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EU-FORA efsa JOURNAL

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Foreword

I am very glad to introduce the sixth collection of reports of EFSA's Fellowship Programme. EU-FORA was conceived in 2016 as part of the ambition shared by EFSA and Member States to address the increasingly growing need for qualified and trained food risk assessors and it is a key initiative for ensuring preparedness for future risk analysis needs. For the past 6 years, EFSA and Member States have been supporting the development of future generations of Europe's experts in food safety and risk assessment. Throughout this time the Programme has evolved to meet the challenges of the ever-changing landscape of food risk assessment. The successful completion of the first cycle under the new EU-FORA 2.0 is proof of EFSA's commitment to this unique opportunity.

These special issues are a recognition to the work and dedication of the fellows, the enthusiasm with which involved organisations embrace the programme, and the support provided by EFSA's colleagues across different departments. Partnership and cooperation are core values for EFSA, and it is gratifying to see their importance shared by our partners.

"We will continue to support the developmet of food safety professionals to build together a common EU culture for risk assessment."

Lastly, I would like to express EFSA's thanks to all participating organisations, as well as our partners for the successful cycle. I congratulate our newly graduated fellows and hope to see them soon in other prominent roles in the risk-assessment ecosystem. We will continue to support the development of food safety professionals to build a common EU culture for risk assessment.

Barbara Gallani

Declarations of interest: If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

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Introduction

Victoria Villamar

I am very happy to present the sixth special issue of the EFSA Journal dedicated to EFSA's Fellowship Programme (EU-FORA). As its predecessors, it gives an insight of the work programmes and activities performed by our fellows in various areas of food risk assessment. The successful completion of these programmes is the result of the good cooperation and dedication of all the actors involved – fellows, institutions, competent organisations and other partners of the wider EU-FORA community.

I applaud the newly graduates and would like to acknowledge their professionalism, engagement and desire to become part of the EU-FORA community. Together with my team, we hope they continue to invest in the Community as Alumni, and in themselves as food risk assessment experts.

This cycle that has ended now has also meant a new chapter in EU-FORA: EU-FORA 2.0. The evolved programme has brought the participation of many new organisations and expanded the outreach across Member States. We hope that the Programme continues to widen the networks, to promote the exchange of knowledge and best practices, and to contribute to achieving a harmonised EU risk assessment culture.

Finally, we would like to thank everyone who was involved and contributed to this cycle of EU-FORA; extending our gratitude to the EFSA and Member States for shaping it, and, of course, to the training consortium and the fellows.

On behalf of the EU-FORA Programme,
Victoria Villamar

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ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLA LOMBARDIA E DELL'EMILIA ROMAGNA "BRUNO UBERTINI" ENTE SANITARIO DI DIRITTO PUBBLICO

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Improvement of quantitative microbiological risk assessment (QMRA) methodology through integration with gaenetic data

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Abstract

Quantitative microbiological risk assessment (QMRA) methodology aims to estimate and describe the transmission of pathogenic microorganisms from animals and food to humans. In microbiological literature, the availability of whole genome sequencing (WGS) data is rapidly increasing, and incorporating this data into QMRA has the potential to enhance the reliability of risk estimates. This study provides insight into which are the key pathogen properties for incorporating WGS data to enhance risk estimation, through examination of example risk assessments for important foodborne pathogens: *Listeria monocytogenes* (*Lm*), *Salmonella*, *Campylobacter* and Shiga toxin-producing *Escherichia coli*. By investigating the relationship between phenotypic pathogen properties and genetic traits, a better understanding was gained regarding their impact on risk assessment. Virulence of *Lm* was identified as a promising property for associating different symptoms observed in humans with specific genotypes. Data from a genome-wide association study were used to correlate lineages, serotypes, sequence types, clonal complexes and the presence or absence of virulence genes of each strain with patient's symptoms. We also investigated the effect of incorporating WGS data into a QMRA model including relevant genomic traits of *Lm*, focusing on the dose–response phase of the risk assessment model, as described with the case/exposure ratio. The results highlighted that WGS studies which include phenotypic information must be encouraged, so as to enhance the accuracy of QMRA models. This study also underscores the importance of executing more risk assessments that consider the ongoing advancements in OMICS technologies, thus allowing for a closer investigation of different bacterial subtypes relevant to human health.

Keywords: quantitative microbiological risk assessment, whole genome sequencing, food-borne pathogen properties, farm-to-fork, pathogen subtypes, genotype–phenotype relationships, random forest

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Summary

The quantitative microbiological risk assessment (QMRA) methodology aims to estimate and describe the transmission of pathogenic microorganisms from animals and food to humans. QMRA can enhance the understanding of the impact of interventions in reducing the public health burden, as well as identify the most significant transmission routes for specific pathogens. However, there are important limitations associated with QMRA. Estimated numbers of human cases often greatly exceed what is found by epidemiological studies suggest. Further, subtypes of pathogens are not distinguished, even though certain subtypes may dominate the food chain but are rarely found in humans, or the other way around. In microbiological literature, the availability of whole genome sequencing (WGS) data is rapidly increasing, and incorporating this data into QMRA has the potential to enhance the reliability of risk estimates. This study aimed to improve the QMRA methodology by using WGS data for different subtypes of a food-borne pathogen. The initial phase of the study focused on investigating the variability of pathogen properties among strains and their impact on risk. Four farm-to-fork QMRAs were studied, for *Listeria monocytogenes* (*Lm*), *Salmonella*, *Campylobacter* and Shiga toxin-producing *Escherichia coli*. The goal was to identify the most critical pathogen properties (e.g. growth, virulence) and related parameters that could be used to improve QMRA through the inclusion of genotype information. Therefore, a comprehensive list of properties and parameters used throughout the food chain was made, and the sensitivity of each parameter in every QMRA was evaluated to assess the magnitude of their impact on model outcomes. Furthermore, the magnitude of variability of each parameter was examined through a review of relevant literature studies, while aiming also to establish connections between the properties/parameters of interest and the genome variability observed in different strains. Studies investigating the relationships between subtypes, genes and the variation in pathogen properties by examining the phenotypes of different subtypes were considered crucial for improving QMRA. Therefore, the set of identified pathogen properties considered valuable for QMRA improvement was compared with the available literature data, so as to gain a comprehensive understanding of the WGS data gaps from the perspective of QMRA. This knowledge is essential for identifying the key aspects that warrant attention in future research. The virulence of *Lm*, which impacts the dose–response phase of the QMRA model, was identified as a promising candidate for associating different symptoms observed in humans with specific *Lm* genotypes. This was investigated further using a genome-wide association study to correlate lineages, serotypes, sequence types, clonal complexes and the presence or absence of virulence genes of each strain with disease outcomes.

Finally, the study aimed at investigating the size and nature of the effect of incorporating WGS data into the QMRA using a QMRA model that integrated the genomic traits into the virulence property of *Lm*. The results highlighted that additional studies focusing on these aspects are needed to enhance the knowledge and refinement of the QMRA methodology.

This study highlighted the importance of developing improved risk assessment methodology in response to the continuous advancements in OMICS technologies, which enables a more detailed examination of various bacterial subtypes relevant to human health.

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1. Introduction

The quantitative microbiological risk assessment (QMRA) is recognised as a robust mathematical modelling approach for estimating human exposure risks to pathogenic microorganisms through food, environment or direct contact with infected animals. QMRA provides valuable insights into the effectiveness of interventions aimed at reducing public health burden and helps to identify the most important transmission routes for specific pathogens (Evers and Bouwknegt, 2016). In the context of food-borne transmission, farm-to-fork models estimate the number of illness cases resulting from pathogen ingestion following the consumption of contaminated food. These models provide a comprehensive description of the entire food chain, starting from the farm phase and ending with food preparation by consumer (Chardon and Evers, 2017). A critical component of QMRA models is the estimation of the number of human cases for the specific disease under consideration. Therefore, there is growing interest in improving the precision of these estimates since QMRA values often exceed epidemiological estimates. In QMRA studies, there is an increasing awareness to differentiate pathogen subtypes based on phenotypic characteristics that are related to genotypic traits. This distinction allows for the identification of strains that may be dominant in the food chain but rarely found in humans, or strains that prevail in humans compared to their presence in food sources. The availability of whole genome sequencing (WGS) data in the microbiological literature is expanding rapidly, and its incorporation into QMRA enables the development of more targeted risk assessments and management strategies (Nielsen et al., 2017; Fritsch et al., 2018, 2019). Therefore, the aim of this work was to improve the QMRA methodology by using WGS data to discriminate between different subtypes of a food-borne pathogen. This subtype distinction, based on the analysis of genomes, aims to provide a deeper understanding of the potential of integrating genotypic information to improve the reliability of risk estimates.

2. Data and methodologies

2.1. Study setting

The study of four farm-to-fork QMRAs on four important food-borne pathogens (*L. monocytogenes*, *Salmonella*, *Campylobacter* and Shiga toxin-producing *E. coli* – STEC) was performed to determine which pathogen properties and related parameters are the most important candidates for QMRA improvement through the integration of genomic data. A list of properties and parameters used throughout the food chain was made, evaluating the magnitude of the effect of changing each parameter on the model outcome (sensitivity) in every QMRA studied. Moreover, the relation between the presence of particular genomic traits and phenotypic differences (variability) and the magnitude of this variability was evaluated studying literature. For each property with both a high sensitivity and variability, the WGS data gaps were assessed from the viewpoint of QMRA, to understand which aspects are important to focus on in future research.

An in-dept study was performed on the virulence property of *Lm* in the dose–response (or case/exposure) phase of the QMRA model using a RIVM (Rijksinstituut voor Volksgezondheid en Milieu, the Netherlands) *Lm* database. The database included *Lm* strains collected in humans and in food in the Netherlands. For each strain were reported the lineage, serotype, sequence type, clonal complex (CC), presence or absence of virulence genes (as reported by the 'Virulence factor database', http://www.mgc.ac.cn/VFs) and the allelic number for each virulence gene, obtained analysing the complete genome (Coipan et al., 2023). Concerning human strains, the patient's characteristics were included in the database (such as age, gender, decease, pregnancy status, underlined disorders, systematic use of antacids or immunosuppressive). Moreover, when available, the symptoms following the *Lm* infection were reported, as sepsis, meningitis, gastrointestinal disease, lung inflammation, encephalitis, endocarditis. Concerning food strains, for each isolate was reported the food source, which included vegetable, pork, fish, beef and veal, chicken, turkey, mutton/lamb and game meat.

2.2. Methodologies

2.2.1. Random forest

The random forest method was applied to human isolates from the *Lm* database. The goal was to predict specific symptoms during *Lm* infection based on isolate characteristics, including CC, patient status (e.g. deceased, pregnant), underlying disorders, immunosuppressive or antacid use, gender,

age and presence or absence of certain virulence genes. The random forest method is a machine learning algorithm able to combine the outputs of multiple decision trees, considering the isolate characteristics, to predict the occurrence of specific symptoms resulting from the ingestion of *Lm*-contaminated food. Additionally, a heatmap was generated and correlation coefficients were calculated, considering virulence genes and symptoms as variables, to better understand the most significant characteristics to focus on. The analysis was performed using Rstudio software (version 4.2.1, http://www.rstudio.org).

2.2.2. Swift quantitative microbiological risk assessment

To assess the impact of incorporating WGS data into QMRA, a QMRA model was expanded to include relevant genomic features obtained from the analysis of the *Lm* database. Further, various scenarios were examined using the sQMRA (swift QMRA) tool, to study the implications of dividing exposures based on the prevalence of each CC in chicken products (Chardon and Evers, 2017). The sQMRA tool is a risk assessment model that provides a comprehensive description of the transmission and propagation of a pathogen in or on a food portion throughout part of the food chain, and its impact on consumers. Uncertainty of prevalence was included in our calculations using @Risk (version 8.3.2, https://www.palisade.com/risk).

3. Results

A comprehensive study of farm-to-fork QMRAs was performed for four important food-borne pathogens: *Lm*, *Salmonella*, *Campylobacter* and STEC. The risk assessment reports for each pathogen and each phase of the assessment were thoroughly examined to identify the key pathogen properties and related parameters that could be targeted for QMRA improvement. A list of properties and parameters used throughout the food chain was made, and the sensitivity of each parameter in the QMRAs was evaluated to determine the extent of the impact of parameter changes on the model outcomes. Moreover, the variability of each parameter among different strains, reflecting differences in genotype, was assessed based on available literature studies. The pathogen properties with significant variability and sensitivity were identified, highlighting the need to prioritise them in future QMRA improvements.

Concerning *Campylobacter*, a farm-to-fork QMRA model focusing on *Campylobacter* in broiler meat was studied (RIVM, 2005). Within the processing phase of the model, the invasiveness and colonisation potential of *Campylobacter* was found to have an important impact on the risk outcomes, and this can also be influenced by genotype variations among different strains (Chaloner et al., 2014). Moreover, during the storage phase at home, the survival or inactivation of *Campylobacter* due to refrigerated storage conditions was identified as an important property affecting the overall risk. Several studies reported variation in viability profiles of different strains at various temperatures, potentially linked to differences in their genotypes, but further studies are needed to clarify this aspect (Murphy et al., 2006). In the ingestion phase, the ability of *Campylobacter* to cause illness was found an important property; studies indicated that variations in the expression of virulence genes can lead to differences in adherence, invasion, intracellular survival capacities and toxin production (Janssen et al., 2008). Other noteworthy properties included environmental persistence, biofilm formation capacities and *Campylobacter* inactivation through heating.

The study of *Salmonella* was performed using a farm-to-consumption model on *Salmonella* in slaughter and breeder pigs (Hill et al., 2010). The assessment of *Salmonella* transmission during the process determined some promising properties and parameters involving differences in virulence, pathogenesis and intestinal persistence based on the genomic traits of the strains (Cui et al., 2021). Additionally, literature reported a significant diversity in stress resistance among various *Salmonella* strains due to the expression of different stress-induced genes (Wang et al., 2020). However, further studies are needed to understand whether the model risk outcome is sensitive to these properties. Finally, the ability to survive cleaning processes due to biofilm formation capacity can be influenced by differences in genotype characteristics that lead to better persist in the environment, but it was shown to have a minimal impact on the model risk outcome (Dantas et al., 2020). This study did not produce important pathogen properties for our aim and based on our criteria.

The study of STEC involved a farm-to-fork QMRA focused on steak tartare (Nauta, 2001). The STEC intestinal colonisation potential can affect the excretion rate, resulting in a possible contamination of the carcass in the slaughterhouse phase of the model, but it can also affect the dose–response phase because it can be important for the STEC virulence. This property had a high sensitivity in relation to

the model outcome, and some studies reported that the adherence and gut colonisation capacities may be associated with differences in the expression of virulence and adherence genes (Pielaat et al., 2015; Barth et al., 2020). The STEC ability to cluster and their susceptibility to inactivation during storage were identified as important properties due to their high sensitivity in relation to the model outcome, but further studies are needed to determine if these properties are associated with specific genetic characteristics.

The study of *Lm* involved the analysis of two risk assessments which focused on estimating the risks of listeriosis associated with the consumption of ready-to-eat (RTE) foods (FDA and FSIS, 2003; Interagency Workgroup, 2013). This provided valuable insights into the properties and parameters of *Lm* important for enhancing the accuracy of QMRA models and risk estimation. In particular, the ability of *Lm* to grow at storage temperature showed high variability attributed to genotypic traits, even if its sensitivity was not quantified in terms of its impact on the outcome variation (Cordero et al., 2016). The thermal inactivation during food preparation showed differences based on the lineage of diverse *Lm* strains, but also in this case, its sensitivity remains unknown (Liu et al., 2021). The ability of *Lm* to persist in the environment and form biofilms was important during food preparation, with high sensitivity related to the risk outcome, and numerous studies suggested that persistent *Lm* strains have distinct genotypes characterised by the presence or absence of specific genes (Ferreira et al., 2014; Mazza et al., 2015). In the dose–response phase, variation of the ability of *Lm* to cause infection was identified as an important property, although the impact of varying this property on the risk outcome remains unknown. Recent studies highlighted differences in virulence among *Lm* strains based on the presence or absence of a truncated *inlA* gene, suggesting that the severity of illness should be considered when improving QMRA models using WGS data (Pouillot et al., 2015; Chen et al., 2020). Moreover, several studies reported differences in virulence between strains based on variations in lineages, serotypes and CCs (Nielsen et al., 2017; Fritsch et al., 2019; Maury et al., 2019; Quereda et al., 2021; Cardenas-Alvarez et al., 2022; Muchaamba et al., 2022).

In conclusion, considering the four pathogens studied, further research is required to establish possible connections between specific phenotypic and genotypic features and to gain a better understanding of how variations in these properties impact the overall risk outcome.

Given that the *Lm* virulence property affecting the risk characterisation phase of the model was found as a promising property, it was further investigated using the RIVM *Lm* database to find a relationship between the symptoms experienced by patients and specific genotype features. To identify the most promising symptom to be predicted, the data were visualised using a heat map, and correlation coefficients were calculated. The random forest method was then performed to predict the symptoms based on the presence or absence of specific virulence genes, but the results were not favourable. The error rate was determined to be 65%, indicating a high level of misclassification. The model accuracy (which equals 100 minus the error rate), representing the proportion of correctly predicted outcomes, was thus only 35%, which suggested that the predictive power of the gene presence/absence for symptoms in the database was limited. Further, random forest analysis was performed to predict symptoms based on isolate characteristics such as age, gender, decease, pregnancy status, underlined disorders, systematic use of antacids or immunosuppressive and an analysis using only CCs as predictors was also conducted. However, in both cases, the error rate remained high, and the model accuracy was low. These findings indicated that the presence or absence of specific virulence genes, isolate characteristics and CCs have no predictive power for symptoms in this database. Further studies and possible evaluation of additional factors are necessary to improve the prediction of symptoms to be used possibly in QMRA models.

The study used prevalence data from the *Lm* database, including both human and food isolates, to investigate the impact of splitting up exposures over CCs, using a QMRA model. To estimate the total exposure for each CC, the fraction of positive food isolates per CC and food in the RIVM database was used. A stochastic approach was used to calculate the uncertainty of these fractions. The number of human cases per CC per year in the Netherlands was determined using the *Lm* incidence in humans in 2021 (Benincà et al., 2022). By calculating the case/exposure ratio, it was possible to identify the CCs that exhibited higher virulence compared to others, despite the high uncertainties calculated. It proved that our results were consistent with existing literature reporting certain CCs as primarily found in food environments and considered hypo-virulent (Quereda et al., 2021). On the other hand, some CCs were predominantly associated with human cases and characterised as hyper-virulent due to their enhanced ability to colonise the intestine and invade the intestinal mucosa (Quereda et al., 2021).

Calculations with a baseline sQMRA model provided a reference point for comparing the model with and without genetic information, useful for a better understanding of the specific contributions of

genomic characteristics to the risk estimates. Investigation of different scenarios for some CC's of interest were performed, to provide insights into the specific risk outcomes associated with different genomic characteristics of *Lm* strains.

4. Conclusion

This study highlighted that by incorporating WGS data into the QMRA model, a more comprehensive and accurate assessment and understanding of the risks associated with pathogen contamination throughout the food chain may be achieved. This integration will allow for a better understanding of the potential differences in risk estimates due to the specific genomic traits of pathogen strains. Such a model can assist in developing more targeted risk management strategies and interventions to mitigate the risks associated with food-borne pathogen contamination. In this study the most important pathogen properties to focus on were determined, and first attempts were made to combine WGS with QMRA.

5. Recommendations

The findings of this study emphasised the importance of including WGS data in QMRA. However, further research and in-dept studies that combine genomic data with phenotype analysis are indispensable to make progress in this. Such studies will allow for incorporation of pathogen subtypes based on genomic traits into QMRA models, so that more accurate risk assessments and risk management strategies can be achieved. Otherwise this will not be possible.

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Assessing Portuguese health risks: *Anisakiks* parasite in Atlantic chub mackerel (*Scomber colias*) sold in Portuguese markets

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Abstract

Anisakiosis is a significant zoonotic disease caused by parasitic nematodes of the *Anisakis* genus. It can be contracted by humans through the consumption of raw or undercooked fish contaminated with the parasite, leading to gastrointestinal and allergic symptoms. While anisakiosis is not frequently documented in Portugal, the presence of allergic reactions to *Anisakis* in Spain suggests ongoing exposure in the Iberian Peninsula. To address this concern, the Interdisciplinary Centre of Marine and Environmental Research in Porto, Portugal, in collaboration with the Biology Centre of Czech Academy of Sciences in Ceske Budejovice, Czech Republic, has proposed a project entitled 'Assessing Portuguese Health Risks: *Anisakis* Parasite in Atlantic Chub Mackerel (*Scomber colias*) Sold in Portuguese Markets' under the European Food Risk Assessment Fellowship Programme. The primary objective of the project is to gather valuable epidemiological data on the host, Atlantic chub mackerel (*S. colias*) and the parasitic nematode (*Anisakis* spp.) with the focus on assessing contamination levels and evaluating potential health risks associated with anisakiosis in the Portuguese population. By conducting this research, the project aims to contribute to the understanding of anisakiosis and its impact on public health in Portugal. Investigation of the presence of the *Anisakis* parasite in Atlantic chub mackerel sold in Portuguese markets will provide crucial insights into the risks associated with consuming raw or undercooked fish. Ultimately, our findings will aid in the development of preventive measures and guidelines to ensure the well-being of the Portuguese population.

Keywords: *Anisakis*, fish parasite, risk assessment, disease, EFSA

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Summary

Risk assessment is an integral component in upholding the safety and quality of food within the European Union, adopting a methodical and scientific approach to evaluate potential hazards related to food products. This process facilitates informed decision-making and the implementation of effective risk management strategies. Acquiring a solid understanding of the principles and foundation of risk assessment is crucial for establishing a robust food safety system.

The evaluation of risks associated with parasites in fishery products is a fundamental process in protecting public health and ensuring the safety of seafood. By systematically assessing the risks linked to parasite contamination, appropriate measures can be employed to safeguard consumers and maintain elevated standards of food safety within the fishery industry.

By conducting meticulous risk assessments pertaining to parasites in fishery products, regulatory authorities and stakeholders can implement suitable control measures. These measures encompass the use of adequate processing techniques, application of freezing treatments and dissemination of consumer education. Their purpose is to mitigate risks and ensure the safety of seafood. Continuous monitoring and surveillance programmes further play a vital role in the detection and management of parasite presence, thus enhancing the safety and quality of fishery products.

This study aims to provide valuable insights into the potential risks associated with the consumption of untreated or undercooked mackerel. The findings of this study will contribute to the development of appropriate risk management strategies, thereby safeguarding public health. Ultimately, this project exemplifies the commitment of the fellow and collaborating institutions to address crucial food safety concerns and fulfil the objectives outlined within the EFSA programme.

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1. Introduction

1.1. European food risk assessment fellowship Programme

The European Food Risk Assessment Fellowship Programme (EU-FORA), established under the auspices of the European Food Safety Authority (EFSA), aims to bolster the scientific assessment capacity and knowledge community within the European Union, in line with EFSA's strategic goals for 2020. This initiative provides scientists employed in food safety organisations throughout Europe with a valuable opportunity to expand their expertise and practical skills in food risk assessment through immersive training experiences. In this case, the fellowship recipient was hosted by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) in Porto, Portugal.

