, was added to the cell cultures to induce the formation of cells with two nuclei (binucleated cells) (Fenech, 2020). This protocol focuses the analysis of micronuclei to cells divided only once after treatment. The binucleated cells were examined under an optical microscope to count the number of micronuclei present per thousand binucleated cell (OECD TG 487, 2016).

3.1.4 | GC–MS-based metabolomics

HepaRG cells were seeded at an initial density of 5×10^5 cells/mL in T25 culture flasks and incubated at 37°C with 5% CO_2 for 24 h to allow adherence. Culture media without cells (blanks) were maintained under the same conditions. On the following day, cells were exposed to MMS and EMS at concentrations of 0.25, 1 and 4 µg/mL, and incubated for an additional 24 h. Sample collection for metabolomic analysis of intracellular and extracellular metabolites were performed according to established protocols (Amaro et al., 2025; Lima et al., 2018). Four independent experiments using cells from different passages were performed for each condition. For exometabolome analysis, culture media were collected from blanks, controls and HepaRG cells exposed to MMS and EMS. For endometabolome analysis, cells underwent metabolic quenching and metabolite extraction prior to sample collection. Quality control (QC) samples for both intra- and extracellular metabolomic analyses were prepared by pooling aliquots from individual samples and analysed by GC–MS under the same conditions as the experimental samples to assess analytical reproducibility. Analytical-grade distilled water was added to all samples and QCs to facilitate freezing prior to lyophilisation. Samples were then stored at −80°C until analysis. For GC–MS analysis, samples were shipped to Toxicology Laboratory at the Faculty of Pharmacy, University of Porto (Porto, Portugal) on dry ice.

On the day of GC–MS analysis, the internal standard (desmosterol) was added to all samples and QCs, followed by drying under a nitrogen stream. For both endometabolome and exometabolome analyses, derivatisation was carried out following previously described protocols (Amaro et al., 2025; Lima et al., 2018), involving methoximation with methoxyamine hydrochloride (MOX) and silylation using N,O-Bis(trimethylsilyl)trifluoroacetamide +1% Trimethylchlorosilane (BSTFA + TMCS). All samples were injected into the GC–MS system (Bruker 436-GC model coupled to a SCION single quadrupole (SQ) mass spectrometer). The analytical conditions followed those reported in the referenced protocols (Amaro et al., 2025; Lima et al., 2018). Samples were analysed in random order, with QC samples injected every six runs to monitor and ensure analytical performance.

GC–MS data from the endometabolome and exometabolome were converted to netCDF format and analysed using PARADISe software (v6.0.1). Peaks were selected, deconvoluted and tentatively identified by comparing mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library (version 2.2, built in 2014). Identified metabolites were normalised to the total chromatogram area, and contaminants or low-quality peaks (signal-to-noise ratio below 10 and a relative standard deviation (RSD) greater than 30% in the QCs) were excluded. Metabolite annotation was supported by retention index comparison and, when possible, confirmed using commercial standards. Identification confidence followed recommended classification levels (Viant et al., 2017).

3.1.5 | Statistical analysis

Cell viability data were analysed using GraphPad Prism 8 (v8.2.1) with the Kruskal–Wallis test, and results were shown as column bar graphs. Metabolomic data were processed in MetaboAnalyst 6.0, with Pareto scaling applied to reduce the impact of high-abundance metabolites. PCA was used to assess analytical reproducibility and overall metabolic variation, while partial least squares discriminant analysis (PLS-DA) helped identify metabolites contributing to group separation. Volcano plots and Mann–Whitney *U* tests were used for univariate analysis, and significant metabolites ($p < 0.05$) were visualised with boxplots in GraphPad Prism.

4 | ASSESSMENT

4.1 | Cell viability assay

To select appropriate concentrations for the genotoxicity and metabolomics analyses, a range of MMS and EMS concentrations was tested to evaluate their effects on the viability of HepaRG cells using the MTT assay. Cells were also exposed to DMSO at the highest volume used to prepare the MMS and EMS solutions (maximum 0.6%); no significant changes in viability were observed (data not shown). Figure 1A,B present the column bar graphs of cell viability measured following exposure to MMS and EMS, respectively. A statistically significant reduction in cell viability – approximately 16% compared to the vehicle control – was observed at the highest concentration of MMS.

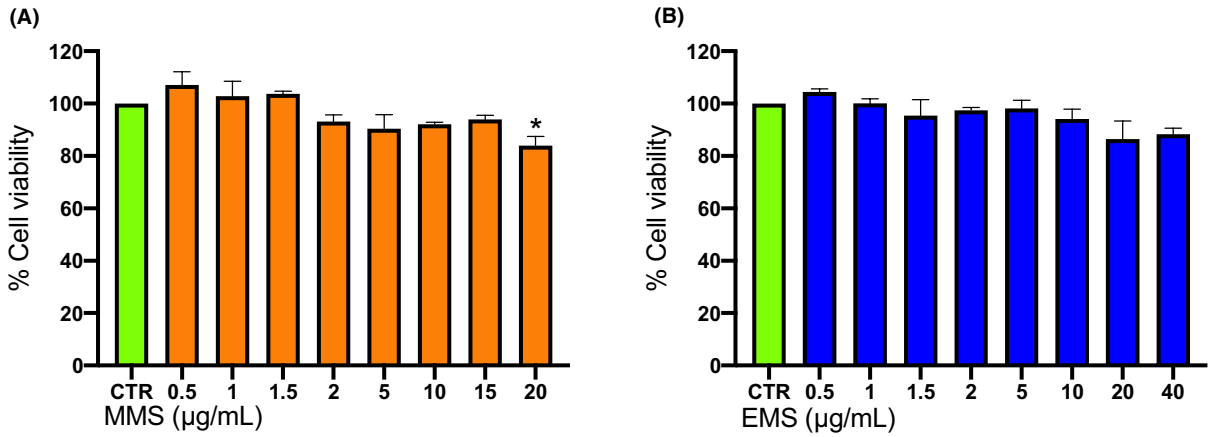

FIGURE 1 Cell viability of HepaRG cells after 24-h exposure to increasing concentrations of methyl methanesulfonate (MMS) (A) and ethyl methanesulfonate (EMS) (B), as assessed by the MTT assay. Data are expressed as mean ± standard deviation from three independent experiments ($n = 3$). Statistical significance was assessed using the Kruskal–Wallis test (*$p < 0.05$).

4.2 | Genotoxicity testing

Three concentrations of MMS and EMS to be used for the experiments of metabolomics were selected based on the results of cytotoxicity and genotoxicity testing. The aim was (i) not to observe cytotoxicity, that could bias the metabolomic analyses and (ii) to induce genotoxic effects at two out of three concentrations. Therefore, based on the results of preliminary experiments on cytotoxicity and genotoxicity, 0.5, 1 and 2 µg/mL of MMS or EMS were tested with the micronucleus assay No significant cytotoxicity was detected up to the highest concentrations tested. Besides, the lowest concentration was negative for the induction of DNA damage, while a statistically significant and concentration-related increase in the frequency of micronuclei was observed at the mid and high concentrations ($p < 0.05$).

4.3 | Metabolic profiling of the HepaRG endometabolome

Metabolic profiling of the endometabolome of HepaRG cells enabled the identification of 36 metabolites, spanning various chemical classes such as amino acids, sugars, organic acids, fatty acids, among others, as detailed in Table A1 (Appendix A). GC–MS analysis demonstrated high analytical reproducibility, as indicated by the clustering of QC samples in the PCA model (data not shown).

To investigate metabolic alterations induced by MMS and EMS exposure, multivariate analysis using PLS-DA was performed. Figure 2A,B shows the overall PLS-DA models comparing controls and MMS and EMS-exposed cells, respectively. Additionally, pairwise PLS-DA models were constructed to compare control samples with each individual concentration of MMS and EMS. These models also failed to show meaningful separation, as indicated by Q^2 values near zero, suggesting low predictive power (data not shown). These results suggest that, under the conditions of this study, the intracellular metabolic profiles of control cells and those exposed to EMS and MMS for 24 h are similar.

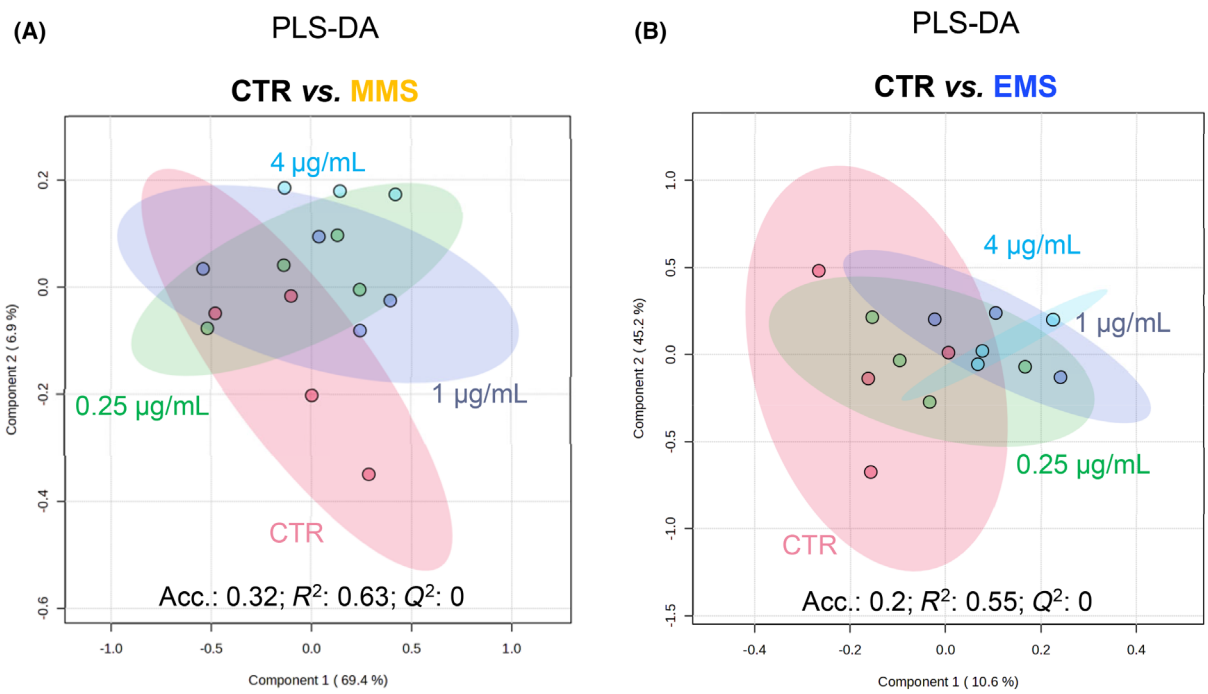

FIGURE 2 Partial least squares discriminant analysis (PLS-DA) score scatter plots of intracellular metabolic profiles of HepaRG cells after 24-h exposure. (A) Control versus methyl methanesulfonate (MMS)-exposed cells; (B) Control versus ethyl methanesulfonate (EMS)-exposed cells. Acc., R^2 and Q^2 values were obtained with two components.

4.4 | Metabolic profiling of the HepaRG exometabolome

Metabolic profiling of the exometabolome of HepaRG cells was also performed. A total of 50 metabolites were identified in the extracellular culture medium of cells (Table A2, Appendix A). Similar to the endometabolome analysis, the GC–MS profiling of the exometabolome demonstrated high analytical reproducibility (data not shown).

Figure 3A,B display the PLS-DA models constructed to provide an overview of the metabolic profiles of extracellular media from control cells and those exposed to MMS and EMS, respectively. No separation was observed in these models, with Q^2 values close to zero, indicating low predictive power. Further pairwise PLS-DA models were generated to compare control samples with each individual concentration of MMS and EMS. However, all models showed weak or no separation (data not shown). Only the comparison with the highest EMS concentration (4 µg/mL) yielded a PLS-DA model with a superior Q^2 value (Figure 4A). Univariate analysis using Volcano plot (Figure 4B) revealed a statistically significant alteration (p-value (FDR) = 0.037) in the level of one metabolite when comparing control cells with those exposed to EMS at 4 µg/mL. No reliable identification was achieved for this compound despite comparison with the NIST database. Figure 4C illustrates the levels of this unknown metabolite in cells exposed to all studied EMS concentrations (0.25, 1 and 4 µg/mL), as well as in the blanks (culture medium without cells). The metabolite was not detected in the blanks or in control cells but was present exclusively in the EMS-exposed samples, with increasing levels corresponding to higher EMS concentrations. This pattern suggests that the metabolite could be a degradation product or derivative of EMS.

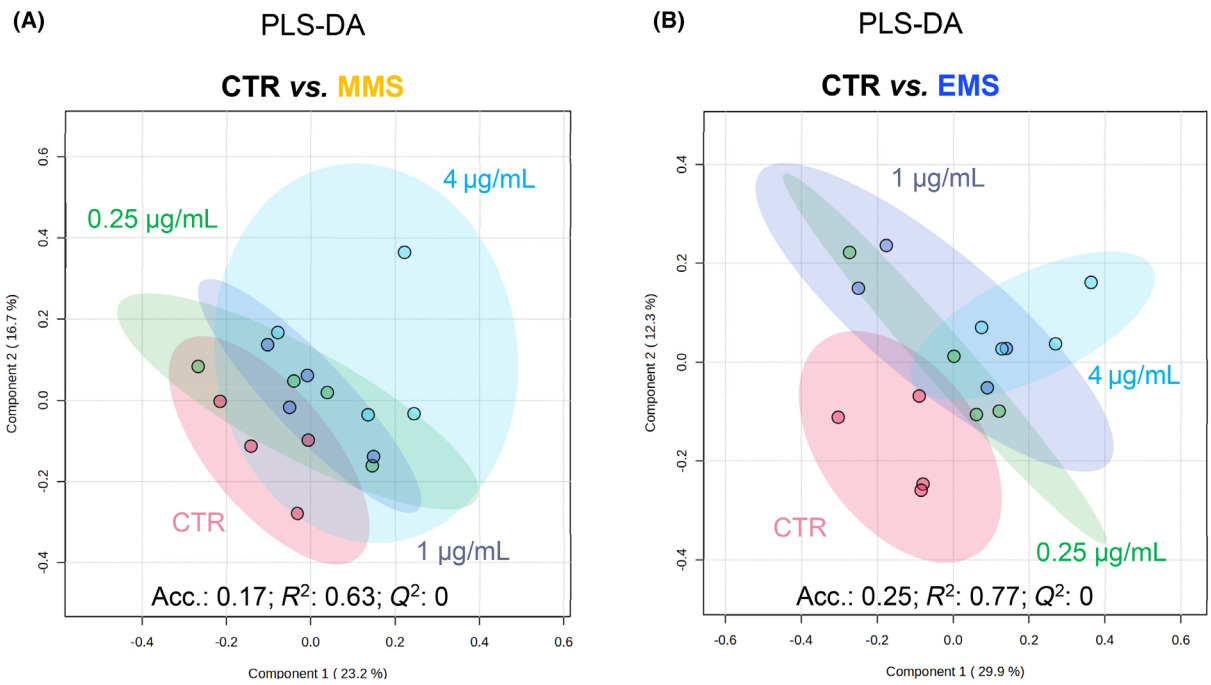

FIGURE 3 Partial least squares discriminant analysis (PLS-DA) score scatter plots of extracellular metabolic profiles of HepaRG cells after 24-h exposure. (A) Control versus methyl methanesulfonate (MMS)-exposed cells; (B) Control versus ethyl methanesulfonate (EMS)-exposed cells. Acc., R^2 and Q^2 values were obtained with two components.

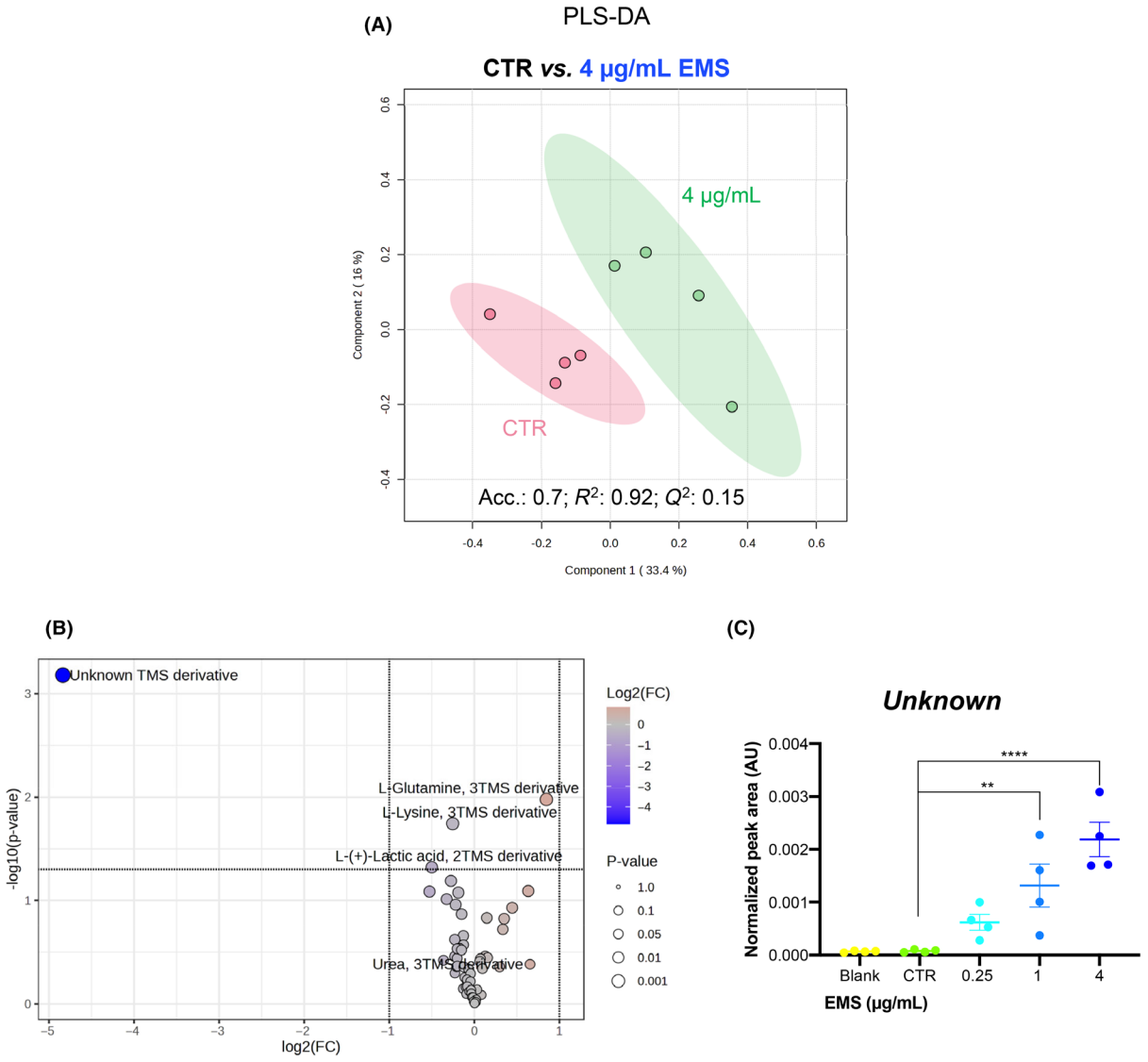

FIGURE 4 (A) Partial least squares discriminant analysis (PLS-DA) score scatter plot of extracellular metabolic profiles of HepaRG cells after 24-h exposure to 4 µg/mL ethyl methanesulfonate (EMS). (B) Volcano plot comparing control HepaRG cells with those exposed to 4 µg/mL EMS. (C) Boxplot of an unknown compound found to be significantly altered between control and 4 µg/mL EMS-exposed cells. Statistical significance was assessed using the Mann–Whitney test (**$p < 0.01$; ****$p < 0.0001$). Acc., R^2 and Q^2 values were obtained with two components.

5 | CONCLUSION

This study used a GC–MS-based metabolomics approach to evaluate in vitro whether two reference genotoxic compounds induced biochemical changes. The aim was to support alternative strategies that reduce the need for animal testing in the risk assessment of food and feed. Concentrations that induced genotoxic effects without causing cytotoxicity were selected. No significant changes in the cellular metabolome were observed under the tested conditions, likely because the 24-h exposure period was insufficient, as metabolomic changes occur downstream of genomic, transcriptomic and proteomic changes. Nevertheless, metabolomics remains a valuable tool as it might reflect the phenotypic consequences of genotoxic stress and provide insights closer to the functional cellular outcome. Further studies involving extended exposure times and different cell types are required to fully assess the potential of metabolomics in genotoxicity evaluation.

ABBREVIATIONS

Acc	accuracy
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
EMS	ethyl methanesulfonate
FBS	fetal bovine serum
GC–MS	gas chromatography–mass spectrometry
HepRG	human liver-derived cells
MMS	methanesulfonate
MOX	methoxyamine hydrochloride
NAMs	new approach methodologies

NIST	National Institute of Standards and Technology
PCA	principal component analysis
PLS-DA	partial least squares discriminant analysis
QC	quality control
RSD	relative standard deviation
RT	retention time
SQ	single quadrupole
TMCS	trimethylchlorosilane

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APPENDIX A

GC–MS metabolomics data

TABLE A1 List of intracellular metabolites identified in HepaRG cells.

Compound name	RT (min)	*m/z*	Match	R match	Prob. (%)	RI_T	RI_E	RSD QCs (%)
Lactic Acid, 2TMS derivative[L1]	5.60	73, 117, 147, 191	932	940	45	1055	1060	19.6
Glycolic acid, 2TMS derivative [L2]	5.80	73, 133, 147, 177	820	904	61.6	1072	1077	53.7
L-Alanine, 2TMS derivative[L1]	6.10	73, 116, 147, 190	927	929	77.2	1095	1103	20.7
L-Valine, 2TMS derivative[L1]	7.24	73, 144, 218, 246	894	903	74.8	1224	1211	8.8
Urea, 2TMS derivative[L1]	7.47	73, 147, 189, 204	607	787	47.5	1243	1234	4.8
L-Leucine, 2TMS derivative[L1]	7.78	73, 147, 159, 232	750	833	40.7	1272	1271	7.3
L-Isoleucine, 2TMS derivative[L1]	8.00	73, 158, 218, 232	924	936	73.3	1301	1287	18.2
L-Proline, 2TMS derivative[L1]	8.07	73, 142, 216, 244	853	885	96.4	1302	1294	19.3
Succinic acid, 2TMS derivative[L1]	8.18	73, 147, 172, 247	894	945	79	1314	1305	8.7
Serine, 3TMS derivative[L1]	8.60	73, 147, 204, 218	877	893	80.5	1342	1352	17.9
L-Threonine, 3TMS derivative	8.84	73, 117, 218, 191	863	921	95.9	1367	1378	9.1
Malic acid, 3TMS derivative[L1]	9.71	73, 147, 233, 245	884	934	96.9	1498	1477	8.1
Meso-erythritol, 4TMS derivative[L2]	9.87	73, 103, 147, 217	796	868	34.2	1500	1494	12.5
L-Aspartic acid, 3TMS derivative[L1]	9.99	73, 100, 147, 232	942	945	97.5	1502	1508	11.6
L-5-Oxoproline, 2TMS derivative[L1]	10.04	73, 147, 156, 230	927	932	80.9	1520	1515	16.8
L-Cysteine, 3TMS derivative[L1]	10.30	73, 200, 220, 294	926	950	81.4	1565	1547	12.8
L-Glutamic acid, 3TMS derivative[L1]	10.80	73, 128, 147, 246	929	934	97.2	1612	1609	13.5
Phenylalanine, 2TMS derivative[L1]	10.90	73, 147, 192, 218	910	919	65.3	1625	1623	7.7
Asparagine, 3TMS derivative[L1]	11.18	73, 116, 188, 231	894	928	87.2	1655	1660	12.2
L-Lysine, 3TMS derivative[L2]	11.48	73, 84, 147, 156	828	842	95.6	1742	1700	36.6
L-Glutamine, 3TMS derivative[L2]	11.96	73, 147, 156, 203	908	912	96.6	1764	1764	21.4
Citric acid, 4TMS derivative[L1]	12.26	73, 147, 273, 347	851	912	92.4	1839	1804	19.9
Sugar derivative 1[L3]	12.62	73, 147, 217, 231	–	–	–	–	1854	13.5
Sugar derivative 2[L3]	12.69	73, 10, 147, 189	–	–	–	–	1865	13.8
Sugar derivative 3[L3]	12.74	73, 147, 189, 273	–	–	–	–	1872	14.7
Sugar derivative 4[L3]	12.83	73, 147, 205, 273	–	–	–	–	1885	12.0
d-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1E)-[L2]	12.80	73, 147, 205, 319	859	916	20.8	1897	1881	27.0
Sugar derivative 5[L3]	12.89	73, 147, 205, 273	–	–	–	–	1895	23.8
L-Tyrosine, 3TMS derivative[L2]	13.12	73, 100, 147, 218	914	963	89.7	1952	1928	10.6
Scyllo-inositol, 6TMS derivative[L2]	13.69	73, 147, 217, 318	747	804	61.7	1999	–	5.0
Palmitic Acid, TMS derivative[L1]	13.83	73, 117, 220, 313	714	770	75	2039	–	16.2
Myo-inositol, 6TMS derivative[L2]	14.11	73, 147, 217, 305	941	941	82.6	2153	–	8.3
Stearic acid, TMS derivative[L2]	15.23	73, 117, 132, 341	866	942	89.2	2186	–	19.3
Sugar derivative 6[L3]	19.20	73, 204, 319, 361	–	–	–	–	–	33.6
Sugar derivative 7[L3]	19.34	73, 204, 319, 361	–	–	–	–	–	33.0
Cholesterol, TMS derivative[L1]	23.22	73, 129, 329, 368	908	908	82.1	2654	–	37.3
Desmosterol, TMS derivative (IS)[L1]	23.63	69, 73, 129, 253	929	929	90.7	2703	–	15.9

TABLE A2 List of extracellular metabolites identified in HepaRG cells.

Compound name	RT (min)	*m/z*	Match	R match	Prob. (%)	RI_T	RI_E	RSD QCs (%)
Lactic acid, 2TMS derivative[L1]	5.61	73, 117, 147, 191	941	947	33.4	1055	1061	18.7
Glycolic acid, 2TMS derivative[L1]	5.77	73, 133, 147, 177	906	942	82.2	1072	1075	11.7
L-Alanine, 2TMS derivative[L1]	6.09	73, 116, 147, 190	919	920	75.2	1037	1103	7.8
Glycine, 2TMS derivative[L2]	6.27	73, 102, 147, 176	928	932	90.5	1105	1119	16.0
Oxalic acid, 2TMS derivative[L2]	6.48	73, 133, 147, 190	702	781	20	1124	1139	38.2
3-Hydroxybutyric acid, 2TMS derivative[L2]	6.67	73, 103, 147, 177	833	934	52.2	1160	1157	12.2
L-2-Aminobutyric acid, 2TMS derivative[L2]	6.80	73, 130, 147, 158	854	887	42.9	1167	1169	6.1
L-Valine, 2TMS derivative[L1]	7.24	73, 100, 144, 218	854	863	77	1234	1211	19.0
Urea, 2TMS derivative[L1]	7.48	73, 147, 189, 204	920	943	93.5	1243	1235	19.0
Unknown, TMS derivative[L4]	7.84	73, 106, 136, 180	918	925	64.2	1274	1271	4.7
L-Isoleucine, 2TMS derivative[L1]	8.00	73, 158, 218, 232	796	826	74.2	1272	1287	39.2
L-Proline, 2TMS derivative[L1]	8.07	73, 133, 142, 216	880	891	97.3	1302	1294	16.5
Glycine, 3TMS derivative[L2]	8.13	73, 147, 174, 248	906	909	86.7	1317	1300	5.1
Succinic acid, 2TMS derivative[L1]	8.18	73, 129, 147, 172	895	954	84.3	1314	1306	9.9
Glyceric acid, 3TMS derivative[L2]	8.30	73, 147, 189, 292	856	940	83	1342	1319	9.9
Uracil, 2TMS derivative[L2]	8.39	73, 99, 147, 241	829	860	80.2	1326	1329	8.4
Serine, 3TMS derivative[L1]	8.60	73, 100, 209, 218	943	944	92	1322	1352	20.1
L-Threonine, 3TMS derivative[L1]	8.83	73, 117, 218, 291	926	955	97.6	1367	1377	17.5
Malic acid, 3TMS derivative[L1]	9.71	73, 147, 233, 245	883	916	93.8	1498	1476	11.5
Meso-Erythritol, 4TMS derivative[L2]	9.86	73, 147, 205, 217	898	915	32.8	1500	1493	9.4
L-Aspartic acid, 3TMS derivative[L1]	9.98	73, 147, 218, 232	878	918	85	1502	1508	11.7
L-5-Oxoproline, 2TMS derivative[L1]	10.04	73, 147, 156, 230	926	933	85.1	1520	1515	4.5
L-Threonic acid, tris(trimethylsilyl) ether, trimethylsilyl ester[L2]	10.14	73, 147, 185, 292	770	861	51	1525	1527	9.2
L-Cysteine, 3TMS derivative[L1]	10.30	73, 100, 147, 220	863	827	75.5	1562	1547	7.8
L-Ornithine, 3TMS derivative[L2]	10.76	73, 102, 142, 348	924	926	96	1596	1604	12.4
L-Glutamic acid, 3TMS derivative[L1]	10.79	73, 128, 147, 246	945	949	96.3	1612	1608	4.1
Phenylalanine, 2TMS derivative[L1]	10.89	73, 147, 192, 218	944	945	79	1625	1622	11.6
Asparagine, 3TMS derivative[L1]	11.18	73, 116, 132, 231	946	948	92.1	1665	1660	16.7
L-Lysine, 3TMS derivative[L2]	11.47	73, 84, 156, 230	874	888	97.7	1742	1699	19.3
L-Glutamine, 3TMS derivative[L2]	11.95	73, 156, 245, 347	850	881	93.5	1755	1762	21.2
Citric acid, 4TMS derivative[L1]	12.25	73, 147, 211, 273	892	896	96.5	1839	1802	10.7
D-(−)-Fructose, pentakis(trimethylsilyl) ether, methyloxime (anti)[L2]	12.62	73, 147, 217, 307	934	934	19.9	1858	1855	7.5
D-(−)-Fructose, pentakis(trimethylsilyl) ether, methyloxime (syn)	12.69	73, 147, 217, 307	839	843	10	1868	1860	8.5
d-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-[L2]	12.80	73, 147, 205, 319	935	955	29	1900	1881	18.1
Sugar derivative 1[L3]	12.90	73, 147, 205, 273	–	–	–	1895	–	10.4
Sugar derivative 2[L3]	12.97	73, 147, 205, 319	–	–	–	1905	–	10.7
D-Mannitol, 6TMS derivative[L2]	13.08	73, 147, 205, 319	906	909	27	1979	1922	8.0
L-Tyrosine, 3TMS derivative[L2]	13.12	73, 100, 218, 280	949	953	94.8	1952	1928	6.7
Sugar derivative 3[L3]	13.18	73, 147, 217, 361	–	–	–	–	1937	14.3
D-Glucose, 5TMS derivative[L2]	13.32	73, 147, 191, 204	926	930	8.4	1970	1959	19.4
Sugar derivative 4[L3]	13.64	73, 204, 217, 319	–	–	–	–	2000	32.5
Palmitic Acid, TMS derivative[L1]	13.83	73, 117, 129, 313	850	876	94.9	2039	–	13.5

TABLE A2 (Continued)

Compound name	RT (min)	*m/z*	Match	R match	Prob. (%)	RI_T	RI_E	RSD QCs (%)
Myo-inositol, 6TMS derivative[L2]	14.10	73, 147, 217, 305	938	939	79.8	2153	–	11.3
L-Tryptophan, 3TMS derivative[L2]	15.01	73, 202, 218, 291	899	935	82.8	2208	–	16.0
Stearic acid, TMS derivative[L2]	15.22	73, 117, 132, 341	757	839	78.2	2236	–	11.1
L-Cystine, 4TMS derivative[L2]	15.63	73, 146, 218, 266	836	847	95.5	2312	–	23.7
D-Lactose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)	19.20	73, 147, 204, 361	924	924	25.6	2658	–	24.4
D-Lactose, octakis(trimethylsilyl) ether, methyloxime (isomer 2)[L2]	19.35	73, 147, 204, 361	926	927	25.7	2674	–	45.0
Cholesterol, TMS derivative[L1]	23.20	73, 129, 329, 368	880	880	76.1	2654	–	28.2
Desmosterol, TMS derivative (IS)[L1]	23.63	69, 73, 129, 253	926	927	89.5	2703	–	17.2

Note: [L1]: identified metabolites (confirmed using a chemical reference standard), [L2]: putatively annotated compounds (NIST14 database), [L3]: putatively annotated compound classes, [L4]: unknown compound. The experimental RI could be calculated up to a RT of 20 min using an alkane standard solution C8–C20.

Abbreviations: IS, internal standard; Prob., probability; RI_E, experimental retention index; RI_T, theoretical retention index (reported in NIST14 database); RSD QCs, relative standard deviation in quality control samples; RT, retention time.

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Computational characterisation of ecological hazard and risk of environmental mixtures

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Abstract

The project *Computational characterisation of ecological hazard and risk of environmental mixtures* focused on two key priorities in advancing a next-generation risk assessment (NGRA) workflow: (i) transitioning to assessing mixtures and (ii) utilising mechanism-based hazard assessment based on new approach methodology (NAM) data. Through a case study, an enhanced component-based mixture risk assessment (CBMRA) framework, integrating high-throughput-screening (HTS) bioactivity data combined with a quantitative adverse outcome pathway (qAOP) approach for hazard and risk assessment was demonstrated. The case study utilised a previously published qAOP based on the proposed AOP-Wiki AOP#245 'Uncoupling of photophosphorylation leading to reduced ATP production associated growth inhibition' (Xie et al., 2018), which models growth inhibition via uncoupling of mitochondrial oxidative phosphorylation from a reference chemical on a model aquatic plant (Moe et al., 2021). It used pesticide monitoring data from the European Environmental Agency's Pesticide Indicator dataset (WISE statistics – Pesticides) in freshwater environments (European Environment Agency, 2023). Relevant bioactivity data from ToxCast and Tox21 were mapped to the target pesticides to derive equipotent mixture compared to the reference chemical used to parametrised the qAOP (i.e. 3,5-dichlorophenol), enabling assessment of the potential initiation of the AOP cascade. In silico methods were used to fill bioactivity data gaps and probabilistic modelling using a Bayesian network (BN) was designed to incorporate various uncertainties into the current NGRA workflow. The study assessed the strengths and limitations of a NAM-based CBMRA, with a particular attention on equipotency assessment as a means to extend the chemical domain of qAOPs for mechanistic ecological risk assessment. It highlighted both conceptual and technical innovations and identified research needs to improve the approach towards future regulatory adoption.

KEYWORDS

adverse outcome pathways, Bayesian network, environmental monitoring data, high-throughput screening, mixture risk assessment, new approach methodologies, pesticides

CONTENTS

SUMMARY

The next-generation risk assessment (NGRA) framework emphasises assessing chemicals and mixtures using mechanism-based hazard evaluation leveraging data from ethically acceptable and cost-efficient new approach methodologies (NAMs). The adverse outcome pathway (AOP) framework, in particular quantitative AOPs (qAOPs), further allows hazard predictions based on NAM data.

The EU FORA work programme (WP) titled *Computational characterisation of ecological hazard and risk of environmental mixtures* investigated a NAM-based component-based mixture risk assessment approach (NAM-based CBMRA), which integrates high-throughput screening (HTS) in vitro bioactivity data, qAOP modelling and in silico gap-filling into the CBMRA framework. It also explored the use of probabilistic modelling, as Bayesian network modelling, to integrate and propagate uncertainty through the process. The WP's specific scientific goals were: (i) conceptualising the NAM-based CBMRA methodology and (ii) applying it to a case study for evaluating strengths, limitations and improvements. The WP's training objectives for the fellow were: (i) mastering ecological risk assessment guidance and cross-cutting methods (i.e. uncertainty analysis); (ii) developing probabilistic modelling skills (e.g. Bayesian networks); (iii) understanding NAMs and AOPs and using related tools/databases; (iv) advancing R programming skills for data handling and modelling; (v) strengthening critical evaluation of strengths and uncertainties in ERA to support decision-making.

The NAM-based CBMRA is a modular, two-part framework, consisting of the equipotent mixture concentration assessment and the qAOP modelling construct. Based on specific assumptions, the equipotent concentration assessment converts the water concentration of each chemical in the mixture into an equivalent concentration of the reference chemical – used to parametrise the qAOP – that is expected to have comparable potency at the molecular initiating event (MIE) of the target AOP. The MIE represents the initial interaction between a chemical and its biological target that can trigger the AOP, and equipotency at this event is determined from relative activity of reference and target chemicals in high-throughput screening (HTS) in vitro assays (i.e. ToxCast, Tox21) informative for the MIE. Summing equipotent concentrations allows assessment of whether the MIE is sufficiently active to trigger the downstream AOP events.

The NAM-based CBMRA requires nine steps: (1) exposure data consolidation, (2) assay endpoint selection from ToxCast/Tox21 database; (3) bioactivity data filtering; (4) bioactivity data coverage assessment; (5) bioactivity data-gap filling (i.e. in silico methods); (6) bioactivity data consolidation; (7) equipotency assessment; (8) equipotent mixture concentration assessment; (9) qAOP-based prediction of adverse outcomes. Uncertainty can be introduced at any step of the process.

The case study considered pesticide concentrations in European freshwaters from the European Environmental Agency's Pesticide Indicator dataset (WISE statistics – Pesticides). It applied qAOP based on the proposed AOP-Wiki AOP#245 'Uncoupling of photophosphorylation leading to reduced ATP production associated growth inhibition', developed by the hosting organisation from *Lemna minor* in vivo exposures to 3,5-dichlorophenol (3,5-DCP). Hazard characterisation used ToxCast/Tox21 data informative for potential inhibition of mitochondrial oxidative phosphorylation (i.e. the MIE of the target AOP), comparing a rich-data assay (aeid 1854) with a poor-data assay (aeid 52) under increasingly strict filtering schemes. The exercise evaluated whether pesticides in European freshwaters pose cumulative risk to *Lemna minor* and it was intended as low-tier assessment for screening, prioritisation and hypothesis-generation.

The findings highlighted the strengths and limitations of using equipotency assessment based on HTS bioactivity data and in silico method to extend the chemical applicability of qAOPs and assess the cumulative impact of mixtures through a specific mode of action (MoA). They also emphasised the importance of bioactivity data availability and the careful selection of reference chemicals in qAOP models.

1 | INTRODUCTION

Conventionally applied methodology on risk assessment of chemicals has fallen out of step with technological advancements and regulatory requirements, challenging effectiveness and accuracy of the assessments (de Jong et al., 2022; Escher et al., 2022; Marx-Stoelting et al., 2023; Sousa et al., 2022). The European Food Safety Authority's (EFSA) vision of a next-generation risk assessment (NGRA) emphasises two pivotal changes: (i) the transition from evaluating individual substances to assessing mixtures and (ii) the move towards a mechanism-based hazard assessments and informative risk characterisation of chemicals by incorporating new approach methodologies (NAM) into the process (de Jong et al., 2022; Escher et al., 2022; Sousa et al., 2022).

NAMs have become important components in NGRA, as these alternative approaches can greatly reduce the needs for conventional laboratory animal testing, fill data gaps and increase cost-efficiencies in chemical hazard assessment. NAMs generate toxicological data for hazard assessments that can be integrated with the biological pathway information through adverse outcome pathway (AOP) frameworks. AOP is a conceptual construct that describe the scientifically credible causal links between a molecular initiating event (MIE., i.e. initial interaction of a chemical with a biological target), a cascade of downstream key events (KEs) at various levels of biological organisation and an adverse outcome (AO, generally defined at the individual or population level) of regulatory concern (Ankley et al., 2010). While qualitative AOPs can already support the use of NAM data in mechanistically based risk assessment, quantitative understanding of the AOPs (i.e. qAOPs) further allows prediction of chemical hazards based on NAM data with uncertainty estimates (Cao et al., 2023; Perkins et al., 2019).

Research efforts worldwide continue to develop new NAMs, establish AOPs and explore their potential for integration within comprehensive risk assessment frameworks and other assessment domains (e.g. environmental surveillance) (Blackwell et al., 2017; Bradley et al., 2019; Corsi et al., 2019; Escher et al., 2020; Loken et al., 2023; Mitchell et al., 2023; Oliver et al., 2023; Schroeder et al., 2016). For instance, the ToxCast and Tox21 in vitro high-throughput screening (HTS) programs have made publicly available data for ~10,000 chemicals in hundreds of bioassays targeting a wide range of mechanistic endpoints like gene expression, enzyme activities, transcription factor activation, in cell-based or cell-free systems (Richard et al., 2021). The AOP knowledgebase (AOP-Wiki; https://aopwiki.org) has been systematically cataloguing AOPs according to a key set of principles and internationally harmonised guidance. Several qAOPs have been published, and research is ongoing to refine methods for their development (Moe et al., 2021).

The present study built on this context and explored the potentials of integrating NAMs in the domain of ecological risk assessment (ERA) and with a focus on a specific methodology: the screening of the cumulative pressure of pollutants in aquatic environments through the application of component-based mixture risk assessment (CBMRA) methodology (EFSA Scientific Committee et al., 2019). It incorporated two advances within the CBMRA framework: (1) HTS bioactivity data and (2) a qAOP for hazard prediction. To enable this integration, the project proposed a NAM-based equipotency assessment method to extend the chemical domain of the target qAOP beyond the original reference chemical used for its parametrisation, therefore making it applicable to potentially any chemicals active in the target MIE, including their mixtures. To further support this, it can employ in silico methods to fill data gaps in the equipotency assessment. Together, these enhancements formed a refined CBMRA approach, hereafter referred to as the 'NAM-based CBMRA'.

The project explored NAM-based CBMRA through an environmentally relevant case study. The methodology for performing the equipotency assessment was fully conceptualised, including the identification of assumptions and the sequential steps involved. Particular attention was given to understanding the uncertainty underlying the equipotency assessment and the possible need to address it using probabilistic modelling. The overall NAM-based CBMRA was critically evaluated to highlight potentials and limitations, and suggest priorities to continue improving in this line of research.

2 | DATA AND METHODOLOGIES

The work programme was conducted within the EU FORA framework at two institutions: the hosting institution, Norwegian Institute for Water Research (NIVA) and the fellow's home institution, Italian Institute for Environmental Protection and Research (ISPRA). It centred on a case study that served both as (i) a hands-on training ground for mastering skills in NAM methodologies and tools in RA and (ii) a proof-of-concept for advancing use of NAM data, AOP frameworks and modelling in CBMRA.

The case study was designed as a 'NAM-based CBMRA' exercise based on a real-world dataset of pesticide concentrations in the aquatic environment. As better detailed below, the study considered the pesticide levels measured in European freshwaters, as reported in the European Environmental Agency's (EEA) Pesticide Indicator dataset, 'WISE Statistics – Pesticides (1990–2021), 2023' (European Environment Agency, 2023). It focused on the proposed (not endorsed) AOP-Wiki AOP#245 'Uncoupling of photophosphorylation leading to reduced ATP production associated growth inhibition' (Xie et al., 2018). A Bayesian network-based qAOP model (BN-qAOP) for this AOP was developed by NIVA (Moe et al., 2021), based on data from in vivo exposure of the macrophyte species *Lemna minor* to the model uncoupler 3,5-dichlorophenol (3,5-DCP), which delineates its applicability domain. The current study used ToxCast and Tox21 bioactivity data relevant for the MIE of this AOP for hazard characterisation. In this context, the assessment addressed whether pesticides with potential for stimulating the target MIE, as 3,5-DCP, pose cumulative risk to the macrophyte *Lemna minor* in European freshwaters. The assessment was intended as a low-tier evaluation for screening, prioritisation and hypothesis-generation purposes.

2.1 | Data

Exposure. We used measured environmental concentrations (MECs) aggregated for sampling year and site reported in the table AggregatedData_Pesticides (downloaded from https://discomap.eea.europa.eu/App/DiscodataViewer/?fqn=[WISE_Indicators].[v4r1].[AggregatedData_Pesticides]). The dataset collects pesticide levels in European surface water and groundwater bodies between 2013 and 2022. Originally, the dataset was compiled and consolidated by EEA with the scope of developing a EU level indicator based on the MECs of pesticides in rivers, lakes and groundwater and assessed against effect thresholds (European Environmental Agency, 2024).

qAOP. We considered the portion of the target AOP (Xie et al., 2018) describing the causal relationships between the MIE of oxidative phosphorylation (OXPHOS) uncoupling, reduction in electron transfer rate (ETR) and the AO of growth inhibition in *Lemna minor*. The qAOP was constructed as a Bayesian network model (BN-qAOP) (Moe et al., 2021).

Bioactivity data. The publicly available U.S. Environmental Protection Agency's (US EPA) database InvitroDB (v4.26) was used as a source to obtain relevant bioactivity data. It is a curated database of HTS in vitro bioactivity data, primarily from the programs ToxCast and Tox21. It compiles results from thousands of chemical assays to assess the biological activity of chemicals across multiple molecular targets and pathways. Data were accessed via the API and R package rcdk (Guha, 2007).

2.2 | Methodology: NAM-based CBMRA

Concept and structure. The structure of the NAM-based CBMRA is represented in Figure 1. NAM-based CBMRA has a modular structure which allows for application to mixtures as well as single substances. It is composed of two sequential parts: (i) the equipotent (mixture) concentration assessment and (ii) the qAOP model.

With specific reference to the case study, the NAM-based equipotency assessment linked to the qAOP calculated the equipotent concentrations (EPCs) of each EEA pesticide by multiplying their MECs by a NAM-based equipotency factor (EPF). The EPF reflected the pesticide's in vitro potency on the MIE (i.e. OXPHOS uncoupling) relative to the reference compound 3,5-DCP. EPFs were estimated using relevant in vitro bioactivity potency data (i.e. ToxCast, Tox21). Then, for each target mixture, individual EPCs were summed to yield a total equipotent mixture concentration (EPMC), which was entered into the qAOP model (more precisely, into the concentration-response model for the MIE).

Besides assuming the concentration addition model, the NAM-based equipotency assessment introduces two additional assumptions within the CBMRA framework: (i) relative in vivo potency is not influenced by toxicokinetics and (ii) relative in vitro potency is not influenced by the test system (cell, organ, species).

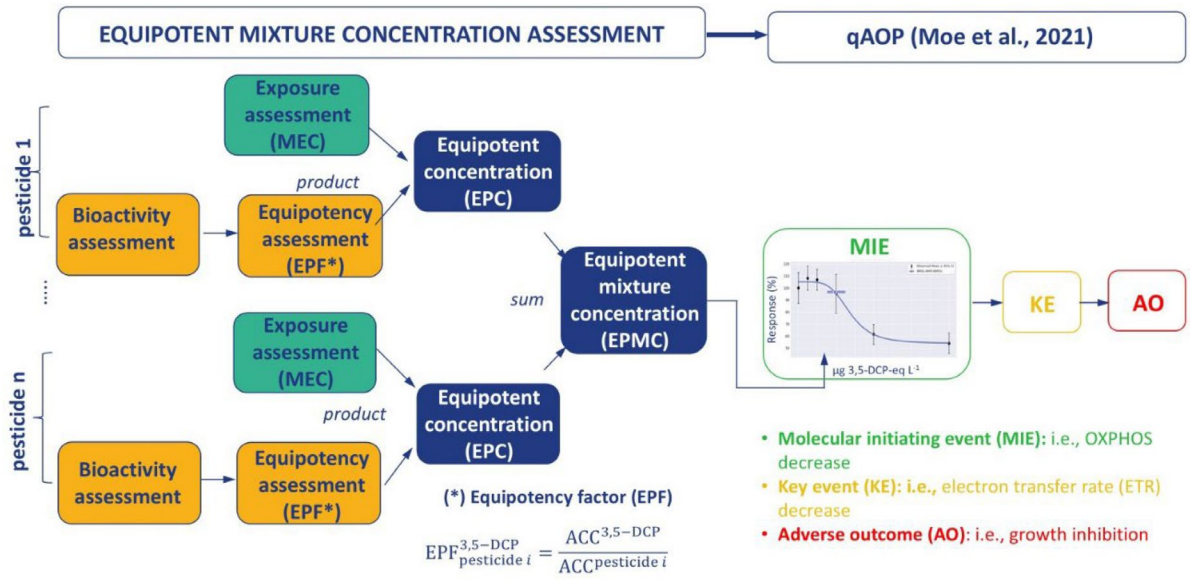

FIGURE 1 Structure of the NAM-based CBMRA applied to the case study.

Stepwise implementation. The implementation of the overall NAM-based CBMRA assessment involved the 9 key sequential steps described below. Data processing and analysis were conducted in R (R Core Team, 2025).

Step 1: EXPOSURE DATA CONSOLIDATION. Levels of pesticides in European freshwaters were downloaded from the Pesticides Aggregated Dataset. Mixtures were constructed by aggregating the maximum annual concentration of each pesticide detected at individual monitored sites. The consolidated exposure dataset referred to 261 pesticides counted 146,418 annual maximum concentration records referring to 21,212 annual mixture samples across 6723 sampling stations over 9 years (2013–2021).

Step 2: ENDPOINT SELECTION. We mapped the assay endpoints available in InvitroDB relevant for the MIE, i.e. OXPHOS uncoupling. Since mitochondrial membrane potential (MMP) has been widely accepted as an indicator of OXPHOS (Song & Villeneuve, 2021), we first selected four MMP assays: Tox21_MMP_ratio (aeid 1854), the APR_HepG2_MitoMembPot_72hr (aeid 52), the APR_HepG2_MitoMembPot_24hr (aeid 32) and the APR_HepG2_MitoMembPot_1hr (aeid 12). For the exercise, we finally used data from two of the assays: i.e. aeid 1854 and aeid 52, representing a rich- and a poor-data case, respectively.
Step 3: BIOACTIVITY DATA FILTERING. We downloaded the entire datasets available in InvitroDB for aeid 1854 and aeid 52. The activity concentration at cut-off (ACC) was selected as the potency metrics. To consolidate the bioactivity datasets, we referred to some quality-related parameters resulting from the ToxCast data pipeline (tcpl) (Filer, 2025), such as 'chid_rep' (identifying the representative sample among analytical replicates), 'fitc' (denoting fit quality categories) and 'Level 6 flags' (highlighting quality issues). Since no standardised method currently exists for filtering InvitroDB data, we applied a sequential filtering strategy to explore the associated uncertainty: Filter 1 performed a basic consolidation of the dataset (basic filtering scheme); Filter 2 excluded borderline records (loose filtering scheme); and Filter 3 additionally removed cautionary flagged records (strict filtering scheme).
Step 4: BIOACTIVITY DATA COVERAGE ASSESSMENT. We mapped the target EEA pesticides onto the aeid 1854 and aeid 52 datasets consolidated according with the three filtering schemes. Under each setting, we identified active and inactive pesticides, as well as data gaps to be addressed in Step 5.
Step 5: BIOACTIVITY DATA-GAP FILLING. In silico methods were used to address missing data within the bioactivity datasets through a stepwise approach that predicts: (1) hitcall (activity vs. inactivity) and (2) the potency (ACC) for the substances predicted to be active. Both steps involved calculating the similarity-weighted activity of selected source analogues (nearest neighbours) using the Jaccard (Tanimoto) index. This strategy is conceptually aligned with to the Generalised Read-Across (GenRA) framework (Beal et al., 2022; Patlewicz & Shah, 2023). The applied methods were selected based on a grid-search aimed at evaluating the performance across different settings of chemicals fingerprints and similarity thresholds.
Step 6: BIOACTIVITY DATA CONSOLIDATION. We merged the InvitroDB- and predicted- bioactivity data for each scenario and we identified the pesticides with the potential for uncoupling activity.
Step 7: EQUIPOTENCY ASSESSMENT. For uncouplers, we computed their associated equipotency relative to the model chemical 3,5-DCP (i.e. EPF).
Step 8: EQUIPOTENCY MIXTURE CONCENTRATION ASSESSMENT. Based on the concentrations in the consolidated EEA pesticide dataset, we computed the equipotent concentrations for the uncoupling pesticides and then their cumulative concentration at mixture level.
Step 9: qAOP-BASED PREDICTION OF ADVERSE OUTCOME. We ran the qAOP model using the equipotent mixture concentration values as input, and we screened their probability to cause adverse outcomes in macrophytes (i.e. growth inhibition in *Lemna minor*).

Uncertainty. Probabilistic BN modelling (Kjærulff & Madsen, 2008) was selected as the method of choice to deal with uncertainty arising in the different components of the NAM-based CBMRA and propagating throughout the whole process. More specifically, a model was designed to integrate uncertainty associated with exposure assessment, NAM-based bioactivity characterisation (arising from the choice of assay endpoints and filtering strategies) and qAOP model. The model was implemented through the software Hugin Researcher v9.5 (Hugin Expert A/S, Denmark).

3 | ASSESSMENT

The case study allowed for an in-depth exploration of each phase of the NAM-based CBMRA approach under a real-life scenario, testing its practicability and identifying areas for improvement. More specifically, the selected assay endpoints enabled the methodology to be challenged under two case-scenarios: a rich-bioactivity data scenario (aeid 1854) and a poor-bioactivity data scenario (aeid 52), thereby better highlighting both strengths and limitations.

Data for aeid 1854 covered a substantial portion of EEA pesticides as well as the reference chemicals and provided a larger pool of source analogues for in silico prediction, enabling data-gap filling and the finalisation of the full assessment. In contrast, data for aeid 52 provided limited coverage of the EEA pesticides and did not include the reference chemical 3,5-DCP. Furthermore, the in silico methods tested in the grid search for predicting bioactivity of untested pesticides performed poorly, preventing their use for filling data gaps. Overall, these limitations prevented the accomplishment of the NAM-based CBMRA.

Bioactivity characterisation showed that assay endpoints for the same biological target can yield different results due to inherent biological differences. For instance, the classification of EEA pesticides as active or inactive, when tested under both aeid 1854 and aeid 52, was partly inconsistent, with aeid 1854 showing more activity. This highlighted that the choice of assay endpoints (aeid) can be a potential source of uncertainty.

The applied filtering strategy did not significantly affect the identification of actives for the tested aeids. In contrast, for aeid 1854, it had a greater influence on the activity-inactivity prediction through in silico method, likely as a consequence of the alteration of the relative proportion of actives and inactives in the source analogue pool. This suggested that filtering, as a source of uncertainty, should be evaluated on a case-by-case basis, as it can strongly depend on the specific composition of data available in InvitroDB.

Screening equipotency of the EEA pesticides relative to the reference chemicals 3,5-DCP was possible only for the aeid 1854, as already mentioned. The equipotency assessment highlighted that some pesticides exhibited even stronger activity at the MIE (i.e. OXPHOS inhibition) than the reference chemical, supporting the mechanistic relevance of the target AOP in pesticides toxicity.

The full aeid 1854 bioactivity characterisation-based CBMRA identified a subset of mixtures with cumulative potential for OXPHOS uncoupling. However, under the applied setup, chosen to reflect relevant assessment conditions (i.e. qAOP, reference compounds and InvitroDB assay), the estimated EPMC values were too low to significantly trigger the MIE. This suggested that pesticide mixtures in European freshwaters are unlikely to pose a significant risk to *Lemna minor* via direct uncoupling of OXPHOS. Nevertheless, this did not rule out the possibility of adverse outcomes mediated by the same chemicals through other MIEs and AOPs.

4 | CONCLUSION

4.1 | Conclusions from the scientific study

The study explored the NAM-based CBMRA as a supplementing methodology for evaluating the cumulative environmental risk posed by pollutants, while providing mechanistic insight into the assessment process. The approach showed promise for low-tier applications, such as screening, prioritisation and hypothesis generation, where higher uncertainty and reliance on assumptions are more acceptable. The study proposed the equipotency assessment based on in vitro bioactivity data as a practical approach to expand the chemical applicability domains of qAOP, which are inherently restricted to the experimental conditions that underpinned their development. Moreover, the applied in silico method was shown to provide a viable strategy for further extending the chemical applicability domain.