The fellow's specific work programme spanned 3 months of hands-on-practice and focused on 'Assessing Portuguese Health Risks: *Anisakis* Parasite in Atlantic Chub Mackerel (Scomber colias) Sold in Portuguese Markets.' This comprehensive EU-FORA programme entailed a 3-week induction training course, followed by four 1-week modules, each dedicated to distinct facets of risk assessment, identification, perception and communication. While the majority of modules were conducted online, Module 3 took place at EFSA's headquarters in Parma, Italy.

Upon the completion of the induction training, the fellow assumed responsibility and took over as the subsequent substitute for the EFSA project, overseeing its continuity and implementation.

1.2. Overview of risk assessment for parasites in fishery products

1.2.1. Importance of risk assessment in ensuring seafood safety

The risk assessment process for parasites in fishery products plays a pivotal role in safeguarding public health and ensuring the safety and quality of seafood consumed within the population. Parasitic worms, such as *Anisakis* species, can be found in various fishes commonly used in fishery products. Ingestion of such parasites can lead to health risks, making it imperative to conduct comprehensive risk assessments to mitigate potential hazards and protect consumers. The risk assessment encompasses several key steps, including hazard identification, exposure assessment and risk characterisation.

1.2.2. Hazard identification: identifying parasites of concern

The first step in the risk assessment process is hazard identification, where the focus lies on identifying the parasites that pose a significant risk to human health. In the case of fishery products, this step involves evaluating the presence and prevalence of parasites across different fish species and potential impact on consumers.

1.2.3. Exposure assessment: evaluating consumer exposure to parasites

Once the parasites of concern are identified, the exposure assessment stage aims to quantify the level of exposure that consumers may face when consuming fishery products. This involves assessing factors such as consumption patterns, cooking methods and storage practices to estimate the likelihood and frequency of parasite ingestion. Understanding the potential routes and levels of exposure is crucial in determining the overall risk associated with consuming fishery products.

1.2.4. Risk characterisation: assessing the health risks and establishing safety measures

Risk characterisation is the final step in the risk assessment process, where the collected data on hazard identification and exposure assessment are combined to evaluate the actual health risks posed by parasites in fishery products. This step involves quantifying the risks and establishing safety measures such as tolerable levels of parasites, maximum acceptable limits and guidelines for processing techniques to ensure the safety and quality of seafood.

1.2.5. The role of risk assessment in regulation and risk management

Comprehensive risk assessments serve as a crucial foundation for the development of regulatory frameworks and risk management strategies in the fishery industry. The findings from risk assessments provide valuable information to regulatory authorities, enabling them to establish appropriate control

measures, such as adequate processing techniques and freezing treatments, that effectively reduce the risks associated with parasites in fishery products. Additionally, risk assessments support the development of consumer education programmes to raise awareness about proper handling and cooking practices, further enhancing seafood safety.

1.2.6. Ongoing monitoring and surveillance programmes

Risk assessment is not a one-time process but requires continuous monitoring and surveillance programmes to detect and manage the presence of parasites in fishery products (EFSA, 2010). Regular monitoring allows for timely identification of emerging risks and the implementation of appropriate control measures. These programmes contribute to the overall safety and quality of fishery products by ensuring ongoing compliance with established safety standards.

1.3. Overview of quantitative risk assessment in fishery products

Quantitative risk assessment (QRA) is a rigorous methodology utilised to estimate the probability and severity of hazards, providing a numerical expression of risk. In the context of fishery products, the presence of *Anisakis* spp. L3-stage larvae present a significant health concern for human consumption. Anisakiosis can manifest in various forms, ranging from mild to severe, affecting the gastric, intestinal, ectopic and allergic systems. Due to the nonspecific symptoms and infrequent outbreaks, anisakiosis is often misdiagnosed or underdiagnosed, and person-to-person transmission is considered improbable. A notable example of QRA application is evident in a study conducted by Lindqvist and Westöö (2000) concerning smoked fish in Sweden. The predicted annual number of illnesses attributed to *Listeria monocytogenes* ranged from 47 to 2,800 for consumers at the highest risk category. Bao et al. (2017) undertook the first QRA specifically focusing on fishery products contaminated by parasitic nematodes, utilising anchovies as the subject within the Spanish population. The study evaluated the risks associated with the consumption of raw, marinated and undercooked anchovy fillets. On average, the Spanish population was estimated to consume 0.66 *Anisakis* larvae per untreated (non-frozen) raw or marinated anchovy meal. The calculated probability of anisakiosis per meal was determined to be 9.56×10^{-5}, resulting in the prediction of annual anisakiosis cases requiring medical attention ranging between 7,700 and 8,320.

1.4. Additional information about the host species

The Atlantic chub mackerel (*S. colias*) is a pelagic species of moderate size (30–60 cm) mostly inhabiting warm waters. Population abundance and distribution in Portuguese waters significantly fluctuate over time, with maximum values observed in the middle of the last 3 decades. This rise is possibly correlated with the growing economical (canning industry, feeding of aquaculture species) and gastronomical interest on the species. The majority of chub mackerels caught in Iberian waters are juveniles, up to 2–3 years old, while older individuals are seldom encountered during surveys or by the commercial fleet. The Atlantic chub mackerel serves as a host for a diverse range of parasites. The symptoms exhibited by infected fish can vary depending on the parasite species and the severity of the infection. Common indications of parasitic infestation include lethargy, rapid breathing, reduced appetite, weight loss, fin clamping, laboured or accelerated respiration, the presence of yellow to rust-coloured dust on the fish's body, severe cases of skin peeling, sunken belly, the appearance of white stringy faeces, decreased appetite, respiratory distress, swollen and mucus-coated gills, irritation manifested through scratching behaviour (flashing) and diminished feeding activity.

2. Project description

2.1. Aims

A) Gaining basic knowledge on *Anisakis*.

   a) Morphological identification, life cycle and known hosts of *Anisakis*.
   b) Detection of parasites in the fish muscle using UV-Press method.
   c) Infectivity and vulnerability of the parasite *Anisakis* to humans.

B) Quantification of worms in fish individuals.

   a) Determination of infection levels (prevalence, mean intensity, mean abundance).

b) Identification of Anisakids species found in host by methods of molecular biology.
c) Assessment of food safety Microbiological Risk.

2.2. Methodology

2.2.1. Biometrics of the fish sample

One hundred and one fish samples were purchased from an auction in Aveiro (Portugal) in a time period from January to April 2023. Morphological identification of *S. colias* was conducted to separate *Scomber scombrus* from the samples. Fish biometric data was measured, including fork length, total length and total weight. The fish samples were placed in individual bags separately, with numerical identification and subsequently preserved in a freezer for 2 days before fish dissection and detection of infection by *Anisakis* spp.

2.2.2. Visual inspection of host

To visualise the viscera, fish samples were opened from the most ventral area of the opercula to the anal area. Sex and sexual maturity were evaluated on a scale of 1 (immature) to 5 (very mature, ready to spawn). Visual inspection of the abdominal organs was also evaluated by observation and estimation of overall level of *Anisakis* spp. infection on a scale of 0 (no parasites), 1 (slightly infected), 2 (infected) and 3 (very infected). Parasites found on the liver and in the viscera were counted. The visually detectable parasites were placed onto Petri dish with physiological solution. Encapsulated worms were cleaned, and morphological identification of Type I and Type II of *Anisakis* L3-stage larvae was carried out under a light microscope. The number of parasites per fish individual were counted and recorded. The fish viscera were measured for weight, extracted and placed in individual transparent bags for the UV-pressing method (described in Section 2.2.4).

2.2.3. Isolation of worms from the fish muscle

Each fish fillet, divided into two pieces (right and left side), was scaled, separated into four fragments (dorsal, ventral, anterior, posterior) from each side (eight pieces in total), skinned out and placed in individual transparent bags for the UV-pressing method (described in Section 2.2.4).

2.2.4. UV-pressing

After pressing, the viscera and muscle fragments changed their shape to a 'monolayer' with the thickness of 1–2 mm range. This structure allows investigation of the worm infection in muscles under UV light (Karl and Leinemann, 1993). Blue-fluorescing worms were counted and their precise localisation in the monolayer was marked. After thorough washing, the worms from liver and viscera or worms from fillet were used for morphological or molecular identification, respectively.

2.2.5. Morphological identification of *Anisakis* spp. from the fish liver and viscera

Isolated and cleaned worms were individually mounted on a microscopy slide, with a coverslip. The worm taxonomy (genus *Anisakis* or *Hysterothylacium*) and the identification of the type of *Anisakis* larvae were identified under the light microscope by observing anatomical characteristics, such as the type of mucron, ventricle and intestine.

2.2.6. Molecular identification of *Anisakis* spp. from the fish muscles

Each isolated *Anisakis* spp. sample found in different parts of the fish musculature was processed individually for DNA isolation and amplification of the Internal Transcribed Spacer rDNA (ITS) (Gasser et al., 1993; Gasser and Hoste, 1995) and the mitochondrial encoded Cytochrome C oxidase subunit 2 (COX-2) (Nadler and Hudspeth, 2000). HinfI endonuclease was used for the ITS region PCR product digestion and the product of restriction fragment length polymorphism (RFLP) was detected on agarose gel. COX-2 product was used for the Sanger sequencing. The sequences were trimmed in the Molecular Evolutionary Genetics Analysis (MEGA) software and compared with sequences available in the NCBI database.

2.3. The risk assessment analysis of *Anisakis* spp. in the fish *Scomber colias*

2.3.1. Quantification of infection parameters

Quantitative Parasitology (QPweb) online tool was applied to calculate prevalence, mean intensity and mean abundance of the isolated worms in the analysed fish samples. The fish samples were grouped by time points of the fish collection (January, February and March) and the fish size ('small fish' or 'big fish'). The 'small fish' is of the length from 20.7 to 24.2 cm and the weight from 85.39 to 95.26 g, while the 'big fish' length is from 24.3 to 31.0 cm and the weight is from 100.14 to 268.01 g. The infection parameters were calculated for each tissue, including liver, fillet and viscera.

2.3.2. Quantitative risk assessment

Anisakis spp. L3-stage parasitic larvae can pose a health hazard to humans when ingested in raw fish meal. To evaluate the likelihood of contracting anisakiosis from a regular portion of mackerel fish meal served in local restaurants, we initially employed the @RISK software (trial version) as a QRA tool. However, due to licensing limitations, we subsequently conducted all essential calculations using Microsoft Excel. In addition to the collected experimental data, two types of input data were utilised in the QRA. The first type of data was the annual consumption of mackerel in Portugal, which was extracted from webpages of the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA). The second type of data was from a survey published in Golden et al. (2022), which focused on the total number of meals containing untreated raw mackerels consumed by Portuguese respondents. The current number of Portugal population as of 25 July 2023 was taken from online resources (https://www.worldometers.info/world-population/portugal-population).

3. Conclusion

Despite the initial turnover of fellows during the project, the current fellow, in collaboration with the sending and hosting site supervisors, has established all the necessary materials and requirements for conducting successful experiments at the hosting institution.

The process of isolating parasites from the host provided a novel experience for the fellow. Within the limited time spent at the hosting institution, the fellow acquired valuable skills in fish dissection, parasite isolation and differentiation of morphological characteristics among closely related host species (*S. colias* and *S. scombrus*), as well as the worm parasite species (*Anisakis* type I and II). The implementation of the 'UV-pressing' method proved instrumental in detecting parasites within the host fillet. Additionally, the fellow utilised molecular techniques to identify parasite species. By collecting biometric data on the host and determining parasite distribution in different body regions, the fellow was able to independently analyse the acquired data.

Under the guidance of the sending institution supervisor, the fellow categorised the studied fish samples based on size and collection time points, evaluating the infection parameters in each specific tissue (liver, fillet and viscera) both individually and collectively. This categorisation is crucial for understanding the influence of both seasonal variations (collection time) and host traits (size) on the levels of infection in *S. colias*. The sending institution supervisor has signed up for the European Association of Fish Pathologists (EAFP) conference in 2023 to present the project's results through a poster. This poster presentation at the EAFP conference serves as a platform for sharing the fellow's work with a broader scientific community, providing an opportunity to emphasise the significance of considering host variables and seasonal variations in comprehending the infection dynamics of the fish species *S. colias*. These novel findings contribute to the expanding knowledge in fish pathology and showcase the fellow's unwavering dedication to research in this field.

The QRA has yielded significant information regarding the potential health risk for the Portuguese population associated with consuming untreated (marinated, cold smoked) or undercooked mackerel. Since the fellow did not have the opportunity to attend the induction training, she familiarised herself with the @RISK software with assistance from the @RISK customer service and independently learned the analytical approaches employed in similar risk assessment studies. The principle of the risk model was discussed with Miguel Boa (Institute of Marine Research in Norway). Despite encountering licensing limitations, we proceeded with conducting all essential calculations using Microsoft Excel. The objective of this study is to establish a foundational knowledge base for food safety in Portugal under the auspices of the EFSA programme.

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Abbreviations

CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
COX-2	Cytochrome C oxidase subunit 2
EAFP	European Association of Fish Pathologists
EU-FORA	European Food Risk Assessment Fellowship Programme
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
ITS	Internal Transcribed Spacer rDNA
L3	Third-stage larvae
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
QPweb	Quantitative Parasitology online tool
QRA	Quantitative risk assessment
RFLP	Restriction Fragment Length Polymorphism

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Toxicometabolomics as a tool for next generation environmental risk assessment

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Abstract

Traditionally applied methodology in environmental risk assessment (ERA) has fallen out of step with technological advancements and regulatory requirements, challenging effectiveness and accuracy of the assessments. Extensive efforts have been focused towards a transition to a more data-driven and mechanistically-based next generation risk assessment. Metabolomics can produce detailed and comprehensive molecular insight into affected biochemical processes. Combining metabolomics with environmental toxicology can help to understand the mechanisms and/or modes of action underlying toxicity of environmental pollutants and inform adverse outcome pathways, as well as facilitate identification of biomarkers to quantify effects and/or exposure. This Technical Report describes the activities and work performed within the frame of the European Food Risk Assessment Fellowship Programme (EU-FORA), implemented at the section 'Environmental Chemistry and Toxicology' at the Department of Environmental Science at Aarhus University in Denmark with synergies to an ongoing H2020 RIA project 'EndocRine Guideline Optimisation' (ERGO). In accordance with the 'training by doing' principles of the EU-FORA, the fellowship project combined the exploration of the status of scientific discussion on methodology in ERA through literature study with hands-on training, using the metabolomics analysis pipeline established at Aarhus University. For the hands-on training, an amphibian metamorphosis assay (OECD test no.231) was used as a proof-of-concept toxicometabolomics study case. Both a targeted biomarker – and an untargeted metabolomics approach was applied.

Keywords: next generation environmental risk assessment, systems-toxicology, toxicometabolomics, endocrine disrupting chemicals, untargeted metabolomics, new approach methodology, thyroid-disrupting chemicals

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1. Introduction

EFSA performs environmental risk assessments (ERAs) as part of its evaluations of 'regulated products', including pesticides, genetically modified organisms and additives in animal feed. Traditionally, the ERAs of these products have been performed on a single-substance basis, single exposure route and for a specific type of use (Sousa et al., 2022). These traditional approaches bear several limitations, challenging accuracy, efficiency and relevance of the risk assessments. Driven by significant advancements in scientific knowledge, analytical techniques and computational capabilities, which have expanded the understanding of toxicology, biology and exposure assessment, in addition to ethical considerations and a desire to reduce animal testing, several initiatives have been established in Europe to support a shift towards next generation risk assessment in order to overcome limitations and improve the quality of ERAs (Cozigou et al., 2015; Moné et al., 2020; Miccoli et al., 2022; Sousa et al., 2022; Marx-Stoelting et al., 2023).

Based on the current framework, traditional ERAs often focus on a limited set of endpoints (e.g. mortality, immobility) and may not capture the full range of potential hazards and risks associated with chemicals and environmental stressors. Next generation risk assessment seeks to provide a more comprehensive evaluation of potential adverse effects and exposure scenarios. Consequently, a special emphasis is placed on improving the understanding of underlying mechanisms of toxicity and adverse outcomes.

High-throughput technologies, such as omics techniques, allow for the rapid screening of large numbers of samples or chemicals, but the application of omics can also support a mechanistically-based approach (Brockmeier et al., 2017). Omics produce detailed and comprehensive molecular insights into the biochemical processes occurring in stressed microbes, plants and animals, enabling us to understand more about how these organisms are responding to environmental stresses.

In the past two decades, metabolomics has emerged as a promising alternative and/or supplementary tool to traditional toxicological assays, focusing on the qualitative and quantitative study of small molecules (< 1,500 Da) in biological samples or organisms to identify key metabolites involved in various biological processes. As metabolic changes can be influenced by environmental factors, but also diet, sex and disease, the high degree of controlled conditions in toxicological models present suitable platforms for metabolomics analyses with diverse applications (da Silva et al., 2021). Environmental toxicometabolomics, a subfield of metabolomics, aims to provide insights into how environmental stressors and chemical contaminants perturb the metabolic pathways of organisms, leading to altered physiological responses and potential adverse effects. The identification and quantification of specific metabolites or patterns of metabolites that serve as biomarkers of effect and/or exposure provides valuable information for risk assessment, regulatory decision-making and pollution management (Fowler, 2012), while global profiling of metabolic changes in response to chemical exposures allows for the characterisation of the metabolic networks and biological processes affected. As metabolomics captures the end products of cellular processes, it provides direct information on metabolic responses and the physiological state, and therefore gives the closest reflection of the phenotype of an organism or biological system. This knowledge helps in understanding the mechanisms of toxicity and adverse effects induced at sub-lethal environmental doses. Sublethal effects may not be readily apparent through traditional toxicological endpoints but can have long-term consequences for organisms and ecosystems.

Adverse outcomes resulting from chemical toxicity are rarely caused directly by dysregulation of individual molecules or pathways; rather, they are often caused by system-level perturbations that occur in networks of molecular events (Ravichandran et al., 2022). Recognising the interactions of molecules, pathways and biological processes within networks is fundamental for gaining a comprehensive understanding of the mechanism of action of chemical toxicity in complex biological systems. The adverse outcome pathway (AOP) concept, developed by the US Environmental Protection Agency in 2011, provides a framework to collect, organise and evaluate relevant information on biological and toxicological effects of chemicals (Ankley et al., 2010; Villeneuve et al., 2014). Existing mechanistic knowledge is organised and used to link a molecular-level perturbation of a biological system triggered by a chemical (a molecular initiating event; MIE) through a sequence of causally linked key biological events (key event; KE) to an adverse health or ecotoxicological outcome of regulatory concern (adverse outcome) including population-level responses (Ankley et al., 2010, 2023; Kramer et al., 2011; Villeneuve et al., 2014). Recent advancements in various omics technologies, and integrative multi-omics approaches, have facilitated the identification of MIEs and KEs within AOPs, offering a more comprehensive understanding of toxicity pathways and the connections between

different levels of molecular organisation (Bedia, 2022). Metabolites affected by chemical exposure can provide valuable data to support the development of AOPs. On one hand, metabolite levels can offer critical insights into mechanisms underlying KEs, helping to define the mode of action of an environmental toxicant. On the other hand, particular metabolites may directly represent KEs within an AOP, causally linking the components of the pathway to an AO. Furthermore, metabolomics data can be compared across different species and even extrapolated to predict the effects of stressors in other organisms. Cross-species applicability is essential for the development of AOPs with relevance to multiple species and ecological contexts (Ankley et al., 2010; Brockmeier et al., 2017; Haigis et al., 2023). Metabolomics-based read-across and grouping approaches, utilising structural similarity, have been shown to successfully support evidence through reducing uncertainty in the characterisation of the toxicity profile of analogue chemicals (e.g. phenoxy herbicides (van Ravenzwaay et al., 2016; Sperber et al., 2019).

Despite the growing agreement on the potential metabolomics offer for informing risk assessments when applied as a part of an integrated systems biology approach or when considered in the context of the AOP framework (Brockmeier et al., 2017; EFSA, 2018), it is still not clear how omics datasets can be used in regulatory applications in the risk assessment of chemicals (Viant et al., 2019). While the validation of metabolomics studies still needs to overcome a number of challenges in order to be widely implemented in routine ERAs, the potential of omics techniques to produce new evidence and facilitate the development of alternative testing methods, collectively referred to as new approach methodologies (NAMs), has been recognised (EFSA, 2022a,b; Otto et al., 2023). Progress towards a harmonised reporting framework has been made to support regulatory acceptance of metabolomics data (Buesen et al., 2017; Viant et al., 2019; Harrill et al., 2021; Miccoli et al., 2022). EFSA aims to adopt omics and associated bioinformatic approaches as routine tools in relevant RAs by 2030 (EFSA, 2022b).

2. Description of the work programme

2.1. Aims

The aim of the work programme was for the fellow to gain insights into the current scientific discussion in ERA methodology through literature study. Additionally, the fellow received hands-on training in toxicometabolomics as a potential tool for next generation systems-based ERA approaches, using the analysis pipeline established at the Environmental Metabolomics lab at the Department of Environmental Science (ENVS) at Aarhus University (AU; Denmark) on a case study.

2.2. Activities/methods

The fellow was integrated into the work at AU through a work programme consisting of four defined modules covering both a theoretical and practical introduction to environmental toxicometabolomics and its potential applications in ERA. The work programme was based on on-going project work and previous research interests at the Environmental Metabolomics lab. Between January and July 2023, the fellow spent a total of 3-months physically present at the hosting site. Further data processing and analysis could be performed remotely at the sending site using cloud-based infrastructure.

2.2.1. Theoretical introduction to practices and challenges in environmental risk assessment

As a part of the working group, the fellow participated in weekly lab meetings, learning about the different applications of environmental metabolomics through the various ongoing research projects of the group members.

As an integral part of the group's activities within an on-going HORIZON 2020 project at the host institution ('EndocRine Guideline Optimisation' (ERGO) project; https://ergo-project.eu/), which aims to investigate mechanisms of endocrine disrupting chemicals (EDCs) and improve identification and hazard assessment of EDCs using standardised test guidelines based on optimised approaches, the fellow participated in an ERGO project meeting, held in Amsterdam in January 2023. Moreover, the preceding annual meeting of the European Cluster to improve identification of endocrine disruptors (EURION cluster) provided a broad overview over current research efforts to support the development and improvement of test systems for EDCs. Further insights into challenges with current ERA practises

with respect to data requirements were gained and deepened through a collaboration with a PhD candidate on a manuscript discussing suitability of standard tests, and the potential of a mechanistic approach using NAMs with respect to chemical properties of the test compound, taking cationic polymers as an example (A. M. B. Hansen et al., 2023, Manuscript submitted for publication).