The case study findings suggested that the availability of bioactivity data and the ability to effectively fill data gaps through in silico methods are key for the application of the method. In this regards, particular attention should be given to the reference chemical used to develop the qAOP model, whose robust bioactivity characterisation is essential for performing the equipotent assessment.

The uncertainty associated to the whole bioactivity and equipotency assessment adds to existing uncertainty in exposure data (spatial–temporal variability of water concentrations, analytical uncertainty) and in the qAOP itself. Beside assumptions, uncertainty in the equipotency assessment module can arise from the choice of the assay endpoints, filtering strategy and the data-gap filling. The extent of uncertainty should be evaluated on a case-by-case basis, and the use of probabilistic approaches, including the probabilistic Bayesian network modelling, considered.

4.2 | Conclusions from the participation in the fellowship programme

The EU-FORA programme enabled the fellow to achieve five key learning objectives: (i) mastering knowledge on ecological risk assessment guidance and cross-cutting methodologies; (ii) developing probabilistic modelling skills, especially Bayesian networks; (iii) understanding NAMs and AOPs and accessing relevant tools and databases; (iv) advancing R programming skills for data handling and modelling; and (v) maturing critical evaluation abilities to assess strengths and uncertainties of these approaches for decision-making in ERA.

The fellow presented the EU FORA project at the following national and international conferences and meetings:

- Italian Platform on Alternative Method (IPAM) – Annual meeting 2024 – 'Possible applications of "New Approach Methodologies (NAM)" in ecotoxicology', Naples (IT), 3 December 2024 (poster presentation);
- Norwegian Society of Pharmacology and Toxicology (NSFT) – Winter Meeting 2025, Beitostolen (NO), 23–26 January 2025 (poster presentation);
- Society of Environmental Toxicology and Chemistry (SETAC) – 35th Annual Meeting – Europe 'Innovation for Tomorrow: Progress in Safe and Sustainable Concepts', Vienna (AT), 11 May 2025 (poster presentation);
- Presentation of the EU FORA project to representatives of the Norwegian Scientific Committee for Food and Environment (VKM). Oslo (NO), 21 August 2025.

The fellow participated in the following training courses:

- SETAC Training Course on Introduction to In Silico Modeling Approaches for Regulatory Ecotoxicological Hazard Assessment, Vienna (AT), 11–15 May 2025 (physical attendance).
- EFSA Webinar on the open MCRA Tool for prospective cumulative risk assessment, 26 May 2025 (online).
- 16th EAWAG Summer School in Bayesian Methods for Environmental Modelling, Kastanienbaum (Luzern, CH), 15–20 June 2025 (physical attendance).
- AOP-Wiki Virtual Training, presented by the U.S. EPA's Center for Computational Toxicology and Exposure, 18 September 2024 (online).

- SeqAPASS Virtual Training, presented by the U.S. EPA's Center for Computational Toxicology and Exposure, 1 May 2025 (online).
- Recorded trainings on CompTox Chemical Dashboard, ToxCast/InvitroDB and GenRA from the U.S. EPA's catalogue of NAMs training materials (https://www.epa.gov/chemical-research/new-approach-methods-nams-training).

5 | RECOMMENDATIONS

To continue improving the NAM-based CBMRA approach, it is recommended that the development of qAOP considers, from the very start, the actual applicability of the equipotency assessment module to expand the chemical domain. This largely depends on the availability of data that are informative for bioactivity at the MIE, in particularly for the reference chemical used to parametrise the qAOP model. In addition, the use of reference species that are highly responsive to chemical exposure through MIE induction ('MIE/AOP sentinels') would enhance the overall sensitivity of the assessment. Finally, gaining evidence to support the method's assumptions would further increase its acceptability for practical applications.

ABBREVIATIONS

3,5-DCP	3,5-dichlorophenol
ACC	activity concentration at cut-off
aeid	assay endpoint ID (InvitroDB terminology)
AOP	adverse outcome pathway
CBMRA	component-based mixture risk assessment
EEA	European Environmental Agency
EPC	equipotent concentrations
EPF	equipotency factor
EPMC	equipotent mixture concentrations
ERA	ecological risk assessment
ETR	electron transfer rate
HTS	high-throughput-screening
KE	key event
MEC	measured environmental concentration
MIE	molecular initiating event
MMP	mitochondrial membrane potential
MoA	mode of action
NAM	new approach methodologies
NGRA	next-generation risk assessment
OXPHOS	oxidative phosphorylation
qAOP	quantitative adverse outcome pathway
US EPA	U.S. Environmental Protection Agency
WP	work programme

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EU-FORA SERIES 8

Integrating quantitative chemical and microbial risk assessments to optimise the disinfection of fresh products

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

The fellowship, titled 'Integrating quantitative chemical and microbial risk assessments to optimize the disinfection of fresh products' was implemented at the National and Kapodistrian University of Athens (Greece). The fellowship aimed to provide practical training on the quantitative chemical risk assessment of emulsified nano-components used for washing fresh vegetables. This is motivated by the fact that, although a washing step is essential for the safety of fresh vegetables, chlorine-based solutions may have negative effects on the health of workers and consumers. Nanoemulsified D-limonene can thus present an alternative solution with fewer side effects. The fellowship focused in the chemical risk assessment of D-limonene residues after washing. This fellowship provided the fellow with the necessary skills in risk assessment and taught him mass spectrometry techniques for sample analysis.

KEYWORDS

chemical risk assessment, D-limonene, margine of exposure, nanoemulsion

CONTENTS

1 | INTRODUCTION

Foodborne illnesses continue to pose a major societal issue, with food safety remaining a primary concern within the food industry. The risk of contracting such illnesses varies across food categories, with minimally processed and ready-to-eat (RTE) products generally carrying a higher risk (Zwietering et al., 2021). This creates a paradox, as many high-risk food products are also those most recommended for their nutritional benefits. Fruits and vegetables are a prime example: although they are rich in health-promoting bioactive compounds (Agulló et al., 2022; Anand et al., 2008), they have also been implicated in multiple foodborne outbreaks (Hamilton et al., 2006; Possas et al., 2021).

Tomatoes illustrate this dilemma well. They are widely recognised for their nutritional value, being rich in vitamins and bioactive substances (Beecher, 1998), yet they have been involved in several outbreaks of foodborne disease (Bennett et al., 2015; Gupta et al., 2007; Hedberg et al., 1999). A key challenge in ensuring tomato safety is the sensitivity of their quality attributes to heat treatments, which limits the use of thermal pasteurisation methods that are commonly used to ensure food safety. As a result, washing becomes the primary method for reducing microbial contamination on tomato surfaces (Van Haute et al., 2020).

To enhance the antimicrobial efficacy of washing, industries often use sanitising agents – commonly chlorine-based compounds, especially in southern Europe (Banach et al., 2021). However, concerns have been raised about these compounds due to the formation of harmful by-products such as trihalomethanes and chloramines, which pose potential health risks (Coroneo et al., 2017). Consequently, several European countries, including Belgium, Switzerland and the Netherlands, have banned chlorine for decontaminating fresh-cut produce, and others like Spain have recently tightened regulations by lowering the maximum allowable concentration.

These regulatory shifts have driven the search for alternative antimicrobial agents. Among the most promising candidates are essential oils (EOs), which are plant-derived secondary metabolites with demonstrated bactericidal and bacteriostatic properties (Burt, 2004). Their natural origin and classification as 'generally recognised as safe' (GRAS) (Pizzo et al., 2023) make them attractive to the food industry (Battacchi et al., 2020). Nonetheless, their practical use on an industrial scale remains limited because of several challenges. EOs are hydrophobic, which restricts their activity in aqueous environments where most bacteria reside and their intense aromas can affect the organoleptic qualities of food at high concentrations (De Souza Pedrosa et al., 2021).

Recent research suggests that formulating EOs as nanoemulsions can overcome these limitations (Donsì et al., 2011). Nanoemulsification enhances their antimicrobial efficacy by increasing surface area and improving interaction with microbial membranes, potentially leading to membrane depolarisation, altered fluidity and cytoplasmic leakage. These mechanisms contribute to the antibacterial effect of EO nanoemulsions, as documented in various studies (He et al., 2022; Jiang et al., 2025; Majeed et al., 2016; Maurya et al., 2021).

A previous study successfully developed an EO-based nanoemulsion washing solution that served as an alternative to commercial chlorine-based disinfectants for cherry tomatoes, with no observed increase in illnesses related to *Escherichia coli* (Bodea et al., 2023). However, additional research is needed to address remaining challenges before such a solution can be scaled up for industrial use. This study focuses on the chemical risk assessment of these nanoemulsions and especially of the compound D-limonene, to evaluate the safety of these washing solutions for consumers. Different scenarios were evaluated to analyse the transfer of D-limonene from the washing solution to the tomatoes. The margin of exposure (MOE) was calculated for each scenario considered.

2 | DATA AND METHODOLOGIES

2.1 | Data

The project was carried out in two distinct phases. The first phase took place at the Polytechnic University of Cartagena (UPCT) in Spain. During this phase, *Listeria monocytogenes* 4032 and two adapted variants of the same strain to different temperatures (58 and 62°C) from Spanish Collection were used. Nanoemulsions of D-limonene were used for the disinfection of cherry tomatoes inoculated with the wild strain of *L. monocytogenes* and the two adapted variants. The tomatoes were inoculated with the wild strain and with the two adapted variants of the same strain, and washed with nanoemulsified D-limonene solutions. The antimicrobial efficacy of this solution against the wild strain and the two adapted variants was evaluated. This study allowed to estimate too the MIC of D-limonene for all the variants of the studied strains.

In the second phase, which took place in the Department of Chemistry of the Kapodistrian and National University of Athens (Greece), the chemical risk assessment of D-limonene nanoemulsions was developed with the help of spectrophotometric and chemometric analysis. The risk assessment was mainly aimed at evaluating possible critical thresholds in D-limonene values within tomatoes treated with these solutions, as well as searching for other compounds resulting from chemical reactions such as hydroperoxides, peroxides and other reactive radicals. In addition, the chemical risk assessment was developed to evaluate the possible carcinogenicity and genotoxicity of the nanoemulsions due to the presence of high concentrations of D-limonene.

2.2 | Methodologies

During the second phase of the program, which took place at the Department of Chemistry of National and Kapodistrian University of Athens, the fellow learnt new techniques of hybrid mass spectrometry, such as triple quadrupole combined with linear trap spectrometry (QTRAP) tandem with liquid chromatography, as well as dynamic light scattering (DLS) for the particle size analysis of nanoemulsions. He also learnt the use of specific software tools for chemical risk assessment, such as the ImproRisk platform (https://sglcy.shinyapps.io/improrisk/).

2.2.1 | Preparation of nanoemulsions

D-Limonene nanoemulsions were prepared using a high-energy ultrasonic device, following a protocol previously used in similar studies (Bodea et al., 2023) with minor modifications. Briefly, an oily phase consisting of 8 mL of Limonene (Sigma-Aldrich, Steinheim, Germany) and 6 mL of Tween 80 (PanReac AppliChem, Barcelona, Spain), was mixed with an aqueous phase consisting of 13.75 mL of propylene glycol (Guinama, Valencia, España) and 37.5 mL of sterile distilled water. Once the two different phases had been prepared, the aqueous phase was combined with the oily phase and stirred mechanically with a magnetic stirrer for 10 min. After this time, the two phases were homogenous, creating a water-in-oil emulsion. The intention was to reverse the two phases by ultrasonication, creating a oil-in-water emulsion. By applying such energy, thanks to the action of ultrasonic cavitation that breaks the particles into very small sizes, nano-sized oil-in-water emulsions were created, which guarantee their stability. Ultrasonication was applied with a ultrasonic processor (Hielscher UP400St Teltow Berlin, Germany) equipped with an S24d7 sonotrode. The emulsions were subjected to continuous sonication working at a constant amplitude of 100% with a maximum power of 400 W, until the imposed energy limit of 12,000 W-s was reached. The treatment period was set to 3 s with a pause of 1 s between operation times to produce the required disruptive forces.

2.2.2 | Preparation of washing solutions

The washing solutions consisted of a total of 300 mL in which the concentration of D-limonene in nanoemulsion form was 50 mM. To achieve this concentration within the 300 mL, 15 mL of D-limonene nanoemulsions were added to 285 mL of sterile distilled water.

2.2.3 | Washing of cherry tomatoes

The cherry tomatoes were bought from a local supermarket (Cartagena, Spain). For each experiment, three tomatoes (aprox. 30 g) were used in a 1:10 ratio with the washing solution. The tomatoes were immersed in the washing solution and left in it for 5 min. At the end of the 5 min, they were chopped with a blender and were collected with their juice in a 15 mL falcon to analyse the content of D-limonene after washing.

2.2.4 | Analysis of compounds by LC–MS/MS (QTRAP) spectrometry

The samples were analysed after extraction with an organic solvent (hexane) in equal proportions to water (50%). Once the organic components have been extracted from the samples, they were analysed with a LC–MS/MS mass spectrometer (AB Sciex, QTRAP 6500+, Toronto, Canada). Twelve samples were analysed, including a sample of nanoemulsion, a sample of limonene essential acid alone, samples of tomatoes before and after washing treatment, and the washing solutions before and after treatment.

2.2.5 | Chemical risk assessment

Chemical risk assessment through the ImproRisk online platform, which included the Food Consumption values provided by EFSA in Spain (https://www.efsa.europa.eu/en/microstrategy/foodex2-level-7) regarding the consumption of cherry tomatoes in different populations (adolescents, adults and the elderly). The proposed scenarios considered various possible transf ers of D-limonene from the washing solution to the tomatoes, i.e. percentages ranging from 0% to 100%. The concentrations of the D-limonene solutions tested were 50, 100 and 200 mM of D-limonene in the washing solution. For each of these solutions, different transfer scenarios (from 0% to 100%) of D-limonene from the solution to the tomatoes were evaluated. Using the ImproRisk online software, food consumption data for the three populations considered (adolescents, adults and the elderly) were entered. By entering this consumption data, along with the occurrence data values calculated for each percentage of transfer tested, the MOE was obtained. This is an index used by risk assessors to analyse possible safety concerns arising from the presence in food and feed of substances that are both genotoxic (i.e. that can damage DNA, the genetic material of cells) and carcinogenic.

The MOE represents the ratio between two factors and, for a given population, assesses the dose at which a small but measurable adverse effect is first observed and the level of exposure to the substance under consideration. Once the reference point/exposure ratio has been calculated, the result indicates the level of concern associated with the exposure to the substance. The minimum magnitude of the MOE needed for concluding that the actual human exposure is of low toxicological concern differs for the two different categories of substances.

- Substances that are neither genotoxic nor carcinogenic but the uncertainty about their effects does not allow establishing a HBGV – the minimum magnitude of an MOE for these substances is usually 100 or larger, i.e. a ratio that is below 100 would be considered a concern for public health.

Figure of 100 is based on long-standing principles in toxicological risk assessment about how to account for uncertainties and differences among and within species (https://www.efsa.europa.eu/it/topics/topic/margin-exposure#:~:text=Il%20MOE%20%C3%A8%20il%20rapporto,possibili%20timori%20per%20la%20sicurezza). Essentially, a MOE below 100 indicates a risk to consumer health. A MOE between 100 and 500 indicates a moderate risk depending on the substance, and an MOE > 500 indicates a low risk to human health for that substance.

3 | RESULTS

Experimental studies by time-of-flight spectrometry already indicated a very low chemical risk for the D-limonene washing solution, as the concentration of target compounds was below the detection limit of the spectrophotometer set at 70 ppb.

The results of the risk assessment when considering a level of 70 ppb (equal to the detection limit of the analytical method) are illustrated in Figure 1, with Table 1 providing numerical values. Table 1 shows all the tested scenarios. Values in green indicate an MOE > 500, which means a very low risk to human health. Values in orange indicate a MOE between 100 and 500, which means a low but moderate risk. Values in red, on the other hand, are values where the MOE is < 100, which indicates a relatively high risk to human health. Among the scenarios tested, a MOE < 100 was only obtained for the adult population, when exposed to a solution with a 200 mM D-limonene concentration with transfer rates higher than 94%.

TABLE 1 MOE values at different % of D-Limonene transfers to the tomatoes.

Concentration	50 mM	50 mM	50 mM	100 mM	100 mM	100 mM	200 mM	200 mM	200 mM
Population	Adolescents	Adults	Elderly	Adolescents	Adults	Elderly	Adolescents	Adults	Elderly
% transfer	MOE	MOE	MOE	MOE	MOE	MOE	MOE	MOE	MOE
0.1	814,597.6	268,817.2	1,075,269	557,041	187,575	735,294.1	278,172.8	93,684.19	367,242
1	81,459.76	26,881.72	107,526.9	55,704.1	18,757.7	73,529.41	27,821.36	9368.42	36,724.2
5	16,291.95	5376.34	21,505.38	11,140.82	3751.2	14,705.88	5563.59	1873.45	7343.94
7.5	10,861.3	3657.38	14,366.2	7427.21	2501	9803.92	3709.06	1248.97	4895.96
10	8145.98	2743.03	10,752.69	5570.41	1875.75	7352.94	2781.83	936.74	3672.02
12.5	6516.78	2194.43	8602.15	4456.33	1500.6	5882.35	2225.44	749.38	2937.58
15	5430.65	1828.69	7168.46	3713.61	1250.6	4901.96	1854.53	624.48	2447.98
17.5	4654.84	1567.45	6144.39	3183.09	1071.86	4201.68	1589.6	537.27	2098.27
20	4072.99	1371.52	5376.34	2785.2	937.88	3676.47	1390.9	468.36	1835.99
22.5	3620.43	1219.13	4778.97	2475.74	833.67	3267.97	1236.35	416.32	1631.99
25	3258.39	1097.21	4301.08	2228.16	750.3	2941.18	1112.72	374.69	1468.79
50	1629.2	548.61	2150.54	1114.8	375.15	1470.59	556.1	187.35	734.05
75	1086.13	365.74	1433.69	742.72	250.1	980.36	370.91	124.9	489.6
90	905.11	304.78	1194.74	618.93	208.42	816.99	309.09	104.08	408
91	895.16	301.43	1181.61	612.13	206.13	808.02	305.69	102.94	403.51
92	885.43	298.16	1168.77	605.48	203.89	799.23	302.37	101.82	399.13
93	875.91	294.95	1156.2	598.97	201.69	790.64	299.12	100.72	394.84
94	866.51	291.81	1143.9	592.6	199.55	782.23	295.94	99.65	390.64
100	814.6	274.3	1057.27	557.04	187.58	735.29	278.18	93.67	367.2

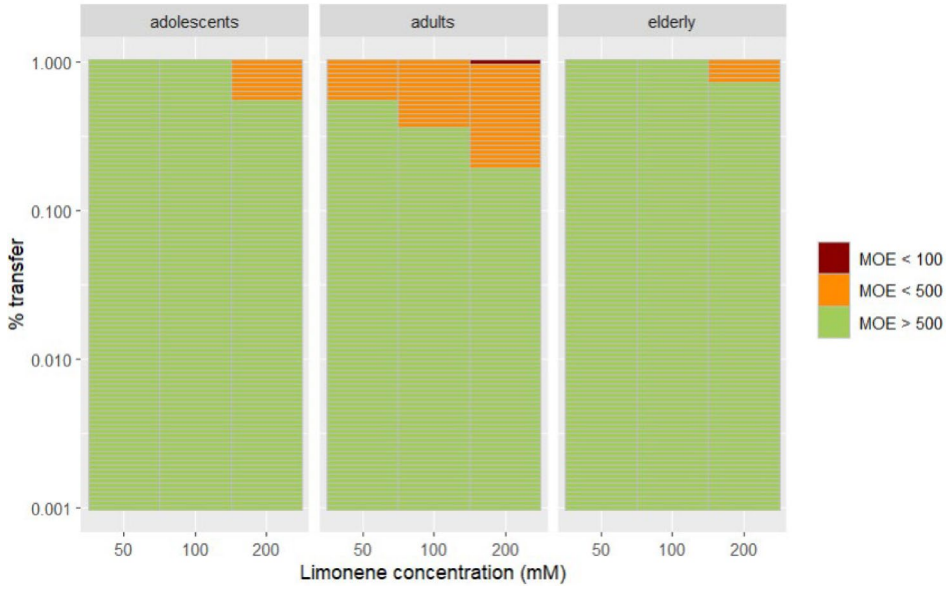

FIGURE 1 Heat map of MOE values of the different scenarios.

4 | CONCLUSION

The EU-FORA program granted the fellow the possibility to develop skills on chemical risk assessment, with hands-on training on the application of experimental techniques (spectrometry) and software applications (ImproRisk). The training revolved around a case study on the chemical risk of cherry tomatoes washed by a nanoemulsified solution of D-limonene. The results show that there is no significant risk of genotoxicity or carcinogenesis attributable to D-limonene. This demonstrates the safety of D-limonene, which is generally recognised as safe (GRAS). These results support these washing solutions as an alternative to current methods for surface decontamination of produce.

ABBREVIATIONS

DLS	dynamic light scattering
EOs	essential oils
GRAS	generally recognised as safe
MOE	Margin of Exposure
QTRAP	triple quadrupole combined with linear trap spectrometry
RTE	ready-to-eat
UPCT	Polytechnic University of Cartagena

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Training in metataxonomics-integrated risk assessment for foodborne pathogens in the Polish and Spanish dairy chain (DAIRYPOL)

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Abstract

Food safety is a key concern in the European Union, with the harmonisation of risk assessment methodologies being a strategic priority. The EU-FORA programme, coordinated by EFSA, promotes standardised approaches to microbial risk assessment across Europe. Metagenomic sequencing has advanced the understanding of microbial ecosystems in food production, with Illumina (short-reads) and Oxford Nanopore Technologies (ONT) (long-reads) playing significant roles in detecting pathogens and characterising microbial communities. These technologies differ in accuracy, read length and resolution, potentially influencing risk profiles when used in quantitative microbiological risk assessment (QMRA). This fellowship, conducted at UPCT in Spain and IBPRS-PIB in Poland, compares the two platforms in assessing microbial populations in Polish and Spanish dairy chains. It explores how sequencing strategy affects hazard identification and QMRA outcomes, while also demonstrating how metagenomic data can enhance predictive modelling and support practical improvements in food safety management.

KEYWORDS

dairy production chains, metataxonomic, quantitative microbial risk assessment, whole genome sequencing

CONTENTS

SUMMARY

Food safety is a critical concern within the European Union, with the harmonisation of risk assessment methodologies being an important priority. The EU-FORA programme, coordinated by EFSA, aims to strengthen cooperation and promote the standardisation of food safety risk assessment practices across Europe. One of these areas of interest is the integration of metagenomic sequencing technologies into microbial risk assessment, transforming the understanding of microbial ecosystems in food production. Sequencing platforms such as Illumina and Oxford Nanopore Technologies have become essential tools for identifying pathogens, resistance genes, and microbial community dynamics. However, these platforms differ significantly in read length, accuracy, throughput and resolution, potentially introducing variability into microbial risk profiles, particularly when applied in quantitative microbiological risk assessment (QMRA).

This fellowship was developed jointly by UPCT in Spain and IBPRS-PIB in Poland, two institutions with extensive expertise in food safety and biotechnology. This technical report presents the work the fellow developed to establish a comparative evaluation of the Illumina and Oxford Nanopore sequencing platforms in analysing microbial populations across Polish and Spanish dairy production chains. It investigates how variations in sequencing strategies may impact the accuracy of microbial hazard identification and the outputs of downstream QMRA models. The findings will contribute to improved food safety management protocols in the dairy sector and beyond.

1 | INTRODUCTION

Advances in molecular microbiology have revolutionised the detection and characterisation of microbial communities in food environments. There are different sequencing approaches used in microbial analysis, each with distinct features and applications. Whole genome sequencing (WGS) provides the complete DNA sequence of an individual organism, allowing for precise identification, strain-level resolution and detection of virulence and antimicrobial resistance genes. It is highly informative but typically requires pure isolates. Metagenomic sequencing, or shotgun metagenomics, sequences all DNA present in a sample, facilitating taxonomic and functional characterisation of complex microbial communities without the need for culturing. While metagenomics is powerful for exploring microbiomes and detecting pathogens in mixed samples, it is more computationally demanding.

A third approach, 16S ribosomal RNA (rRNA) gene sequencing has become a frequent tool for identifying bacterial populations (metataxonomics) in complex matrices like food products and processing sites. This conserved genetic marker contains hypervariable regions facilitating identification at genus-level and, sometimes, species-level resolution. As a culture-independent method, it detects both culturable and non-culturable microorganisms, which is crucial for comprehensive food safety assessments.

16S rRNA sequencing remains a widely used (Johnson et al., 2019). In dairy production, it can help to effectively characterise the microbial composition of raw materials and processing environments. Dairy microbiota includes beneficial lactic acid bacteria, spoilage organisms and opportunistic pathogens (Quigley et al., 2012), all influencing product safety and quality. As genomic tools become routine in dairy safety monitoring, understanding the strengths and limitations of 16S sequencing is essential for protocol standardisation and improved risk assessments. This is especially relevant across countries like Spain and Poland, where variations in processing, hygiene and climate shape microbial profiles.

Despite its advantages, 16S rRNA sequencing has limitations. Short-read platforms like Illumina MiSeq usually target 1–2 variable regions (e.g. V3–V4), limiting resolution and risking misclassification of closely related taxa at species level. Long-read platforms like Oxford Nanopore Technologies (ONT) cover near full-length genes, improving resolution but introducing higher error rates and interpretation challenges (Ciuffreda et al., 2021). Additionally, DNA from dead cells may inflate risk estimates unless paired with viability assays (Emerson et al., 2017), one of the issues for accurate QMRA (Quantitative Microbiological Risk Assessment) exposure and risk characterisation. Thus, platform choice and methodological differences can significantly alter microbial community profiles and hazard identification outcomes.

QMRA provides a structured, science-based approach to estimate and manage microbial risks along the food chain. It includes hazard identification, exposure assessment, hazard and risk characterisation. Applied to spoilage (Koutsoumanis et al., 2021), QMRA helps the food sector to estimate the probability that a product becomes unacceptable due to microbial growth, helping with shelf-life decisions and reducing waste. Probabilistic models simulate spoilage under varying conditions and identify microbial control points. While traditionally used for pathogens, QMRA is increasingly applied to spoilage microorganisms, enhancing risk-based decision-making. Integrating molecular methods, especially metagenomics, improves precision in hazard identification, vital for estimating prevalence and assessing risk.

In this project, we investigate how the platform of choice (short-read Illumina or long-read ONT) affects the 16S-based pathogen hazard identification and QMRA results in Polish and Spanish dairy chains. Ultimately, this work will contribute to the development of a harmonised risk assessment across Europe.

2 | BACKGROUND AND TERMS OF REFERENCE

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. The fellowship project, entitled Training in metagenomics-integrated risk assessment for foodborne pathogens in the Polish and Spanish dairy chain (DAIRYPOL), was developed through a partnership between the Institute of Agricultural and Food Biotechnology-State Research Institute (IBPRS-PIB)(Poland) as the sending institution of the fellow Dr. Joanna Bucka-Kolendo, and the Technical University of Cartagena (UPCT) (Spain) as the hosting institution, with Dr. Enriqueta Garcia-Gutierrez and Prof. Pablo S. Fernández as supervisors.

3 | DESCRIPTION OF WORK PROGRAMME

3.1 | Aims

The fellowship was a multidisciplinary, hands-on training combining theory and practice in food safety, bacterial genomics analyses and microbial risk assessment. Its goal was to examine how the choice of sequencing strategy (short-read Illumina vs. long-read Oxford Nanopore) can affect the microbial hazard detection in dairy environments and the resulting impact on QMRA outcomes.

3.2 | Activities

At the start of the fellowship, the fellow underwent training at the UPCT, focusing on microbiological risk assessment methodologies and software used by food safety authorities, industry and academia. The training covered databases, statistical tools for experimental design and data interpretation, and UPCT-developed web platforms such as bioinactivation, biogrowth and bioOED.

3.3 | Laboratory experience

Following the initial theoretical phase, the fellow conducted experimental work. Sampling was conducted in two factories, one in Murcia (Spain) and one in Warsaw (Poland). Swabs were collected on surfaces at different points in the cow farm and in the dairy-processing factories. Finally, final products from both companies were collected and analysed. Subsequently, DNA was extracted and sequenced, applying next-generation sequencing (NGS). The fellow expanded her expertise by using the Oxford Nanopore MinION, complementing prior experience with Illumina platforms. Sequencing microbial DNA from milk, dairy products and surfaces allowed her to assess how platform choice affects taxonomic resolution and microbial community profiling. These activities generated key data for predictive model development and deepened her understanding of how experimental design affects variability and uncertainty in microbial risk assessment.

The resulting data supported the construction and validation of predictive models describing microbial behaviour in the dairy chain. Using R and the bioinactivation, biogrowth and biorisk packages, both sequencing datasets were integrated to examine how platform-related differences impact growth predictions and spoilage risk estimates. This process also strengthened the fellow's skills in model development, selection, validation and the integration of uncertainty.

In the final phase, the fellow implemented a full QMRA for dairy production in Poland and Spain, defining scope, identifying contamination pathways and quantifying hazards using both experimental and public data. The models enabled comparative risk assessments, showing how regional differences in production, environment and hygiene affect consumer exposure and risk. This cross-country comparison helped contextualise findings within EU-wide food safety goals.

4 | DATA AND METHODOLOGIES

The fellowship combined experimental, bioinformatics and quantitative modelling approaches to assess how different WGS strategies affect microbial hazard identification and QMRA outcomes in the dairy production chain.

4.1 | Sampling in polish and Spanish dairy environments

The primary data consisted of microbial samples collected from raw milk, dairy products at various processing stages, and contact surfaces in farms and production facilities across Spain and Poland. These samples reflected typical environmental and product-related microbiota in dairy settings. DNA was extracted using standardised protocols optimised for dairy matrices and surfaces. Sequencing libraries were then prepared following Baker's protocols (https://quadram.ac.uk/wp-content/uploads/2021/06/5.-16S-Illumina-Library-Preparation-Protocol-v1.0.pdf) for two platforms: Illumina MiSeq (short-read, high-accuracy) and ONT MinION (long-read, portable). Using both platforms enabled comparison of taxonomic resolution and community composition biases.

4.2 | Bioinformatics analyses and predictive modelling

Sequencing data were processed using standard pipelines: Illumina reads via QIIME2 (DADA2, SILVA/Greengenes), ONT reads via MinKNOW, Guppy, EPI2ME and NanoCLUST. Special attention was given to ONT error correction and taxonomic resolution. Comparative analysis focused on community structure, diversity indices and detection of key pathogens and spoilage organisms, assessing cross-platform consistency.

The sequencing-derived microbial data were integrated into predictive models describing microbial behaviour in the dairy supply chain. Metataxonomics abundance data were translated into location mapping to evaluate the changes across the production lines.

4.3 | Quantitative microbiological risk assessment (QMRA)

The QMRA framework followed the Codex Alimentarius four-step process: hazard identification, exposure assessment, hazard characterisation and risk characterisation. Both deterministic and probabilistic methods were applied. Exposure models estimated microbial loads at consumption, accounting for contamination sources, processing/storage growth and surface cross-contamination. They included variability in storage times, temperatures and hygiene practices in Polish and

Spanish dairy chains. Risk characterisation results quantified consumer exposure and the probability of adverse health effects or spoilage.

4.4 | Methodological integration

This project highlights the strong influence of NGS strategy on microbial community detection and its downstream effects on QMRA. Combining both platforms may offer the most comprehensive QMRA outcomes. QMRA models should account for sequencing biases, and regulatory frameworks could benefit from hybrid approaches and cross-validation to ensure reliable food safety evaluations. Further innovation of the work programme was the integration of metagenomic data, traditionally used for descriptive microbial ecology, into predictive microbial risk models. This interdisciplinary approach will bridge the gap between molecular microbiology and quantitative food safety assessment, enabling a more nuanced understanding of how sequencing methodologies influence hazard identification and risk estimates.

4.5 | Secondary scientific activities during the fellowship

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

- The fellow attended an online Academy of the Manager organised by Horizon Europe Framework Programme (HE) National Contact Point for EU Research Programmes at the National Centre for Research and Development together with the network of Horizontal Contact Points on 18.9.2024–14.11.2024.
- A hands-on course on microbial risk assessment using R, enhancing proficiency in modelling tools used in food safety risk assessment, delivered by Dr. Alberto Garre (UPCT, Spain).
- Presentation of the 'Latest achievements and experiences in next-generation sequencing' at the Rapid Microbiology Testing Schoolorganised by Merck on 23.10.2024–24.10.2024.
- On-site workshop on Microbial risk assessment and use of Oxford Nanopore Technology at UPCT on 30.1.2025–31.1.2025.
- Visit of the headquarters of the Spanish Agency for Food Safety and Nutrition (AESAN) in Madrid and the National Centre for Food Laboratory, on 18.2.2025–19.2.2025. During the visit, the fellow benefited from AESAN's presentation on risk assessment, management and communication.
- On-site Mycology training – microscopy and bioinformatics on 7.3.2025 and 29.3.2025, organised at Warsaw University, Poland.
- On-site Illumina NGS workshop on 28.4.2025, conducted by David Baker (Quadram Institute Bioscience) at IBPRS-PIB.
- The results from the project are planned to be presented as a poster at the Joint International Conference of MIKROBIOKOSMOS & Central and East Europe Symposium on Microbial Ecology, which will take place on 22–24 September 2025 in Thessaloniki, Greece.

5 | ASSESSMENT

This fellowship project investigated how various NGS strategies might impact microbial hazard identification and QMRA outcomes in the dairy sector. Comparing short-read Illumina and long-read ONT data showed that platform choice strongly shapes our understanding of microbial communities along the food chain. Illumina provided high accuracy and genus-level resolution for dominant taxa, confirming its value in food microbiology. ONT, despite higher error rates, captured broader microbial diversity, detecting taxa missed by Illumina. However, ONT data requires careful error correction and validation before informing food safety decisions.

These differences affected hazard identification, contamination estimates and exposure assessments. Predictive models developed during the fellowship, revealed that microbial risk estimates varied by platform. Spoilage organisms and opportunistic pathogens found only in ONT data influenced initial contamination assumptions, sometimes increasing risk levels.

QMRA models for Poland and Spain revealed both common and region-specific risks. While critical control points were similar, differences in microbial prevalence, contamination sources and storage conditions led to distinct profiles. This highlights the need to adapt risk assessments to local contexts and supports harmonised and flexible tools across Europe.

The fellow actively participated in both institutions, joining meetings, seminars and collaborations, supporting professional development and building connections in the European food safety and microbiology community. Practically, the project provides useful insights for food safety authorities and the dairy industry. It supports development of more robust QMRA models, improving hazard detection, shelf-life prediction and food safety strategies.

6 | CONCLUSION

In conclusion, this fellowship will contribute to strengthened European food safety capabilities by integrating genomic identification of bacterial communities and quantitative risk modelling. The collaborative work between UPCT and IBPRS-PIB is an example of the value of cross-border partnerships in addressing complex food safety challenges. By training future risk assessors in cutting-edge analytical and modelling techniques, the programme supports EFSA's goal of harmonising risk assessment practices and enhancing food safety across the EU.

This study highlights the critical influence of the NGS strategy on microbial community detection and its downstream impact on QMRA models. The choice between Illumina and Oxford Nanopore affects not only the microbial diversity captured but also functional gene predictions and ultimately risk estimates. Combining data from both platforms may yield the most comprehensive QMRA insights. Differences in microbial compositions between Polish and Spanish dairy products also underline the need for region-specific risk assessments. QMRA models should account for sequencing biases, and regulatory frameworks may benefit from hybrid sequencing and cross-validation methods to ensure reliable food safety assessments.

ABBREVIATIONS

EU-FORA	European Food Risk Assessment Fellowship Programme
IBPRS-PIB	Institute of Agricultural and Food Biotechnology-State Research Institute
NGS	next-generation sequencing
ONT	Oxford Nanopore Technologies
QMRA	quantitative microbiological risk assessment
rRNA	ribosomal
UPCT	Polytechnic University of Cartagena
WGS	whole genome sequencing

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DISCLAIMER

The results of these studies are intended to be published in other scientific journals. To avoid copyright claims, they were described only very briefly in this report.

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Training in tools and modelling to develop risk ranking and quantitative microbial risk assessment for cheese along the Polish and Spanish food

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

To ensure that ready-to-eat products are safe to eat, it is important to develop new tools to prevent and control bacterial contamination. Risk assessment has been gaining popularity in recent years and provides tools with which we can develop mathematical models for robust food safety management tools for use by health authorities, consumer protection agencies and industry stakeholders This training programme was developed under the EU-FORA programme and had the goal of training a fellow in modern quantitative microbiological risk assessment (QMRA) methods through a hands-on approach within the Unit of Microbiology and Risk Assessment at Universidad Politecnica de Cartagena (UPCT). The programme involved working on a case study focused on the microbiological safety of artisanal cheese, covering every step of a QMRA, including hazard identification, exposure assessment, hazard characterisation and risk characterisation. The case study was related to listeriosis, which constitutes a significant public health concern. Ready-to-eat foods, such as artisanal cheeses, provide an ideal environment for growth *Listeria monocytogenes*. The fellow developed skills in microbiological techniques such as preparation of thermal and growth experiments, development of a protocol for preparing artisanal cheeses at laboratory scale, as well as data analysis using the R programming language through the *bioinactivation*, *biogrowth* and *biorisk* packages. Experimental findings demonstrated that thermal treatment of milk at 60°C for 9 min causes more than 6 log-reductions of *Listeria* below the detection limit, with similar kinetics in cow's and goat's milk. Conversely, storing the finished cheese at elevated temperatures (e.g. above 25°C) was shown to promote *Listeria* proliferation. This information was combined into kinetic models based on predictive microbiology, and applied in an academic exercise to evaluate the exposure of the consumer to the hazard and to estimate the illness per serving.

KEYWORDS

artisanal cheese, artistenal cheese, predictive microbiology, QMRA model

CONTENTS

1 | INTRODUCTION

The growing demand from consumers for healthy and safe food products has led to increased interest in developing and implementing effective quality and safety management systems for ready-to-eat products (RTE) (Koutsoumanis et al., 2021). Dairy-derived foods represent a common preference among consumers, with cheese being one of the most popular ones in terms of production volume within European Union countries (Possas et al., 2022). As RTE products lack a cooking step by the consumer, it is important to adopt a broader perspective on the microbiological risks and infection-related challenges associated with such products.

Listeria monocytogenes is a pathogenic bacterium that causes listeriosis, one of the most serious diseases affecting consumers. It is one of the foodborne diseases with the highest mortality rate (EFSA BIOHAZ Panel, 2018; Garre et al., 2019), mainly in immunocompromised people, pregnant women, newborns and the elderly (Martín et al., 2022). Although listeriosis was the fifth most commonly reported zoonosis in humans in the European Union (EU), it caused the highest number of hospitalisations and deaths (EFSA and ECDC, 2023; Wang et al., 2025). In 2022, there were 2738 confirmed cases of invasive listeriosis in the EU, resulting in 1330 hospitalisations and 286 deaths (EFSA and ECDC, 2023). The bacterium shows high tolerance to low temperatures and has the ability to grow in various environments (Bonanno et al., 2024; Martín et al., 2022). Fresh cheeses provide an optimal environment for listeriosis to develop. Even when stored in refrigerated conditions, they provide the bacteria with the ideal environment for growth, given that Listeria can grow at low temperatures and pH, provided the water activity remains high (Falardeau et al., 2021; Martín et al., 2022).

Listeria spp. may be affected by natural competition in ready-to-eat products, in what is often called the 'Jameson effect'. Particularly in cheese, there is competition between lactic acid bacteria (LAB) and *L. monocytogenes*. In practice, the dominant microorganism limits the growth of others, reducing the concentration of *L. monocytogenes* (Serra-Castelló et al., 2022). This is due to LAB producing antimicrobial compounds, such as lactic acid, hydrogen peroxide and bacteriocins, which can inactivate or inhibit the growth of pathogens (Cadavez et al., 2019; Serra-Castelló et al., 2022).

Predictive microbiology is a tool that enables the prediction of the behaviour of microorganisms under specific conditions that may occur at various stages of the food production chain – from preparation and processing to distribution and storage. Temperature plays a key role in shaping the growth dynamics of microorganisms, and this parameter is an integral part of predictive modelling (Thomas et al., 2019). In the context of risk analysis, the focus of the food safety management system is on assessing the health risks associated with food consumption, and on selecting and implementing effective strategies to reduce these risks (Koutsoumanis et al., 2021).

The work programme focused on training the fellow on methodologies related to quantitative microbiological risk assessment (QMRA). The fellow joined the research group at the host institution, gaining practical experience in experimental methods (inactivation and growth experiments), statistical analysis, mathematical modelling and stochastic simulations. The programme covered all stages of risk assessment, including hazard identification, exposure assessment, hazard characterisation and risk characterisation.

2 | DATA AND METHODOLOGIES

2.1 | Methodologies

2.1.1 | Strain preparation

L. monocytogenes EGD-e was selected, as this is a common reference strain for *L.* monocytogenes. The strains were individually activated by transferring one colony of the stored culture to 5 mL of TSB and incubated at 37°C during 24 h before either growth or inactivation experiments.

2.1.2 | Thermal treatments

Thermal treatments were carried out using a Mastia thermoresistometer (Conesa et al., 2009), developed by the host group. The Mastia thermoresistometer enables the resistance of microorganisms to high temperatures to be measured under both isothermal and non-isothermal conditions (Conesa et al., 2009). The Mastia thermoresistometer is designed to maintain a constant temperature within the analysed sample throughout the entire process. This is made possible by the design of the vessel, which is equipped with a stirrer. The sample is poured into the vessel and the stirrer maintains the set temperature by stirring the sample throughout the experiment (Conesa et al., 2009).

Isothermal experiments were applied at 57°C, 60°C and 62°C for 45, 9 and 4.5 min, respectively. Experiments were done in tryptic soy broth (TSB, Scharlau) as laboratory medium, as well as goat's milk (from a local store). Both media were poured into the vessel of the thermoreistometer at a volume of 400 mL, heated to the set temperature and inoculated 0.2 mL of *L. monocytogenes* EDG-e, resulting in a concentration of ~6 log CFU/mL. Samples were collected at set timepoints, serially diluted and spread on TSA plates. The plates were then stored for 24 h at 37°C before counting. The experiments were conducted on three independent replicates in both media for all three temperature profiles.

2.1.3 | Growth experiments

2.1.3.1 | *Development of a laboratory-scale cheese model*

The first stage involved the development of a laboratory protocol for a model cheese from unpasteurised goat milk. The raw milk was provided by a local supplier (Cartagena, Spain) and microbial rennet from a Polish supplier (Browin, Poland). The cheeses were produced on a laboratory scale in accordance with a protocol developed by the fellow holder and the UPCT group. Milk used to produce cheese was tested for pH and water activity, then salted, enriched with calcium chloride, warmed and combined with rennet to form curd, which was cut, drained in moulds and left to set. The resulting cheese was portioned and prepared for storage. The final cheese product was portioned into 10 g pieces and transferred to Falcon tubes. To artificially contaminate the test samples, each Falcon tube was inoculated by placing 10 µL-aliquots of strain *L. monocytogenes* culture (3 log cfu/g) in the central perforation of each test sample using a micropipette. Samples were grouped into control and test samples and stored at 25°C for 48 h. Each test tube represented a specific point in time. A temperature of 25 degrees was chosen as the worst-case scenario for consumers.

2.1.3.2 | *Microbiological analysis*

The growth of *L. monocytogenes* (in test samples) and LAB (both in test and control samples) was studied at 25°C. Samples were taken after 0, 2, 4, 6, 14, 16, 18, 20, 22, 24 and 48 h. Test samples were placed into sterile bags (LABOLAN S.L., Navarra, Spain) with the addition of 90 mL of peptone water 1.0% (dilutor system, IUL Instruments®, Barcelona, Spain) and homogenised for 1.5 min using a Stomacher. The homogenised sample was serially diluted in peptone water before plating. The concentration of *L. monocytogenes* was quantified on Chromogenic Listeria Agar, Ottaviani and Agosti (ALOA, Condalab®, Spain), whereas the concentration of LAB was measured in MRS. Plates were incubated at 37°C (48 h) for *L. monocytogenes* and 30°C (72 h) for LAB. Colonies were counted and results were expressed as log cfu/g.

2.1.3.3 | *Physicochemical analyses*

The pH and aw of the control samples were measured throughout storage. The measurement for pH was made by immersing the electrode in the sample 10 g of sample was mixed with 10 mL of distilled water at 40°C, according with PN-73/A-86232 'Milk and dairy products. Cheese. Test methods'. After determining electrode readings, the result was read with an accuracy of 0.01. Three measures of each physicochemical parameter were made for each sample. Water activity (aw) was measured at 25 ± 0.2°C using an AquaLab 4TE device (MeterGroup, USA) after allowing samples to reach equilibrium in the sealed chamber.

2.1.4 | Predictive microbiology models

2.1.4.1 | *Microbial inactivation*

The data obtained from isothermal treatments was analysed using the Bigelow and Geerards model. As presented in Equation (1), the Bigelow model assumes that the logarithm of the microbial concentration (N) decreases linearly over time during the treatment, relative to the initial concentration (N_0). In this approach, the rate of inactivation is described by the D-value (D).

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

In the context of secondary models, the Bigelow model describes a log-linear dependence between the D-value and the treatment temperature (T). As illustrated in Equation (2), the microorganism's sensitivity to temperature variation is quantified by the z-value (z). To enhance the identifiability of the model parameters, a reference temperature (T_{ref}) is introduced, with D_{ref} representing the D-value at T_{ref}.

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

Geeraerd's model (Geeraerd et al., 2000) is a logarithmic-linear model of microorganism inactivation, extended by an adaptation phase (shoulder) and an asymptotic value (tail). As the data obtained did not show a clear tail, it was described using the algebraic solution for the Geeraerd model without tail (Equation 3).

$$N = N_0 e^{-kt} \frac{e^{kS_l}}{1 + \left(e^{kS_l} - 1\right)e^{-kt}}. \tag{3}$$

In this equation, the shoulder length is described by parameter S_l. This model introduces a log-linear secondary model for k, similar to the one defined within the Bigelow model (Equation 4). This introduces k_{ref} as the value of k at T_{ref}.

$$\log k(T) = \log k_{\text{ref}} + \frac{T - T_{\text{ref}}}{z}. \tag{4}$$

The models were fitted using the online version of *bioinactivation* (Garre et al., 2025) available at https://foodlab-upct.shinyapps.io/bioinactivation4/.

2.1.4.2 | *Microbial growth*

The data obtained from growth experiments was analysed using the Baranyi model. This model takes into account the adaptation phase (lag phase), the logarithmic growth phase and the stationary phase. Under isothermal conditions, the Baranyi model is defined by Equation (5)

$$\ln N = \ln N_0 + \mu_{\max} A(t) - \ln\left(1 + \frac{e^{\mu_{\max} A(t)} - 1}{e^{\ln N_{\max} - \ln N_0}}\right), \tag{5}$$

$$A(t) = t - \lambda + \frac{1}{\mu_{\max}} \ln\left(1 - e^{-\mu_{\max} t} + e^{-\mu_{\max}(t-\lambda)}\right).$$

The model is parameterised by the maximum specific growth rate ($\mu_{\max}$), the lag phase duration (λ) and the maximum concentration in the stationary phase ($N_{\max}$), as well as the initial concentration (N_0). These parameters was estimated from the experimental data using the online version of the *biogrowth* package (Garre et al., 2023) available online at https://foodlab-upct.shinyapps.io/biogrowth4/.

2.1.5 | QMRA

The final stage of the project was to build a QMRA model as an academic exercise, combining the predictive microbiology models developed with literature data. The exercise included four different scenarios:

- Cheeses produced from pasteurised milk including bacterial competition **(Baseline)**
- Cheeses produced from pasteurised milk without bacterial competition (**P^+C^-**)
- Cheeses prepared from unpasteurised milk including bacterial competition (**P^-C^+**)
- Cheeses prepared from unpasteurised milk without bacterial competition (**P^-C^-**)

The QMRA was developed as a Modular Process Risk Model (Nauta, 2008) Besides the predictive models for growth and inactivation, the model requires probability distributions for the logistic parameters of the food supply chain (pasteurisation time and temperature; storage time and temperature), the initial concentration (prevalence and concentration of *L. monocytogenes* in raw milk) and the consumer behaviour (serving size). These data were taken from the scientific literature and described using Normal, Uniform, Empirical and Triangular distributions.

For the initial concentration, we used data according to Dalzini et al. (2016), ranging from < 1 to > 1000 cfu^{-1}. Due to the lack information of storage time in 25°C data for artisanal cheese in literature, we assumed the best-case scenario was 3 h and the worst-case scenario was 48 h. We assumed the serving size to be 100 g.

The QMRA model was evaluated using Monte Carlo simulations (Pouillot & Guillier, 2020) to perform forward uncertainty propagation. All calculations were performed in R (version 4.2.3) using the biorisk package, which is available at https://github.com/albgarre/biorisk. One million Monte Carlo simulations were calculated for each scenario.

Figure 1 illustrates the elements of the QMRA model built for the baseline scenario (green). The P^-C^+ and P^-C^- do not include a pasteurisation step. Therefore, the concentration of *L. monocytogenes* in raw milk reported by Dalzini et al. (2016) is used as direct input for the growth element (red outline in Figure 1).

Regarding the competition, our laboratory results showed that the presence of LAB only influenced the maximum concentration of *L. monocytogenes* in stationary phase (probably due to Jameson effect). Therefore, scenarios without competition (P^+C^- and P^-C^-) assigned $\log N_{\max}$ a constant value of 9 log CFU/g (based on expert opinion), whereas scenarios with competition (P^+C^+ and P^-C^+) assigned the parameter values estimated from experimental data.

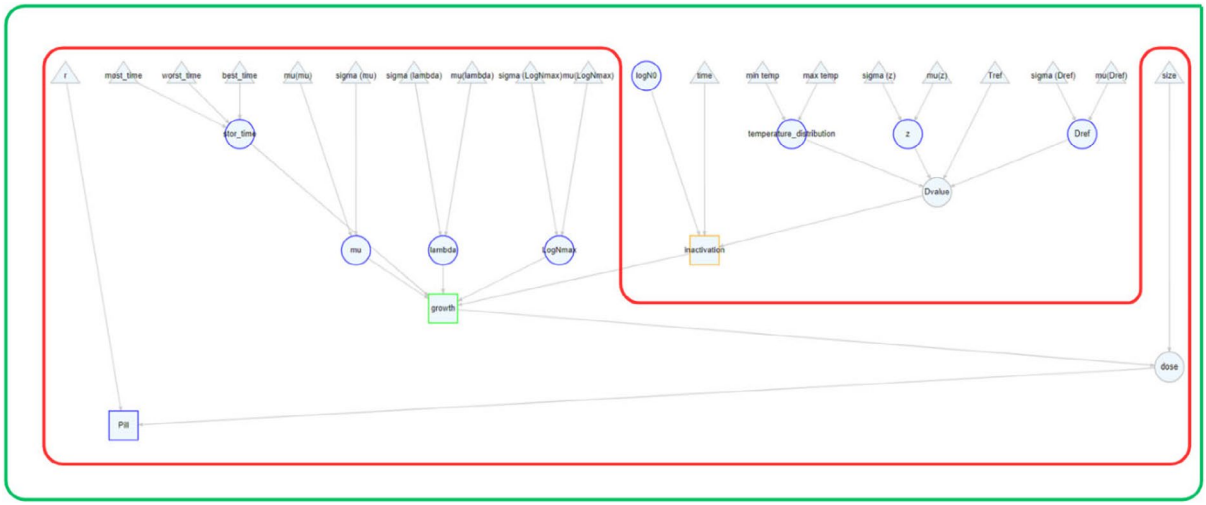

FIGURE 1 Illustration of the elements of the QMRA model developed as an academic exercise within the training programme.

3 | RESULTS

The first part of the training programme generated the experimental data required to build growth and inactivation models for *L. monocytogenes* in cheese and milk, respectively. Table 1. summarise the parameters of the Geeraerd and Baranyi model fitted to these observations.

TABLE 1 Parameters of the Geeraerd and Baranyi models obtained for the inactivation kinetics of *Listeria monocytogenes* in goat's milk and for its growth in goat's cheese.

Response	Parameter	Unit	Estimate ± SD error
Inactivation	log D_{ref}	min	0.30 ± 0.11
	z	°C	5.69 ± 1.73
Growth	log $N0$	log10 CFU/g	3.10 ± 0.19
	μ (mu)		0.30 ± 0.12
	λ (lambda)	h	3.51 ± 1.69
	logN max	log10 CFU/g	5.71 ± 0.10

The models summarised in Table 2 was the basis for the QMRA model. The QMRA model was used to estimate the exposure of the consumer to *L. monocytogenes* in fresh cheeses, as well as the associated probability of illness during storage at 25°C. For scenarios Baseline and $C^{-}P^{+}$, a large number of simulations (75%) had a microbial dose of 0 cells. However, only 0.005% of the simulations in scenarios $C^{+}P^{-}$ and $C^{-}P^{-}$ would have a dose of 0 cells. This shows the importance of the pasteurisation treatment for the risk in this academic scenario, as it would ensure the absence of the microorganism in the serving.

Table 2 summarises the results of the QMRA model. The baseline scenario has an expected number (median of 1,000,000 MC simulations) of listeriosis cases per million servings of zero. On the other hand, the worst-case scenario has an expected number of 61 cases per million. This demonstrates the relevance of competition and/or the pasteurisation treatment on the risk of listeriosis in cheese for this academic exercise. The scenario without pasteurisation ($C^{+}P^{-}$) had a higher expected number of cases per million servings (25 cases) than the one without competition ($C^{-}P^{+}$; 0 cases). This implies that the pasteurisation treatment is more relevant than the competition for the expected number of cases.

TABLE 2 Number of cases per million servings estimated from the academic QMRA model built for the risk of listeriosis in cheese.

Type of scenario	Median	90th percentile	99th percentile
Baseline	0	0	59
$C^{-}P^{+}$	0	0	80,634
$C^{+}P^{-}$	25	61	84
$C^{-}P^{-}$	61	92,979	131,801

Baseline: pasteurised milk including bacterial competition.
$C^{-}P^{+}$: pasteurised milk without bacterial competition.
$C^{+}P^{-}$: unpasteurised milk including bacterial competition.
$C^{-}P^{-}$: unpasteurised milk without bacterial competition.

The output in Table 2 presents uncertainty by including the 90th and 99th percentile besides the median number of cases. The comparison between the Baseline and Worst-case scenario show similar results as before, with the baseline having a 99th percentile of 59 cases and the worst case 131,801 cases.

However, the interpretation of the results for the two intermediate scenarios is not so evident. Scenario $C^{+}P^{-}$ has a higher number of expected cases (25 cases) than $C^{-}P^{+}$ (0 cases), but a lower number in the 99th percentile (84 vs. 80,634 cases). This can be explained based on the microbial concentration at exposure, illustrated in Figure 2. The pasteurisation treatment has a big impact on the average microbial concentration, reducing the overall risk. However, the competition defines a maximum threshold for the microbial concentration. As a result, scenario $C^{+}P^{-}$ has overall higher exposure, resulting in higher cases. However, competition prevents exposure to extremely high microbial concentrations. On the other hand, the $C^{-}P^{+}$ scenario does not define that threshold. Therefore, extreme events are more likely, despite a lower overall risk.