In addition, the fellow attended focused workshops and meetings on relevant topics:

- EFSA Risk Assessment Research Assembly (7.12.22, Berlin, Germany)
- EURION omics working group meeting (15.3.23, online)
- ENVS Research seminar on Emerging environmental pollutants and public health evaluations (19.4.23, AU campus Risø, Roskilde, Denmark)
- International Summer School on Non-Targeted Metabolomics (21–25.8.23, Copenhagen, Denmark)

The training was complemented through the 3-weeks EU-FORA induction training (September 2022), and three completed training modules (December 2022, March 2023 and June 2023), covering also topics around ERA, the application of omics in risk assessment and the AOP framework (Training module 2). The fellowship training will be concluded with a last training module taking place in August 2023.

2.2.2. Practical introduction to environmental toxicometabolomics- case study

For hands-on experience with metabolomics approaches, an amphibian metamorphosis assay (AMA; Organisation for Economic Co-Operation and Development (OECD) test guideline 231) was used as a proof-of-concept toxicometabolomics study case. The study was made in close collaboration with academic partners within the ERGO project. Both a targeted and untargeted approach was applied.

Disclaimer

Detailed results obtained from the metabolomics analyses are not included in this report, as the study is blinded, and parts of the analyses are still on-going. Further collaboration on the study and data is planned subsequent to the finalised fellowship-programme, with the aim to publish the results in peer-reviewed scientific journals. The EU-FORA fellowship and funding will be acknowledged in any manuscript submitted for peer-reviewed scientific journals.

2.2.2.1. Background

Endocrine disrupting chemicals are substances that can interfere with the hormonal system of living organisms, potentially leading to adverse effects on human health and the environment. Over the past decade, the European Union (EU) has taken significant measures to address the issue of EDCs (EC, 2018), and specific provisions have been included in the legislation on pesticides (EC 1107/2009[1]), biocides (EC 528/2012[2]), chemicals in general ('REACH Regulation', EC 1907/2006[3]), medical devices[4] and for aquatic environments according to the 'Water Framework Directive' (2000/60/EC[5]), in line with the different requirements laid down in the relevant legislation.

Based on the definitions for EDCs proposed by the WHO in 2002 and 2009 (WHO/IPCS, 2002, 2009), the EU introduced specific criteria for the classification of EDCs, particularly for pesticides and biocidal products in 2017 (EC, 2017), allowing for more stringent regulation and monitoring of these substances. The specific scientific criteria for identification of EDCs address three key elements: (i) chemical-induced adverse effects on the endocrine system of humans or non-target organisms (adversity), (ii) chemical-specific endocrine modes of action (MOAs) and (iii) the scientifically plausible causal link between the adverse effects observed and the endocrine activity of the substance (causality/plausibility). According to EDC criteria, all available scientific data must be considered in the assessment, and a weight of evidence approach should be applied (EC, 2017, 2018; ECHA/EFSA, 2018).

The amphibian metamorphosis assay

The AMA is a screening test, identifying substances that interfere with thyroid-mediated pathways or the function of hypothalamic–pituitary-thyroid (HPT) axis in vertebrates (OECD, 2009). The test is

[1] OJ L 309, 24.11.2009, pp. 1–50.
[2] OJ L 167, 27.6.2012, pp. 1–123.
[3] OJ L 396, 30.12.2006, pp. 1–849.
[4] OJ L 117, 5.5.2017, pp. 1–175.
[5] OJ L 327, 22.12.2000, pp. 1–73.

conducted with larval stages (tadpoles) of the African clawed frog, *Xenopus laevis*. The assay is designed as a dose–response, exposing tadpoles at Nieuwkoop and Faber (NF) developmental stage 51 (http://www.xenbase.org/, RRID:SCR_003280) to a minimum of three different concentrations of a test chemical in addition to a control for 21 days. The developmental stage, hindlimb length, snout to vent length measurement and wet weight are recorded as apical endpoints on day 7 and day 21 of the assay, in addition to thyroid gland histopathology at test termination.

Anuran metamorphosis is triggered by thyroid hormones and highly regulated by the HPT axis. The regulation of thyroid hormone-dependent molecular and physiological processes during metamorphosis, which occurs following a precise sequence and timing, is highly susceptible to disruptions caused by environmental and chemical factors (ECHA/EFSA, 2018). As the development of *X. laevis* is well-characterised, with distinct stages from egg to tadpole to adult (Zahn et al., 2022; Fisher et al., 2023), it is considered a validated test species for the AMA, providing a standardised framework for assessing effects (adversity) of chemicals on amphibian metamorphosis.

However, although the relevance of the AMA for tier 1 identification of thyroid-disruptive chemicals is largely recognised (ECHA/EFSA, 2018), the assay is exclusively based on morphological endpoints, and specificity of the thyroid responsive endpoints with respect to thyroid activity of chemicals has been questioned (Dang, 2019). While the AMA is not designed to indicate a molecular target of a chemical, the incorporation of biochemical or molecular biomarkers, such as thyroid hormone or expression of thyroid hormone related genes, into the guideline as mechanistic endpoints has been proposed, informing the MoA criterion and facilitating the development of a targeted test strategy (Dang, 2022).

2.2.2.2. Sample preparation – thyroid hormone and metabolite extraction

Two types of anuran tissues from the AMA were chosen for the study: Thyroid tissue was chosen for the analysis to characterise and explore thyroid-related metabolite changes. Moreover, experimental evidence suggests that alterations in the thyroid hormone system can influence eye development in vertebrates, but the chain of events from the molecular interaction of thyroid hormone system disruption to adverse outcomes to eye morphology and vision (the AOP) are not yet fully understood (Gölz et al., 2022). To further explore a potential relationship between thyroid- and eye metabolism, also eye tissue was included for the study. The samples were prepared for both targeted and untargeted metabolomics.

For each tissue, three individual samples of each of the four treatment replicates (n = 4 replicate tanks per treatment × three replicates per tank; developmental stages at day 21 between NF 58–62) were taken and kept (−80°C) for the additional metabolomics analyses. Treatment groups were labelled using an unknown colour code to provide blinding of all experimenters during endpoint recording, tissue sampling, sample preparation and analyses. Metabolites were extracted from the samples in random order, as previously reported (Pannetier et al., 2023), following a modified version of the Matyash method (Matyash et al., 2008; Sostare et al., 2018), which is based on a methyl-tert-butyl ether (MTBE)/methanol/water (2.6/2.0/2.4) biphasic solvent system to extract both polar and non-polar compounds separately. To enhance the extraction of thyroid hormones to the polar phase, 1% ammonium hydroxide (v/v) was added to the polar solvents (methanol and water). Procedural blanks were included from the start of the extraction protocol.

Both samples and procedural blanks were spiked with isotopically labelled 13C-thyroid hormone internal standards. The polar compounds (methanol/water phase) were enriched using solid-phase micro-extraction and reconstituted in 5% methanol containing an instrument control standard. As an additional quality control (QC) measure, a pool of all study samples was prepared (QC sample) to monitor stability throughout the run.

The extracts of the polar phase were used for both targeted TH determination and untargeted metabolomics. For metabolomics analyses, a mix of metabolite standards prepared in QC sample was added to the sequence. The separated non-polar phase (MTBE) was evaporated to dryness and reconstituted in 8Bu + solvent (Danne-Rasche et al., 2018) containing a mix of 14 isotopically labelled internal standards of all major lipid classes to enable quantification and/or normalisation.

2.2.2.3. Data acquisition and thyroid hormone quantification

Following a previously developed protocol, the targeted analysis of THs was performed on an Agilent 6495c triple-quadrupole system with a hyphenated Agilent 1290 Infinity II ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies, Santa Clara, CA USA) as described by (Hansen et al., 2016; Pannetier et al., 2023). Twelve THs were included into the targeted

analysis. The targeted THs were quantified in reference to external standard multi-point calibration curves, constructed using a serial dilution of an equimolar mix of neat standards. The data were analysed using the MassHunter Quantitative Analysis software (Agilent Technologies, Santa Clara, CA USA).

Detection and quantification of several THs was successful in the anuran tissues (e.g. thyroxin (T4); Figure 1). Incorporation of TH measurements with the results of standard AMA apical endpoints, will help to confirm whether observed effects on metamorphosis (development of limbs and tail) are indeed due to alterations in TH signalling pathways and not caused by general toxicity, secondary effects or unrelated mechanisms of action. Also, the quantification of targeted compounds allows for determining the potency and concentration at which a chemical affects thyroid function, as well as comparison of the potency across chemicals. Thus, the generated data may give valuable additional information, which minimises uncertainties around ambivalent results on standard assay endpoints and can be used in a regulatory context. With amphibians representing sensitive indicators for ecosystem health and changes, EDC screening with the AMA has environmental relevance. However, the thyroid system is highly conserved among vertebrates. Therefore, a more accurate identification of thyroid-related mechanisms through the AMA may also be relevant for hazard identification for human exposures.

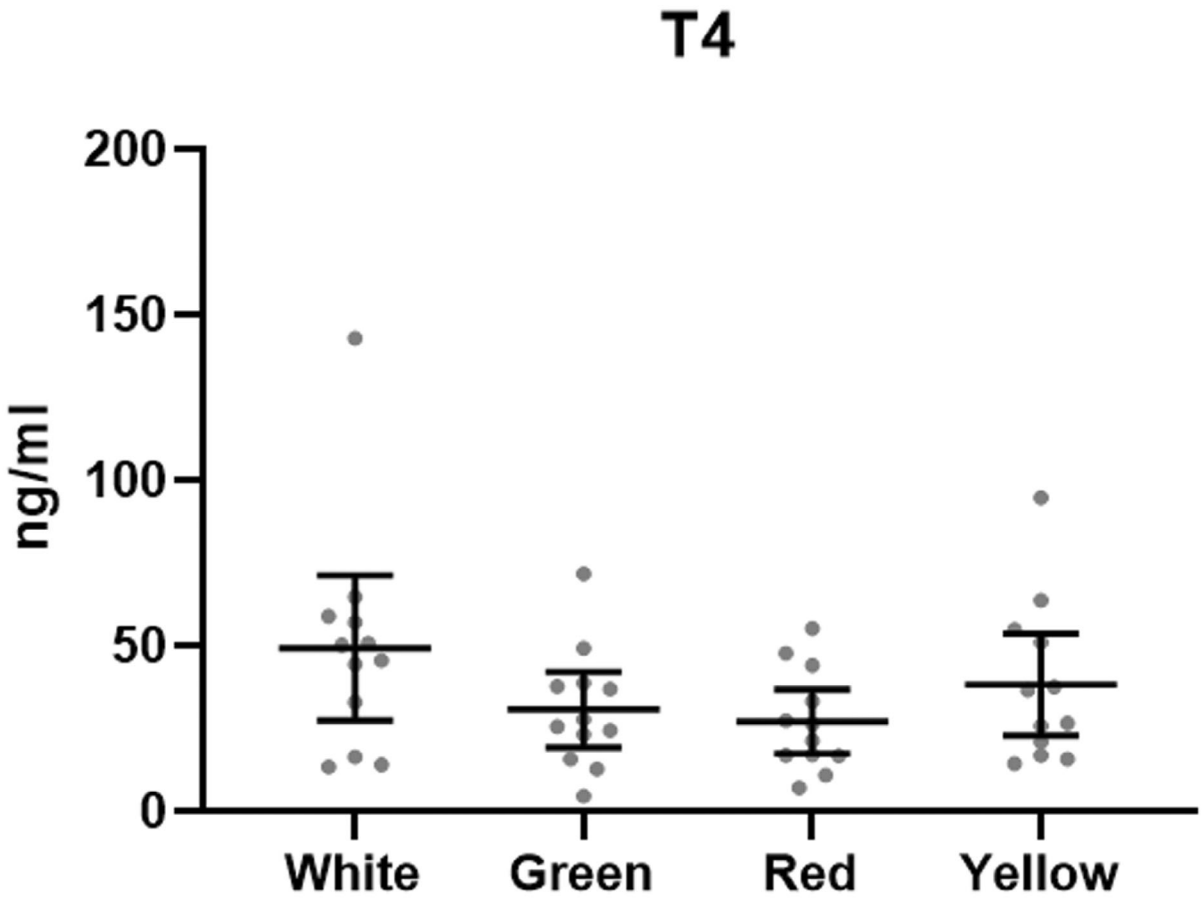

Figure 1: Thyroxine (T4) concentrations in tissue of tadpoles exposed to three different doses of the test compound for 21 days or unexposed. The experimental groups are colour-coded to maintain experimenter blinding regarding the treatment until the analysis is concluded

2.2.2.4. Data acquisition untargeted metabolomics

To further explore sub-lethal effects on the thyroid and a potential relationship between thyroid and eye metabolism, in a second step, untargeted metabolomics and lipidomics were performed using UHPLC Orbitrap high resolution tandem mass spectrometry system (Q Exactive HF, ThermoFisher Scientific). The samples were analysed in a randomised order. To account for potential instrument fluctuations and detect systematic errors, a pool of all study samples was interspersed as a QC regularly between individual samples during the injections.

2.2.2.5. Preprocessing of untargeted data

Four untargeted metabolomics datasets were generated, to explore lipid-changes and general metabolic pathways. The fellow became acquainted with the different steps necessary for the data processing from raw data files to a list of features, and learned how to compose, customise and run an identification workflow using the Compound Discoverer software (version 3.3; Thermo Scientific). The identification workflow was based on a pre-existing workflow template, including retention time alignment and unknown peak detection, compound detection and grouping, gap filling and merging of features, background correction based on instrument and procedural blanks, and normalisation based on the included QC samples. Initial compound identification was based on formula and accurate mass data from both custom mass lists and publicly available databases, as well as mass spectral library matching. The pre-processed datasets, each containing more than 1000 detected features, were preliminarily explored using the statistical tools available in the Compound Discoverer software (Figure 2).

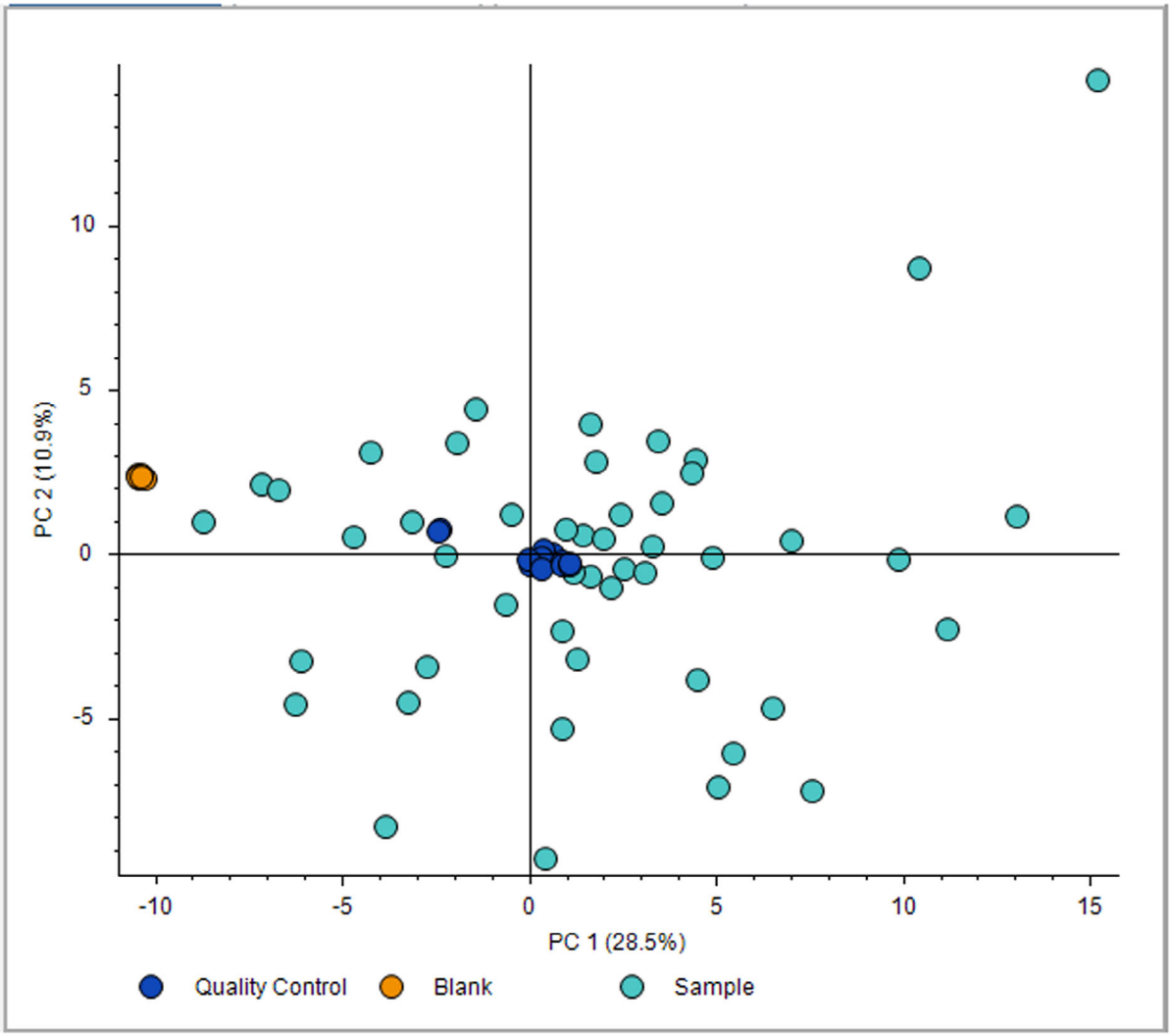

Figure 2: Principal component analysis filtered by sample type. To validate stability and reliability of the analytical method used, a pool of all included samples (Quality Control (QC); dark blue circles) was injected repeatedly throughout the acquisition of tadpole samples (light blue circles). Solvent blanks (orange circles) were also included. Apart from an additional QC sample containing a mix of standards (also dark blue), the QC samples show close clustering.

Correct identification of the large number of compounds detected in the individual datasets is essential for accurate and reliable interpretation of metabolic pathways and organismal responses.

Identification and annotation of the compounds from the untargeted metabolomics datasets is still on-going at the time of writing. Finalising the work programme, the fellow will participate in the Summer School for non-targeted metabolomics data mining, which is co-organised by the section Environmental Chemistry and Toxicology and hosted by Statens Serum Institute in August 2023. The collaboration between the fellow and the hosting site will continue subsequent to the fellowship programme, in order to conclude the statistical and pathway analyses, and biological interpretation of the generated datasets.

3. Conclusions

During the EU-FORA fellowship programme, the fellow was introduced to the current issues in ERA methods, challenges arising from traditional approaches, as well as the potential of metabolomics as a tool to support the transition to a more data-centric, mechanistically based next generation ERA. Being fully integrated into the Environmental Chemistry and Toxicology section at the Department of Environmental Science at AU, synergies between the fellowship working programme and on-going research activities were enabled and established, allowing for direct application of new knowledge and greatly enhancing the 'training by doing' character of the fellowship programme.

Moreover, the physical placement at AU provided a unique opportunity for the fellow to gain very valuable first-hand experience, and to familiarise herself with environmental toxicometabolomics applying both a targeted and untargeted metabolomics workflow, based on the protocols established at the hosting site. In addition to the scientific insights from the studies, the fellow learned best practice approaches for quality assurance and omics-based data analysis, and strengthened personal skills related to laboratory techniques, mass spectrometry technologies, application of bioinformatic computational tools and data management. The practical work provided transferable knowledge, which can be incorporated to add value to on-going research into food and feed safety at the fellow's home institution, the Institute of Marine Research (IMR), Norway, and built a foundation for future collaboration between the IMR and AU.

Both through the training modules, exchange among the fellows as well as opportunities created by the fellow's supervisor Martin Hansen during the implementation of the working programme, participation in the EU-FORA programme allowed to expand the scientific network within (eco) toxicology research, food and environmental safety and risk assessment, and provided a very valuable personal and professional experience.

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Abbreviations

AMA	amphibian metamorphosis assay
AOP	adverse outcome pathway
AU	Aarhus University
EDCs	endocrine disrupting chemicals
ENVS	Department of Environmental Science, Aarhus University
ERA	environmental risk assessment
EU-FORA	European Food Risk Assessment Fellowship Programme
HPT	hypothalamic–pituitary-thyroid
KE	key event
MIE	molecular initiating event
MOA	mode of action
MTBE	methyl-tert-butyl ether
NAMs	new approach methodologies
NF	Nieuwkoop and Faber
OECD	Organisation for Economic Co-Operation and Development
QC	quality control
UHPLC	ultra-high performance liquid chromatography

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

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Abstract

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

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

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1. Introduction

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

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

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

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

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

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

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

2. Description of work programme

2.1. Aims

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

2.2. Activities/methods

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

2.2.1. Laboratory experience

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

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

Preparation of nanoemulsions

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

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

Decontamination treatments of cherry tomatoes

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

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

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

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

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

Training on bacterial inactivation

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

2.2.2. Training in risk assessment

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

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

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

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

2.3. Secondary scientific activities during fellowship

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

- The fellow attended a hybrid Workshop held by the University of Cordoba (Spain) on 27–28 October 2022: 'Use of Quantitative Microbial Risk Assessment Tools. Case studies on foodborne pathogens in ready-to-eat foods'.
- The fellow visited the headquarters of the Spanish Agency for Food Safety and Nutrition (AESAN) in Madrid and the National Centre for Food laboratory, in Majadahonda on 17 and 18 January. During the visit, the fellow benefited from the presentation of AESAN's work on risk assessment, management and communication. She also had the opportunity to present her work and activities to the AESAN Scientific Committee.
- The fellow was invited as a guest lecturer and delivered a seminar for Food Quality Management Master students enrolled in the biological hazard assessment and control in food quality management course at USAMV CN. She presented her research activities under the EU-FORA programme.
- The fellow also attended the presentations of two Final MsC Reports related to decontamination with nanoemulsions of RTE fresh produce at the hosting organisation (UPCT).

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

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

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

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

3. Conclusion

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

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

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Abbreviations

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

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

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Training in the evaluation of pesticides (plant protection products and active substances) according to Regulation (EC) No 1107/2009

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Abstract

Plant Protection Products, also called 'pesticides', are intended to protect crops by controlling pests, weeds and diseases. This Technical Report contains a description of the activities within the work programme of the EU-FORA Fellowship on the training in the evaluation of pesticides (active substances and plant protection products) according to Regulation (EC) No 1107/2009. The purpose of the Plant Protection Product Regulation is to ensure a high level of protection of both human and animal health and the environment and at the same time to improve the functioning of the internal market through the harmonisation of the rules on the placing on the market of plant protection products, while improving agricultural production. The data requirements for active substances and plant protection products are set out in Regulations (EC) 283/2013 and 284/2013, covering active substance(s), safeners or synergists. The scope of this work programme was to help the fellow to gain knowledge and experience in order to understand the evaluation process of the data submitted by the Applicants at each different section of an active substance and a plant protection product dossier in a regulatory and scientific basis.