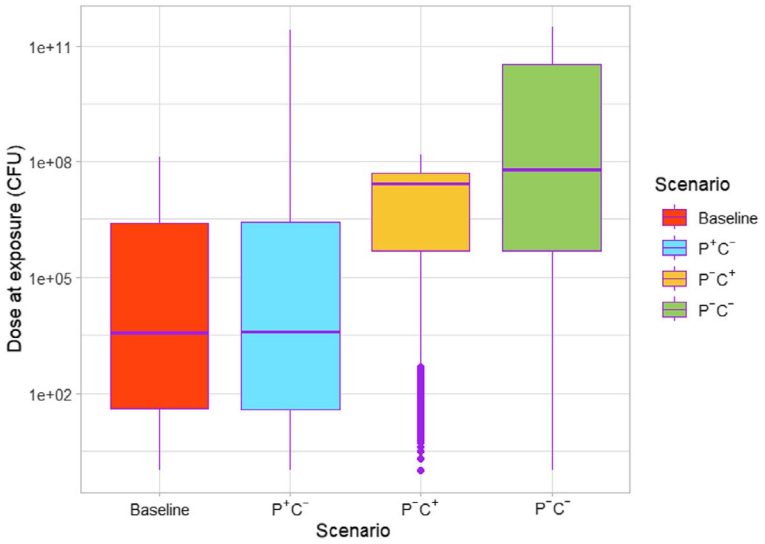

FIGURE 2 Microbiological concentration at the consumption stage for all scenarios considered for cases where the dose was higher than zero. Baseline – baseline model + competition (75% simulations with 0 dose); $C^{-}P^{+}$ – baseline model no competition (75% simulations with 0 dose); $C^{+}P^{-}$ – raw milk + competition (0.005% simulations with 0 dose); $C^{-}P^{-}$ – raw milk no competition (0.005% simulations with 0 dose).

4 | CONCLUSION

The EU-FORA programme gave the fellow the chance to learn novel methodologies for microbiological risk assessment. The programme provided theoretical and hands-on training on microbiological risk assessment through the participation in various training courses and activities at the Polytechnic University of Cartagena. Building on this experience, the fellow conducted microbial growth and inactivation experiments, analysing the data to build predictive microbiology models that were the basis for an academic QMRA model implemented in the R programming language. For this, the fellow also became familiar with relevant databases for food safety. The EU-FORA programme also equipped the scholarship holder with valuable skills in project management, planning and conducting experiments.

5 | ADDITIONAL ACTIVITIES

The fellow joined a research group involved in various microbiological risk assessment projects, gaining comprehensive experience in QMRA, from data collection to developing food safety recommendations. The fellow participated in various seminars and practical classes on food safety.

She will also be visiting the Spanish Agency for Food Safety and Nutrition (AESAN) in Madrid, and the National Centre for Food laboratory, in Majadahonda on 18–19 February. The presentation covered AESAN's activities in the areas of risk assessment, management and communication. The fellow also shared information about her work and activities within the EU-FORA programme.

The grantee participated in the IX Day of Scientific Coopertaion with Spanish organisations collaborating with EFSA on April 24 in Madrid, where she presented her experience in the EU-FORA programme. In the second half of the year, the fellow will present the results of his work at an International Conference.

ABBREVIATIONS

AESAN	Spanish Agency for Food Safety and Nutrition
ALOA	Chromogenic Listeria Agar, Ottaviani and Agosti
aw	water activity

Baseline	pasteurised milk + bacterial competition
BIOHAZ	Panel on Biological Hazards
C^+P^-	raw milk + bacterial competition
CFU	colony forming units
C^-P^-	raw milk - bacterial competition
C^-P^+	pasteurised milk - bacterial competition
LAB	lactic acid bacteria
MRS	de Man, Rogosa and Sharpe agar
QMRA	quantitative microbiological risk assessment
RTE	ready to eat food
RTEF	refrigerated ready to eat food
TSA	tryptic soy agar
TSB	tryptic soy broth
UPCT	Universidad Politecnica de Cartagena

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Training in metagenomics-integrated risk assessment for food-borne pathogens in the Slovenian and Spanish meat chain (METAMEAT)

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Abstract

Next-generation sequencing (NGS) has become an essential tool for antimicrobial resistance (AMR) surveillance, enabling comprehensive detection of AMR determinants in both bacterial isolates and complex microbial communities. Metagenomic sequencing enables culture-independent profiling of antimicrobial resistance genes (ARGs) in different environments, while whole-genome sequencing (WGS) is widely used in AMR surveillance laboratories to predict phenotypic resistance in major food-borne pathogens. AMR risk assessment usually considers factors such as the pathogenicity of the ARG-carrying bacterial host, the abundance of ARGs and their mobility potential inferred from association with plasmids or other mobile genetic elements that facilitate horizontal gene transfer. Clinical relevance of antimicrobials and the severity of clinical outcomes can further be implemented in AMR risk assessment. Exposure assessment contextualises hazards within real-world scenarios by estimating consumer exposure to AMR bacteria or their ARGs through food or other routes. Despite challenges in fully quantitative assessments, the integration of NGS-based surveillance with risk modelling represents a critical step towards proactive AMR risk management. In this study, broiler samples from different stages of a Slovenian and a Spanish slaughterhouse were analysed using conventional microbiology, shotgun metagenomic sequencing and WGS of isolates of selected pathogenic species. A modular, semi-quantitative risk assessment model was developed that combines (meta)genomic data with key risk factors and, where available, exposure assessment. This approach prioritises AMR risks in broiler meat processing and supports evidence-based decision-making in the areas of food safety and public health.

KEYWORDS

antimicrobial resistance, broiler meat processing chain, Illumina technology, metagenomics, next-generation sequencing, semi-quantitative risk ranking

CONTENTS

1 | INTRODUCTION

Antimicrobial resistance (AMR) is one of the most pressing global public health crises of the 21st century. The rise of bacteria resistant to commonly used antimicrobials threatens to undermine decades of medical progress, making once-treatable infections potentially lethal (World Health Organization, 2022). The emergence and spread of AMR is driven by complex and dynamic interactions between pathogens, commensals and environmental bacteria that can exchange antimicrobial resistance genes (ARGs) through horizontal gene transfer (HGT). Successfully and comprehensively addressing AMR risks in interconnected human, animal and environmental systems requires a multi-method approach that is consistent with the One Health framework. Furthermore, there is a lack of science-based risk assessment frameworks that can both identify AMR-related hazards and transparently rank and quantify the associated risks. Such integration of (semi)quantitative data into AMR risk assessment models could be used to evaluate the impact of different hypothetical scenarios (targeted control measures) to combat AMR.

Next-generation sequencing (NGS), also known as high-throughput sequencing or massively parallel sequencing, has transformed the field of AMR detection, monitoring and characterisation. Whole-genome sequencing (WGS) of bacterial isolates enables the identification of known and putative novel AMR determinants in bacterial genomes. On the other hand, shotgun metagenomic sequencing of complex communities provides insight into the entire repertoire of AMR determinants within a given sample (i.e. the resistome) without the need for prior cultivation of AMR isolates.

In WGS, an entire genome of an isolate is sequenced, providing insight into the entire gene repertoire of a given isolate, including its ARGs and virulence genes. It has become essential in current AMR surveillance in public health, particularly for priority food-borne bacteria such as *Escherichia coli* and *Salmonella* Enterica. In these species, genotype–phenotype correlation of AMR profiles is high; therefore, prediction of phenotypic AMR profiles based on the presence of specific ARGs and AMR-associated mutations is highly accurate (Boolchandani et al., 2019; Clausen et al., 2016). Metagenomics bypasses the culturing step and enables culture-independent profiling of the entire resistome of complex microbial ecosystems such as microbiomes of the animal gut, soil, water, wastewater or food (Fitzpatrick & Walsh, 2016). Metagenomic approach can uncover potential hidden reservoirs of ARGs in non-pathogenic environmental and commensal bacteria that may serve as sources for the transfer of ARGs to pathogens through HGT. In addition, it captures the overall diversity and abundance of ARGs in all bacteria and offers insights into the environmental drivers and dissemination pathways of AMR that are not considered in isolate-based surveillance strategies. Consequently, it enables the tracking of resistomes across the One Health continuum (humans, animals, environment and food) and helps to identify AMR hotspots and transmission pathways (Martak et al., 2024).

While AMR surveillance identifies the presence and possibly the characteristics (class, type or group) of AMR-related hazards, risk assessment is the strategy that allows these hazards to be prioritised and resources to be allocated. Current AMR risk assessment frameworks that rely on NGS typically consider several interrelated key factors such as the pathogenicity of the bacterial host, the abundance of ARGs and their mobility potential. First, host pathogenicity is associated with the ability of the AMR host to cause disease. In other words, an ARG in a highly virulent and invasive pathogen poses a greater threat to human health than the same gene in a non-pathogenic environmental bacterium. It is usually measured by the number of known virulence or pathovar-associated genes. Second, a higher abundance of ARGs in a given sample or in the genome of the AMR isolate indicates a higher AMR risk. Third, ARGs can be transferred to other bacterial hosts through HGT, which increases the risk of AMR spread. The ability of an ARG to spread through HGT is influenced by its association with mobile genetic elements such as plasmids, transposons and integrons that facilitate such transfer (Partridge et al., 2018). In bioinformatics, ARG mobility is usually measured by their proximity to mobile genetic elements. Recently, long-read sequencing technologies such as Oxford Nanopore Technologies and PacBio SMRT sequencing have enabled the reconstruction of gap-free plasmids and the identification of ARGs associated with mobile genetic elements, improving the accuracy of predicting ARG mobility.

Two additional characteristics that can be included in the framework are the clinical relevance of the antimicrobial agent to which the bacterial host is resistant and the severity of the clinical outcome (EFSA and ECDC, 2025). AMR-related risk is of particular interest when it concerns antimicrobials critical for human medicine. In humans, resistance to last-resort antimicrobials such as extended-spectrum cephalosporins, carbapenems, fluoroquinolones and polymyxins (e.g. colistin) is of particular concern due to limited or no alternative treatment options, particularly because they can lead to increased mortality, prolonged hospitalisation, treatment failure and more severe disease outcomes (Zanichelli et al., 2023). In livestock, veterinary important antimicrobials for food-producing animals such as aminoglycosides, amphenicols, cephalosporins and macrolides can be prioritised (World Health Organisation for Animal Health, 2024).

A comprehensive farm-to-fork risk assessment model should include exposure assessment that estimates the likelihood and magnitude of human (or animal) exposure to AMR bacteria or ARGs through relevant pathways such as consumption of contaminated food/feed or water or transmission between individuals (EFSA BIOHAZ Panel, 2021; World Health Organization, 2021). Due to the currently limited data available, such an assessment is often semi-quantitative and based on several simplifications. Nonetheless, a farm-to-fork quantitative risk assessment model for cephalosporin-resistant *Salmonella* Heidelberg in broilers was developed (Collineau et al., 2020), which was used to assess the impact of different hypothetical interventions in broiler farms on the likelihood of human disease. Major gaps remain in the understanding of the pathogenicity, growth and survival of AMR strains compared with their susceptible counterparts.

Another challenge in reliable quantitative AMR risk assessment based on NGS data is the lack of standardisation of bioinformatic pipelines and databases for the detection and characterisation of ARGs. In addition, the interpretation of the

functional impact of novel ARG variants, the integration of different data sources (experimental, genomic, metagenomic, transcriptomic, epidemiological, clinical and consumption data) and the modelling of the complex transmission pathways are challenging. The integration of NGS data into AMR surveillance and risk assessment is a crucial step in fighting AMR. By providing insights into the genetic basis, distribution and mobility of ARGs, NGS facilitates the transition from reactive detection to a proactive risk management and mitigation.

Despite all the discussed technologies that have substantially strengthened AMR surveillance and hazard identification, there is still a need to translate (meta)genomic data into actionable, context-specific risk assessments that can guide decision-making and targeted interventions in the food chain. To address this, in this project, broiler neck skin and caecal samples were collected from different stages of a Slovenian and a Spanish slaughterhouse and analysed using both conventional microbiology and NGS to detect ARGs, mobile genetic elements and (putative) host pathogenicity. All broiler samples underwent shotgun metagenomic sequencing using Illumina technology. DNA from selected zoonotic pathogenic isolates was sequenced using Oxford Nanopore or Illumina technology. A modular, semi-quantitative AMR risk assessment model was developed to rank complex broiler samples and AMR isolates with respect to their AMR-associated risk, incorporating key factors such as host pathogenicity as well as the abundance and mobility of ARGs, while simultaneously weighting the clinical importance of antimicrobials and, in the case of selected AMR isolates, estimating consumer exposure through contaminated broiler meat.

2 | BACKGROUND AND TERMS OF REFERENCE

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. The fellowship project, entitled 'Training in metagenomics-integrated risk assessment for food-borne pathogens in the Slovenian and Spanish meat chain (METAMEAT)', was developed through a partnership between the University of Ljubljana (Slovenia) as the sending institution of the fellow Dr. Bojan Papić, and the Technical University of Cartagena (Spain) as the hosting institution, with Dr. Enriqueta Garcia-Gutierrez and Prof. Pablo S. Fernández acting as supervisors.

3 | DATA AND METHODOLOGIES

3.1 | Sampling in Slovenian and Spanish broiler slaughterhouses

One Slovenian and one Spanish broiler slaughterhouse were included in the study, and the same sampling strategy was applied in both. Pooled neck skin samples were collected from three stages of the slaughter process, i.e. before and after evisceration and after the exit of the carcasses from the cooling tunnel.

Evisceration was chosen because it is one of the critical steps in the slaughter process for microbiological contamination of broiler carcasses. Namely, automated evisceration systems can rupture the intestine, leading to contamination of carcasses, equipment and surfaces (Gruntar et al., 2015).

Different slaughter batches (corresponding to different flocks) were analysed, and technical replicates were also analysed at each sampling point to ensure that potential significant differences between the study groups were reliably and consistently identified. Broiler caecal contents were also sampled.

3.2 | Sample preparation and microbiological examination

The neck skin samples (25 g) were first supplemented with 225 mL of buffered peptone water and homogenised for 2 min. Homogenised samples then underwent the following microbiological examinations:

- thermophilic *Campylobacter* count: direct cultivation on mCCDA agar (ISO 10272-2:2017);
- total *E. coli* count: TBX agar;
- total aerobic count: Petrifilm aerobic count plates.

In addition, non-selective pre-enrichment of the samples (37°C, 18 h) was performed, followed by cultivation on selective media:

- motile *Salmonella*: semi-solid MSRV agar for an additional selective pre-enrichment, followed by confirmation of *Salmonella* presence on chromogenic agars XLD and Rambach (ISO 6579-1:2017);
- Extended-spectrum beta-lactamase (ESBL)-producing *E. coli*: chromID ESBL agar;
- Carbapenemase-producing *E. coli*: chromidID CARBA SMART agar (CARB/OXA).

3.3 | DNA extraction and next-generation sequencing

Total DNA was extracted from neck skin and caecal samples using PowerSoil Pro kit (Qiagen). Shotgun metagenomic sequencing of the samples was performed using Illumina paired-end (2 × 150 bp) technology on a NovaSeq X Plus system.

Total DNA from the obtained bacterial isolates was extracted using DNeasy Blood & Tissue kit (Qiagen). WGS of isolates was performed using Illumina paired-end (2 × 150 bp) technology on a NovaSeq X Plus system or Oxford Nanopore's MinION sequencer.

3.4 | AMR risk assessment

For resistome risk ranking, we used a slightly modified model implemented in the MetaCompare 2.0 tool (Rumi et al., 2024). This model is intended for short-read NGS data generated with Illumina technology and takes into account the abundance of ARGs, their mobility and host pathogenicity. Originally, ARG abundance is represented by the number of ARG-carrying contigs weighted by the total number of contigs. ARG mobility is represented by the number of MGE-carrying ARG contigs weighted by the total number of contigs. Host pathogenicity is represented by the species-level taxonomic classification of contigs to a given list of bacterial pathogens. Each of these variables represents a dimension in a 3D (or 4D) hazard space, and the final risk score is calculated using the Euclidean distance of the sample to the maximum point in the 3D hazard space (Figure 1). It should be noted that this resistome risk ranking model does not include exposure assessment data but rather focuses on hazard identification and characterisation.

MetaCompare 2.0 defines two different resistome risk scores. Human health resistome risk focuses on clinically important (high-risk) antimicrobials and pathogens (e.g. ESKAPEE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *E. coli*). Ecological resistome risk focuses on all ARGs and all potential pathogenic hosts (Rumi et al., 2024).

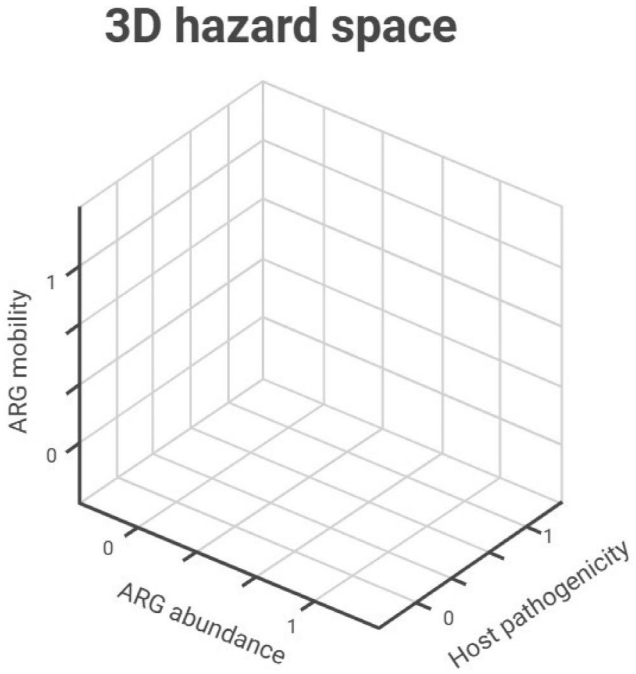

FIGURE 1 A concept of a 3D hazard space that is implemented in the MetaCompare 2.0 tool for resistome risk ranking.

A conceptually similar model was used in this training to rank the AMR isolates from Slovenia and Spain according to their AMR risk and broiler metagenomes according to their resistome risk. ARG abundance was estimated either by the number of ARGs (isolate-based AMR risk) or by the number of ARG-carrying contigs, normalised by the total number of contigs (resistome risk). ARG mobility was estimated as the number of MGE-carrying ARG contigs, normalised by the number of ARG-carrying contigs in resistome risk only. Host pathogenicity was assessed either through taxonomic classification of the ARG-carrying contigs to putative pathogen species (resistome risk) or by quantifying pathovar- or species-associated virulence genes (isolate-based AMR risk).

3.5 | Secondary scientific activities during the fellowship

The fellow participated (or will participate) in the following activities:

Invited speaker – conference:

- CESAR2025 (https://hmd-cms.hr/cesar2025/) – 22–25 September 2025, Zadar (Croatia), invited talk entitled 'Integrating (meta)genomic sequencing data into AMR risk assessment'.

Invited speaker – lecture:

- Seminar for the Advanced Techniques of Agrifood R&D PhD programme (TAIDA) entitled 'Integration of »omics« into risk assessment'.

Hands-on workshops:

- A course on microbial risk assessment using R, enhancing proficiency in modelling tools used in food safety risk assessment, delivered by Dr. Alberto Garre (UPCT, Spain).
- A wet-lab training on Oxford Nanopore sequencing, covering sample preparation, sequencing and bioinformatic workflows as well as troubleshooting, delivered by Mr. David Baker (Quadram Institute Bioscience, United Kingdom).

Webinars:

- ECDC GenEpi-BioTrain - Virtual training 14 - R data analysis and visualisation for beginners.
- ECDC GenEpi-BioTrain - Virtual training 18 - Empowering AMR Research through R: Analysis and Visualisation.

4 | ASSESSMENT

4.1 | Microbiological examination

Microbiological examination of broiler neck skin samples revealed different trends in microbial counts between the slaughterhouses examined. A linear mixed model showed that biological repetition (flock) had a significant influence on microbial counts in both slaughterhouses. In one slaughterhouse, *E. coli* counts increased after evisceration, indicating contamination from gut contents during the process; however, the counts subsequently decreased after chilling. These differences in microbial trends between slaughterhouses may be attributed to differences in meat processing technologies or hygiene practices. In addition, flock-level variation (e.g. gut microbiota composition, health status or on-farm management) likely also contributed to the observed differences, as shown by the significant influence of biological repetition in the model.

4.2 | Isolate-based AMR risk assessment

The risk assessment model was successfully applied to rank the obtained AMR isolates based on their AMR risk. It integrated the 3D hazard space concept (hazard characterisation) with national data on annual chicken meat consumption and portion size (exposure assessment). As a result, the model effectively differentiated between isolates of the same species based on their ARG repertoire and national consumption patterns.

4.3 | Resistome risk ranking

The resistome risk of caecal samples was very comparable between the biological repetitions. Resistome risk scores differed significantly between the analysed slaughterhouses, with the ecological resistome risk scores being higher than the human health resistome scores. In contrast, resistome risk ranking of the broiler neck skin samples differed markedly between the biological repetitions. A major methodological limitation in assessing the resistome risk of neck skin samples was the low abundance of detected ARGs, primarily due to the high (>98%) proportion of host (*Gallus gallus*) DNA in the obtained sequences. This finding suggests that a host depletion strategy should be employed to increase the proportion of microbial DNA in shotgun metagenomic sequencing. Potential approaches include neutrase–saponin pretreatment of food samples prior to DNA extraction, depletion of eukaryotic DNA post-extraction (e.g. using the NEBNext Microbiome DNA Enrichment Kit, NEB) or adaptive sampling during nanopore sequencing to reject reads that map to the host genome.

5 | CONCLUSION

The project confirmed the usefulness of metagenomic sequencing as a powerful tool for comprehensive AMR (resistome) surveillance in food systems, enabling high-resolution profiling of AMR determinants and supporting evidence-based risk modelling. The comparison between Slovenian and Spanish broiler processing chains revealed notable differences in ARG prevalence and microbial community structure, which may reflect national or flock-based differences in antimicrobial use, farming/slaughtering practices and biosecurity measures. The developed risk assessment model combines NGS data with public health considerations to produce a semi-quantitative framework for ranking AMR risks. Despite its semi-quantitative nature, this integrative approach improves the transparency and effectiveness of AMR risk prioritisation in the context of food safety and public health.

6 | RECOMMENDATIONS

We recommend the integration of metagenomic surveillance into routine AMR surveillance programmes in all EU Member States to complement conventional culture-based methods and enable comprehensive resistome profiling. We also recommend supporting the development and validation of modular, (semi)quantitative risk assessment tools that can be adapted to different food production and processing systems and countries to improve transparency and prioritisation in AMR risk management. AMR surveillance should prioritise ARGs conferring resistance to critically important antimicrobials, especially those associated with high prevalence, mobility potential and/or host pathogenicity, to better guide intervention strategies to protect public health. Finally, we aim to promote the open sharing of sequencing data, analytical pipelines and risk models to strengthen an integrated European AMR surveillance network and a collective risk assessment capacity. The investigation also revealed a critical lack of publicly available (genomic) data on antimicrobial susceptibility of food-borne pathogens from human clinical cases in national and joint EU reports. Accurate estimation of AMR profile occurrence across sectors would enable more precise attribution of human cases caused by AMR strains to broiler meat, especially when isolates from different sectors are typed using WGS.

ABBREVIATIONS

AMR	antimicrobial resistance
ARG	antimicrobial resistance gene
ESBL *E. coli*	extended-spectrum beta-lactamase-producing *Escherichia coli*
HGT	horizontal gene transfer
NGS	next-generation sequencing
WGS	whole-genome sequencing

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DISCLAIMER

The results of these studies are intended to be published in other scientific journals. To avoid copyright claims, they were described only very briefly in this report.

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EU-FORA SERIES 8

Ensuring ethical production of beef: A comprehensive risk assessment of animal welfare during transportation and slaughter processes

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

Animal welfare is a recognised and important issue within the European Union, addressed through comprehensive regulations such as Regulations 1/2005 and 1099/2009, which emphasise the necessity to respect and protect the welfare of animals, particularly during transport and slaughter procedures. These legislative measures are part of an ongoing European commitment to ensure ethical treatment and minimise suffering for food-producing animals, aligning with broader food safety and public health goals. This project aimed to create a prototype of an updated cattle path designed to minimise stress and reduce reliance on coercive means such as electric prods, while also decreasing the risk of injury due to trauma or falls. The approach focused on implementing evidence-based methods and integrating preventative design features that support both animal behaviour and safety in handling environments. The ultimate goal was to guarantee the welfare and protection of animals as specified in European guidelines, while also achieving improvements in meat quality. By advancing these objectives, the project demonstrates how compliance with animal welfare standards can be harmonised with operational efficiency and product quality, benefitting both animals and consumers across the European Union.

KEYWORDS

animal welfare, beef production, cattle, slaughter process, transport of animal

CONTENTS

SUMMARY

The European Union has developed a comprehensive and stringent regulatory framework to ensure both food safety and animal welfare throughout the food supply chain. Beginning with Regulation (EC) No 178/2002, the EU established core principles for food law, created the European Food Safety Authority (EFSA) and outlined essential procedures for protecting public health. This foundation was expanded by the 2004 'hygiene package', which harmonised hygiene and control standards from animal farming through food distribution.

Animal welfare is now an integral part of food safety legislation, notably emphasised in Regulations 853/2004, 625/2017 and 1099/2009. These laws mandate coordinated, transparent and science-based actions across all food chain actors and require continual updates to incorporate new scientific knowledge. Official veterinarians play a key role in enforcing compliance, taking corrective action and upholding welfare standards, especially during animal transport and slaughter.

To enhance animal welfare and reduce stress during handling, an innovative prototype cattle path made from multilayer pressed cardboard was developed. This eco-friendly, recyclable material was tested in real slaughterhouse conditions for resistance to compression, tearing, humidity and trampling. Cattle using the prototype path exhibited fewer slips, stops and required no electric prods. Biochemical measurements further showed lower levels of cortisol and beta-endorphins – markers of stress – in these animals, indicating improved welfare outcomes.

The results demonstrate that the integration of proper handling practices and sustainable materials can effectively reduce animal stress, improve welfare and enhance both meat quality and operational safety in abattoirs. This project aligns with EU legal requirements, scientific best practices and sustainability objectives, offering a valuable model for modernising livestock management across Europe.

1 | INTRODUCTION

The European Union has established a robust regulatory framework to ensure food safety and animal welfare throughout all stages of the food supply chain, beginning with Regulation (EC) No 178/2002. This regulation laid down the core principles of food legislation, created the European Food Safety Authority (EFSA) and defined procedures to safeguard public health. The 'hygiene package' adopted in 2004 reorganised regulations, taking effect from 1 January, 2006, and ensuring safety in animal farming, production and distribution.

EFSA was founded to protect consumer health and restore confidence after public health crises in the 1990s, such as BSE and dioxin contamination events. Its mandate is clear: provide scientific opinions and risk assessments for European institutions, thereby guiding laws affecting consumer safety. EFSA's impartial advice underpins regulatory acts – an example being Regulation 854/2004, which simplified rules for official controls on animal-origin products based on EFSA's guidance.

Animal welfare has become increasingly central to EU food legislation. Regulation 882/2004 asserted that animal health and welfare significantly influence food quality and safety, disease prevention and humane treatment. European law requires communication between all actors in the food supply chain to ensure coordinated, science-based practices. Regulation 853/2004 reiterates the need for efficient communication across food business operators, while Regulation 625/2017 and 1099/2009 further stress adaptable controls, transparency and continual updates reflecting new scientific knowledge (Anonymus, 2002, 2004a, 2004b, 2017, 2019). Official veterinarians, under these laws, ensure immediate corrective action for non-compliance, enforce proportionate responses and cooperate with other authorities to uphold welfare standards, especially during transport and slaughter (Čobanović et al., 2024; Ferri et al., 2023; García-Díez et al., 2023; Harley et al., 2012; Kautto et al., 2023; Valkova et al., 2022).

Protecting animals at the time of slaughter is legislatively framed as minimising anxiety and suffering. Regulation 1099/2009 acknowledges that even under optimal conditions, slaughter can cause pain or fear. Thus, personnel must implement advanced techniques and comply with sector best practices to reduce suffering. Continuous improvement is encouraged, recognising that better animal protection also leads to improved meat quality and safer working conditions for slaughterhouse staff (Anonymus, 2013; Arzoomand et al., 2019; Guardone et al., 2020; Stärk et al., 2014; Vidal et al., 2016).

Despite regulatory advances, there is no full scientific consensus on the best parameters for assessing unconsciousness in slaughtered animals. Regulation 1099/2009 recommends using indicators such as loss of posture, lack of voluntary movement and absence of reflexes or responses to stimuli as practical measures of unconsciousness and insensitivity. Ongoing research aims to refine these benchmarks and improve the effectiveness of stunning methods.

Large-scale European studies – such as the Dialrel project and BoRest – have tackled these complex issues. Dialrel, funded by the European Commission, brought together academics, industry stakeholders and public authorities to examine religious, legislative and welfare standards, striving to harmonise divergent views and practices. BoRest evaluated containment systems and practices across EU and third countries, assessing welfare impacts of slaughter with and without stunning. Key findings have informed updated recommendations and equipment designs, balancing animal welfare, meat quality, work safety and socio-economic constraints (https://www.dialrel.net, https://op.europa.eu/en/publication-detail/-/publication/02969f61-ca0a-11e9-992f-01aa75ed71a1#).

Practical guidelines have emerged from EU research and field observations to guide slaughterhouse design and operation (Anonymus, 2013, 2016, 2020; Antunović et al., 2021; Bonardi et al., 2021; Ferri et al., 2023). Key principles include:

- Layout, construction and equipment: Well-designed fences, corridors, ramps and flooring reduce animal stress and risk of injury. Non-slip, well-drained floors and gentle slopes enable animals to move independently and calmly. Corridors should allow group movement, avoid dead ends and sharp turns and use solid sides to prevent distractions.
- Lighting and noise: Animals prefer moving from dark to lighter areas but are frightened by sudden or intense lights and loud noises. Uniform lighting and minimising sudden noises help prevent panic.
- Water, ventilation, pens: Ready access to clean water, ample space to turn and separation of different animal types reduces aggression and distress. Effective ventilation counters heat and gas build-up.
- Equipment for containment and handling: Mechanical restraints should be tailored to species and used only by trained staff. Unacceptable practices include suspending, immobilising with electric shocks or causing pain intentionally – such actions are strictly prohibited.
- Handling best practices: Calm, confident and gentle handling avoids causing bruising or distress. Handlers should use natural animal behaviours and avoid obstructing or frightening animals. Injured animals unable to move should be euthanised where they are to avoid further suffering.

The correct application of stunning must always be verified. After stunning, operators check for immediate collapse, lack of regular breathing, absence of righting reflex and other signs to confirm unconsciousness. EFSA recommends specific criteria for monitoring and, if necessary, immediate corrective procedures.

The project also explored the stress response in cattle, noting that both short- and long-term stress (measured biochemically by cortisol and beta-endorphins) affects welfare and meat quality. Chronic stress disrupts immunity, growth and behaviour, while acute pain is moderated by neuropeptides such as beta-endorphin. Good design and handling reduce negative stress effects and improve outcomes.

A particularly innovative aspect of this research was the prototyping of cattle paths and traps using eco-sustainable materials such as corrugated cardboard. This material, commonly used for packaging, proved to be 100% recyclable,

biodegradable and customisable. It was tested in two thicknesses (1.5 and 3 cm) as flooring and side panels. Repeated tests with increasing numbers of cattle evaluated resistance to compression, tearing, humidity and trampling. Observation showed that, after trial runs with groups of 5, 15 and 25 animals, the material maintained functionality, though some damage was evident – providing valuable data for future refinements and confirming the promise of sustainable solutions in the improvement of animal handling environments.

In conclusion, the body of EU legislation, supported by scientific research and practical innovation, continues to drive improvements in animal welfare and food safety. Projects like these underscore the value of combining legal requirements, evidence-based best practices and sustainable design to ensure the ethical and efficient management of livestock in the European food industry.

2 | BACKGROUND

This project aimed to create a prototype of an updated cattle path that minimizes stress and reduces the use of coercive means such as electric prods, while also decreasing the risk of injury due to trauma or falls. The ultimate goal was to ensure the welfare and protection of the animals, as outlined by European guidelines, while simultaneously improving the quality of the meat produced.

3 | DATA AND METHODOLOGIES

3.1 | Data

Stress, especially long-term, can negatively affect the welfare of an animal. Stress is a widely used term in human society and may be defined as 'the non-specific response of the body to any demand for change'. In general, stress is a change in the psychological, physiological and/or physical characteristics of an organism. During stress, the animal's behaviour is altered to increase alertness, increase cardiac output, respiration and catabolism, as well as to divert blood flow to provide full perfusion of the brain, heart and muscles. In response to a stressor, the hypothalamic–pituitary–adrenal axis is activated, resulting in the secretion of its terminal hormones, the glucocorticoids. One of these glucocorticoids – and the most common stress hormone in mammals – cortisol, plays a key role in the release of energy, immune and mental activity, development and growth, as well as reproductive functions. In its important role in the stress response, cortisol is sensitive to both physical and emotional stimuli; its release is based on a circadian rhythm. Under physiological stress, cortisol modulates the immune system and mobilises energy stores, making more resources available to respond to a particular stressor. While glucocorticoids are useful for short-term survival, prolonged/increased release (chronic stress) can lead to metabolic, immune and physiological dysfunctions. Therefore, the cumulative occurrence of stressors could lead to changes in the welfare of an animal, changes in its social behaviour and the development of pathologies. It has been shown that chronic stress in domestic animals leads to altered levels of cortisol, reduced growth and reduced play behaviour in the young. High levels of cortisol are also linked to many diseases and behavioural, physiological, nutritional disorders, as well as obesity and diabetes caused by increased plasma glucose concentrations due to cortisol (Andoni et al., 2023; Ferri et al., 2023; García-Díez et al., 2023; Stella et al., 2022; Valadez-Noriega et al., 2022; Valkova et al., 2022).

Beta-endorphin is an endogenous opioid neuropeptide and peptide hormone produced in certain neurons within the central and peripheral nervous systems. Beta-endorphin's function is known to be associated with hunger, fear and pain, as well as maternal care, sexual behaviour and reward cognition. Broadly speaking, beta-endorphin is mainly used in the body to reduce stress and maintain homeostasis. In behavioural research, studies have shown that beta-endorphin is released in response to a wide variety of stimuli. Beta-endorphins have been studied primarily for their influence on nociception (i.e. pain perception). Beta-endorphins modulate pain perception in both the central and peripheral nervous systems. When pain is perceived, pain receptors (nociceptors) send signals to the dorsal horn of the spinal cord and then up to the hypothalamus, via the release of a neuropeptide called substance P. In the peripheral nervous system, this signal causes T lymphocytes – white blood cells from the immune system – to be recruited to the area where pain was perceived. The T lymphocytes release beta-endorphin in this localised region, allowing it to bind to opioid receptors, causing direct inhibition of substance P. In the central nervous system, beta-endorphins bind to opioid receptors in the dorsal root and inhibit the release of substance P in the spinal cord, reducing the number of excitatory pain signals sent to the brain. The hypothalamus responds to the pain signal by releasing beta-endorphins through the periaqueductal grey network, which acts primarily to inhibit the release of GABA, a neurotransmitter that prevents dopamine release. Therefore, inhibition of GABA release by beta-endorphin allows for greater dopamine release, contributing in part to the analgesic effect of beta-endorphin itself. The combination of these pathways reduces the sensation of pain, allowing the body to stop a painful impulse once it has been sent (Čobanović et al., 2024; Earley et al., 2017; García-Díez et al., 2023; Guardone et al., 2020; Hartung, 2003; Padalino & Raidal, 2020; Valadez-Noriega et al., 2022; Valkova et al., 2022).

Corrugated cardboard is the material mainly used for packaging. It consists of two flat paper surfaces, known as liners, and a corrugated sheet, all held together by natural adhesives. The combined action of the liners and the inner wave gives rigidity and strength to the cardboard and, as a result, to the packaging. There are many types of cardboard that differ based on the papers used, weights, wave heights (single, double or triple) and the coefficient of corrugation. Corrugated

cardboard is 100% recyclable and biodegradable and allows versatile protection and transport of the products it wraps. The different types of waves that can be combined give rise to an extremely versatile product, making infinite uses, development and customisation possible thanks to the use of state-of-the-art digital printing and cutting technologies, ongoing research into materials and finishes and the design and creativity of designers.

3.2 | Methodologies

The resistance to compression, tearing, humidity and trampling were evaluated both in the single-layer version and with multiple layers coupled together for repeated tests with a high number of animals.

3.3 | Resistance test to compression, tearing, humidity and trampling

The chosen product was delivered by the manufacturing company in two types: thickness 1.5 cm and thickness 3 cm. In short, the test consisted of placing the flooring along the corridor that leads the animals from the resting stalls to the trap and a panel at the curve leading to the final section of the path. The condition of the material, pending more precise laboratory assessments, is illustrated in the following images (Photograph 1–15).

PHOTOGRAPH 1 Folding floor of pressed cardboard, thickness 3 cm in three layers.

PHOTOGRAPH 2 Folding floor of pressed cardboard, thickness 1.5 cm in two layers.

PHOTOGRAPH 3 Side panel for prototyping the curved path.

PHOTOGRAPH 4 Installation of supports for side panels.

PHOTOGRAPH 5 Cardboard floor in situ.

PHOTOGRAPH 6 Side panel in situ.

PHOTOGRAPH 7 Condition of the floor after the passage of 5 animals.

PHOTOGRAPH 8 Condition of the floor after the passage of 15 animals.

PHOTOGRAPH 9 Condition of the floor after the passage of 25 animals.

PHOTOGRAPH 10 Condition of the side panel after the passage of 25 animals.

PHOTOGRAPH 11 Details of damage to the side panel after the passage of 25 animals.

PHOTOGRAPH 12 Side panel still intact and able to perform the function after the passage of 25 animals.

PHOTOGRAPH 13 Condition of the materials at the end of the resistance test.

3.4 | Material

The material is beige and is well tolerated by the animals (cf. the indications from the Fear Free colour palette association: (https://www.dvm360.com) (Figure 1).

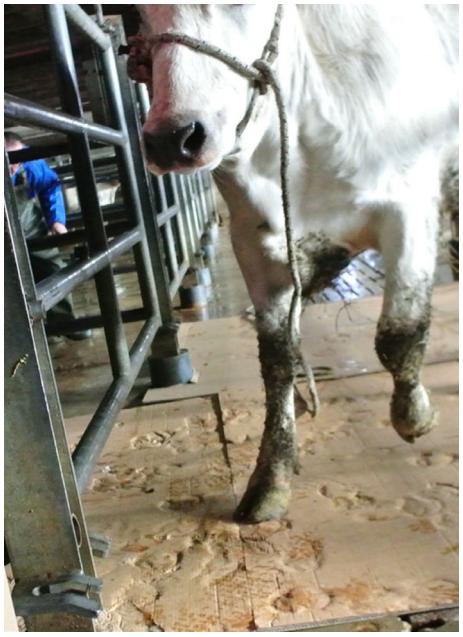
FIGURE 1 Palette of colours that do not frighten animals, with indication of the colour of the material used in our prototype.

After the transit of 25 animals, with wet concrete underneath, the material did not experience damage that would render it unusable or hinder the advancement of the animals. The same can be said for the side panel, positioned at the point of greatest contact and pressure from the animals (it is a narrowing, also a critical point in the corridor where two animals often present themselves simultaneously).

The uniformity of the material ensures the animals proceed linearly and without hesitation. It should be noted here that the current configuration of the concrete floor has a grill that, alternating dark and light areas, makes animal movement uncertain.

Another important note is the difficulty animals have walking on the existing slippery floor. The cardboard prototype instead allows animals to move forward without stress, to the point that after contacting the surface their gait becomes more secure and brisk.

Similar considerations apply to the panelling, which makes the wall uniform and limits distractions for the animals (Ceccarelli et al., 2018; García-Díez et al., 2023; Guardone et al., 2020).



PHOTOGRAPH 14 Brisk and safe gait on the floor covered by the prototype.

The material can be used successfully in the creation of the definitive prototype, which should then be evaluated by measuring animal welfare and meat quality parameters with reference to control animals (see PSR project filed at the time of the call) (Figure 2).

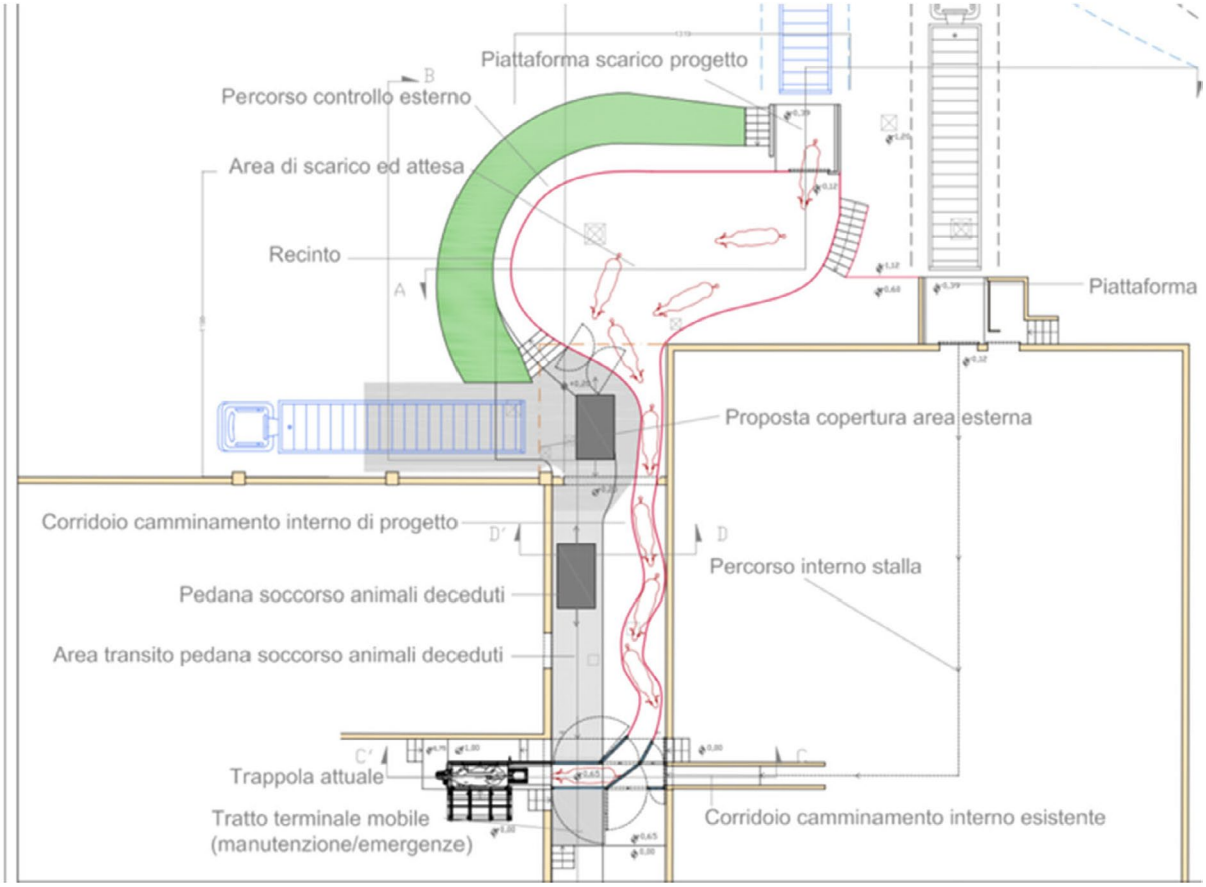
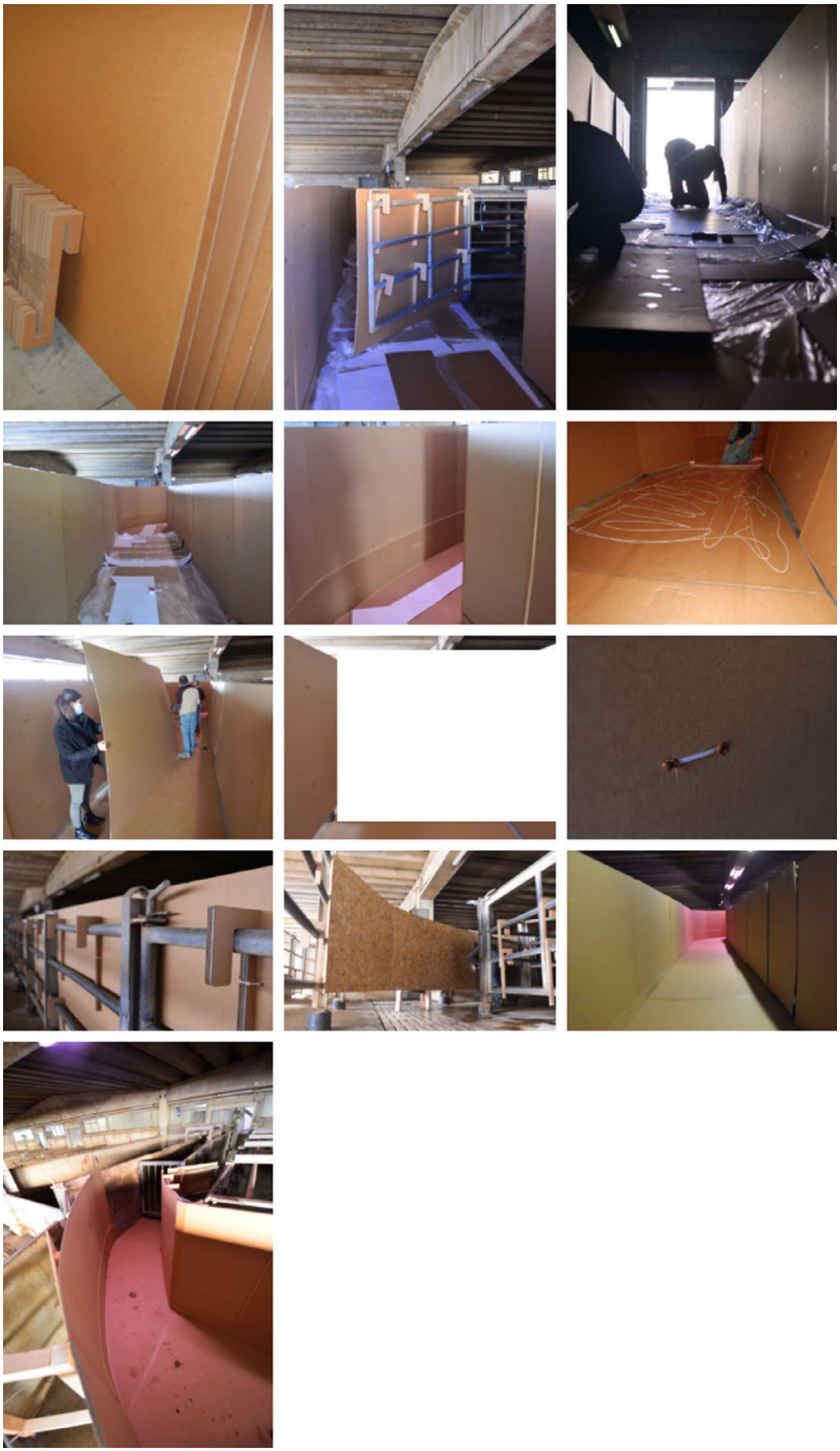


FIGURE 2 The path with a curved layout.



PHOTOGRAPH 15 Photos showing the construction of the prototype. The utmost care was taken to ensure uniformity of the structure and to limit light infiltration through the use of adhesives of the same colour as the panels.

3.5 | Statistical analysis

Data Analysis Software. All statistical analyses were performed using StatView (version X, Abacus Concepts Inc., Berkeley, CA, USA) for Mac OS. StatView is an integrated statistical analysis and graphics package offering comprehensive capabilities for descriptive and inferential statistics, including ANOVA and post-hoc comparisons. StatView was selected for its intuitively clear interface and low learning curve, which are especially advantageous for teaching purposes, despite being an older software package designed to run exclusively on PowerPC Macintosh systems with Mac OS 9.

Statistical Analysis. Descriptive statistics (mean and standard deviation) were calculated for each group. Group comparisons for serum cortisol and beta-endorphin concentrations were conducted using a one-way analysis of variance (ANOVA), provided parametric assumptions (normality and homogeneity of variance) were met. Whenever ANOVA indicated a significant overall effect ($p < 0.05$), pairwise group differences were further assessed using Tukey's Honestly significant difference (HSD) post-hoc test to control for multiple comparisons. The significance threshold for all statistical tests was set at $p < 0.05$. All reported p-values correspond to two-tailed tests unless otherwise specified.

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4 | ASSESSMENT

4.1 | Observed welfare parameters

Number of activities/behaviours indicative of non-compliance with animal welfare for each animal; mean of 25 animals per group (Table 1).

TABLE 1 Observational parameters.

	Prod	Falls/slips	Stoppage in march
Control group ($n = 25$)	0.56	0.75	0.50
Prototype group ($n = 25$)	0.00	0.00	0.04

Analysis of the table shows that in the control group, the operators had to resort to using the electric prod once every two animals (0.56 times per animal) and the animals often suffered falls or slips (0.75 times per animal) or stopped (0.50 times per animal).

In the group of animals examined with the prototype installed, it was never necessary to use the electric prod and no animal ever slipped. Only in one case did an animal make a 'U-turn', but this is attributable to the excessive width of the pre-existing structure, which with the prototype could only be partially reduced.

4.2 | Blood chemistry parameters

The animals under study were divided into three groups. The first group (A), made up of 17 animals, used the prototype path to reach the capture trap. The second (B), made up of 24 animals, used the normal path on a day characterised by low stress levels. The third (C), made up of 30 animals, used the normal path on a day characterised by high stress levels. The animals in these groups were heterogeneous in breed, age, sex and origin. Blood samples were taken during jugulation using tubes without anticoagulant. Samples were transported to the laboratory and centrifuged at 3000 rpm for 10 minutes. The serum thus obtained was collected and placed in sterile Eppendorf tubes for subsequent analyses. Quantification of cortisol and beta-endorphins was carried out on the serum using commercial ELISA kits (Abbexa LTD, Cambridge, UK). These are kits based on a competitive binding technology between enzyme and antibody. Standards and samples to be analysed are introduced into the available wells. At the end of the procedure, the optical density of the individual wells was measured spectrophotometrically at a wavelength of 450 nm and the analyte concentration was calculated.

As regards cortisol, group A (prototype) had the lowest average value (375.96 ng/mL), followed by group B and group C (controls) with average values of 397.71 ng/mL and 1029.26 ng/mL respectively. The difference in cortisol concentration

in the serum was very large and statistically significant between groups A and B compared to group C, with a *p*-value < 0.0001. As for beta-endorphins, the lowest serum values were those of group A (prototype) averaging 8.03 pg/mL, followed by groups B and C (controls) with mean values of 9.35 pg/mL and 31.14 pg/mL respectively. Again, the difference between control group C and the other two groups was statistically significant with a *p*-value < 0.05.

Cortisol is considered an important blood chemistry indicator of stress. In stressful conditions, the animal's behaviour is altered to increase alertness, cardiac output, respiration and catabolism, as well as to divert blood flow to ensure full perfusion of the brain, heart and muscles. In response to a stressor, the hypothalamic–pituitary–adrenal axis is activated, resulting in the secretion of its terminal hormones, the glucocorticoids, among which is cortisol. Beta-endorphins are one of three types of endogenous opioid neuropeptides produced by mammals. Beta-endorphins are mainly used by the body to reduce stress and maintain homeostasis. The results obtained show that proper management of animals aimed at minimising stress factors in the phases prior to slaughter leads to significant reductions in serum levels of these analytes and that the prototype path under study excellently fulfils this function (Figures 3 and 4).

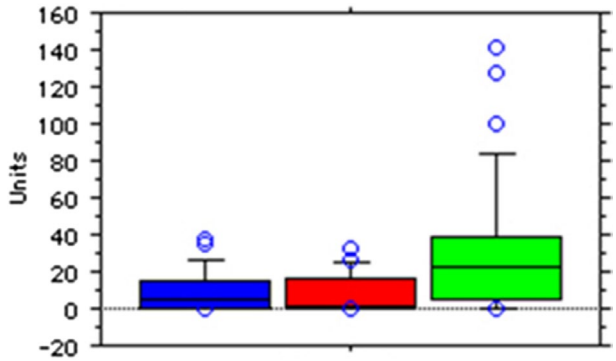


FIGURE 3 Beta-endorphins level in serum samples, expressed in pg. mL^{-1}. Blue: Normal path with correct handling; red: Innovative path; green: Normal path with non-correct handling.

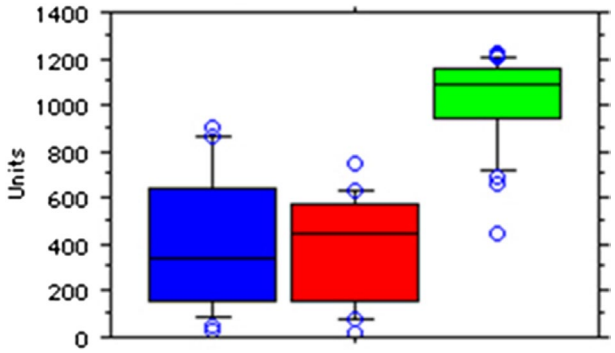


FIGURE 4 Cortisol level in serum samples, expressed in ng mL^{-1}. Blue: Normal path with correct handling; red: Innovative path; green: Normal path with non-correct handling.

5 | CONCLUSION

For the prototype's construction, different materials were evaluated. In the end, the choice fell on multilayer pressed cardboard which guaranteed the right balance between functionality, cost and resilience. The group managed using the innovative prototype path and correct handling procedures exhibited a reduced need for coercive intervention by the operators. Additionally, the animals displayed improved ethological indicators of stress, and their blood levels of cortisol and beta-endorphins were lower.

The results obtained in this study demonstrate that the prototype path enables cattle to move forward more easily, thereby enhancing their welfare and minimising risks for both the animals and operators. The application of the prototype path developed in this study has the potential to significantly improve animal protection conditions and meat quality in numerous abattoirs.

6 | RECOMMENDATIONS

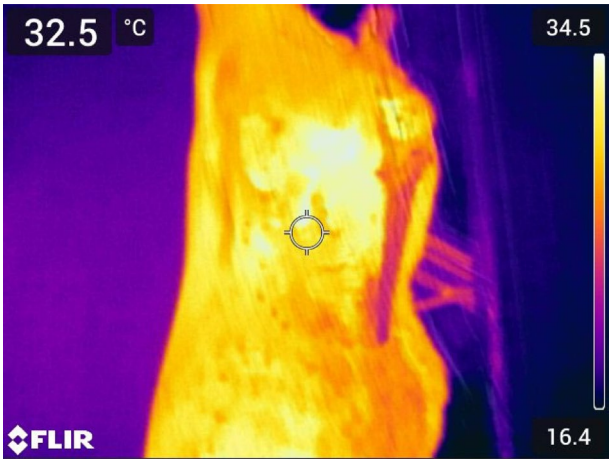
1. Ensure that animal handling pathways are designed with non-slip flooring, gentle slopes and curved layouts to facilitate calm, efficient cattle movement and minimise stress and injury.

2. Replace or cover traditional concrete floors and barriers with eco-sustainable, shock-absorbing materials such as multi-layer pressed cardboard to reduce noise, visual distractions and slips.
3. Avoid the use of electric prods and coercive handling devices whenever possible; instead, rely on environmental and behavioural design to promote voluntary animal movement.
4. Provide uniform, diffused lighting in all areas used for animal holding and transfer, while minimising noise and shadows, to prevent animal agitation and hesitation.
5. Regularly train slaughterhouse staff in humane handling techniques, including recognising indicators of animal stress and unconsciousness, and responding promptly to welfare issues.
6. Maintain ready access to clean water, adequate ventilation and appropriate space for different animal groups to reduce aggression and dehydration during lairage.
7. Continuously monitor animal welfare indicators and blood biochemistry (such as cortisol and beta-endorphin levels) to evaluate the effectiveness of welfare interventions.
8. Adapt controls and procedures based on ongoing scientific research and regulatory updates, ensuring that best practices are routinely implemented and revised.
9. Encourage collaboration and transparent communication among all food chain stakeholders, including veterinarians, operators and competent authorities, to ensure coordinated animal welfare management.
10. Prioritise the use of recyclable, biodegradable and animal-friendly materials in the construction of new abattoir infrastructure to advance both welfare and sustainability goals.