Keywords: pesticides, risk assessment, active substance, product, authorisation

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Summary

This Technical Report focuses on the activities within the work programme of the EU-FORA Fellowship on the training in the evaluation of pesticides (active substances and plant protection products) according to Regulation (EC) No 1107/2009. All the relevant areas of evaluation of an active substance and plant protection product dossier according to Regulations (EC) 1107/2009, 283/2013, 284/2013 have been considered for the training of the fellow student. More specifically, identity, physicochemical properties, methods of analysis, efficacy, residues, mammalian toxicology, fate and behaviour in the environment, ecotoxicology and classification and labelling were discussed through the prism of the existing regulatory framework. Furthermore, the requirements and the EU common approaches, have been scientifically analysed in each section. An introduction in specific requirements (legislation framework – data requirements) for each section, followed by hands-on-training (specific case studies), have been performed.

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1. Introduction

Plant protection products (PPPs), also referred as pesticides, contain at least one active substance. These active substances can be chemicals, micro-organisms, pheromones, botanical extracts or viruses that enable PPPs to perform a controlling effect on the target pests, diseases or weeds. They may also contain other components such as co-formulants, safeners and synergists. The active substance(s), safeners or synergists, are intended for one of the following uses:

- protecting plants or plant products against harmful organisms or preventing the action of such organisms, unless the main purpose of these products is considered to be for reasons of hygiene rather than for the protection of plants or plant products (e.g. fungicides, insecticides);
- influencing the life processes of plants, such as substances influencing their growth, other than as a nutrient (e.g. plant growth regulators, rooting hormones);
- preserving plant products, in so far as such substances or products are not subject to special Community provisions on preservatives (e.g. extending the life of cut flowers);
- destroying undesired plants or parts of plants, except algae unless the products are applied on soil or water to protect plants (e.g. herbicides/weedkillers to kill actively growing weeds);
- checking or preventing undesired growth of plants, except algae unless the products are applied on soil or water to protect plants (e.g. herbicides/weedkillers preventing the growth of weeds).

As an example, PPPs may include insecticides, fungicides, herbicides, molluscicides and plant growth regulators.

Regulation (EC) No 1107/2009 is the main legislative framework regulating the placing on the market of PPPs. The Regulation lays down the rules and procedures for the approval of active substances and the authorisation of PPPs. For active substances to be use in PPPs, they must fulfil the approval criteria while PPPs cannot be placed on the market or used without prior authorisation in an EU Member State.

This specific project '*Training in the evaluation of pesticides (plant protection products and active substances) according to Regulation (EC) No 1107/2009*' in the context of the EU-FORA fellowship programme was implemented by the hosting site Benaki Phytopathological Institute (BPI), which specialises in risk assessment and advice on the safe usage of agricultural chemicals with regards to the protection of human health and that of the environment.

2. Description of the work programme

2.1. Aims

The training programme followed the evaluation of pesticides according to the European Legislation framework and the risk assessment of PPPs and their active substances in the areas of identity and physicochemical properties, methods of analysis, efficacy, residues, mammalian toxicology, fate and behaviour in the environment and ecotoxicology, as well as classification and labelling. As an introduction the training focused on the general aspects of evaluation procedures, existing Legislation framework including guidance documents related to horizontal procedures of the evaluation of PPPs. Then, a comprehensive presentation of the various evaluation sections including evaluated case studies was delivered by key experts in order to obtain a first overview of the evaluation procedure and the interconnections between the different sections of PPPs evaluation dossiers.

2.2. European legislation framework

Regulation (EC) No 1107/2009[1] is the legislation concerning the placing of PPPs on the market in the European Union. This Regulation specifies the cut-off criteria for the approval of active substances and decision-making criteria for the authorisation of PPPs at Member State level. In accordance with the mentioned Regulation, approval of active substances in PPPs including the definition of maximum residue levels is coordinated at the European level with involvement of the European Food Safety

[1] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1–50.

Authority (EFSA). EFSA is responsible for the EU peer review of risk assessments of active substances used in PPPs, in close cooperation with EU Member States and the European Commission. However, the authorisation procedure for PPPs such as zonal and mutual recognition as well as additional applications of authorised PPPs are coordinated by Member States.

The data requirements for the active substance provided for in Article 8(1)(b) of Regulation (EC) No 1107/2009 are set out in the Annex to Regulation (EU) No 283/2013,[2] The respective data requirements for a PPP for in Article 8(1)(b) of Regulation (EC) No 1107/2009 are set out in the Annex to Regulation (EU) No 284/2013[3].

The evaluation and authorisation of PPPs is also subject to Uniform Principles in accordance with Regulation (EU) No 546/2011.[4] These principles include assessment of impact on animal and human health, influence on the environment and physical and chemical properties.

The legislative framework of PPPs also regulates maximum residue levels (MRLs) of active substances in food and feed commodities. The setting and compliance of MRLs in food and feed commodities are regulated by Regulation (EC) No 396/2005.[5] MRLs are set at a low level that poses no health risk to consumers in line with good agricultural practices. In addition, other relevant European Legislations and guidance documents are also applicable of PPPs. These include Regulation (EC) No 1272/2008[6] for the classification and labelling of active substances and PPPs as well as Directive 2009/128/EC[7] establishing a framework for Community action to achieve the sustainable use of pesticides.

2.3. Risk assessment areas of PPPs and active substance

Risk assessment of active substances and PPPs is performed to evaluate efficacy and foreseeable risks, which may have potentially harmful or unacceptable effects on human, animal and the environment health, respectively. For the determination of the approval of active substance and the authorisation of PPPs, specific areas are assessed. The evaluation of active substances and PPPs generally follows the main principles of the risk assessment process by identifying and characterising the hazard, performing an exposure assessment and characterising the risk.

2.3.1. Identity and physicochemical properties

The identity section contains information regarding the applicant and the manufacturing details as well the details of the chemical composition of the technical active substance as manufactured, manufacturing processes and starting materials. In this section, the specifications regarding the minimum purity of the active substance and the maximum content of any impurity are set. For the PPPs the type of PPP, information on co-formulants, manufacturing process and the full composition of the product are described in detail. From a physicochemical point of view, the following characteristics are evaluated for the active substance, i.e. appearance, melting/boiling point, spectra, vapour pressure, solubility (in water and organic solvents) partition coefficient *n*-octanol/water, dissociation in water, safety properties (oxidising and explosive properties, flammability/flash point, self-heating), surface tension. For a PPP the respective physicochemical properties are required, i.e. appearance, safety properties (oxidising and explosive properties, flammability/flash point, self heating), surface tension and viscosity, acidity/alkalinity and pH value and relative/bulk density. In addition the technical characteristics of the product need to be addressed (e.g. wettability, persistent foaming, suspensibility,

[2] Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.

[3] Regulation (EU) No 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of pl.

[4] Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products.

[5] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. OJ L 70, 16.3.2005, p. 1–16.

[6] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. OJ L 353, 31.12.2008, p. 1–1355.

[7] DIRECTIVE 2009/128/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. OJ L 309/71

particle size distribution, etc.). The Commission Communication (2013/C 95/01)[8] (European Commission, 2013) provides the list of test methods and guidance documents relevant to the implementation of the data requirements Regulation as well as the applicable OECD test guidelines. It is highlighted that besides the safety of the active substance and/or PPP and the technical performance of the PPP, several physicochemical properties are important for the risk assessment in human health and environment (e.g. vapour pressure, water solubility, UV/VIS absorption, partition in *n*-octanol/water).

2.3.2. Methods of analysis

Fully validated and adequately sensitive analytical methods are necessary for the determination of active substance, relevant metabolites and impurities in technical active substance and PPPs and for the determination of pesticide residues. These are generally required for pre-registration and post-registration control and monitoring purposes. Methods of analysis for pesticides covers different substrates and matrices such as plants, plant products, food, animal feed, soil, water and air including both risk assessment and monitoring methods. The training focused on the two relevant guidance documents SANCO/3030/99 (European Commission, 2019b) and SANTE/2020/12830.

2.3.3. Efficacy

Efficacy data are evaluated to assess the nature and extent of benefits that accrue following the use of the PPP on particular crop/pest combination. Submitted data for risk assessment reflect several factors including variability in plant health conditions, climatic differences, the range of agricultural practices, the uniformity of the crops, the mode of application, the type of pest and the type of PPP. Relevant trials on the effectiveness and phytotoxicity should be conducted in accordance with the general and specific standards of the European and Mediterranean Plant Protection Organisation (EPPO). The EPPO standards describe the procedures covering all general aspects of the efficacy evaluation such as analysis and number of trials, minimum effective dose, phytotoxicity/crop safety, effects on succeeding crops or adjacent crops, analysis of resistance, minor uses and climatic considerations. In terms of the efficacy section, extrapolation of results to other crops can be also possible. Such extrapolation for effectiveness and crop safety should follow the available EPPO extrapolation tables on a case-by-case manner and expert judgement.

2.3.4. Residues

Residues are traces of pesticides that can be found in food and feed after treatment with a PPP. Under Regulation (EC) 396/2005, MRLs of these residues are set and a risk assessment is performed as to ensure the safety for all European consumer groups, including the vulnerable groups. Therefore, the setting of MRLs is completed before an authorisation for PPPs is granted. As a general principle, MRLs are the upper legal level of a concentration for a pesticide residue in or on food or feed, based on good agricultural practice (GAP) and the lowest consumer exposure necessary to protect vulnerable consumers. The critical GAP meaning, for example, the maximum number of proposed applications, the shortest interval between applications, the maximum application rate and the shorter preharvest interval is taken into consideration when conducting residue trials. The assessment of residues includes the evaluation of relevant studies such as storage stability studies, nature, and magnitude of residues in plants, livestock, processed commodities and succeeding crops. Data can be extrapolated among corps and tolerances from the intended GAP can be acceptable following specific the residue section, guidelines (SANTE/2019/12752) (European Commission, 2019a).

Following the evaluation of these data, a consumer risk assessment is performed as to ensure that food is safe for consumption.

2.3.5. Mammalian toxicology

In the area of mammalian toxicology, risk assessment includes hazard and exposure assessment. Hazard assessment (identification and characterisation) is carried out considering all available information and data on endpoints in line with data requirements as described in Regulations (EC) No

[8] Commission Communication in the framework of the implementation of Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market (2013/C 95/01).

283/2013 or 284/2013 for an active substance or a PPP, respectively. These endpoints may include studies on acute toxicity, irritancy and sensitisation conducted on both the active substance and the product as well as studies on genotoxicity/mutagenicity, carcinogenicity, reproductive toxicity (fertility, development), neurotoxicity, target organ toxicity, endocrine disruption, etc., carried out with the active substance. Additional toxicological information on co-formulants is considered, as available. Most of the toxicity studies are performed using experimental animals, although effort is made in developing alternative approaches for Replacement, Reduction and Refinement of animal use. Mechanistic information provides insight on the mechanism of action and human relevance of observed effects. Where relevant, health-based guidance values (HBGVs), i.e. acceptable daily intake (ADI), (acute) acceptable operator exposure level ((A)AOEL) and acute reference dose (ARfD), are established on the basis of the results of animal studies and considering appropriate uncertainty factors for extrapolation to humans ensuring sufficient protection of vulnerable groups of the population.

The non-dietary exposure assessment is carried out on the active substance of a specific PPP for different population groups, i.e. operators, workers, residents and bystanders, according to the EFSA (2022) guidance document and considering different exposure scenarios depending on the proposed uses of the PPP. Operators, workers, residents and bystanders may be exposed to PPPs either directly through contact with the concentrate, spray dilution, spray drift or dust (via dermal or inhalation routes) or indirectly through contact with drift deposits (dermal or ingestion) or vapour drift arising from volatilisation of deposits. In addition, exposure may be acute (during a single day) or short-term (repeated).

At risk characterisation, the acute and short-term exposure estimates (E) are compared to the relevant HBGVs to conclude if the underlying risk is acceptable ($E < HBGV$) or not ($E > HBGV$). In cases of unacceptable risk, different risk mitigation measures may be proposed for consideration in decision making during risk management.

2.3.6. Environmental fate and behaviour in the environment

The environmental fate and behaviour of pesticides session has been divided in two parts. In the first part, how the fate and behaviour of an active substance and its metabolites, in different compartments (soil, surface water, sediment groundwater and air), is addressed according to the data requirements of Regulation (EC) 283/2013, based on appropriate OECD test guidelines. Special attention was given in microbial degradation of the active substance in different compartments. The second part focused on the environmental exposure following the use of a PPP. The relevant entry paths to environmental compartments have been discussed (e.g. precipitation, dry deposition, run-off, drainage, drift, leaching). In addition, mechanisms and factors influencing environmental exposure, such as transport mechanisms, use pattern, substance related factors, and environmental conditions have been considered regarding their impact on the exposure. The estimation of predicted environmental concentrations (PECs) of active substances and their metabolites in the relevant environmental conditions is necessary for the evaluation of the exposure in soil, surface water, groundwater, sediment and air according to Regulation (EC) 284/2013. PEC calculations are estimated taking into consideration the realistic worst-case scenario for active substances and relevant metabolites. A worst-case scenario is considered based on the intended uses. If more than one use is proposed the 'risk envelope' approach in accordance with SANCO/11244/2011 rev.5 is recommended (European Commission, 2011). EU agreed models are available to calculate appropriate PEC in soil, surface water, sediment and groundwater (FOCUS, 1997, 2001, 2014). Relevant PEC calculations have been performed by considering specific active substances and GAP tables for better understanding the rationale behind and the impact on the environmental risk assessment. Moreover, the available and EU acceptable risk mitigation measures according to SANCO/10422/2005 (European Commission, 2007) have been discussed and implemented where necessary.

2.3.7. Ecotoxicology

An ecotoxicological evaluation is carried out to assess the impact on non-target organisms (flora and fauna). An impact on non-target organisms can result from single, prolonged or repeated exposure and can be reversible or irreversible. For the determination of an acceptable or non-acceptable risk, the assessment for PPPs is carried out taking into consideration ecotoxicity studies and data from the fate and behaviour section for soil, surface water and sediment. Following the evaluation of relevant studies, the risk assessment is performed using ecotoxicity endpoints and guidance documents for different categories of non-target organisms. These categories include birds and

mammals, aquatic organisms, soil organisms, bees and other non-target arthropods and non-target terrestrial plants.

For birds and mammals, currently the EFSA (2009) guidance is taken into consideration for the risk assessment. An updated guidance document has been recently published by EFSA (2023), which is not yet legally in force. The effects on mortality reproduction (effects at population level) from exposure of birds and mammals to the pesticide/ active substance are assessed by an acute risk assessment and long-term/reproductive risk assessment, respectively. A tiered approach in risk assessment is followed taking into consideration a screening step of 'indicator species' in terms of the worst-case assumption regarding exposure, a first-tier risk assessment for 'generic focal species' in terms of feeding guilds and BBCH stages and a higher-tier/refined risk assessment of 'focal species' that represents the real species found in a particular crop at a particular time (BBCH stage) to determine realistic exposure estimates.

For aquatic organisms, risk assessment is necessary to compare toxicity levels with exposure and is conducted according to the EFSA PPR Panel (2013) guidance. A tiered assessment is carried out, in which the exposure and in some cases the toxicity (e.g. with the use of microcosm studies) are gradually refined from a worst-case towards a more realistic approach. For aquatics, the risk is estimated on basis of EU active substance data tested on aquatic organism groups and PEC calculations for surface water and sediment for the product uses/application rate. Introduction of risk mitigation measures can take place in order for a safe risk to be demonstrated.

For soil organisms and non-target plants, the risk assessment is carried out according to the SANCO/10329/2002 (European Commission, 2002). The risk assessment for soil organisms is performed on the basis of chronic exposure data to estimate the long-term reproductive toxicity effects taking into consideration also the maximum PEC calculations for soil. Furthermore, the risk assessment is performed for earthworms and other non-target soil meso- and macro-fauna. For the PPPs categorised as non-herbicides only a screening test is performed in order to investigate the phytotoxic effects on the non-target plants outside the treated field. For the PPPs that are categorised as herbicides or plant growth regulators, studies with seedling emergence and vegetative vigour examining the effects on non-target plants are required in order for the risk assessment to be completed. For bees and other non-target arthropods, the risk assessment is carried out according to the SANCO/10329/2002 (European Commission, 2002). For non-target arthropods, exposure toxicity is estimated following a tiered approach starting with glass-plate studies on sensitive indicator species at tier 1 and extended laboratory studies on sensitive and additional indicator species at tier 2. In this case, exposure is calculated for both in-field and off-field area (Candolfi, 2002). Currently for honeybees, the potential acute toxicity is assessed in accordance with the Terrestrial Guidance Document and the potential chronic toxicity for honeybee larvae and worker bee is assessed following the EPPO scheme (2010). Generally for PPPs, the risk assessment in accordance with the EFSA (2013) is currently being performed for illustrative purposes given that it is not yet legally inforce.

3. Conclusion

The training programme was developed and implemented by the BPI. The work plan provided training and knowledge in areas of the evaluation of pesticides (PPPs and active substances) according to Regulation (EC) No 1107/2009. The work programme allowed the fellow to get a comprehensive overview into the risk assessment of active substance and PPPs. It also provided the opportunity to develop important skills within critical aspects of the risk assessment framework of different areas of pesticide evaluation. This was done specifically by theoretical sessions and experiencing practical examples of evaluated case studies to identify critical areas of concern. The end result allows for the fellow to efficiently use the gained experience and knowledge to contribute to work involving pesticides in particular PPPs.

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Abbreviations

AAOEL	acute acceptable exposure level
ADI	acceptable daily intake
AOEL	acceptable operator exposure level
ARfD	acute reference dose
BBCH	Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie
BPI	Benaki Phytopathological Institute
EPPO	European and Mediterranean Plant Protection Organisation
GAP	Good Agricultural Practice
HBGV	Health-based guidance levels
MRL	maximum residue level
OECD	Organisation for Economic Cooperation and Development
PEC	predicted environmental concentration
PPP	plant protection product

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Determination of plastic polyester oligomers in real samples and their bioeffects

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Abstract

Plastics are ubiquitously, becoming part of our everyday life. Recently, the issue of human exposure to micro- and nanoplastic particles and potentially resulting toxicological consequences has been broached, triggered by the discovery of microplastics in foodstuff and dietary exposure via contaminated food and beverages. Within this EU-FORA fellowship project, a determination and quantification of plastic polyester plastics oligomers in food samples was performed to assess exposure at these categories of 'nanoplastics', evaluating them as potential contaminants or as indicators and marker compounds of the exposure to specific nanoplastics/microplastics from polyesters, such as polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). UHPLC-TOF–MS/MS analysis has been set-up for 10 PET and PBT oligomers and analysis has been performed in foods and drinks. Moreover, the project focused also on the effects of these oligomers in *in vitro* and *ex vivo* experiments. These data would be combined with EFSA Comprehensive Food Consumption Database, for the exposure and risk assessment of these 'Nanoplastics'.

Keywords: plastic oligomers, nanoplastics, food, occurrence, toxicity, risk assessment

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1. Introduction

Packaged food can contain regulated additives or intentionally added substances (IAS), allowed to be used for the production of plastic food contact materials (FCM). However, apart from IAS, also non-intentionally added substances (NIAS) can occur in a plastic FCM, as a result of reaction and degradation processes or due to the presence of impurities in the raw materials used for the packaging production. An important subset of the aforementioned group of substances are the oligomers, a class of substances deriving from plastic FCM that have been found in recent years to migrate in substantial amounts from the packaging to the food itself. Recently, both DG SANTE and EFSA showed an interest in this category of compounds, but real chemical, migrating and toxicological data are not available for most of these substances as no comprehensive toxicological studies have been performed. This fact has a clear impact in the determination of their safety and potential health risks.

2. Description of work programme

2.1. Aims

Legislation for microplastics and nanoplastics as contaminants in food is lacking as the knowledge is limited. There is hardly any scientific data on the exposure and the toxicity of plastic oligomers. As a result, risk assessment cannot be estimated. This EU-FORA fellowship project called 'QUantitative determination of Plastic polyester OLIGOmers in real samples' (QUPOLIGO) aimed to conduct a comprehensive framework on this topic, including data mining and systematic analysis of the literature to cover knowledge on the occurrence and potential toxicity of micro- and nanoplastics. These results would offer the base for risk assessment calculation. The project has also included experimental work for the determination of oligomers in *in vivo*, *ex vivo* samples and foods. A particular focus have been given to three categories of plastic FCM oligomers, which include a regulated group of cyclic oligomers from polystyrene (PS), polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). For PS oligomers, EFSA published a Scientific Opinion regarding Assessment of the impact of the IARC Monograph on the safety of the styrene substance (FCM No 193) and its respective oligomers in plastic FCM, for which no genotoxicity data nor mechanistic data or comparative toxicokinetics and analysis of species differences are existing at the moment. All PS oligomers are considered NIAS. Regarding PET, these cyclic oligomers can potentially migrate from plastic FCM to foods. Typical examples of cyclic oligomers found frequently are those resulting from reactions between adipic acid (AA) and phthalic acid (PA) with diethylene glycol (DEG) from multilayer materials, as well as terephthalic acid (TPA) with ethylene glycol (EG). However, no data on real food samples exist nor potential toxicological effects. Furthermore, it shall not be omitted that at the moment only PET is allowed to be mechanically recycled at EU level, where EFSA deals with a large number of FCM recycling applications to be assessed. All PET cyclic oligomers are considered as NIAS. Finally, PBT oligomers (FCM 885) are regulated and allowed to be used with certain limitations for the production of plastic FCM, according to European Commission Regulation No 10/2011. This mixture of PBT cyclic oligomers consists of the dimer, trimer, tetramer and pentamer at a specific ratio and are considered as IAS. No data are available for the concentrations of these compounds into real foods and analysis methods to detect them are missing.

2.2. Activities/methods

2.2.1. Revision of available data

A comprehensive literature search has been performed in order to retrieve peer-reviewed articles referring to the presence of the selected oligomers in foods, including beverages, as well as for their toxicity, either *in vivo* or *in vitro*. Scientific databases such as 'PubMed', 'Scopus', 'Google Scholar' and 'Web of Science' were searched using various keyword combinations (e.g. 'polymer', 'oligomers', 'cyclic oligomers', 'PS', 'PET', 'PBT', 'food ', 'toxicity', 'in vivo', 'in vitro'). Then, a more focused research has been performed to restrict the field with a combination of words related to the effects of food contact materials on children ('risk assessment', 'toxicity', 'analysis and occurrence', 'oligomers', 'children').

2.2.2. Analytical work/evaluation of concentrations

The scope of this part of the fellowship was to train the applicant by participation in research through the development and validation of methods for analysis of the oligomers of interest in various specimens. Work has been built upon the development of a method for ultra-high performance liquid chromatography-time-of-flight tandem mass spectrometry (UHPLC-TOF-MS/MS) analysis for the determination of oligomers in real samples. Methods for sample preparation developed in the lab have been set up (Diamantidou et al., 2022; Diamantidou et al., 2023). The method determined the following monomers: (1) PET dimer, (2) PET trimer d12 (internal standard), (3) PET trimer, (4) PBT dimer, (5) PET tetramer, (6) PET pentamer, (7) PBT trimer, (8) PBT tetramer with high sensitivity (limit of quantifications in the area of 5–12 μg L^{-1}). A calibration and preparation of solutions in standard mixtures has been done. Stock solutions of the PET dimer and the isotope internal standard were prepared at a concentration of 5000 mg L^{-1} in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) whereas PET trimer, tetramer and pentamer stock solutions were at 2,500 mg L^{-1}. PBT mix solution in HFIP was at a concentration of 20 mg L^{-1}, in HFIP as solvent. A multi-component solution (2.5 mg L^{-1}) containing equal concentrations of every analyte was prepared by mixing the appropriate volumes of the stock solutions. Serial dilutions of this mix solution followed in amber vials with ethanol:H_2O 50:50 (v/v), to prepare working standard mixtures at 10 concentrations (5, 10, 25, 50 100, 250, 500, 750, 1,000, 2,500 μg L^{-1}). Stock solutions and working standards were both kept at −20°C. Calibration curves were constructed by plotting the means of ratios of the compound peak areas to the internal standard peak areas against concentrations of the analytes. Four different 0.5-L PET bottles intended for contact with beverages and edible oils were supplied from local market. Two of them were from 100% virgin PET (vPET) and the other two from 100% recycled PET (rPET). In the first test, the migration experiment was performed according to the Reg. EU No 10/2011 (European Commission, 2011). For water samples, compliance testing has been carried out using ethanol (20%) in water. Then, both the vPET and the rPET plastic bottles containing the food simulants were stored in an incubator at a temperature of 60°C for 10 days. Each sample volume was adjusted to 400 mL and two replicates were prepared. Test time and temperature were selected to cover a storage period of more than 6 months at room temperature or below, mimicking potential household conditions. For the migration measurements in tap water, time and temperature storage conditions representative of household conditions/real life were selected. Thus, in this second migration experiment, PET bottles were filled with 400 mL of 'blank' tap water sample. Three replicates for each type of PET bottle (virgin and recycled) were prepared and were stored for 12 months at room temperature (~ 20°C). Samples were collected at three time points (2, 6 and 12 months) and analysed. An UHPLC system (Bruker, Germany) was used for chromatographic separations of samples using a Waters BEH C18 (150 × 2.1 mm, 1.7 μm) analytical column, protected by a UPLC BEH C18 (5 × 2.1 mm, 1.8 μm) VanGuard pre-column. The method is fully described in Diamantidou et al. (2022). Briefly, the mobile phase consisted of solvent A: H_2O with 0.1% formic acid and solvent B: ACN with 0.1% formic acid at a flow rate of 0.3 mL/min. The elution was performed in a 12-min gradient as follows: 0–1 min: 50–70% B; 1–12 min: held to 90% B. The composition was returned to the initial (50% B) in 0.1 min. An equilibration time of 4 min was set for the column before the next injection. The analytical column was temperature-controlled at 60 °C and the injection volume was 5 μL. The system was operated by Compass HyStar 5.1 software (Bruker, Germany) and was hyphenated to a timsTOF mass spectrometer (Bruker, Germany) operating in positive ionisation mode at a 3.5 kV capillary voltage. The source operated at 300°C and nitrogen was used as drying (10.0 L/min) and nebulising gas (2.0 Bar). The Funnel 1 RF, Multipole RF, Deflection Delta and Collision RF were set at 200 V, 200 Vpp, 50 V and 700 Vpp, respectively. The acquisition mode was set at full scan acquiring data over the range of 300–1,000 m/z at a rate of 3 spectra/s. For individual recalibration of the chromatograms, sodium formate solution was injected before every analysis from 0.1 to 0.3 min. Data Analysis 5.3 software was used for data handling (Bruker, Germany).

2.2.3. *In vitro* experiments

The potential of oligomers for cellular viability has been assessed. For this purpose, Caco-2 cell line has been used to mimic small intestine conditions. Based on the results of experiment described in the previous paragraph, three PBT oligomer concentrations (no oligomers as control; low concentration; high concentration) has been applied. All experiments have been performed in triplicate. The cells were exposed and incubated for 24 and 48 h (Figures 1 and 2).

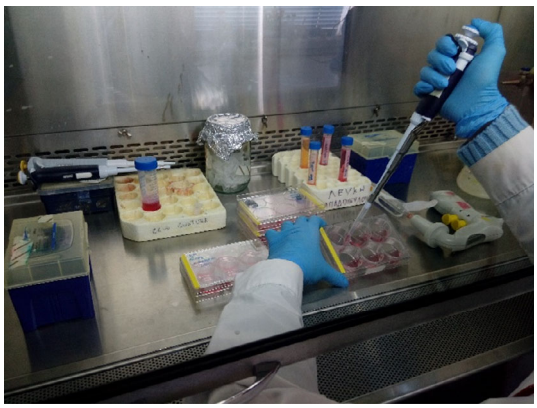

Figure 1: Preparation of cell cultures for the experiments

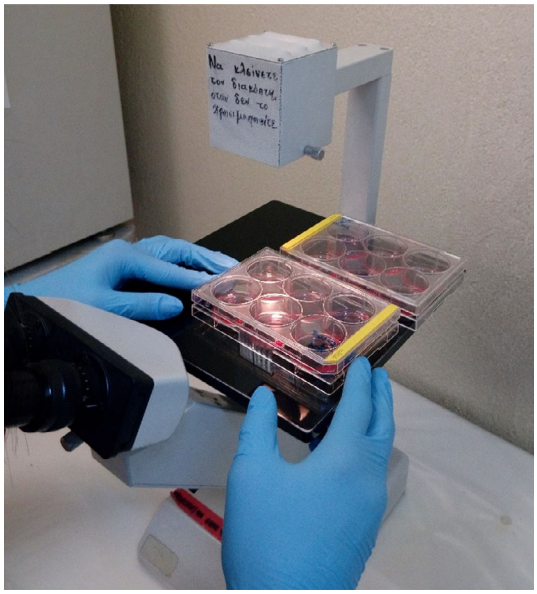

Figure 2: Part of the procedure to check the confluency of cells in each well/plate with the invertoscope

After 24 h and 48 h, cells were tested for apoptosis and cytotoxicity level.

Apoptosis were checked by using Fluoroisothiocyanate Annexin V Apoptosis Detection Kit (BioLegend; Cat# 640914). Annexin V (or Annexin A5) is a member of the annexin family of intracellular proteins that binds to phosphatidylserine in a calcium-dependent manner. Phosphatidylserine is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane asymmetry is lost and phosphatidylserine translocates to the external leaflet. Fluorochrome-labelled Annexin V can then be used to specifically target and identify apoptotic cells. To help distinguish between the necrotic and apoptotic cells, propidium iodide solution has been used. Early apoptotic cells will exclude phosphatidylserine, while late stage apoptotic cells stained positively, due to the passage of the dyes into the nucleus where they bind to DNA. When excited by 488 nm laser light, propidium iodide, a fluorescent dye that binds to DNA, can be detected and it is commonly used in evaluation of cell viability or DNA content in cell cycle analysis by flow cytometry.

Cytotoxicity was tested by lactate dehydrogenase assay (Biolegend, Cat# 426401). The determination of cytotoxicity has been performed by measuring lactate dehydrogenase activity released from damaged cells. Lactate dehydrogenase catalyses dehydrogenation of lactate to pyruvate thereby reducing NAD^+ to NADH. NADH reduces water-soluble tetrazolium salt in the presence of an electron mediator to produce an orange formazan dye.

2.2.4. *Ex vivo* or permeability experiments

Rat gut sac is a paradigm widely employed for determination of absorption kinetics of drugs along with evaluation of effects of absorption enhancers. In this case, the potential of oligomers for permeation through the membrane tract (8 cm) has been evaluated after 15, 30, 60, 90 and 120 min from the exposition to different oligomer concentrations at 37°C. The same concentrations of the *in vivo* experiments have been adopted (Figures 3 and 4).

Figure 3: Preparation of the rat gut for the permeability experiment

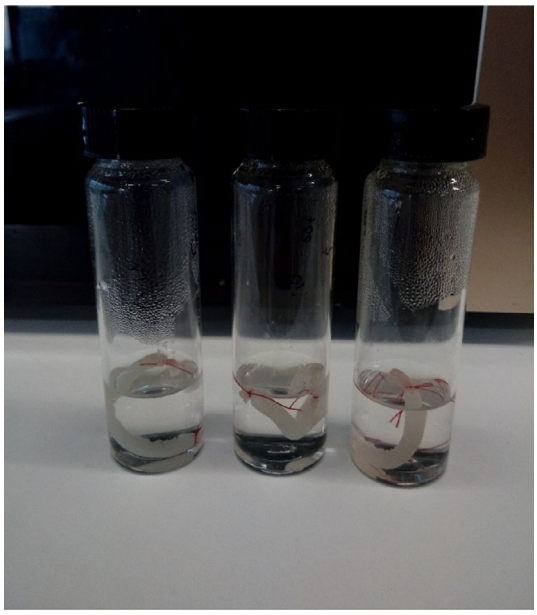

Figure 4: *Ex vivo* permeability study evaluated using the non-everted gut sac method

Then, the released oligomers out the lumen have been carried out by using UHPLC-TOF–MS/MS.

2.2.5. Training in risk assessment

Risk assessment training had the aim to train the applicant in calculating the risk assessment of target oligomers. The applicant has attended training activities from EFSA. In addition, the host has provided some training activities to try to calculate the risk assessment of PET, PS and PBT oligomers based on the concentrations that will be found in the experiments described in the above paragraphs. In the future, all the results of these activities are going to be maximised by knowledge transfer in participating to workshops, conferences, panels and working groups meetings at the hosting site as well as at the EU level (EFSA conferences and international workshops) and at fellow sending organisation.

3. Conclusion

Overall, the work programme allowed the fellow to gain knowledge on the analysis of plastic oligomers and their effects. On the one hand, an analysis and evaluation of available data on the occurrence of NIAS in food contact materials and their toxicological effects on children has been performed. On the other hand, the fellow gained knowledge in practical research work, with respect to both oligomer detection and *in vitro* and *ex vivo* toxicity assessment. The work on plastic oligomers was embedded into the overall context of food risk assessment and opportunities for scientific networking and collaboration. Results of the fellow's project will become part of scientific papers to be published in a peer-reviewed scientific journal.

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Abbreviations

AA	adipic acid
BIOMIC	Centre for BIOanalysis and OMICs
DEG	diethylene glycol
EG	ethylene glycol
EU-FORA	European Union Food Risk Assessment fellowship programme
FCM	food contact materials
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HILIC	hydrophilic interaction liquid chromatography
IARC	International Agency for Research on Cancer
IAS	intentionally added substances
NIAS	non-intentionally added substances
PA	phthalic acid
PBT	polybutylene terephthalate
PET	polyethylene terephthalate
PS	polystyrene
rPET	recycled polyethylene terephthalate
RPLC	reversed-phase liquid chromatography
TPA	terephthalic acid
UHPLC-TOF–MS/MS	ultra-high performance liquid chromatography-time-of-flight tandem mass spectrometry
vPET	virgin polyethylene terephthalate

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Cumulative risk assessment with pesticides in the framework of MRL setting

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Abstract

This abstract presents a report on the proposed work programme, focus on cumulative risk assessment (CRA) for chemical risks, specifically pesticide residues in food. While not a scientific publication, this technical report aims to provide insights without including the fellow's data to avoid publication restrictions. This report focuses on addressing the question concerning the trigger value to perform a prospective CRA in case of a new maximum residue level (MRL) setting. The 1,000 margin of exposure (MOE) threshold value was tested and compared to preliminary ANSES results. Alternative thresholds were calculated and explored. The EU-FORA fellow selected two cumulative assessment groups (CAGs) related to acute craniofacial alterations and chronic thyroid effects. The fellow performed exposure assessments, integrating effects data, French monitoring data, processing factors, agricultural uses, MRLs and extrapolations into Monte Carlo risk assessment (MCRA). Retrospective cumulative exposures using MCRA were conducted for children, adults and a vulnerable group of childbearing women based on the French survey INCA3, identifying background levels at P99.9. The fellow also performed prospective assessments with MCRA, analysing results at P99.9 to evaluate the adequacy of the 1,000 MOE threshold. Alternative thresholds are discussed and proposed.

Keywords: cumulative risk assessment, pesticide residues, EU Commission working group trigger value, Monte Carlo risk assessment (MCRA), cumulative assessment groups (CAGs), retrospective and prospective cumulative risk assessment, MRL setting

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Summary

The EU-FORA programme offers motivated candidates to increase their knowledge and hands-on experience in food safety risk assessment. The programme's principal focus is on chemical and microbiological risk assessment. This report will focus on the proposed work programme as the fellow took part in cumulative risk assessment (CRA) work focusing on chemical risks in food, and in particular on pesticide residues in and on food.

For the implementation of CRA in the regulatory context, France represented by the French ministry in charge of agriculture and food, in cooperation with ANSES actively participates to the working group set up at EU Commission level. This group, coordinated by EU Commission, is preparing a note for risk managers to implement CRA in the evaluation process. The input of risk assessors is requested to decide on the parameters to be taken into account and also how to interpret the results obtained with Monte Carlo risk assessment (MCRA).

The harmonised European methodology for retrospective CRA among all Member States was taken note by the European Commission in September 2018 (SANTE-2015-10216 rev. 7).

European experts of the working group discussed the scenarios for CRA in the framework of maximum residue level (MRL) setting (prospective scenarios). As ANSES risk assessors are involved in both risk assessment for setting MRLs (Article 10, Article 12, SCOPAFF, CCPR) and CRA with MCRA, their participation in the working group is valuable for the development and implementation of the CRA methodology together with the participants of the other member states, EFSA and the European Commission.

In 2021, case studies were carried out by ANSES with residue trials in order to perform both chronic and acute prospective cumulative exposure assessments. Several cases with an increase of MRL for different commodities (plant or animal) on several active substance from a specific cumulative assessment group (CAG) has been conducted.

Experts of the EU working group were also asked to propose a threshold that would be used in order to identify the cases where a modification of an existing MRL or the setting of a new MRL will trigger the need to perform a prospective CRA.

This report will focus on this question. The threshold of 1,000 margin of exposure (MOE) was tested in the framework of the EU-FORA programme and another threshold could be suggested.

In order to achieve this objective, one CAG with acute effects on craniofacial alterations and one CAG with chronic effects on thyroid were selected. Data for exposure assessment (effects data, French monitoring data from 3 years, processing factors, agricultural uses, MRLs, extrapolations, etc.) were conducted by the fellow for implementation into MCRA. Retrospective cumulative exposures based on the French survey INCA3 were used for children, adults and a vulnerable group within MCRA. These allowed to identify the background levels at P99.9.

The fellow performed prospective assessments with MCRA Software. He analysed results at P99.9 in order to conclude whether the threshold of 1,000 MOE is an adequate trigger. Other trigger values were tested and discussed.

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1. Introduction

A pesticide is defined as a 'product used to prevent, destroy, or control harmful organisms (pests) or diseases, or to protect plants or plant products during their production, storage, and transport' (European Commission, 2009). The focus of this report was particularly on active substances (AS) used in the composition of plant protection products (PPPs).

The evaluation of PPPs and the active substances present in these PPPs has been regulated in the EU since 1993 by European Directive 91/414/EEC. In June 2011, this directive was updated with the entry into force of Regulation (EC) No 1107/2009. This regulation harmonised the approval procedures for active substances and the placing on the market of preparations among the Member States of the European Union (European Commission, 2009). Regulation (EC) No 396/2005 addresses the maximum residue limits (MRLs) for pesticides present in or on foodstuffs and feed of plant and animal origin (European Commission, 2005). MRLs are defined as 'the maximum legally permitted concentrations of pesticide residues in or on food intended for human consumption'. Their values reflect the authorised agricultural uses or good agricultural practices (GAPs) for PPPs in Europe, while adhering to the ALARA principle (As Low As Reasonably Achievable). Generally, a GAP is defined by the treated crop, the AS application rate, the number of applications, the minimum preharvest interval, and the growth stage. GAPs are production and post-production processes aimed at achieving 'safe and healthy agricultural products while considering economic, social, and environmental sustainability'.

According to Article 3 of Regulation (EC) No 1107/2009, a pesticide residue consists of 'one or more substances present in or on plants or animal products, drinking water, or elsewhere in the environment. It is the remaining result of the use of a plant protection product, including its metabolites and degradation or reaction products'.

Article 4 of Reg. 1107/2009 stipulates that for the approval of active substances 'they shall not have harmful effects on human health, including that of vulnerable groups or animal health, taking into account known cumulative and synergistic effects where the scientific methods developed by the authority to assess such effects are available'.

It was agreed that as soon as the methodology was developed by EFSA, cumulative and synergistic effects should be taken into account when setting MRLs for pesticide residues: Reg. 396/2005 states in Art.14 (2) (b) requirements, 'that for decisions on applications concerning MRLs, account shall be taken of the possible presence of pesticides residues arising from sources other than current plant protection uses of active substances and their known cumulative and synergistic effects when the methods to assess such effects are available'.

For the Retrospective assessment (= post-marketing), based on monitoring data, a consensus for a Tier I and Tier II retrospective approach was taken note during SCOPAFF meeting in September 2018. For the prospective assessment (= pre-marketing, e.g. application for MRLs), questions remained open. When the exposure of the focal substance in the focal commodity accounts for more than 10% of the acceptable daily intake/acute reference dose (ADI/ARfD) or less than 1,000 margin of exposure (MOE), should prospective cumulative risk assessment (CRA) be carried out?

1.1. Background and terms of reference as provided by the requestor

The fellow was already trained for pesticide risk assessment and coordinated European control plans, which is a prerequisite to conduct CRA. For further involvement in cumulative risk assessment, ANSES has already put in place training sessions. The fellow participated in different formations including theoretical presentations, reading and explanation of EFSA reference documents on the topic and practicing on MCRA supervised by experienced risk assessors. A training dealing with uncertainty analysis in accordance with EFSA guidance on EKE (Expert Knowledge Elicitation) was given. These sessions span over the whole period of the fellowship program.

A task of this program was to organize primary and secondary data for exposure assessment with MCRA in accordance with EFSA (EFSA 2020, EFSA 2022). Including individual data of the Third French Individual and National Food Consumption Survey (INCA3) into MCRA. Two databases of the selected acute and chronic toxicological effects (including lists and codes of active substances, NOAELs, Relative Potency Factors). Additionally, French monitoring occurrence data from 3 consecutive years in accordance with the list of active substances included in the CAGs (acute and chronic) were selected for this program. Processing factors from the available European database on processing factors (Scholz et al., 2018, update, Zincke F. 2022), MRLs, authorized used, extrapolation rules, variability factors.

The fellow performed retrospective cumulative exposure assessments with MCRA: for both acute and chronic exposures, Tier II scenario for whole french population or childbearing women depending on the population of interest for the CAG. Bootstrap was used in order to address the sampling uncertainty.

The fellow had to choose criteria and selected case studies in EFSA Reasoned Opinions in accordance with these criteria. Case studies include modification of MRLs on vegetable commodities only. Indeed, in order to calculate prospective cumulative exposure, MCRA uses results of Magnitude of Residue (MoR) trials. This kind of data is only available for crops. The deterministic acute or chronic consumer exposure was calculated with MCRA and the estimated MOE was compared to the threshold of 1,000.

Additionally, for all case studies, acute or chronic prospective cumulative exposure assessments were performed with MCRA using bootstrapping. The GAP scenario as mentioned in RIVM Report 2021 (RIVM, 2021), including the use frequency was used. Calculations were performed for several modifications of MRLs (fictive and real case studies).

The retrospective (= background) and prospective cumulative exposures at P99.9 were considered, the MOET were calculated and compared together as well as with the deterministic calculations.

1.2. Interpretation of the Terms of Reference

Deterministic Approach: Exposure assessment in regulatory dossiers can be conducted using either a deterministic or probabilistic approach. The deterministic approach is used when there is limited data, employing fixed values for statistical analysis to calculate an average or percentile. In contrast, the probabilistic approach considers variations in food consumption and contamination data, utilising a distribution of exposure based on a large data set. Consumer risk assessment in regulatory dossiers is typically performed deterministically. The EFSA's PRIMo model is widely employed at the European level for consumer risk assessment. It estimates short-term and long-term exposures to pesticide residues, comparing them with toxicological reference values (TRVs) to identify potential risks to consumer health. TRVs, established by international bodies, such as WHO, EFSA or national institutions, differ based on exposure duration and route. The PRIMo model incorporates algorithms for calculating dietary exposure to pesticide residues, which vary depending on whether assessing acute or chronic risk.

Cumulative Risk Assessment: The European approach to CRA of pesticide residues in food is guided by Regulation (EC) 396/2005 and Regulation (EC) 1107/2009. It requires consideration of residues from sources other than current pesticide uses and their cumulative and synergistic effects, provided suitable assessment methods exist. Three methods were outlined by the EFSA's Pesticides Peer Review Panel to combine the toxicity of multiple active substances: addition of responses, addition of doses and interaction. However, the addition of responses is deemed irrelevant for pesticide residues, and synergistic effects are considered highly unlikely due to the low levels of consumer exposure. Thus, the EFSA focuses on the additivity of effects (addition of doses) when assessing pesticide residues in food.

In 2009, EFSA published an opinion on the cumulative risk of triazole pesticides, presenting a methodology that involves the creation of a Cumulative Assessment Group (CAG), a tiered approach for hazard characterisation and exposure assessment, and the calculation of the Hazard Index (HI) using deterministic and probabilistic methodologies. This exercise highlighted the need for a tiered approach in CRA, but questions remain about the level of protection provided.

In 2012, EFSA released a guide on probabilistic approaches for modelling dietary exposure to pesticide residues, emphasising the use of tiered approaches with cautious assumptions to estimate exposure (EFSA, 2012).

Cumulative Assessment Groups: In 2013, EFSA published a scientific opinion on the identification of pesticide active substances (ASs) to be included in CAGs based on their toxicological profiles (EFSA 2013a). From 2022, EFSA will develop new CAGs for system/organs that require a refined assessment of cumulative risks (Biesebeek J, Sam M and Sprong R, 2021).

Monte Carlo Risk Assessment: The ACROPOLIS project, coordinated by the RIVM (Klaveren JDV, 2015), aimed to develop a framework for cumulative and aggregate risk assessment of pesticides. As part of this project, the MCRA (Monte-Carlo Risk Assessment) software was developed and validated to assess cumulative exposure to active substances within CAGs, following EFSA guidance (RIVM, 2019a,b). The MCRA software applies the concept of dose additivity through Relative Potency Factors (RPFs), which originated from Toxic Equivalent Factors (TEFs) used to assign toxicological values to mixtures of chemically similar compounds. The MCRA software provides probabilistic assessment for both acute and chronic cumulative exposures using various models and algorithms.

2. Data and methodologies

2.1. Data

To conduct the probabilistic assessment of cumulative risk, a substantial amount of data needs to be imported into MCRA. This data importation is performed through completed databases in Microsoft Excel and Access.