7 | OTHER ACTIVITIES DURING THE EU-FORA FELLOWSHIP

During the fellowship, the fellow had the opportunity to participate to various scientific activities. These included:

a. contact with stakeholders
b. learning about specifics of the Italian meat industry during meetings of the research team and visit to processing plants and collect the data in the industry condition
c. laboratory work in ongoing projects, including: conducting microbiological and biochemical analyses and cultures; preparing microbiological media; and performing minor laboratory work
d. working in a cell culture laboratory
e. weekly meetings with state veterinary services on RA and best practices
f. meetings with the scientists and specialists from the hosting site
g. meeting and training with the Inspectors from Polish National Food Quality Inspection organised by sending site
h. g. active participation to the activities of the master in official control of food, https://www.ispezioneperugia.it and of the PhD program in veterinary medicine, https://medvet.unipg.it/didattica/dottorati-diricerca/sanita-e-scienze-sperimentali-veterinarie that are coordinated by the local supervisor Prof. Cenci Goga)
i. desk work and data analysis
j. participation in classes with students at the slaughterhouse during meat quality evaluation and veterinary inspection
k. an attempt to use a thermal imaging camera FLIR to assess animal welfare.



ABBREVIATIONS

ANOVA	analysis of variance
HSD	Honestly significant difference

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EU-FORA SERIES 8

Risk assessment of replacing synthetic preservatives with natural ingredients

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

The present work, developed within the EU-FORA fellowship programme, assessed the risk assessment of vegetable by-product extracts as natural alternatives to synthetic preservatives in food formulations. Extracts were obtained from onion peel (*Allium cepa* L.), zucchini (*Cucurbita pepo* L.) and kale (*Brassica oleracea* L. var. *acephala*), which represent abundant residues with limited commercial value but high potential for valorisation. Suitable extraction techniques were applied to each matrix, and the resulting extracts were characterised through phenolic profiling, antioxidant activity, cytocompatibility and pesticide residue analysis. Onion peel extract was rich in quercetin glycosides and showed strong antioxidant activity, while zucchini and kale extracts contained quercetin or kaempferol derivatives with moderate effects. Cytocompatibility assays demonstrated acceptable safety margins at lower concentrations, though dose-dependent toxicity appeared at higher levels, particularly in hepatic cells. Pesticide residues were mostly absent or present only at trace levels in zucchini and kale extracts. In onion peel extract, hexachlorobenzene and β-HCH were detected, but remained below EU maximum residue limits (MRLs). Overall, the findings suggest that the benefits of these extracts, including preservative potential and sustainable waste valorisation, outweigh the identified risks.

KEYWORDS

bioactive potential, cytocompatibility, pesticide residues, preservative ingredients, vegetable by-products

CONTENTS

SUMMARY

This report presents the results of a risk assessment of natural ingredients obtained from vegetable by-products, associated with the benefits that can be incorporated, performed within the framework of the EU-FORA fellowship programme. The aim was to explore the potential of onion peel, zucchini and kale extracts as natural alternatives to synthetic preservatives, combining food safety considerations with sustainability goals.

The study followed the EFSA guidance on risk assessment, integrating data on chemical composition, bioactivity, cytocompatibility and pesticide residues. Extracts were obtained using appropriate extraction techniques adapted to each by-product, ensuring efficient recovery of phenolic compounds. The phenolic characterisation showed that onion peel and zucchini were rich in quercetin glycosides, while kale contained kaempferol derivatives. Onion peel extract presented the highest phenolic content and exhibited strong antioxidant activity, whereas zucchini and kale extracts showed moderate antioxidant effects.

The biological evaluation demonstrated that cytocompatibility was dependent of the concentration of the extract. In gastrointestinal Caco-2 cells, all extracts maintained ≥ 70% viability up to 2500 μg/mL, suggesting low intestinal toxicity. In hepatic AML12 cells, onion peel extract preserved ≥ 70% activity only up to 1250 μg/mL, zucchini up to 2500 μg/mL and kale up to 5000 μg/mL, indicating greater sensitivity of hepatic cells to high doses.

Pesticide occurrence was also assessed. Zucchini and kale extracts contained only trace, non-quantifiable residues (< method detection limit (MDL) or < method quantification limit (MQL)). In onion peel extract, low levels of hexachlorobenzene (HCB) and β-hexachlorocyclohexane (β-HCB) were detected, but these concentrations were below the maximum residue limits (0.01 mg/kg) established by EU legislation. Since results were expressed on a dry extract basis, they represent a worst-case scenario compared to raw material levels.

Overall, the integration of results indicates that the benefits of using vegetable by-product extracts outweigh the identified risks. These extracts provide significant antioxidant activity, potential preservative function and sustainability benefits by promoting waste valorisation. Risks are limited to dose-dependent cytotoxicity at high concentrations and low-level pesticide residues in onion peel, which do not exceed regulatory thresholds. The findings support the safe use of onion peel, zucchini and kale extracts as natural ingredients, provided application levels remain within cytocompatible ranges and pesticide monitoring is maintained.

1 | INTRODUCTION

Synthetic preservatives are important ingredients in the food industry that provide shelf life by controlling microbial growth and preventing oxidation and browning of products. In the same intensity that synthetic preservatives are important in the food and pharmaceutical industries, their consumption has been related to various adverse effects in the human body, such as neurological, gastrointestinal and dermatological disorders, as well as the development of different cancerous tumours (Caleja et al., 2016; Carocho et al., 2014).

As a result, consumers are increasingly concerned about synthetic additives and have been seeking products formulated with natural ingredients. This has driven a growing demand for ingredients of natural origin, which, in addition to their preservative action through antimicrobial and antioxidant properties (Shah & Mir, 2022), may contribute to human healthy by combating free radicals and potentially helping to prevent diseases such as cancer, Alzheimer's, diabetes, allergies and cardiovascular and respiratory conditions (Carocho et al., 2018). This growing demand has led to increased interest in plant-derived bioactive compounds, particularly phenolic compounds, which are among the most promising candidates for replacing synthetic preservatives (Shah & Mir, 2022).

Phenolic compounds are obtained from plant sources and are typically safe for human consumption (Tresserra-Rimbau et al., 2018). Their hydroxyl groups confer both strong antioxidant potential and antimicrobial activity, which are desirable in food preservation (Alara et al., 2021). However, this same property can also pose toxicity risks to humans at high concentrations, making it essential to determine safe incorporation limits in food formulations (Chen et al., 2024).

In the development of natural preservatives, a structured risk–benefit assessment is essential to ensure consumer safety while maximising potential functional advantages. Following the guidance provided by the European Food Safety Authority (EFSA) (EFSA Scientific Committee, 2010), this approach requires clear identification of the ingredient, its intended use and the target population, followed by evaluation of both favourable and adverse effects. For phenolic-rich extracts obtained from vegetable by-products, beneficial effects are associated with antimicrobial and antioxidant properties that may enhance food preservation and contribute to human health. Potential adverse effects may arise from contamination with pesticide residues or toxicity at high concentrations. The assessment, therefore, integrates compositional analysis (phenolic profile), determination of potential contaminants and in vitro evaluation of cytocompatibility in relevant human cell models, providing a scientific basis for establishing safe use levels in food applications (Zeng et al., 2023).

Vegetable by-products have therefore been widely studied as potential preservative and functional ingredients. For example, Fernandez et al. (2020) evaluated the stability of beet leaf extract-enriched smoothies over 21 days of storage at 5°C, showing reduced microbial counts and extended shelf life compared to controls, as well as increased total phenolic content without significant losses during storage. Gong et al. (2022) assessed beet extract in cooked pork for *Listeria monocytogenes* control, observing significant microbial reductions without compromising product quality. dos Santos Silva et al. (2022) investigated oxidative stability in beef burgers enriched with microencapsulated grape pomace extract, demonstrating better lipid oxidation control than a conventional synthetic antioxidant, without affecting colour during storage.

Based on these findings, further studies addressing both the preservative efficacy and the safety profile of natural ingredients are essential. Evaluating their chemical composition, possible contamination with pesticides and cytocompatibility is crucial to define safe application levels and to encourage their adoption by the food industry. Such an approach can support the partial or total replacement of synthetic preservatives, promoting more sustainable practices, foods with greater nutritional and functional value and guaranteed safety for consumers.

2 | BACKGROUND AND TERMS OF REFERENCE AS PROVIDED BY THE REQUESTOR

2.1 | Description of work programme

As part of this programme, the overall objective was to assess the health risks associated with the obtention and consumption of natural ingredients derived from vegetable by-products, to support the replacement of synthetic preservatives in food formulations. The study addressed key risk questions, namely the potential benefits of incorporating natural ingredients, the possible risks associated with their consumption, and the likelihood of pesticides being present in vegetable by-products and their extracts.

To achieve this objective, the work programme was divided into four main tasks. First, a literature review was conducted to gather scientific evidence on the use of natural ingredients derived from vegetable by-products, focusing specifically on their preservative potential and safety aspects. Second, extracts were obtained from vegetable by-products, such as onion peel, kale, and zucchini, which did not meet commercial quality standards, using an extraction method designed to maximize the recovery of phenolic compounds. Third, the extracts were submitted to in vitro characterization through

antioxidant activity assay, cytocompatibility tests on gastrointestinal (Caco-2) and hepatic (AML12) cell lines, and chromatographic analysis of phenolic compound profiles and pesticide residues. Finally, the data from the compositional analysis, biological activity, pesticide residue testing, and cytocompatibility assessments were integrated into a risk assessment framework, enabling the evaluation of both potential benefits and adverse effects, and providing a scientific basis for the safe use of vegetable by-products as natural ingredients for food preservation.

2.2 | Additional activities for training and networking

All these activities were conducted as part of the European Food Risk Assessment (EU-FORA) Fellowship Programme for the 2024–2025 cycle. The programme is designed to broaden the expertise available in the field of food safety and to strengthen the European network for food risk assessment. Within the 12-month EU-FORA fellowship, the fellow participated in a structured training scheme, which included an initial three-week introductory course followed by four advanced modules of 1 week each, amounting to 7 weeks of dedicated training. In addition, the fellow completed a 3-month stay at the Nutrition and Food Group (NuFoG) of the Department of Analytical Chemistry and Food Science, Instituto de Agroecoloxía e Alimentación (IAA), University of Vigo, Spain, under the supervision of Dr. Miguel Ángel Prieto Lage, and also visited the Spanish Agency for Food Safety & Nutrition (AESAN) in Madrid and the laboratory in the National Centre for food (CNA) located in Majadahonda, Spain, that was possible learn more about how they conducted the food safety.

The EU-FORA program also allowed the fellow to participate in a conference, through oral communication:

- Oliveira, T.C.G., Chamorro, F., Carpena, M., Prieto, M.A., Raimundo, D., Ferreira, I.C.F.R., Oliveira, M.B.P.P., Pereira, E., and Barros, L. Sustainable bioactive recovery: The most efficient quercetin extraction from onion peel. Presented at XXIII European Conference on Food Chemistry, 11-13 June 2025, Bratislava, Slovakia. Oral presentation.

3 | MATERIALS AND METHODS

3.1 | Plant material

The risk assessment was conducted on extracts obtained from onion peel (*Allium cepa* L.), zucchini (*Cucurbita pepo* L.) and kale (*Brassica oleracea* L. var. *acephala*) (Figure 1), used as representative vegetable by-products. The raw materials were supplied by a company in Portugal and originated from vegetables that did not comply with commercial quality standards for human consumption.

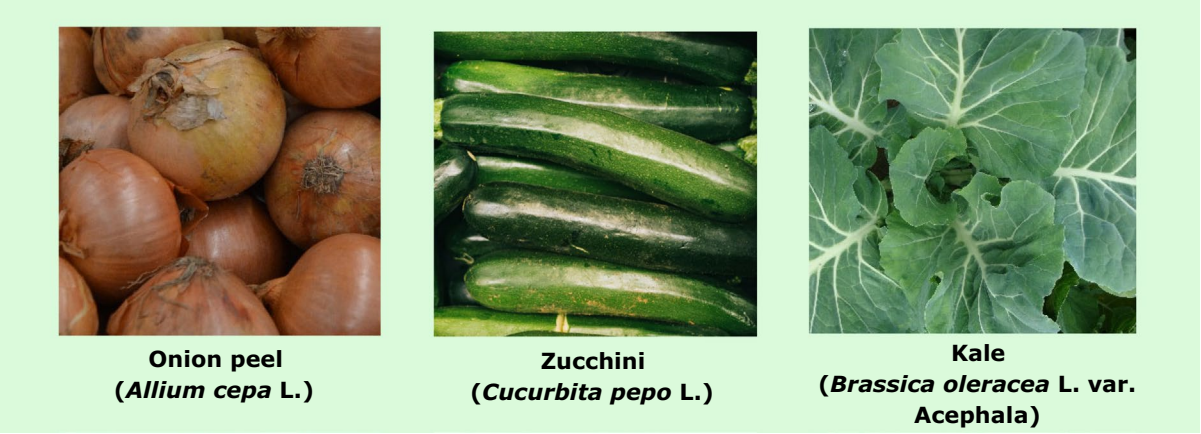

FIGURE 1 Vegetable by-products used in the study, common and scientific names. Images sourced from Unsplash/Pexels (free licence).

3.2 | Obtention of natural ingredients from vegetables by-products

Extracts from onion peel, zucchini and kale were obtained using different extraction techniques selected for each raw material. Microwave-assisted extraction (MAE) was applied for onion peel, while pressurised liquid extraction (PLE) was employed for zucchini and kale. The operational conditions were chosen based on prior experimental design studies aimed at maximising the recovery of phenolic compounds. The extracts obtained under these conditions were subsequently used for the risk assessment analyses.

3.3 | Characterisation of natural ingredients from vegetable by-products

3.3.1 | Phenolic compounds identification

The phenolic profiles of the extracts were analysed using an ultra-performance liquid chromatography system (UPLC, Dionex Ultimate 3000, Thermo Scientific, San Jose, CA, USA) equipped with a diode array detector and coupled to a mass spectrometer (LTQ XL, Thermo Finnigan, San Jose, CA, USA) operating in negative ion mode. Data acquisition was performed with Xcalibur® software. Identification of the main phenolic compounds was based on retention times, UV–Vis spectra and mass fragmentation patterns, compared with available reference standards and reported literature. Only major compounds corresponding to the most intense chromatographic peaks were considered.

3.3.2 | In vitro antioxidant activity

The antioxidant potential of the extracts was assessed using two complementary colorimetric assays: thiobarbituric acid reactive substances (TBARS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging.

3.3.3 | In vitro cytocompatibility

The cytocompatibility of the extracts was evaluated in gastrointestinal (Caco-2) and hepatic (AML12) cell lines, obtained from a commercial supplier. Cells were seeded in 96-well plates and exposed to different concentrations of the extracts for 24 h. Cell viability was assessed using the resazurin reduction assay, and results were expressed as a percentage of metabolic activity relative to the negative control.

3.3.4 | Pesticide occurrence

The presence of organochlorine pesticides (OCPs) in the extracts was assessed. For this, sample cleanup was performed using solid-phase extraction as described by Punín Crespo and Lage Yusty (2006). Analysis was conducted with gas chromatography equipped with an electron capture detector, using a capillary column suitable for pesticide detection. Helium served as the carrier gas and nitrogen as the make-up gas. Chromatographic conditions were optimised to enable the separation and identification of target compounds, including dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethane (DDD), aldrin, dieldrin, endrin, endosulfan I and II, hexachlorobenzene (HCB), various isomers of hexachlorocyclohexane (α-, β-, γ- and δ-HCH) and methoxychlor. Method validation was conducted in accordance with European Commission guidelines, encompassing assessments of linearity, accuracy and detection and quantification limits.

4 | ASSESSMENT

The vegetable by-products chosen for this study – onion peel, zucchini and kale – are important sources of phenolic compounds and show the potential for adding value to raw materials that do not meet commercial quality standards. Besides their bioactive potential, using these by-products promotes sustainability and circular economy strategies by reducing food waste.

Different extraction techniques were applied to each vegetable by-product based on prior optimisation studies. This approach allowed the recovery of extracts with high phenolic content and ensured that the subsequent characterisation reflected the most suitable conditions for each matrix. While this strategy supports maximising bioactive potential, it also emphasises the importance of assessing potential toxicological risks related to higher phenolic concentrations.

4.1 | Characterisation of natural ingredients

4.1.1 | Phenolic compounds identification

The extracts showed distinct phenolic profiles. Onion peel and zucchini extracts were characterised by quercetin glycosides, while kale extracts contained acylated and glycosylated derivatives of kaempferol. Onion peel presented the highest phenolic content, as reflected by the chromatographic peak intensities. These findings are consistent with the literature and support the potential of these compounds to contribute to antioxidant and preservative effects.

4.1.2 | Antioxidant activity evaluation

The antioxidant potential was strong for onion peel extract and moderate for zucchini and kale extracts. This behaviour is consistent with the phenolic composition identified and suggests that onion peel is particularly promising as a source of natural preservatives with high radical-scavenging capacity.

4.1.3 | Cytocompatibility assessment

Cytocompatibility was dose-dependent across the tested concentrations (156–10,000 μg/mL), as shown in Figure 2. In gastrointestinal cells (Caco-2), all extracts maintained ≥ 70% metabolic activity up to 2500 μg/mL, suggesting low intestinal toxicity in this range. In hepatic cells (AML12), onion peel extract maintained ≥ 70% activity up to 1250 μg/mL, while kale extract was tolerated up to 5000 μg/mL. Zucchini extract preserved high viability up to 2500 μg/mL, but above this concentration, cell viability dropped markedly. These results indicate that hepatic cells are more sensitive than intestinal cells, and toxicity is more evident at higher doses, particularly for onion peel and zucchini.

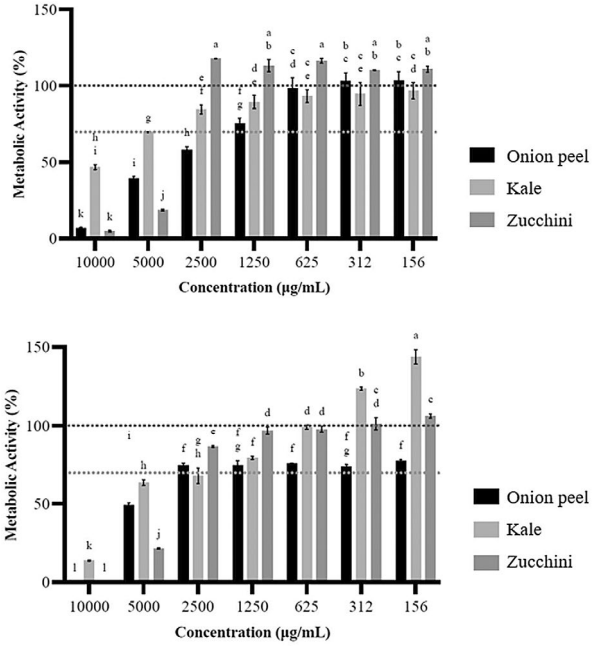

FIGURE 2 Metabolic activity of AML12 (up) and Caco-2 (down) cells after 24 h of exposure to onion peel, kale and zucchini extracts. The horizontal line indicates 70% and 100% cell viability. Values expressed as mean ± standard deviation ($n = 3$). Different letters indicate significant differences ($p < 0.05$; Tukey's test).

4.1.4 | Pesticide occurrence

The residues of OCPs analysed in vegetable by-product extracts are presented in Table 1. It showed that onion peel extract had low levels of HCB (0.4 ± 0.1 mg/kg) and β-HCH (0.5 ± 0.3 mg/kg), while all other compounds were not detected. In zucchini extract, only trace levels of endrin and methoxychlor were observed, below the method detection limit (< MDL). For the kale extract, a trace amount of endrin was detected below the method quantification limit (< MQL).

According to the EU Pesticides Database (European Commission, 2025, Regulation (EC) No 396/2005), the maximum residue limits (MRLs) for the commodities corresponding to onion, zucchini and kale are set at 0.01 mg/kg for these OCPs. In this study, however, results are expressed on a dry extract basis, which concentrates residues relative to the fresh raw material. Therefore, the values detected in onion peel extract are not directly comparable to the regulatory limits but still indicate that contamination is negligible. The presence of persistent compounds such as HCB and β-HCH highlights the importance of continued monitoring, although levels remain very low in the context of risk assessment.

TABLE 1 Concentration of organochlorine pesticides (OCPs) tested in onion peel, zucchini and kale extracts. Results expressed in mg OCPs/kg dry extract.

Compound	Onion peel extract (mg/kg dry extract)	Zucchini extract (mg/kg dry extract)	Kale extract (mg/kg dry extract)
DDE	n.d.	n.d.	n.d.
Aldrin	n.d.	n.d.	n.d.
DDT + DDD	n.d.	n.d.	n.d.
Dieldrin	n.d.	n.d.	n.d.
Endosulfan I	n.d.	n.d.	n.d.
Endosulfan II	n.d.	n.d.	n.d.
Endrin	n.d.	< MDL	< MQL
HCB	0.4 ± 0.1	n.d.	n.d.
α-HCH	n.d.	n.d.	n.d.
β-HCH	0.5 ± 0.3	n.d.	n.d.
γ-HCH	n.d.	n.d.	n.d.
δ-HCH	n.d.	n.d.	n.d.
Methoxychlor	n.d.	< MDL	n.d.

Note: Methoxychlor – LOD = 0.02 mg/kg and LOQ = 0.0514 mg/kg; Endrin – LOD = 0.02 mg/kg and LOQ = 0.065 mg/kg.

Abbreviations: < MDL, below method detection limit; < MQL, below method quantification limit; DDD, dichlorodiphenyldichloroethane; DDE, 2,2-bis(p-chlorophenyl)-1,1-dichloroethene; DDT, dichlorodiphenyltrichloroethane; HCB, hexachlorobenzene; HCH, hexachlorocyclohexanes; n.d., not detected.

4.2 | Risk–benefit integration

The integration of chemical composition, bioactivity, cytocompatibility and pesticide occurrence provides an overall picture of the safety and functionality of the studied extracts.

From the perspective of benefits, onion peel, zucchini and kale extracts presented relevant phenolic compounds, with onion peel showing the highest content of quercetin glycosides and strong antioxidant activity, while zucchini and kale displayed moderate activity. These properties support their potential as natural preservatives and contribute to the valorisation of vegetable by-products in line with circular economy principles.

In terms of risks, cytocompatibility results indicated acceptable safety margins at lower concentrations, with dose-dependent cytotoxicity appearing at higher levels, particularly in hepatic cells. Regarding pesticide residues, zucchini and kale extracts contained only non-quantifiable traces (< MDL or < MQL), while onion peel extract showed low levels of HCB and β-HCH. Although results are expressed on a dry extract basis, and therefore represent a worst-case scenario compared with raw vegetables or food formulations, the concentrations detected were consistent with the EU maximum residue limits of 0.01 mg/kg established for the studied commodities (European Commission, EU Pesticides Database).

Considering both aspects, the risk–benefit balance suggests that the benefits of using these extracts as natural ingredients outweigh the identified risks, provided that concentration ranges remain within cytocompatibility limits and pesticide levels continue to comply with EU safety thresholds. With this, Table 2 summarises the integration of phenolic composition, antioxidant activity, cytocompatibility, pesticide occurrence and the overall risk–benefit evaluation.

TABLE 2 Risk–benefit assessment of extracts from vegetable by-products.

Extract source	Main phenolic compounds	Antioxidant activity	Pesticides residues	Potential hazards	Potential benefits	Risk–benefit conclusion
Onion peel (*Allium cepa* L.)	Quercetin glycosides	Strong	Low levels of persistents OCPs detected, below EU safety limits	Hepatic cytotoxicity at high doses; presence of persistent OCPs	High antioxidant effect; preservative potential; by-product valorisation	Benefits outweigh risks, but continued monitoring of pesticide residues is recommended
Zucchini (*Cucurbita pepo* L.)	Quercetin glycosides	Moderate	Only trace residues (< MDL) negligible for risk assessment	Cytotoxicity above 2500 μg/mL in hepatic cells; negligible pesticide residues	Moderate antioxidant effect; waste valorisation	Benefits outweigh risks, but continued monitoring of pesticide residues is recommended
Kale (*Brassica oleracea* L. var. *acephala*)	Kaempferol acylated/ glycosylated derivatives	Moderate	Trace residues (< MQL), negligible for risk assessment	Cytotoxicity above 5000 μg/mL; negligible pesticide residues	Moderate antioxidant effect; functional ingredient potential; sustainable valorisation	Benefits outweigh risks at applicable concentrations

Note: EU safety limits: 0.01 mg pesticide/kg vegetable.

Abbreviations: < MDL, below method detection limit; < MQL, below method quantification limit; OCPs, organochlorine pesticides.

5 | CONCLUSION

The present work demonstrated that extracts obtained from onion peel, zucchini and kale are rich in phenolic compounds with relevant antioxidant potential, supporting their application as natural alternatives to synthetic preservatives. Cytocompatibility assays indicated safe margins at lower concentrations, while toxicity emerged only at higher doses, particularly in hepatic cells. Pesticide residues were mostly absent or present at trace levels, with low detections in onion peel extract remaining below EU maximum residue limits. Overall, the risk–benefit balance supports the safe use of these vegetable by-product extracts within appropriate concentration ranges, highlighting their potential for food preservation and their contribution to sustainability through by-product valorisation.

6 | RECOMMENDATIONS

The EU-FORA fellowship provided valuable opportunities to build knowledge and practical skills across multiple phases of risk assessment. The interdisciplinary teams established among participating institutions represent a key strength of the programme, laying the groundwork for continued cooperation in risk-related scientific research.

ABBREVIATIONS

AESAN	Spanish Agency for Food Safety & Nutrition
CNA	laboratory in the National Centre for food
DDD	dichlorodiphenyldichloroethane
DDT	dichlorodiphenyltrichloroethane
DPPH	1,1-diphenyl-2-picrylhydrazyl
EU-FORA	European Food Risk Assessment Fellowship Programme
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane (α-, β-, γ-, δ-isomers)
IAA	Instituto de Agroecoloxía e Alimentación
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave-assisted extraction
MDL	method detection limit
MQL	method quantification limit
MRL	maximum residue limit
NuFoG	Nutrition and Food Group
OCPs	organochlorine pesticides
PLE	pressurised liquid extraction
TBARS	thiobarbituric acid reactive substances

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EU-FORA SERIES 8

Algal-derived functional additives for ensured safety and efficacy

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

This document was developed within the framework of the EU-FORA fellowship programme, which aims to improve expertise in food risk assessment through training to ensure awareness for future risk analysis needs. The work aimed to study extracts obtained from macroalgae to be used as functional additives and discusses issues related to their efficacy and chemical safety. Macroalgae have promising potential as a source of bioactive compounds for functional foods due to their antioxidant, antimicrobial, neuroprotective and anticancer properties. However, ensuring safety is of paramount importance due to concerns about the presence of endogenous and exogenous contaminants, such as heavy metals, organic pollutants and other elements like arsenic and iodine.

KEYWORDS

bioactive activities, iodine, macroalgae extracts

CONTENTS

SUMMARY

The technical report of the EU-FORA fellowship programme, entitled Algal-derived functional additives for ensured safety and efficacy, encompass the work programme and a detailed account of the activities executed by the fellow throughout the course of its implementation. These programme by the fellow Maria Aurora Soares da Silva, MSc hosted by the University of Vigo under the supervision of Dr. Miguel A. Prieto awarded by EFSA to the REQUIMTE (University of Porto) in Portugal as sending institution under the supervision of Dra. M. Fátima Barroso. This technical report encompasses some of the methodologies employed and a portion of the evaluative results. In conjunction with the programme, an article is currently in progress; consequently, certain findings are not included within this report.

Macroalgae have promising potential as a source of bioactive compounds for functional foods due to bioactive properties. However, ensuring safety is of utmost importance due to concerns regarding the presence of environmental contaminants, heavy metals and other elements such as iodine. To overcome these challenges, a structured approach is proposed. First, a comprehensive literature review was conducted to identify promising macroalgae species and extraction methods. Subsequently, extraction procedures were optimised to maximise yield and antioxidant capacity while minimising potential negative effects, by applying cutting-edge extraction techniques and non-toxic solvents. In vitro characterisation studies then assessed the bioactive potential and toxicity. The EFSA databases and literature was screened to determine the most relevant risks. In addition, studies on the incorporation of the extracts into food formulations were programmed to evaluate their efficacy. This systematic approach aims to ensure the safety and efficacy of using macroalgae-derived bioactive compounds in functional foods. A list of scientific outputs achieved during the programme is presented in Appendix A.

1 | INTRODUCTION

The willingness of European consumers to adopt alternative diets, e.g. vegan and vegetarian, is high and often motivated by environmental or health reasons. In addition, there is a great demand for food supplements with health-promoting properties and functional foods with biologically active additives that allegedly have a positive effect on various pathological processes.

In this context, it is crucial to recognise that the ocean serves as a vital reservoir of resources, harbouring an array of species that contain bioactive compounds. Among these, macroalgae stand out not only for their nutritional properties but also for their potential as a rich source of bioactive compounds, yet their utilisation remains relatively underexplored in Europe.

Macroalgae are a widespread group of multicellular organisms with an estimated 10,000 species, categorised according to their main pigments into brown (Phaeophyta), green (Chlorophyta) and red algae (Rhodophyta). (Ghanavati et al., 2022; Vega et al., 2020). They are an important source of minerals such as calcium, iron, potassium (Cassani et al., 2022), and they are also rich in other essential nutrients such as fibre, carbohydrates, fatty acids and proteins (Pereira, 2016; Wu et al., 2023) and iodine (Blikra et al., 2022).

Because of their abundance in secondary metabolites like phenolic compounds, including flavonoids and tannins, alkaloids, terpenes, glycosides, pigments and others, macroalgae are well known for their antioxidant capacity. These metabolites play a vital role in the human body by promoting defence responses against reactive oxygen species (ROS) (Piotrowicz et al., 2022; Priyanka et al., 2022). ROS are responsible for causing damaging effects on DNA and other vital molecules, and there is an established link between antioxidant capacity and the reduction of oxidative stress. ROS include free radicals and molecules, such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radicals (HO•) and hydrogen peroxide (H_2O_2). These may lead to conditions like diabetes, atherosclerosis, coronary artery disease, cancer, inflammation, liver diseases, cardiovascular diseases, cataracts, nephrotoxicity and normal ageing-related neurodegenerative processes (Ngo et al., 2011; Rocha De Souza et al., 2007).

Macroalgae biorefinery has recently focused in the production of crude or purified extracts with these antioxidant capacity but also been studied for their potential application as neuroprotective agents acting as acetylcholinesterase (ACHE) and butyrylchlonesterase (BUCHE) inhibitors (Ahn et al., 2012; Kang et al., 2012; Rafiquzzaman et al., 2015; Silva et al., 2019; Wu et al., 2021) suggesting their potential for aiding in the control Alzheimer's disease, anti-inflammatory and oxidative stress-related diseases.

Moreover, antioxidant supplementation alone or in combination with chemotherapeutics hinders further tumour development, prevents chemoresistance by improving the response to chemotherapy drugs and enhances cancer patients' quality of life by alleviating side effects. Preclinical and clinical studies have revealed the efficacy of using phytochemical and dietary antioxidants from different sources in treating chemo and radiation therapy-induced toxicities and enhancing treatment effectiveness (Ferdous & Yusof, 2021). Several breakthrough studies have explored the effects of macroalgal components on cancer cell development (Catarino et al., 2021; Chen & Xue, 2019; Ferdous & Yusof, 2021; Shi et al., 2018; Supraja et al., 2018; Xin et al., 2023; Yuan et al., 2015). However, to harness the bioactive potential of algae secondary metabolites, the extraction procedure is a critical point.

Although extraction techniques and their optimisation have been the focus of many scientific studies, it is not common to evaluate if adverse effects occur. For instance, high temperatures can disrupt thermolabile compounds and cause potential toxicity. On the other hand, the natural algae potential to accumulate in their structure xenobiotics of anthropogenic origin, such as hydrocarbons, aromatic and polycyclic hydrocarbons or heavy metals can lead a concentration effect. Based on this, research was carried out to assess both the advantages and the risk of using macroalgae extracts in food additives and functional foods. This enabled the food industry to incorporate these ingredients into their processes effectively, offering foods with enhanced nutritional and functional benefits while ensuring consumer safety.

1.1 | Work programme

Task 1 – Literature review.
Task 2 – Extraction of bioactive compounds from macroalgae.
Task 3 – In vitro characterisation of the macroalgae extracts obtained, such as their bioactive potential, toxicity, presence of xenotoxics and heavy metals.
Task 4 – Macroalgae extracts incorporation.

1.2 | Aims

The primary aim of the work programme is experiential learning through practical training, which will imply a thorough evaluation of health hazards linked to safety of algae-based extracts, adhering to the methodology proposed by EFSA.

2 | SELECTION OF THE ALGA MATERIAL

2.1 | Occurrence, production and consumption

Macroalgae have been part of the human diet for centuries, especially in Asian countries. Furthermore, there is a growing trend toward increased consumption of this type of food worldwide. The world algae production is presented in Figure 1. According to the FAO, 97% of algae were produced by aquaculture (FAO, 2025).

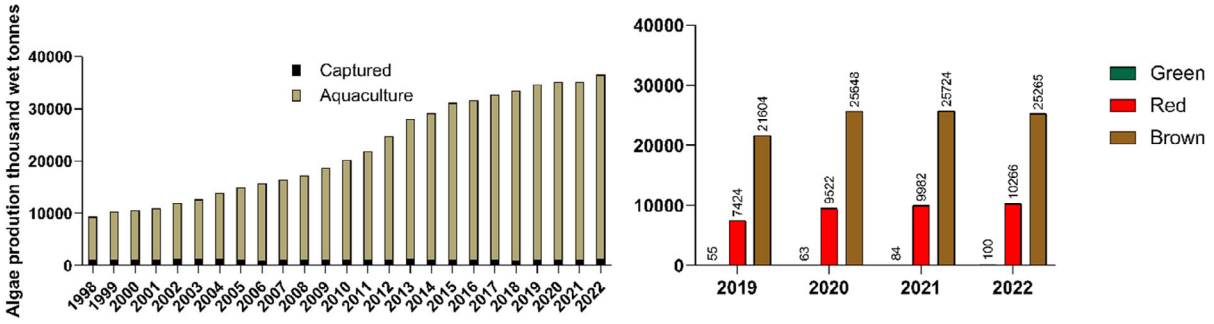

FIGURE 1 World production of algae expressed in thousand tonnes wet weight and the distribution by algae classes (FAO, 2025).

The graphical representation of the algal production categorised by classes was derived from the 10 species with the highest production levels, which account for 94% of the overall production (FAO, 2025). It is evident that the brown algae species are the most relevant in terms of global production, leading to the choice of the Phaeophyceae class for this study. Six species of edible macroalgae from the northwestern region of the Iberian Peninsula were evaluated: *Undaria pinnatifida* (UP), *Himanthalia elongata* (HE), *Fucus spiralis* (FS), *Bifurcaria bifurcata* (BB), *Ascophyllum nodosum* (AN) and *Sargassum muticum* (SM). The common names along with their classifications are shown in Table 1.

TABLE 1 Classification of the selected brown macroalgae.

Macroalga	Common name	Class	Order	Family
Undaria pinnatifida	Wakame	Phaeophyceae	Laminariales	Alariaceae
Himanthalia elongata	Sea spaghetti		Fucales	Himanthaliaceae
Fucus spiralis	Spiral wrack		Fucales	Fucaceae
Bifurcaria bifurcata	–		Fucales	Sargassaceae
Ascoplhylum nodosum	Knotted wrack		Fucales	Fucaceae
Sargassum muticum	Japanese wireweed		Fucales	Sargassaceae

3 | METHODOLOGY

3.1 | Extraction and bioactive evaluation

Extraction was performed using microwave technology (Multiwave-3000 microwave reaction system, Anton Paar, Graz, Austria) as this technique is known for its low extraction times, reduced solvent usage and high extraction rates (Quitério et al., 2022). Ethanol:water mixtures were selected as solvents because they are non-toxic and proper for the food industry. The extraction optimisation was carried out using response surface methodology (RSM), aiming to maximise yield and the antioxidant potential of the extract. The extracts obtained were studied to determine their capacity to scavenge reactive nitrogen and oxygen species (RNOS) such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$), hydrogen peroxide (H_2O_2) and nitroxide ($^{\bullet}NO$) radical. They were also studied for their neuroprotective potential and their effect on cancer cell lines (Cassani et al., 2024; Grosso et al., 2024; Silva, Carpena, et al., 2024; Silva, Cassani, et al., 2024).

3.2 | Assessment of adverse elements

The evaluation of the presence of adverse elements was conducted utilising the methodology established by Millos et al. (2009). An overview of the techniques and instruments employed for quantification is as follows: Microelements were quantified through inductively coupled plasma optical emission spectrometry (ICP-OES): iron (Fe), manganese (Mn), copper (Cu), zinc (Zn). For the quantification of mercury (Hg), cold vapour atomic absorption spectrometry was employed. The

determination of iodine (I), arsenic (As) and lead (Pb) was carried out using inductively coupled plasma mass spectrometry (ICP-MS). Calibration curves were made for each element and ^{115}In was used as an internal standard. All analyses were performed in triplicate.

3.3 | Cytotoxicity

The cytotoxicity of the extracts was evaluated by testing their effect on Vero cells (healthy cells from the kidney of an African green monkey) using the sulforhodamine B protocol (Vichai & Kirtikara, 2006) in a 96-well plate format. Following an incubation phase, the cell monolayers are stabilised using a 10% (wt/vol) solution of trichloroacetic acid and subsequently stained for 30 min, after which surplus dye is eliminated through repeated washings with 1% (vol/vol) acetic acid. The dye that is bound to proteins is then solubilised in a 10 mM Tris base solution, and the optical density is measured at 510 nm using a microplate reader.

3.4 | Data

This investigation employed the Rapid Alert System for Food and Feed (RASFF) to evaluate the prevalence of threats reported on algae in the European Union over 5 years (2020–2025).

4 | RESULTS

4.1 | Extracts bioactive properties

The macroalgae extracts were evaluated for their bioactive properties, namely the RNOS depletion capacity and the inhibition of ACHE and BUCHE enzymes related to Alzheimer's disease. A summary of the key findings is presented in the figure below (Figure 2).

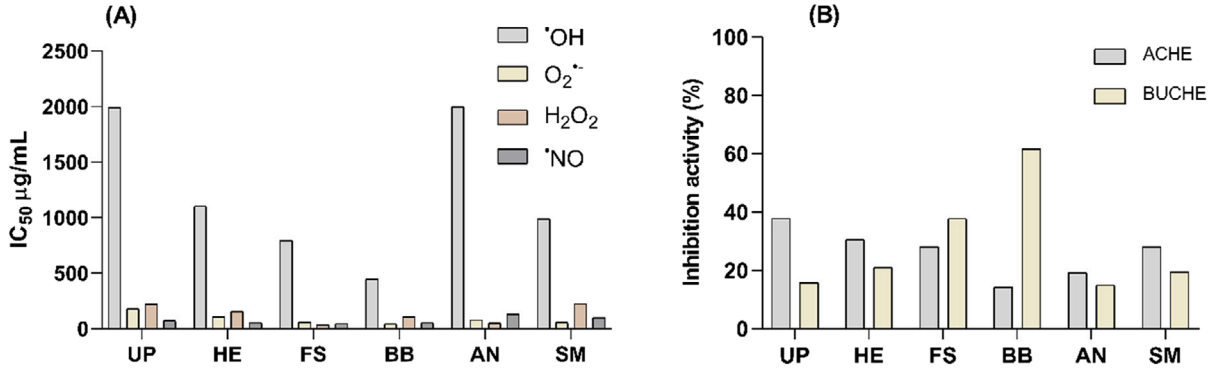

FIGURE 2 Bioactive properties of the algae extracts, (A) RNOS scavenger capacity; (B) neuroprotective activity at the extract concentration of 2 mg/mL.

Analysing the results, the algae that provided the best outcomes as RNOS scavengers were BB and SM, corresponding to the lowest IC_{50} in the four parameters tested. As inhibitors of ACHE and BUCHE, the results indicated that some activity exists, but only at high extract concentrations, specifically at 2 mg/mL.

The assessment of cytotoxicity revealed no impact on Vero cells up to the highest concentration examined for all the algae extracts, with the exception of BB and SM, which showed an average IC_{50} of 133 µg/mL (Silva, Carpena, et al., 2024; Silva, Cassani, et al., 2024). Moreover, previous results regarding the anti-tumoral activity, highlights the performance against lung and gastric adenocarcinoma (A549 and AGS, respectively) by the SM extract, with an IC_{50} lower than 50 µg/mL (Silva, Cassani, et al., 2024). Given the bioactive potential of SM and the large amount of biomass available since it is an invasive species, the investigation of adverse factors was concentrated on this macroalga.

4.2 | Elements analysis

The SM algae were studied for its nutritional composition (Silva et al., n.d.) and the results highlighted the presence of carbohydrates, fibres, lipids and proteins, as well as macroelements. The presence of iodine and other elements was also considered and is summarised in Table 2.

TABLE 2 Quantification of elements in SM and SM extracts.

Element	Algae (mg/kg dw)	Extract (mg/kg dw)
I	106.24	198.81
As	117.88	338.3
Hg	< 0.040	< 0.040
Zn	10.930	3.950
Cu	0.677	0.637
I	57.6	4.39
Pb	< 1.25	< 1.25

The results indicated that while the concentration of heavy metals decreases during the extraction procedures, non-metals such as iodine and metalloids like arsenic exhibit some accumulation. This fact highlights the importance of thorough examination of natural extracts aimed at human consumption.

5 | INCORPORATION INTO FOOD

With the growing interest in functional foods, numerous food items have been created to include extracts from natural sources, and algae extracts have likewise been integrated into food to enhance qualities or serve as functional foods. A variety of examples can be observed in current literature (Table 3).

TABLE 3 Selected examples of incorporation of algae extracts in food.

Food product	Macroalgae	Ref.
Cheese Spread	*Osmundea pinnatifida*	Faustino et al. (2023)
Yogurt	*Laurencia caspica, Saccharina japonica, Undaria pinnatifida, Codium tomentosum*	Tahmasebi and Mofid (2021); Wang et al. (2025); Costa et al. (2025)
Beer	*Fucus virsoides*	Habschied et al. (2024)
Pasta	*Fucus vesiculosus, Ulva rigida, Sargassum marginatum*	Ribeiro et al. (2021); Prabhasankar et al. (2009)

Of the data collected, one article stands out as it describes the nanoencapsulation of algae extract in a phospholipid nanoparticle, creating phytosomes (Costa et al., 2025), a technique also used in some of the algae of this work within the project framework (Portela et al., 2025), which were later incorporated into the formulation of an yogurt. The results substantiate that the encapsulation of phytosomes elevates both lipid and caloric values, increases protein concentrations and decreases sugar content (Costa et al., 2025).

6 | ASSESSMENT

According to the revision of the RASFF, 57 notifications appear when the keywords “algae” or “seaweed” are searched. The compilation of the information extracted from the database is presented in Figure 3.

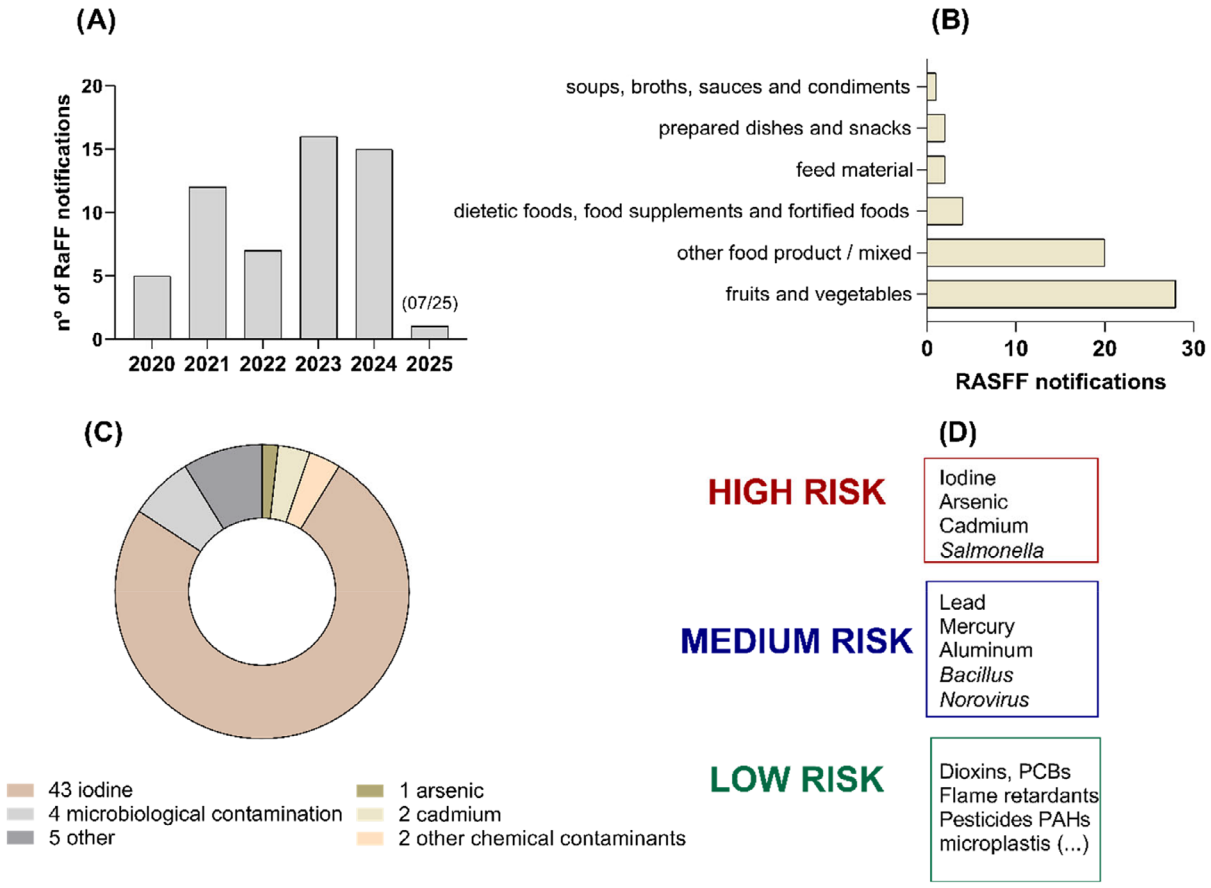

FIGURE 3 Resume of the data extracted from RASFF (A) notification by year, (B) notifications by FoodEx category; (C) problems reported, (D) risk ranking (Banach et al., 2020; FAO & WHO, 2022).

While the overall trend indicates an increase in macroalgae production (Figure 1), the notifications do not reflect a similar pattern (Figure 3A). In terms of the FoodEx category, 49% (28) of the notifications are classified within the group 'fruits and vegetables', closely followed by 35% (20) related to 'other food products/mixed' category (Figure 3B). It is essential to note that there is no information available on algae extracts, and only four notifications were identified in the categories of 'dietetic foods, food supplements, and fortified foods'. This distribution in FoodEx exposes the need of creating a specific category for algae and its derived products, especially considering the current upward production trends.

Most subjects reported (75%, Figure 3C) are associated with elevated levels of iodine. Iodine is an essential micronutrient, vital for producing thyroid hormones, with a daily recommended consumption of 150 μg for adults. Elevated iodine levels can lead to thyroid issues (either hypothyroidism or hyperthyroidism) in susceptible populations such as infants, individuals with renal problems and those with pre-existing thyroid disorders, while it is generally safe for those with healthy thyroid due to the body's inherent regulatory mechanisms (Sohn et al., 2024). On the other hand, iodine is among the most common nutrient deficiencies worldwide, with projections suggesting that it affects nearly 35%–45% of the global population. Iodine deficiency results in goitre, impacting an estimated 2.2 billion people globally. Even in situations of mild iodine deficiency, the goitre prevalence ranges from 5% to 20% (Hatch-McChesney & Lieberman, 2022).

The other two most frequently reported problems are microbiological contamination and other issues such as labelling problems. Despite arsenic being a widely recognised toxic element, there is only one case reported in RASFF database. Arsenic in algae is endogenous and is present in both inorganic and organic forms, a recent EFSA opinion stated that it is considered probable that the dietary exposures to the organic forms of arsenobetaine and glycerol arsenosugar do not pose a significant health risk (Knutsen et al., 2024), highlighting the need of having speciation data on arsenic. Furthermore, research suggests that cooking can significantly lower arsenic concentrations (Ichikawa et al., 2006).

The risk ranking illustrated in Figure 3D was derived from the research conducted by Banach et al. (2020) and a report from FAO experts (FAO & WHO, 2022). It largely aligns with the RASFF data, scoring as high risk the presence of iodine, arsenic and cadmium as chemical contaminants and *Salmonella* presence as microbiological contamination.

7 | CONCLUSION

The primary objective of the work programme was to study macroalgae extracts as potential food additives by integrating advanced extraction methodologies, in vitro bioactivity assessments and toxicological evaluations. The results demonstrated that algal extracts exhibit relevant antioxidant, neuroprotective and antitumoral properties while maintaining low

cytotoxicity under the tested conditions. Nonetheless, the observed enrichment of certain elements during the extraction process underscores the necessity for monitoring both extracts and food enriched products to guarantee their safety. In conclusion, while macroalgae represent a promising and sustainable resource for the development of functional additives that can be extremely important for iodine-deficient populations, their safe application requires toxicological validation and exposure assessment to ensure consumer safety and to support the responsible integration of algal bioactive extracts into the food supply chain.

8 | RECOMMENDATIONS

The EU-FORA fellowship initiative has made a considerable impact in advancing skill in the diverse phases of risk evaluation. The cooperative working groups established among a range of organisations constitute a valuable component of the programme and will facilitate forthcoming collaboration in the research of risk assessment.

ABBREVIATIONS

ACHE	acetylcholinesterase
BUCHE	butyrylchlonesterase
FAO	Food and Agriculture Organization
IC_{50}	half-maximal inhibitory concentration
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry
RASFF	Rapid Alert System for Food and Feed
RNOS	reactive nitrogen and oxygen species
ROS	reactive oxygen species
RSM	response surface methodology

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APPENDIX A

Multidisciplinary activities

Additional relevant activities completed by fellow.

A.1 | COMMUNICATION IN INTERNATIONAL CONFERENCES

Carpena, M., Barciela, P., Chamorro, F., Perez-Vazquez, A., Pereira, A. G., **Silva, A**., Olíva, A., Jorge, S., & Prieto, M. A. Nutritional and chemical profile analysis of *Fucus vesiculosus* and *Pelvetia canaliculata* brown macroalgae. 5th Food Technology, Quality and Safety – FoodTech 2024 Novi Sad, Serbia, October 16–18, 2024.

Silva, A., Chamorro, F., Barroso, M. F., Fernandes, V. V., Soares, C., & Delerue-Matos, C. Safety Assessment of Brown Seaweed Species and Their Extracts. The 5th International Electronic Conference on Foods MDPI Online, 28–30 October 2024.

Carpena, M., **Silva, A**., Chamorro, F., Pereira, A. G., Santos, F., Soares, C., Barroso, M. F., & Prieto, M. A. Innovative Dietary Solutions: Macroalgae Amino Acids for Differentiated Nutrition. The 4th International Electronic Conference on Nutrients. MPDI online, 16–18 October 2024.

Carpena, M., Barciela, P., Perez-Vazquez, A., Chamorro, F., **Silva, A**., & Prieto, M. A. Evaluation of red macroalgae as biomass feedstock for biofuel production 8th AMIFOST – the Future of Food Science & Technology: Innovations, Sustainability, and Health. Noida, India 27–28 March 2025.

Silva, A., Soares, C., Carpena, M., Nogueira-Marques, R., Barroso, M. F., & Prieto, M. A. Evaluation of PAHS traces in macroalgae extracts. 8th AMIFOST-2025 International Conference on the Future of Food Science & Technology: Innovations, Sustainability, and Health Noida India, 27–28 March 2025.

Silva, A., Carpena, M., Echave, J., Grosso, C., Barroso, M. F., & Prieto, M. A. Exploring the Role of Brown Algae as Scavengers of Reactive Oxygen and Nitrogen Species: The 2nd International Electronic Conference on Antioxidants MDPI: Basel, Switzerland online, 7–9 April 2025.

Silva, A., Soares, C., Chamorro, F., Carpena, M., Barroso, F., & Prieto, M. A. Nutritional potential of brown algae extracts: A study on *Bifurcaria bifurcata*, *Fucus spiralis*, and *Ascophyllum nodosum*. XXIII European Conference on Food Chemistry EUROFOODCHEM XXIII Bratislava, Slovakia 11–13 June 2025.

Silva, A., Soares, C., Chamorro, F., Carpena, M., Nogueira-Marques, R., Barroso, M. F., & Prieto, M. A. Cristina Delerue-Matos: Assessing the Nutritional Potential of *Undaria pinnatifida* and *Himanthalia elongata* for Human Consumption Dare2change innovation-driven agrifood business Porto Portugal, 2 April 2025.

A.2 | PROCEEDINGS PAPER PUBLICATIONS

Carpena, M., **Silva, A**., Chamorro, F., Echave, L., Olivia, A., Jorge, S., Barroso, M. F., & Prieto, M. A. (2024). Exploring algal metabolism: Insights from metabolomics and computational approaches. *Biology and Life Science Forum*, *35*, 9. https://doi.org/10.3390/blsf2024035009

Barciela, P., Carpena, M., Perez-Vazquez, A., **Silva, A**., Olivia, A., Jorge, S., & Prieto, M. A. (2024). Bromophenols in red algae: Exploring the chemistry and uncovering biological benefits of these unknown compounds. *Biology and Life Science Forum*, *35*, 11. https://doi.org/10.3390/blsf2024035011

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A.3 | ARTICLES IN INTERNATIONAL JOURNALS

Silva, A., Carpena, M., Cassani, L., Grosso, C., Garcia-Oliveira, P., Delerue-Matos, C., Simal-Gandara, J., Barroso, M. F., & Prieto, M. A. (2024). Optimization and bioactive evaluation of *Bifurcaria bifurcata* antioxidant-rich extracts for functional food and pharmaceutical applications. *Antioxidants*, *13*, 1189. https://doi.org/10.3390/antiox13101189

Carpena, M., Pereira, C. S., **Silva, A**., Barciela, P., Jorge, A. O., Perez-Vazquez, A., Pereira, A. G., Barreira, J. C., Oliveira, M. B., & Prieto, M. A. (2024). Metabolite profiling of macroalgae: Biosynthesis and beneficial biological properties of active compounds. *Marine Drugs*, *22*(10), 478. https://doi.org/10.3390/md22100478

Costa, M., Soares, C., **Silva, A**., Barroso, M. F., Simões, P., Ferreira, M., Gameiro, P., Grosso, C., & Delerue-Matos, C. (2025). Optimization of nanoencapsulation of *Codium tomentosum* extract and its potential application in yogurt fortification. *Marine Drugs 23*(4), 147. https://doi.org/10.3390/md23040147

A.4 | OTHERS

Best oral communication at the 4th International Electronic Conference on Nutrients: Maria Carpena, **Aurora Silva**, F. Chamorro, Antia G. Pereira, Francisca Santos, Cristina Soares, M. Fátima Barroso, M.A. Prieto Innovative Dietary Solutions: Macroalgae Amino Acids for Differentiated Nutrition.

Award – IACOBUS – PUBLICACIÓNS CIENTÍFICAS (PAPERS): Optimization and Bioactive Evaluation of *Bifurcaria bifurcata* Antioxidant-Rich Extracts for Functional Food and Pharmaceutical Applications, Antioxidants 2025.

Visit to the Agencia Española de Seguridad Alimentaria y Nutrición and the Laboratory: National Centre for Food (CNA).

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