The fellow used R-software to create tables according to Standard Sample Description (EFSA 2013b) derived from Monitoring Data sourced from the Nations Surveillance and Control Plans (PSPC), Agricultural use data, Consumption data (INCA3), MRL history of the Pesticide Database. Tables for MCRA had to be established for CAG Database, Effects Database compiling the RPFs, which highlight the relative toxicity of the active substances present in the acute and chronic CAGs, based on the toxicity of a reference substance. Tables for the Variability Factors, Extrapolations, Processing Factors, Substance conversion from measured active substances and residue definitions as well as a food translation table for MCRA had to be created. For the prospective CRA tables including values from supervised residue trials (fictive and from current Art.10 MRL modifications) where uploaded to the MCRA software. Several Settings for the MCRA tool had to be learned and understood in alignment of the presentations from RIVM as well as the already gained knowledge from ANSES experts.

2.2. Methodologies

Cumulative Exposure – acute: The assessment of acute cumulative exposure is conducted using the 'person-day' approach in the MCRA software. This involves randomly selecting an individual and a consumption day from the INCA 3 food consumption diary. For each food item consumed on that day, a sample is randomly selected from the concentration database, taking into account all active substances measured in that sample. The concentrations of active substances are multiplied by the consumption of the food item and weighted by the RPF associated with each active substance. The process is repeated 100,000 times to obtain a distribution curve of the relative cumulative exposure.

Cumulative Exposure – chronic: For chronic cumulative exposure assessment, the MCRA software utilises the Observed Individual Means (OIM) model, which calculates the average exposure for each individual by multiplying their average consumption from the INCA3 food consumption diary with estimated average concentrations of active substances in the consumed food items.

The software also allows for an uncertainty analysis using the bootstrap method, which performs 100 resampling iterations to quantify the sampling uncertainty around the average exposure. This generates a 95% confidence interval, indicating the range within the true exposure value is likely to fall.

The margin of exposure total (MOET) is the chosen method for characterising cumulative risk associated with pesticide residue ingestion in Europe. It is calculated by dividing the no observed adverse effect level (NOAEL) of the substance of reference of the CAG by the cumulative exposure estimate. The MCRA software provides relative cumulative exposure values for different percentiles, with the 99.9th percentile being of particular interest for probabilistic assessment. An MOET value above 100 indicates an acceptable risk to consumer health, while a value below 100 suggests an unacceptable risk.

Retrospective CRA: In retrospective evaluation of cumulative risk, the focus is on examining cumulative exposure using surveillance data over a specific time period. It reflects the level of population exposure to pesticide residues after obtaining authorisation for their use. This is often referred to as background exposure.

Retrospective assessments should be repeated regularly to account for changes in exposure patterns and potential updates to CAGs. It is proposed to repeat retrospective CRAs every 3 years, which aligns with the 3-year cycle of the EU Multi-Annual Control Programme (MACP).

Prospective CRA: The methodology for prospective CRA focuses on the modification of MRLs and considers the existing background exposure to pesticide residues as well as the exposure associated with the potential authorisation of a new use. The MCRA software is used to combine data from residue trials and surveillance plans in five different scenarios. These scenarios involve replacing or appending concentration data, using concentration limit values, or removing measurements for specific active substance/focal commodity pairs. The European Commission and EFSA prioritise the harmonisation of this methodology across EU Member States.

The fellows work programme objectives include understanding the MCRA software, defining a threshold for prospective analysis, assessing the impact of RPF and/or high and low consumed food, and evaluating the cumulative risk resulting from modifications of MRLs. By achieving these objectives, the results aim to contribute to the development of a harmonised approach for CRA in the MRL setting process and support the safety of agricultural practices in the European Union.

3. Assessment

Report and interpretation of the results from MCRA are available. This technical report aims to provide insights without including the fellow's data to avoid publication restrictions.

Several questions were raised, including the relevance of the 1,000 MOE trigger value and the SCORE value proposed by ANSES in identifying cases requiring prospective CRA. Additionally, the limitations of using the MCRA software were discussed. The background contribution is not a reliable criterion for predicting the impact of MRL increases on background noise, but factors such as residue level, RPF, quantity consumed, and consumption frequency play a role. The report concludes by highlighting that the 1,000 MOE threshold and SCORE values are not robust enough, and additional thresholds need to be considered. The results from this work may impact the proposed thresholds, and further research is needed to validate the findings to establish more robust criteria for prospective CRA.

Questions asked:

- Is the 1,000 MOE trigger value a relevant threshold that will identify cases of MRL increases requiring prospective cumulative risk assessment?
- Is the SCORE value proposed by ANSES a relevant threshold that will identify cases of MRL increases requiring prospective cumulative risk assessment? It uses available data *a priori* and of the same nature as those used by MCRA, namely the new MRL, the RPF of the focal active substance, consumption of the focal commodity, and the use frequency.
- If not any of this trigger values is robust, can another threshold be proposed?

The fellow faced limitations associated with using the MCRA software corresponding to unspecific Residue Definition, FoodEx2 Code, Parameter Settings for Multiplication Factor according to the EKE for specific CAGs, double reporting of residue trials according to Occurrence Data/Monitoring Data from the Laboratories.

4. Conclusion

CRA is mandated by Article 14 of Regulation (EC) No 396/2005 on MRLs of pesticides. The methodology for assessing cumulative risks has been under development since 2008. Currently, consumer risk assessment for new MRL setting is conducted on a critical NOAEL of an active substance, conducted on a substance by substance basis using PRIMO.

The methodology for creating CAGs has been validated since 2013. CAGs have been formed by EFSA Expert Groups on Toxicology to be used for the Exposure Experts to use specific NOAELs for each active substance included in the CAG to derive RPFs (that put the toxicology of an active substance included in the CAG in relation to the one of reference within the CAG).

The European Commission, with the agreement of Member States and EFSA, also endorsed the characterisation of cumulative risk related to pesticide residues through the calculation of the cumulative exposure margin (MOET) at the 99.9th percentile, with a threshold of 100. The second part, which involves prospective assessment, is still under development and is a priority for the European Commission.

Retrospective assessment relies on 3-year surveillance data to evaluate past cumulative exposure of consumers associated with authorised pesticide uses.

Prospective assessment will use supervised residue trial data to estimate future cumulative exposure of consumers linked to new MRL setting. A use frequency that can be calculated from the information on monitoring data can be used in the prospective scenario as a way to estimate the extent of the future agricultural use.

Probabilistic assessment of cumulative risk requires substantial data and time. Therefore, it is crucial to identify in advance which data are truly necessary before launching a probabilistic prospective assessment. The fellow's work aimed to address the adequacy of two thresholds, the 1,000 MOE agreed by EFSA, COM and Member States and a SCORE derived and proposed by ANSES.

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Abbreviations

ADI	acceptable daily intake
ALARA	As Low As Reasonably Achievable
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
ARfD	acute reference dose
AS	active substance
CAG	cumulative assessment group
CCPR	CODEX Committee on pesticide residues
CRA	cumulative risk assessment
EKE	expert knowledge elicitation
EU-FORA	European Food Risk Assessment Fellowship Programme
FAO	Food and Agricultural Organization
GAP	Good Agricultural Practice
HQ	hazard quotient

JMPR	Joint Meeting on Pesticide Residues
LOAEL	lowest observed adverse effect level
MCRA	Monte Carlo Risk Assessment
MoA	mechanism/mode of action
MOE	margin of exposure
MOET	combined (total) margin of exposure
MoR	Magnitude of Residue
MRL	maximum residue level
NOAEL	no observed adverse effect level
OIM	Observed Individual Means
PF	processing factor
PPP	plant protection product
PRIMo	Pesticide Residue Intake Model
PSPC	Plan de Surveillance et Plan de Controle
RIVM	Dutch National Institute for Public Health and Environment
RPF	relative potency factor
SCoPAFF	Standing Committee on Plants, Animals, Food and Feed (European Commission)
TRV	toxicological reference value

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Microbiota analysis for risk assessment of xenobiotic exposure and the impact on dysbiosis: identifying potential next-generation probiotics

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Abstract

On-going projects of the team are currently dealing with microbiota, xenobiotics, endocrine-disrupting chemicals (EDCs), obesity, inflammation and probiotics. The combination of diet, lifestyle and the exposure to dietary xenobiotics categorised into microbiota-disrupting chemicals (MDCs) could determine obesogenic-related dysbiosis. This modification of the microbiota diversity impacts on individual health–disease balance, inducing altered phenotypes. Specific, complementary, and combined prevention and treatments are needed to face these altered microbial patterns and the specific misbalances triggered. In this sense, searching for next-generation probiotics (NGP) by microbiota culturing, and focusing on their demonstrated, extensive scope and well-defined functions could contribute to counteracting and repairing the effects of obesogens. Therefore, EU-FORA project contributes to present a perspective through compiling information and key strategies for directed taxa searching and culturing of NGP that could be administered for preventing obesity and endocrine-related dysbiosis by (i) observing the differential abundance of specific microbiota taxa in obesity-related patients and analysing their functional roles, (ii) developing microbiota-directed strategies for culturing these taxa groups, and (iii) design and applying the successful compiled criteria from recent NGP clinical studies. New isolated or cultivable microorganisms from healthy gut microbiota specifically related to xenobiotic obesogens' neutralisation effects might be used as an NGP single strain or in consortia, both presenting functions and the ability to palliate metabolic-related disorders. Identification of holistic approaches for searching and using potential NGP, key aspects, the bias, gaps and proposals of solutions were also considered in this workplan.

Keywords: gut microbiota, obesity, xenobiotics, BPA, next-generation probiotics, food safety

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1. Introduction

The prevalence of obesity and metabolic disorders has emerged as a global public health concern in recent decades ('Obesity and overweight', 2021). Particularly concerning is the escalating worldwide occurrence of childhood overweight and obesity, which has the potential to contribute to metabolic disorders in adulthood (de Onis et al., 2010). Although the primary contributors to obesity are excessive calorie consumption and a sedentary lifestyle, thorough analysis of human data, encompassing the interplay between the microbiome, its functioning and metabolites, have extensively indicated their crucial role in determining obesogenic characteristics (Watanabe et al., 2023). Furthermore, emerging findings indicate that exposure to xenobiotic chemicals capable of interfering with adipogenesis and energy balance may also exert a significant influence. The surge in obesity rates has been correlated with the growth in the production and use of synthetic chemicals, providing evidence that aligns with the hypothesis of the 'environmental obesogen' (Vrijheid et al., 2014).

1.1. Exposome, xenobiotics and endocrine disruptors

It is crucial to give special consideration to the cumulative exposure to xenobiotics, particularly during early life, as they have demonstrated obesogenic effects (Janesick and Blumberg, 2016). One such group of EDCs are bisphenols, which are chemical plasticisers that mimic oestrogen. Bisphenols can be found in the production of packaging materials type polycarbonate plastics, epoxy resins and thermal printing papers (Jalal et al., 2018). Specifically, bisphenol A (BPA) stands out as one of the most extensively studied and controversial EDCs. BPA contamination is prevalent in the environment, including soils, sediments, aquatic environments, and in the form of water, air and dust particles (Louati et al., 2019). Various routes of human exposure to BPA have been identified, including ingestion through the digestive system via food packaging, drinking containers and dental materials; maternofetal transmission; inhalation through the respiratory system; and contact with the skin and eyes through thermal paper used in receipts, contact lenses and feminine hygiene products (Hormann et al., 2014; Chung et al., 2017; Gao and Kannan, 2020; Stoker et al., 2020). The presence of obesogens and other potential harmful compounds, such as microbiota-disrupting chemicals (MDCs), has been confirmed in various human biological samples, including serum, urine, saliva, hair, tissues and blood (Vandenberg et al., 2007). Consequently, there is a growing global interest in removing BPA from the natural environment. Several studies have identified effective biological methods for its removal, involving organisms such as bacteria, fungi, algae and plants. However, the industry's response to the evidence of the impact of dietary exposure to BPA has been to replace it with analogous compounds like bisphenol S (BPS), bisphenol F (BPF) and others. Unfortunately, recent studies have indicated that some of these analogues may be even more detrimental than BPA itself (Thoene et al., 2020).

1.2. Gut microbiota and metabolic disorders

Alterations in the microbiota's composition and abundance can result in the modification or inhibition of crucial bacterial metabolite synthesis, changes in intestinal barrier function and initiation of the inflammatory response (Senchukova, 2023). These changes have been linked to a broad range of diseases, including obesity, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome. A reduction in microbial diversity in the gut has been associated with an increased prevalence of common chronic metabolic disorders, with a lower richness of microbial taxa correlating with a relative increase in adiposity, insulin resistance, inflammation and dyslipidaemia (Le Chatelier et al., 2013).

Since the discovery in 2006 that the microbiota of obese individuals has a heightened ability to extract energy from the diet and that this trait is transmissible (as the transfer of this microbiota with an 'obesogenic' phenotype can induce weight gain in lean mice) (Turnbaugh et al., 2006), subsequent epidemiological studies have revealed differences in the gut microbiota composition between obese and lean individuals. Twin studies conducted at the species level have demonstrated that the abundance of short-chain fatty acid (SCFA)-producing bacteria such as *Eubacterium ventriosum* and *Roseburia intestinalis* is associated with obesity (Tims et al., 2013). Conversely, butyrate-producing bacteria like *Oscillospira* spp. (Gophna et al., 2017) and the methanogenic archaea *Methanobrevibacter smithii* have been linked to thinness (Miller et al., 1982).

1.3. Toximicrobiomics

Human gut microbiota genes encode a wide diversity of enzymes, many of which are uniquely microbial proteins, expanding the repertoire of metabolic reactions that occur within the organism (Koppel et al., 2018). The gut microbiota contributes to various aspects of host pathophysiology, from immunomodulation to drug metabolism. The interactions between environmental factors, diet, pollutants and the gut microbiota are bidirectional. Diet and joint xenobiotics can modify the microbial composition and, in turn, intestinal microorganisms can chemically transform these compounds and therefore alter their activity in the host (Lindell et al., 2022). The microbial community can transform these xenobiotics into new metabolites. However, the effects that these may have on microbial communities and host physiology are not always well-known (Velmurugan et al., 2017). Due to existing limitations in the scientific literature, more research is needed to predict the interaction between gut microbiota-derived metabolites and environmental toxicants (Torres-Sánchez et al., 2023).

1.4. Next-generation probiotics

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), have been empirically selected due to their extensive use in fermented foods for centuries and their safety history of use (Gibson et al., 2017). Next-generation probiotics (NGP) align with the conventional definition of a probiotic. However, in this context, we primarily refer to microorganisms that have not been previously employed to promote health and are more likely to be administered under a health or beneficial substance regulatory framework (EFSA NDA Panel, 2016). NPGs also fit comfortably within the definition of a Live Biotherapeutic Product (LBP) provided by the US Food and Drug Administration (FDA), which states that an LBP is a biological product that contains live organisms, such as bacteria, is applicable to the prevention, treatment or cure of human diseases or conditions, and is not classified as a vaccine. The NGP will allow to increase the microbial genera available to verify their beneficial effects (O'Toole et al., 2017). Moreover, gut microbiota could be a potential source for the search of NGP neutralising xenobiotics and able to modulate gut dysbiosis (López-Moreno et al., 2021a).

2. Description of work programme

2.1. Aims

Interaction among distinct scientific disciplines as microbiology, nutrition, toxicology, analytical chemistry, food safety and personalised medicine are needed to analyse factors and substances that affect health and human microbiota eubiosis/dysbiosis. The main objective is to harmonise and exchange methodologies that could enlarge and enrich European food microbiological risk assessment practice and specifically microbiota and probiotics assessments.

- **Objective 1** – **Sending Institution.** To learn main available methods and omics technologies for gut microbiota analysis (composition/activity patterns) while exposed to different level of diet hazardous substances (e.g. BPA and analogues).
- **Objective 2** – **Sending Institution.** To obtain upmost information about human microbiota variability and dysbiosis associated and/or putatively caused by diet hazardous substances exposure and consumption.
- **Objective 3** – **Sending/Hosting Institution.** To reveal candidate microbiota-based strategies for guaranteeing strain benefits and to perform safety assessments.
- **Objective 4** – **Hosting Institution.** To characterise the phenotypes for the beneficial microbes and their potential as NGP to be transfer to food chain.
- **Objective 5** – **Sending/Hosting Institution**. To transfer knowledge and extend the international networking on microbiome, probiotics and risk assessment.

2.2. Methodologies

2.2.1. Omics characterisation of gut microbiota

A total of 106 microbiota samples from a panel of children enrolled in the OBEMIRISK study (Aguilera et al., 2022) were selected. The anthropometric classification was performed according to

guidelines from the WHO (de Onis et al., 2007) in normal weight, overweight and obese children. The participants did not have any intestinal disorders and had not taken antibiotics within the previous 3 months. All faecal samples were collected using in-house anaerobic kit and then immediately frozen at −20°C and maintained frozen at −80°C until further experimental assays. Study permission was obtained from the Institutional Ethics Committee from the University of Granada.

For the culturomics analysis: A 0.5 g of faecal samples were suspended in Luria–Bertani media and exposed to different concentrations of BPA (0.5, 10, 20, 50 and 100 ppm) in anaerobic conditions through the Anaerocult® system (Merck, Darmstadt, Germany), according to previous primary searching and screening studies for obtaining microbial BPA-biodegrader species (López-Moreno et al., 2021b). A total of 5 culture media and 25 conditions were used in this study. Genomic DNA from each pure isolated culture was extracted using DNeasy columns (Qiagen®, Hilden, Germany), amplified by polymerase chain reaction (PCR) using the universal primers for 16S rRNA gene and sequenced by Sanger technique. Detailed protocol is described in Figure 1.

For the 16S rRNA analysis: DNA extraction from stools was performed using the PowerSoil DNA Isolation Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions. The V4 hypervariable region of the 16S rRNA gene was amplified in a two-step process, first using the 515F and 806R universal primers, and second using the specific Illumina multiplexing sequencing and index primers. The library was prepared by pooling equimolar ratios of amplicons and sequenced using an Illumina MiSeq platform. Amplification, library preparation and sequencing were performed at RTL Genomics (Lubbock, TX).

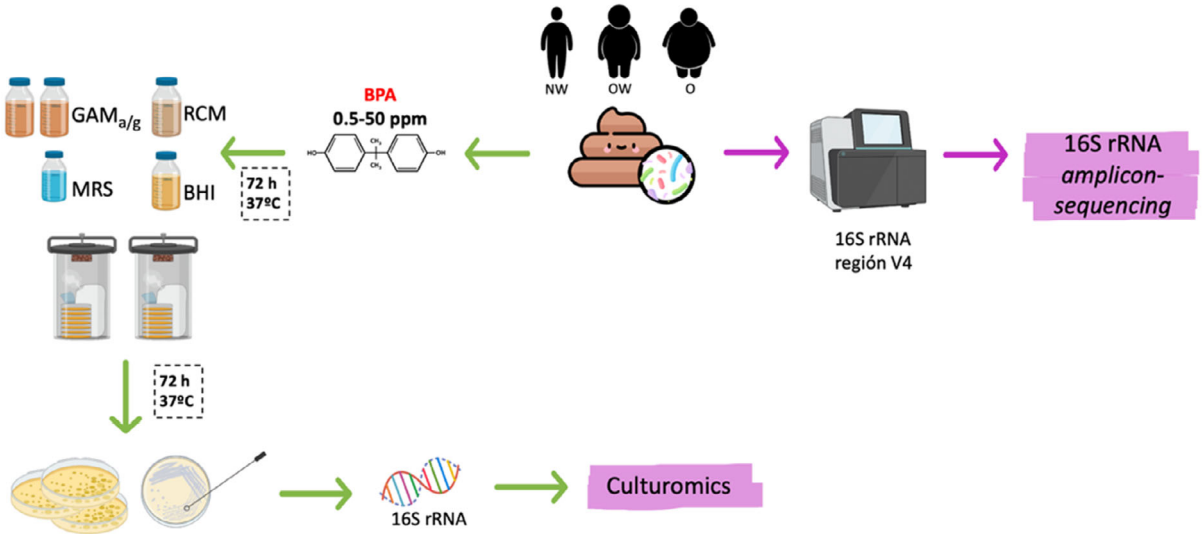

Figure 1: Detailed protocol for the Omics characterisation of gut microbiota of the study population

2.2.2. Characterisation of potential NGP

To characterise the potential beneficial microorganisms as NGP, we evaluated the strains' resistance to the gastrointestinal conditions, antibiotic resistant, SCFA production, the metabolic profile of carbohydrate fermentation and anti-inflammatory assays using HT-29 cell line.

In vitro assays were performed to test the resistance to different pH (pH3 and pH6) and 0.3% bile salts, mimicking the digestive tract. Growth curves with media supplemented with 0.3% and 1% bile acids (Oxoid, ThermoFisher) were performed by measuring at OD 600 nm. Moreover, resistance to pH and bile acid salts shock were tested.

Determination of antibiotic resistant was performed through testing the minimum inhibitory concentration (MIC) following the EFSA guidelines for testing antimicrobial susceptibility (EFSA FEEDAP Panel, 2012) according to EUCAST clinical breakpoint tables and National Committee for Clinical Laboratory Standards (CLSI) criteria (Humphries et al., 2018).

SCFA analysis was performed using gas liquid chromatography (Nelson 1020, Perkin-Elmer, St Quentin en Yvelines, France) as previously described (Lan et al., 2008).

The metabolic profile of carbohydrate fermentation was determinate using API50 CHB/E Medium (BioMérieux™, Marcy-l'Étoile, France) identification system according to the manufacturer's instructions.

Anti-inflammatory assays were carried out following the procedure described by (Kechaou et al., 2013). Human colon adenocarcinoma cell line HT-29 from the American Type Culture Collection (ATCC; Sigma) was seeded in 24-well culture plates in DMEM at 37°C in a 5% CO_2 until 80% confluence was reached. The co-culture day, HT-29 cells were co-incubated with bacteria at a multiplicity of infection (MOI) of 40, stimulated simultaneously with human TNF-α (5 ng/mL; Peprotech, NJ) for 6 h at 37°C in 10% CO_2. After co-incubation, cell supernatants were collected and stocked at −80°C until further analysis of interleukin-8 (IL-8) concentrations by the Human IL-8 ELISA MAX Standard Set (BioLegend™, San Diego, CA, USA) according to the manufacturer's instructions.

2.2.3. Inflammatory assessment of BPA and NGP

One hundred and fifty-four specific pathogen-free (SPF) male C57BL/6 mice (6–8 weeks) (Janvier, Le Genest Saint Isle, France) were housed in cages of 5 under temperature-controlled (20 ± 2°C) environment and a 12-h light/dark cycle with *ad libitum* access to food and water at the animal care facilities of the *Institut national de recherche pour l'agriculture, l'alimentation et l'environement* (IERP, INRAE, Jouy-en-Josas, France). All experiments were performed in accordance with European Community rules for animal care and were approved by the relevant local committee (*Comethea*; protocol number 16744-201807061805486 v2). After a 1-week acclimation, treatments were administered daily for 19 days by intragastric administration. Mice were daily treated orally with 50 μg/kg body weight (bw) per day of BPA or vehicle alone (corn oil) as the control group for 19 days. It was set that dose of BPA since the Environmental Protection Agency (US EPA) had initially established a tolerable daily BPA intake (TDI) of 50 μg/kg bw per day (US EPA, O., 1988), even knowing that EFSA has currently established at 0.2 ng/kg bw per day (EFSA CEP Panel, 2023). Double oral gavage was administered 14 days before dinitrobenzene sulfonic acid (DNBS) injection with BPA and bacteria treatments. Bacterial treatments constituted of 10^9 CFU/mL of *Bacillus* sp. AM1 and *Paeniclostridium* sp. in PBS or PBS alone for control group. The intrarectal injection of DNBS was performed at Day 19 according to Martin et al. with small modifications (Martín et al., 2014).

We assessed the effects of BPA (Figure 2) and bacterial treatments (Figure 3) on DNBS-induced colitis in mice by measuring macroscopic and microscopic scores, myeloperoxidase (MPO) activity levels, percentages of immune cell populations present in the spleen and in the mesenteric lymphoid nodes (MLNs), levels of a panel of 13 pre-selected cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α and GM-CSF) determined with LEGENDplex™ mouse inflammation panel (Biolegend™).

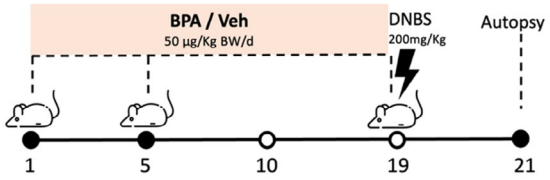

Figure 2: DNBS-induced colitis in mice treated orally with BPA to assess the immunological effects of BPA

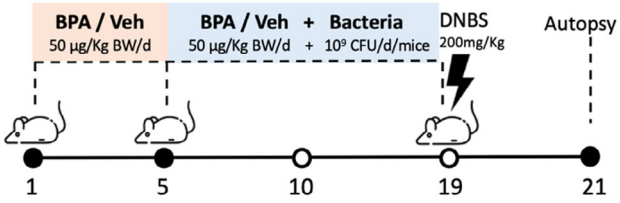

Figure 3: DNBS-induced colitis in mice treated orally with BPA and selected potential NGP to assess the potential anti-inflammatory properties

3. Outcomes

The gut microbiota analysis and characterisation revealed the impact of cultured BPA-tolerant genera on the gut microbial ecology, identifying potential indicators that can trace the effects of BPA

exposure on the composition and dynamics of the microbiota. Additionally, the study explored whether these effects could be associated with obesogenic outcomes. By combining culturomics and metagenomics data analysis, specific taxa affecting the diversity indices of the gut microbial samples were identified. The findings support the hypothesis that certain BPA-associated microbiota drivers, either individually or in consortia, have the capability to establish patterns of lower or higher diversity, thereby could define obesogenic or anti-obesogenic phenotypes, respectively.

The *in vivo* assessment of BPA and selected potential NGP revealed, first an increase in pro-inflammatory biomarkers in mice exposed to BPA compared to the control group, indicating an altered immunological response in BPA-treated mice. These findings suggest a close association between BPA exposure and the activation of innate immune responses. Second, our research also uncovered that certain BPA-tolerant bacteria from the *Paeniclostridium* and *Bacillus* genera possessed potent anti-inflammatory properties. Furthermore, when mice were treated with these BPA-tolerant bacteria, they exhibited limited colon damage, reduced MCP-1 and LCN-2 levels, as well as decreased proinflammatory cytokines (IL-1β and IL-6). These results indicate the potential of these bacteria to counteract the adverse immunological effects caused by BPA exposure. Further investigations are required to fully understand the mechanism by which BPA induces its immunopathological effects and explore potential approaches to mitigate these effects mediated by microbiota.

4. Conclusion

Combined culturing and sequencing data analysis allowed to identify specific taxa influencing the diversity indices of the gut microbial community samples. By integrating complementary omics data, we have enriched our understanding and provided more comprehensive scientific evidence of potential indicators for xenobiotic obesogenicity. This study introduces, for the first time, potential microbiota biomarkers associated with xenobiotic exposure and obesogenic phenotypes. Moreover, the results of this study introduce a promising avenue of research, where the pathophysiology of inflammation exacerbated by BPA could potentially be modified by tolerant bacterial species with anti-inflammatory properties. These bacteria have demonstrated the ability to mitigate the harmful immunological effects triggered by the xenobiotic, offering a potential means to alleviate the adverse impact of BPA on inflammation and colitis.

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Abbreviations

BPS	bisphenol S
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
DNBS	dinitrobenzene sulfonic acid
EDC	endocrine-disrupting chemicals
FAO	Food and Agriculture Organization of the United Nations
FDA	US Food and Drug Administration
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
LBP	Live Biotherapeutic Product
MDC	microbiota-disrupting chemicals
MLNs	mesenteric lymphoid nodes
MOI	multiplicity of infection
MPO	myeloperoxidase
NAFLD	nonalcoholic fatty liver disease
NGP	next-generation Probiotics
PCR	polymerase chain reaction
SCFA	short-chain fatty acid
SPF	specific pathogen-free

Appendix A – Additional relevant activities and learning opportunities completed by the fellow

The following additional relevant activities and learning opportunities were completed by the fellow:

- Metagenomic analysis of the gut microbiota in children in relation to bisphenol A exposure. Ruiz-Rodríguez A; Cerk K; **López-Moreno A**; Rivas A; Monteoliva-Sánchez M; Suárez A; Aguilera M. XIX Reunión Taxon, 2022, Mallorca, Spain. Oral communication.
- Study of the gut microbiota resistant to endocrine disruptors in children with obesity and potential use as Next-generation probiotics. **López-Moreno A**; Moreno MA; Martín R; Monteoliva-Sánchez M; Aguilera M. XIX Taxon, 2022, Mallorca, Spain. Oral communication.
- Gut microbiota dynamics influenced by bisphenol A tolerant taxa in childhood obesity using culturomics and amplicon-sequencing. **López-Moreno A**; Cerk K; Aguilera M; Ruiz-Rodríguez A. XXIX Congreso Sociedad Española de Microbiología, 2023, Burgos, Spain. **Poster communication - Best poster award.** FEMS Meeting Attendance Grants.
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Threat or treat: Exposure assessment and risk characterisation of chemical contaminants in soft drinks and chocolate bars in various Polish population age groups

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Abstract

In the frame of the European Food Risk Assessment (EU-FORA) fellowship programme, two studies on chemical contaminants in food matrices were carried out in Warsaw, Poland, at the Department of Food Safety and Chemical Analysis, Institute of Agricultural and Food Biotechnology. The first study addressed health concerns about the dietary exposure to bisphenol A (BPA) contamination due to consumption of soft drink by Polish population. BPA is an organic additive used in the production of epoxy resins and polycarbonate plastics and because of this it is used in the internal coating of cans and in plastic bottle production. Depending on several factors, BPA can migrate from these materials to the soft drink and so, it can be ingested by consumers causing hormonal and reproductive disorders. To estimate the Polish population exposure to BPA, several soft drinks belonging to different brands were purchased from a supermarket in the city of Warsaw and analysed. The result of the analysis highlight that mean BPA exposure in the Polish population exceeds the tolerable daily intake proposed by the EFSA scientific opinion, raising health concerns. On the other hand, the second study, focused on cadmium exposure due to chocolate consumption by Polish population, did not raise any health concern. Cadmium is a heavy metal that naturally occurs in its inorganic form in the environment and its presence in chocolate derives only from the cocoa beans and not from contamination during processing. Its accumulation in the human body can create several adverse effects, including renal dysfunction and failure. To estimate the Polish population exposure to cadmium, several chocolate bars were purchased from a supermarket in the city of Warsaw and analysed. The results of the analysis show that cadmium exposure in the Polish population does not exceed the tolerable weekly intake proposed by the EFSA scientific opinion.

Keywords: bisphenol A, carbonated beverages, cadmium, cocoa, heavy metals, epoxy resin, food contact material

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1. Introduction

The European Food Risk Assessment (EU-FORA) fellowship programme is an initiative that aims to train a professional in the food risk assessment field in order to expand the pool of food safety experts and to build up a strong food risk assessment network all over Europe. Thanks to this European network, it will be possible to support a common risk assessment culture. The enrolled fellow has carried out for a period of 12 months, both remotely and in presence, the programme of activities offered by the hosting institute (competent organisation), according to the learning-by-doing concept. In parallel, this experience was integrated by several specific training modules distributed throughout the year for a total of 7 weeks that trained the fellow on a wide range of risk assessment-related topics.

The purpose of this paper is to report the activities that the fellow performed during the participation in the 6th EU-FORA cycle (2022–2023). The fellow, belonging to the Department for Life Quality Studies (QuVi) of the Alma Mater Studiorum - University of Bologna (Italy), spent 3 months at the Department of Food Safety and Chemical Analysis, Institute of Agricultural and Food Biotechnology – State Research Institute (IAFB) in Warsaw (Poland). During this period of time, the fellow worked on the exposure to different chemical contaminants present in food matrices and on the related risk for the Polish population. The working activities were related to the risk assessment that is a scientifically based process composed by four steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation (European Commission, 2002). In particular, the working activities were focused on the last two steps. Of those, the former one is essential to estimate the probability that a hazard is present in food and how much of that food is consumed by the population (exposure assessment); the latter one defines whether the found exposure is below or above the recommended safety level (risk characterisation).

Chemical contaminants, that is substances that can potentially harm consumers health and for this reason are considered a hazard, are unwanted substances that may be present in food. Their presence in food might be the result of environmental contamination or can be due to various stages of production, processing, transport or storage of foodstuff. The presence of these contaminants may have some implication for the human health causing, for example: metabolic disruption, immune system disruption, neurotoxicity, hormonal disruption, reproductive disruption or cardiotoxicity. Since the contamination level in food as well as the dietary habits of the population are changing over time, it is important to collect up-to-date data on chemical contaminants in order to monitor and to estimate population exposure to these contaminants and the potential related risks. It is important to note that also the tolerable daily intake (TDI), that represents the safe limit of contaminant amounts that can be consumed over a lifetime, can change over time depending on the availability of new data that may change the hazard identification and characterisation. As a consequence, risk characterisation may change as well.

Good quality data together with a good risk communication are essential to have a well-informed risk assessment and risk management, to ensure a good risk analysis and to achieve a high level of protection of human life and health.

2. Work programme description

2.1. Aim

The aim of the fellow work programme was to transfer knowledge and experience in how to define the occurrence of different chemical contaminants in different food matrices in the Polish market and how to estimate the exposure to these contaminants in different population groups. In particular, the fellow had the opportunity to gain hand-on experience and competence in:

- development and validation of an instrumental analytical method;
- solid phase extraction and microwave-assisted mineralisation protocols to recover the investigated analytes;
- spectrometric analysis by means of liquid chromatography–mass spectrometry (LC–MS) and inductively coupled plasma-mass spectrometry (ICP-MS) to detect and quantify the investigated analytes;
- statistical analysis and result interpretation;
- current legal regulation of food safety.

2.2. Activities/methods

To learn and achieve the above goals, the fellow performed all the laboratory activities at the IAFB (Poland), taking part in the working activities of the hosting institute, while data analysis was performed at the QuVi Department (Italy). In this way, the fellow estimated the risk of chemical dietary exposure related to some specific food consumption in different Polish population groups.

Specifically, the fellow's work was focused on:

i) bisphenol A (BPA) exposure in different Polish population groups consuming carbonated soft drinks;
ii) cadmium exposure in different Polish population groups consuming chocolate bars.

2.2.1. Bisphenol A exposure in different Polish population groups consuming carbonated soft drinks

In order to work on this topic, two assessment questions were posed:

'What is the occurrence of BPA in carbonated soft drinks?';
'How high is the dietary exposure of different Polish population groups to BPA?'

Food matrix

Soft drinks are water-based drinks widely consumed by the world population during the day in many occasions, since they provide refreshment, satisfaction, enjoyment and other benefits. These aspects made soft drinks one of the most popular and consumed products in the modern diet. Due to this high consumption rate, it could be interesting to estimate the risk associated to the consumption of this drink category, and in particular, the research was focused on carbonated soft drinks. Several carbonated soft drinks from different brands were purchased from supermarkets in the city of Warsaw, Poland. The sampling included both canned beverages and beverages in plastic bottles.

According to the EFSA's standardised food classification and description system FoodEx2 (catalogue FoodEx2 Matrix hierarchy (MTX) version 12.0), the selected matrices are labelled as 'Soft drinks with minor amounts of fruits or flavours [A0EQN]', 'Cola-type drinks [A03FQ]' and 'Energy drinks [A03GA]'. The first two matrices belong to the macro category 'Soft drinks [A03DZ]' that is defined as 'Group/item including e.g. Soda Pop or Fizzy drink. The group includes any type of Soft drinks. The part consumed/analysed is by default the whole marketed unit or homogenous representative portion'. The third matrix belong to the broad category 'Functional drinks [A03FZ]' that is defined as 'The group includes any type of Functional drinks, such as energy drinks and isotonic drinks. The part consumed/analysed is by default the whole marketed unit or a homogenous representative part'. All the purchased drinks were stored at room temperature according to market storage conditions.

Soft drinks consumption

Due to the huge economic changes that Poland experienced since 1989, the Polish lifestyle and the access to food drastically changed. For this reason, the Polish government needed a precise assessment on the nutritional situation of the Polish population. Hence, in 2000, the Polish Ministry of Health, together with the National Food and Nutrition Institute – Instytut Żywności i Żywienia (IŻŻ) – and with the technical assistance of the Food and Agriculture Organization of the United Nations (FAO), improved the previous nutrition information system. To do that, from September to November 2000, data on food and nutrient intakes were collected and examined with 24-h recall. To help the assessment of the portion size, a picture book of 201 food items, dishes, soft drinks and alcoholic beverages commonly consumed in Poland was used (Szponar et al., 2001; Sekula et al., 2004). The results of this survey are available in the EFSA Comprehensive European Food Consumption Database with the survey code IZZ FAO 2000.

In this risk assessment, the consumption data for the food category 'Soft drinks' (Exposure hierarchy level 3) for different population groups are based on the IZZ-FAO 2000 survey (Szponar et al., 2001; Sekula et al., 2004). The data, taken from the EFSA Comprehensive Food Consumption Database, are shown in Table 1.

Table 1: 'Soft drinks' consumption in the three different population groups considered (Other children, adolescent and adult)

Population group	g/kg bw
Other children – from 36 months up to and including 9 years of age	1.76
Adolescent – from 10 up to and including 17 years of age	1.89
Adult – from 18 up to and including 64 years of age	0.52

bw: body weight.

Bisphenol A and bisphenol derivatives

2,2-Bis(4-hydroxyphenyl)propane (CAS No 80–05-7), known as bisphenol A (BPA), is an organic additive used in the production of epoxy resins and polycarbonate (PC) plastics. Epoxy resins are widely used as coating material in canned beverages because they create a protective layer that separates drinks from the metal foil used to make the cans. PC plastics, on the other hand, are used to produce common plastic bottles. Residual BPA could potentially migrate from the coating material to the beverage. The migration rate depends from different factors such as temperature, pH, contact time, food composition and packaging material (Russo et al., 2019; Vilarinho et al., 2019; Wang et al., 2019). BPA can alter the functioning of endocrine regulation and cause biochemical changes on proteins, hence, the human exposure to BPA could affect hormone levels and fertility, nervous system, immune system and liver metabolism. Because of these effects on the human health and given the likelihood of such migration, the presence of BPA in food is regulated by the European legislation which allows a maximum of 0.05 mg per kg of food (European Commission, 2018). This strict limit pushed can and bottle manufacturing companies to use other alternative chemicals, known as bisphenol F (BPF – CAS No 620-92-8) and bisphenol S (BPS – CAS No 80-09-1). These are compounds structurally similar to BPA, that can in turn alter the functioning of the endocrine regulation similarly to BPA, but that are not regulated by law (Chen et al., 2016). To avoid adverse health effect in the population due to BPA exposure, in the 2023 EFSA's CEP Panel (EFSA, 2023) established a new TDI of 0.0002 μg/kg body weight (bw), replacing the old temporary tolerable daily intake (t-TDI) of 4 μg/kg bw established by EFSA opinion in 2015 (EFSA, 2015).

BPA and bisphenol derivatives occurrence in soft drinks

To assess the occurrence and, consequently, the levels of BPA, BPF and BPS in carbonated soft drinks, an extraction protocol based on the work of Cao et al. (2021) was optimised. To validate the optimised protocol, the recovery for each one of the considered analytes was evaluated using different fortification levels in the sample. Hence, 10 mL of each sample, fortified with dBPA as internal standard, was subjected to ultrasound treatment followed by solid-phase extraction (SPE) using a C18 sorbent cartridge (500 mg, 6 cc). The elution solvent was removed by evaporation under reduced pressure, and the resulting extract was re-dissolved in 2 mL of a 1:4 (v/v) MeOH:H_2O mixture. To analyse the extracted analytes, a method validated during the study was used. For the ultra-performance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) analysis, 2.5 μL of extract were injected into a Vanquish LC system (Thermo Fischer Scientific, Waltham, MA, USA) connected to a Q Exactive Focus Orbitrap mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA). The LC system was composed by autosampler, oven and reverse phase column (Acquity UPLC BEH C18 1.7 μm, 2.1 mm × 100 mm). The analysis was performed using electrospray ionisation (ESI) on negative mode. Hence, the analytes were separated exploiting a gradient elution with a mobile phase consisting of a 9:1 (v/v) H_2O:MeOH mixture with 1 mM of ammonium fluoride (solution A) and of a 8:2 (v/v) MeOH:H_2O mixture with 1 mM of ammonium fluoride (solution B). The gradient elution, with a flow rate of 0.3 mL/min, was composed as follows: 0–3 min, isocratic elution 0% B; 3–12 min, linear gradient from 0% B to 100% B; 12–20 min, isocratic elution 100% B. Each analyte of interest was quantified using a linear regression model.

The left-censored data were handled according to the upper bound (UB) approach that is the most conservative one. Hence, the occurrence was calculated by replacing all the left-censored data with the limit of quantification (LOQ).

BPA exposure

To estimate chronic exposure to BPA for each population group, the BPA P95 UB occurrence determined in the analysed soft drinks, expressed as μg/g of food, was multiplied by the average soft

drink consumption, expressed as g/kg bw per day, of each population group (see below the reported equation).

$$\text{Exposure}\left[\frac{\mu\text{g of BPA}}{\text{kg bw}}\right] = \text{Occurence}\left[\frac{\mu\text{g of BPA}}{\text{g of food}}\right] \times \text{Consumption}\left[\frac{\text{g of food}}{\text{kg bw}}\right]$$

Risk characterisation

To understand if there is health concern about the exposure to BPA through the consumption of soft drinks, the BPA TDI was compared with the chronic dietary exposure values obtained for each of the considered groups. In all the groups, the exposure to BPA exceeds the TDI set by EFSA in 2023, raising health concerns. It has to be noted that the BPA quantity in the soft drinks is below the maximum limit of 0.05 mg per kg of food allowed by the European legislation (European Commission, 2018).

2.2.2. Cadmium exposure in different Polish population groups consuming chocolate bars

To work on this topic, two assessment questions were posed:

'What is the occurrence of cadmium in chocolate bars?';
'How high is the dietary exposure of different Polish population group to cadmium?'

Food matrix

Chocolate is eaten all over the word and its consumption is associated with pleasure and happiness. Poland is one of the European countries with the highest chocolate consumption. For this reason, it could be interesting to estimate the risk associated with the consumption of chocolate bars. Hence, several chocolate bars, produced from different brands and with different percentages of cocoa, were purchased from supermarkets in the city of Warsaw, Poland.

According to the EFSA's standardised food classification and description system FoodEx2 (catalogue MTX version 12.0), the selected matrices are labelled as 'Bitter chocolate [A034G]' and 'Milk chocolate [A034J]' both of them belonging to the broad category 'Chocolate and similar [A0EQD]'. The Bitter chocolate matrix is described as 'The group includes any type of Bitter chocolate, such as Dark chocolate and Plain chocolate, usually containing at least 35% total dry cocoa solids, with no milk or much less than milk chocolate and with no sugar or small amount of sugar. The part consumed/analysed is by default the whole or a portion of it representing the observed heterogeneity'; the Milk chocolate matrix is described as 'The groups includes any type of Milk chocolate, i.e. a sweet chocolate obtained from cocoa products, sugar and milk or milk products. According to EU legislation (Directive 2000/36/EC; European Parliament and European Union Council, 2000) milk chocolate has to contain not less than 14% dry milk solids and not less than 3.5% milk fat. The part consumed/analysed is by default the whole or a portion of it representing the observed heterogeneity'. All the purchased chocolates were stored at room temperature according to market storage conditions.

Chocolate consumption

As for the 'Soft drinks', the consumption data for the two food categories 'Milk chocolate' and 'Bitter chocolate' (Exposure hierarchy level 5) for different population groups are based on the IZZ-FAO 2000 survey (Szponar et al., 2001; Sekula et al., 2004). The data, taken from the EFSA Comprehensive Food Consumption Database, are shown in Table 2.

Table 2: 'Milk chocolate' and the 'Bitter chocolate' consumption in the three different population groups considered (other children, adolescent and adult)

Population group	Exposure hierarchy (L5)	g/kg bw
Other children – from 36 months up to and including 9 years of age	Milk chocolate	0.18
Adolescent – from 10 up to and including 17 years of age	Milk chocolate	0.07
Adult – from 18 up to and including 64 years of age	Milk chocolate	0.02
Other children – from 36 months up to and including 9 years of age	Bitter chocolate	0.06
Adolescent – from 10 up to and including 17 years of age	Bitter chocolate	0.03
Adult – from 18 up to and including 64 years of age	Bitter chocolate	0.01

bw: body weight.

Cadmium

Cadmium (Cd – CAS number 7440-43-9) is a heavy metal that naturally occurs in its inorganic form in the environment. In parallel to the natural sources of Cd, its anthropogenic sources contribute to increase the level of Cd in the environment. Cd does not have any known biological function in humans, it can be absorbed from diet and accumulates in kidneys and liver, with a biological half-life of 10–35 years. Over time, its accumulation can create several health adverse effects including renal dysfunction, renal failure and osteomalacia (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009).

Cadmium in chocolate derives from the cocoa beans and not from processing contamination (Vanderschueren et al., 2021; Bravo et al., 2022). The cocoa tree (*Theobroma cacao* L.) is particularly effective in up taking Cd from soil. Hence, the Cd content in *T. cacao* depends on its presence in soil and on soil pH, which influences Cd availability (Vanderschueren et al., 2021; Bravo et al., 2022; Wade et al., 2022). Because Cd tends to accumulate in cocoa shells rather than in nibs, the processing steps where shells are removed from the nibs is essential to reduce Cd content in cocoa products (Bravo et al., 2022). Since after this step Cd content in chocolate depends only by the percentage, the quality and the origin of cocoa used (Kruszewski et al., 2018; Vanderschueren et al., 2021; Bravo et al., 2022; Wade et al., 2022), the European legislation (European Commission, 2006, 2021) set a maximum level of Cd allowed in different type of chocolate (Table 3).

Table 3: Maximum level of cadmium allowed in chocolate according to European legislation

Foodstuff	Maximum level (μg/g wet weight)
Milk chocolate with < 30% total dry cocoa solids	0.10
Milk chocolate with ≥ 30% total dry cocoa solids Chocolate with < 50% total dry cocoa solids	0.30
Chocolate with ≥ 50% total dry cocoa solids	0.80

bw: body weight.

To protect the population from adverse health effect, in the 2009 the EFSA's CONTAM panel (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009) established a tolerable weekly intake (TWI) of 2.5 μg/kg bw.

Cadmium occurrence in chocolate bars

According to the European standard EN 13805:2014 (European Committee for Standardization, 2014), to determine the occurrence of Cd in chocolate, 0.250 g of sample were weighted and subjected to microwave-assisted mineralisation after adding 5 mL of 67–69% nitric acid, 1 mL of 30% hydrogen peroxide and 200 μL of yttrium solution (10 μg/mL) as an internal standard. The microwave-assisted mineralisation was performed with a power of 1800 W to reach the temperature of 210°C in 20 min and to maintain it constant for other 20 min, for a total mineralisation time of 40 min using an Ethos Up high-performance microwave digestion system (Milestone). Two blank samples were subjected to the mineralisation process, as well. After the mineralisation process, real and blank samples were transferred and recovered into a 25 mL volumetric flask. The final volume was made up to 25 mL with water. In parallel, two standard mixtures were prepared in a final volume of 25 mL. The first solution was prepared by adding 1 mL of 67–69% nitric acid and yttrium to obtain a final concentration of 0.08 μg/mL. The second standard solution was prepared by adding 1 mL of 67–69% nitric acid, yttrium to obtain a final concentration of 0.08 μg/mL and cadmium to obtain a final concentration of 4 ng/mL.

To measure the cadmium level in chocolate, it was decided to use the ICP-MS analysis method, as it is one of the methods that fulfil the performance criteria set by Regulation No 333/2007 (European Commission, 2007). The Cd analysis was performed using an iCAP RQ ICP-MS (Thermo Scientific) according to an in-house method based on the European standard EN 15763:2009 (European Committee for Standardization, 2009). A kinetic energy discrimination (KED) module filled with helium was used and Cd quantification was performed with external standard calibration curve. Five readings for each sample were performed. The LOQ of this analysis method is 0.001 μg/g.

No left-censored data were present. The occurrences resulted from the analysis of the two different chocolate groups are reported in Table 4.

Table 4: Median and P95 of Cd occurrence in the two analysed chocolate matrices

Matrix	Median (μg/g wet weight)	P95 (μg/g wet weight)
Milk chocolate	0.018	0.029
Bitter chocolate	0.126	0.149

The cadmium occurrence in each chocolate group resulted to be lower than the maximum level of cadmium allowed in chocolate by the European legislation (European Commission, 2006, 2021). It has to be noted that the 'bitter chocolate' group can be subdivided into 'Chocolate with $<$ 50 % total dry cocoa solids' and 'Chocolate with $\geq$ 50% total dry cocoa solids'. To compare the Cd level in chocolate with the maximum level of Cd allowed by the European legislation, the Cd level of each of these two subcategories was considered separately.

Cadmium exposure

As for BPA, to estimate the chronic exposure to Cd for each population group, Cd P95 occurrence determined in the analysed chocolates, expressed in μg/g of food, was multiplied by the average chocolate consumption, expressed in g/kg bw per day, of each population group. As Cd is a contaminant that accumulates in the body, the exposure was multiplied by 7 to have a weekly exposure. See below the reported equation. The weekly exposure values are reported in Table 5.

$$\text{Exposure}\left[\frac{\mu\text{g of Cd}}{\text{kg bw}}\right] = \text{Occurence}\left[\frac{\mu\text{g of Cd}}{\text{g of food}}\right] \times \text{Consumption}\left[\frac{\text{g of food}}{\text{kg bw}}\right] \times 7$$

Table 5: Weekly Cd exposure values for the two chocolate matrices for each of the three different population groups. Occurrence and consumption are also reported.

Population group	Foodstuff	Occurrence (μg/g ww)	Consumption (g/kg bw)	Weekly exposure (μg/kg)
Other children – from 36 months up to and including 9 years of age	Milk chocolate	0.029	0.18	0.037
	Bitter chocolate	0.149	0.06	0.063
Adolescent – from 10 up to and including 17 years of age	Milk chocolate	0.029	0.07	0.014
	Bitter chocolate	0.149	0.03	0.031
Adult – from 18 up to and including 64 years of age	Milk chocolate	0.029	0.02	0.004
	Bitter chocolate	0.149	0.01	0.010

Risk characterisation

To understand if there is health concern about the exposure to Cd through the consumption of milk chocolate and bitter chocolate, the Cd TWI was compared with the chronic dietary exposure values obtained for each of the considered groups. In all the groups, the exposure to Cd is lower than the TWI of 2.5 μg/kg bw set by EFSA in 2009. Hence, the Cd exposure by chocolate consumption does not currently raise any health concern.

3. Conclusion

During the year, the fellow had the opportunity to gain knowledge and experience in the chemical risk assessment field through a theoretical and a practical training. With the bibliographic research, the fellow stepped into the chemical risk assessment dealing with the regulatory part of food safety and understanding the chemical contaminant risk, the contaminant types and their possible negative health effects. During the laboratory work, the fellow gained a practical knowledge on ICP-MS and LC–MS analysis, on developing and validating an analytical method and on sample preparation for heavy metal analysis. All this was possible thanks to the learning-by-doing approach that allowed the fellow to use both the hosting site expertise and the new knowledge acquired during the EU-FORA training modules to deal with practical problems and to perform BPA and Cd risk characterisation in different Polish population groups. The data produced during the internship will be exploited for scientific paper writing, that will contribute to reinforce cooperation between research institutes and to develop and define the fellow's future career.

In addition, the programme allowed the fellow to grow from a professional point of view giving a scientific base and all the necessary tools to understand and critically analyse food-related toxicity issues. All in all, the EU-FORA programme was an important meeting point where to network with international experts and fellows to exchange ideas and build useful scientific connections.

4. Other activities

In addition to the work activities carried out at the hosting site, the opportunity to attend several conferences during this year was important in order to share with the scientific community the collected data and ideas developed in the framework of this project. The main relevant conferences are listed in Table 6.

Table 6: Main conference attended by the fellow during the EU-FORA programme

Type of event	Title	Contribution	Date	Place
Conference	21th National Congress of Italian Society of Toxicology (SITOX)	Poster presentation: Determinazione del livello di contaminazione di bisfenolo A e dei suoi derivati in bibite gassate in lattina presenti sul mercato polacco	From 20 February 2023 to 22 February 2023	Bologna, Italy
Conference	9th EBF Young Scientist Symposium	Poster presentation: BPA exposure in different population groups consuming canned carbonated beverages	From 11 May 2023 to 12 May 2023	Hasselt, Belgium

Disclaimer

To avoid copyright issues, all BPA data were not showed in this technical report as these data are intended to be published in a peer-reviewed journal.

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Abbreviations

BP	bisphenol
BPA	bisphenol A
BPF	bisphenol F
BPS	bisphenol S
bw	body weight
dBPA	deuterated BPA
EU-FORA	European food risk assessment
FAO	Food and Agriculture Organization of the United Nations
IAFB	Institute of Agriculture and Food Biotechnology – State Research Institute of Warsaw
ICP-MS	inductively coupled plasma-mass spectrometry
IŻŻ	Instytut Żywności i Żywienia (National Food and Nutrition Institute)
KED	kinetic energy discrimination
LC	liquid chromatography
LOQ	limit of quantification
MTX	FoodEx2 Matrix hierarchy
MW	microwave
PC	polycarbonate plastics
SPE	solid phase extraction
t-TDI	temporary tolerable daily intake
TDI	tolerable daily intake
TWI	tolerable weekly intake
UB	upper bound
UPLC-HRMS	ultra-performance liquid chromatography high-resolution mass spectrometry

EU-FORA SERIES 6

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Results of multi-actor collaboration in risk analysis: a simplified risk assessment toolkit for rapid detection of emerging risks

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Abstract

The dynamic field of food safety faces continuous challenges, prompting stakeholders to develop collaborative actions for improved food safety systems. As part of these actions, the EU-FORA fellowship programme was dedicated to a multi-actor collaboration addressing risks of the unregulated mycotoxins T-2 and HT-2 toxins in oats. Critical gaps in risk assessment procedures were identified, leading to a joint effort to develop a strategy for rapid data collection and risk assessment, including the development of a risk assessment toolkit comprising of a training manual and two intuitive Microsoft® Excel files. The toolkit enables efficient data collection and processing, facilitating risk assessment calculations and rapid risk detection. Applying the toolkit to assess T-2 and HT-2 toxin risks in Belgian oats revealed minimal concerns, except for children aged 3–9 years, likely due to an overestimation. The toolkit is available on the FoodSafety4EU Platform and will be refined based on user feedback, promoting better risk assessment practices. This approach empowers stakeholders, from professionals to policymakers, fostering collaboration and enhancing food safety practices.

Keywords: T-2 toxin, HT-2 toxin, oats, dietary exposure, data collection, toolkit

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Summary

The field of food safety is constantly evolving, with new challenges and emerging risks posing significant concerns for both public health and the food industry. As part of the EU-FORA programme of EFSA, a work programme was set up aiming to provide a comprehensive overview of the chemical risk assessment process, focusing on the harmonisation of enforcement practices and addressing unregulated hazards and emerging issues.

During the programme, the fellow was actively involved in the 'Food Safety Operational Lab' (FSOLab), a kind of living lab. This is a dynamic and collaborative environment where various stakeholders within the food safety system work together to diagnose current challenges, generate innovative ideas, implement pilot actions and evaluate the outcomes. This involvement allowed the fellow to contribute as a member of the Lab management team, alongside the project coordinator Dr. Veronica Lattanzio and other dedicated team members.

One of the key areas of focus within the FSOLab was the harmonisation of risk assessment strategies in relation to unregulated (i.e. no regulatory maximum limit) mycotoxins, particularly T-2 and HT-2 toxin. These mycotoxins, which can contaminate various food commodities such as oats, present a significant health risk to consumers. However, the current risk assessment procedures for these toxins suffer from several critical gaps that need to be addressed. Within the work programme, it was decided to focus on two issues: hindered analytical data sharing and the need for rapid risk assessment.

A strategy for rapid data collection and risk assessment was outlined, including the development of a simplified risk assessment toolkit comprising of a training manual and 2 intuitive Microsoft® Excel files. One file helps with rapid data collection and is based on the standard sample description format (SSD2) of EFSA and can be customised for specific contaminants and specific food matrices, while the other file contains spreadsheets to process the data and to perform the risk assessment, applying deterministic calculations.

The toolkit can be used for chemical contaminants and was tested to assess the risk of the sum of T-2 and HT-2 toxin in oats for the Belgian population. No risks were identified, except for the upper bound scenario in children between 3 and 9 years old. However, this is very likely an overestimation.

The toolkit is freely available on the Foodsafety4EU Platform (www.foodsafety4.eu) as a beta version, and will be further optimised based on user feedback.

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1. Introduction

The EU-FORA programme focused on identifying and improving issues in risk assessment using a multi-actor approach. The fellow was actively involved in the 'Food Safety Operational Lab' (FSOLab), which is a social living lab. This is a dynamic and collaborative environment where various stakeholders within the food safety system work together to diagnose current challenges, generate innovative ideas, implement pilot actions and evaluate the outcomes (Hossain et al., 2019).

In preparatory work executed before the start of the work programme, two multi-actor workshops were organised to bring together stakeholders from science, policy and society and discuss current constraints and challenges in risk assessment. Emerging and/or unregulated mycotoxins were identified as a main issue, and the group decided to zoom in on T-2 and HT-2 toxins in oats, as their occurrence is high in West, East and Northern Europe and oats are prone to contamination. The perceived challenges in risk assessment among stakeholders were: hindered data sharing, unclear communication between actors, complex risk assessment procedures and insufficient knowledge, changing dietary patterns and climate, unclear mycotoxin mitigation strategies, and lack of analytical capacity and human resources for control. Within the EU-FORA work programme, it was decided to dive deeper into the issues of data sharing and complexity of risk assessment.

Mycotoxins, which are fungal metabolites of low molecular weight, often play a critical role in plant pathogenesis and the spread of fungal infections. *Fusarium* species utilise various compounds, including specific trichothecene mycotoxins, as virulence factors to infect cereals like wheat and barley (Desjardins and Thomas, 1997). These two crops contribute to ~ 80% of small-grain production in Europe and can be heavily contaminated with trichothecenes (Bottalico and Perrone, 2002). T-2 and HT-2 toxin are type A trichothecenes produced under cool and moist conditions before harvest (Janik et al., 2021). Cereal grains, particularly oats, and their derivatives are the primary sources where T-2 and HT-2 toxin are predominantly found (EFSA, 2017a). Rapid deacetylation at the fourth carbon position is the primary metabolic pathway of T-2 toxin, leading to the formation of HT-2 toxin, regardless of the animal species involved (Nathanail et al., 2015). T-2 and HT-2, like many other trichothecenes, not only hinder protein synthesis and cell proliferation in plants but also induce acute or chronic intoxication in humans and animals. These toxins have various detrimental effects such as growth retardation, myelotoxicity, haematotoxicity, and the formation of necrotic lesions at contact sites (Rocha et al., 2005). Due to their toxic nature, EFSA has established a tolerable daily intake (TDI) value of 20 ng/kg body weight (bw) per day for the sum of T-2 and HT-2 (EFSA, 2017a). Additionally, the European Commission Recommendation 2013/165/EU provides indicative levels for the combined presence of these two toxins in cereals and cereal products, ranging from 15 μg/kg for cereal-based foods intended for infants and young children, up to 2,000 μg/kg for oat milling products (European Commission, 2013).

2. Description of the work programme

2.1. Aim

The overall aim was to generate solutions for identified needs in current emerging mycotoxin risk assessment procedures, focusing on a multi-actor approach. These needs included (among others) the lack of high-quality occurrence data of mycotoxins and the need for rapid assessment of (emerging) risks using risk assessment procedures, understandable for non-experts. To address these needs, following sub-goals were set:

1) To execute a small pilot to address the identified needs. This included the generation of a simplified data format to collect data and the development of a simplified risk assessment framework to make it accessible to non-experts and to rapidly detect emerging risks in the form of a toolkit.
2) To make the results of the pilot public for further exploitation on the digital FoodSafety4EU platform.

2.2. Activities

2.2.1. Pilot action to address specific needs in mycotoxin risk assessment

Development of a standard data collection format.

There is a lack of data on mycotoxins, including T-2 and HT-2 toxin and other emerging/ unregulated mycotoxins, as many stakeholders do not share acquired results, such as universities and

industrial actors. While there are requests from EFSA to submit data using a standard format, not many institutes provide all data. The fellow studied the data collection process of EFSA and aimed to simplify the SSD2 format specifically for data collection of T-2 and HT-2 toxin in specific oat types, so that data providers do not need to go through the supplementary guidance files. A Microsoft® Excel file was created including the following fields: Sampler, Sample point, Sample country, Region/ province/department, Sampling year, Sample FoodEx code (Oat groats: A002Z; Oat bran: A003B; oat rolled grains: A00DH; Rolled oats, instant: A00DJ), Sample country of origin, Year of analysis, Lab accreditation, Code analyte (T-2 or HT-2), Method of analysis, Result unit, Result LOD, Result LOQ, Result value and result type (Appendix A.1). While this data format cannot be used for official data submission to EFSA, it allows researchers to rapidly collect data on a specific food contaminant in predefined matrices and perform preliminary risk assessments to rapidly detects potential risks.

Development of a simplified risk assessment toolkit and application to T-2 and HT-2 toxin in oats

A risk assessment toolkit was generated consisting of two Microsoft® Excel files. One file is the data collection format as described above for optional use, and the other file contains a template to perform a risk assessment step-by-step, including simple deterministic calculations (Appendix A.2). The template also includes a sheet for the input of contamination data, where lower and upper means of the concentrations are calculated, and a sheet to insert consumption data for acute and/or chronic consumers of interest. Finally, the toolkit also comes with a short manual, with instructions on how to use the template, with relevant resources.

Hazard identification – The toolkit starts with hazard identification, where the user needs to insert up-to-date information about the contaminant. For T-2 and HT-2 toxin, EFSA performed a hazard identification in 2011. Briefly, T-2 induces ribotoxic and oxidative stress and inhibits DNA, RNA and protein synthesis. T-2 has been shown to cause apoptosis and lipid peroxidation, affecting cell membrane integrity. Recent investigations also suggest that T-2/HT-2 induces anorexia/emesis via alteration of pro-inflammatory cytokines and satiety hormones (EFSA, 2011). The available information on the toxicokinetics of T-2 and HT-2 toxins is incomplete. T-2 toxin is rapidly metabolised to a large number of products, HT-2 toxin being a major metabolite. The metabolic pathways include hydrolysis, hydroxylation, de-epoxidation, glucuronidation and acetylation. Distribution and excretion of T-2 toxin and its metabolites are rapid. There are no significant data available on the toxicity of most metabolites. De-epoxidation is believed to be a detoxification process.

Hazard characterisation – Next, the user needs to find information on the hazard characterisation of the contaminants. The manual provides guidance and resources to search for genotoxicity, reference points (such as the benchmark dose lower confidence level ($BMDL_{10}$)) and health-based guidance values (HBGVs, for instance tolerable daily intake (TDI) and the acute reference dose (ARfD)) in the OpenFoodTox database.[1] There is also guidance for compounds for which no reference values have been identified. The manual helps to distinguish between genotoxic carcinogens and non-genotoxic compounds, as the risk assessment will be different for both classes. T-2 and HT-2 toxin are currently characterised as not genotoxic and carcinogenic. The EFSA Panel on Contaminants in the Food Chain (CONTAM) established a group TDI for T-2 and HT-2 toxin of 0.02 μg/kg bw per day based on an in vivo subchronic toxicity study in rats that confirmed that immune- and haematotoxicity are the critical effects of T-2 toxin and using a reduction in total leucocyte count as the critical endpoint. An ARfD of 0.3 μg for T-2 and HT-2 toxin/kg bw was established based on acute emetic events in mink.

Exposure assessment – The next step is to calculate the exposure to the contaminant in the population of interest. For this step, the additional sheets for contamination and consumption data need to be completed first. The user is guided through the steps.

Collection of T-2 and HT-2 toxin contamination data in oat bran

The data collection format was sent to partners of the project, and in total, 126 analytical results were received, of which 38 for oat bran specifically which could be used for further risk assessment. The sum of T-2 and HT-2 toxin was considered. Only two samples contained toxins in a concentration above the limit of quantification (LOQ). Strategies have been proposed to address these situations, considering the presence of so-called non-detects in calculations and accounting for the potential low concentration of the contaminant. It is therefore important to organise the data into different scenarios. When dealing with contamination data, this involves creating a lower bound scenario, where

[1] https://zenodo.org/record/3693783

non-detects are assumed to be zero, and an upper bound scenario, where non-detects are replaced with the limit of quantification (LOQ) of the analytical method used (EFSA, 2010). The mean concentrations of the sum of T-2 and HT-2 toxin were 0.55 μg/kg and 7.79 μg/kg in the lower and upper bound scenario, respectively. The contamination levels and respective LOQs need to be filled in. The tool will calculate the mean for the lower and upper bound scenarios. These values appear automatically in the risk assessment sheet.

Collection of oat consumption data

Consulting the EFSA food consumption database,[2] statistical descriptors for oat bran were searched for Belgium, for all age categories. Oat bran was found to be classified in L4 as follows: Grain and grain-based products (L1) > Cereal grains and similar and primary derivatives thereof (L2) > Cereal bran (L3) > Oat bran (L4). Only the most recent data (i.e. from the Belgian food consumption survey of 2014) were used. There were only results available for 'other children' (36 months to 9 years), 'adolescents' (10 to 17 years) and 'adults' (18–65 years). The average and 95th percentile (P95) of acute and chronic consumption were used (see Table 1). In the consumption data sheet, the values found in databases for acute and chronic consumption need to be filled in, which will also appear automatically in the risk assessment sheet. The tool will then calculate the exposure for the populations for which consumption data were available and display the corresponding values. The calculated exposure values for T-2 and HT-2 toxin are presented in Table 2.

Table 1: Consumption of oat bran in Belgium

Population	Acute		Chronic	
	Mean (kg/kg bw per day)	P95 (kg/kg bw per day)	Mean (kg/kg bw per day)	P95 (kg/kg bw per day)
Other children (36 months-9 years)	0.0019	0.00397	0.00119	0.0028
Adolescents (10–17 years)	0.00073	0.00158	0.00039	0.00082
Adults (18–64 years)	0.00063	0.00132	0.00042	0.00102

bw: body weight.

Risk characterisation – Finally, the tool will calculate if there is a potential risk associated with the contaminant or not. For acute exposure, the outcome of the exposure assessment is compared with the ARfD. If the exposure exceeds this dose, there is a potential risk identified. The tool calculates the hazard quotient (HQ), which is the exposure value divided by the ARfD. If this is larger than 1, it means that the exposure value exceeds the ARfD, and a certain risk is identified. The tool generates a red colour when a risk is identified. A similar methodology is applied for chronic exposure to non-genotoxic compounds. The outcome of the exposure assessment is compared with the chronic HBGV. If the exposure exceeds this value, there is a potential risk identified. The tool calculates the HQ, which is the quotient of the chronic exposure value and the HBGV. If this is larger than 1, it means that the exposure value exceeds the HBGV, and a certain risk is identified. The tool generates a red colour when a risk is identified. For genotoxic carcinogens, it is recommended to work with margins of exposure (MOEs), rather than hazard quotients. The MOE is the ratio calculated by determining a level of exposure in which harm to human health is not expected to occur (e.g. $BMDL_{10}$), and then dividing that by an estimated level of human exposure (EFSA, 2017b). For genotoxic carcinogens, a value above 10,000 is considered as low risk. When the obtained value is smaller than 10,000, it means that the outcome lies too close to the level at which harm can occur and a potential risk is identified. The MOE is not a HBGV, i.e. it is not a safety threshold below which the daily intake is considered as safe. When there is evidence of harmful effects but not enough to confirm how much is safe, the MOE tells us if current intakes are likely to be harmful or not: a low MOE represents a greater risk than a higher MOE. For T-2 and HT-2 toxin, the risks after both acute and chronic consumption were characterised, using the ARfD and the group TDI. The acute exposure ranged between 0.0003 and 0.0309 μg/kg bw per day throughout all available age categories, consumption patterns and scenarios. At a first glance, it is already clear that no value exceeds the ARfD of 0.3 μg/kg bw per day. Therefore, no potential risk after acute exposure is identified with the available data. This is confirmed by the tool at the risk characterisation step, where no HQ of > 1 was calculated, hence no cell turned red for potential

[2] https://www.efsa.europa.eu/en/data-report/food-consumption-data

risk indication. For chronic exposure, the calculations are similar, but now, the TDI is used. As T-2 and HT-2 toxin are not on the list of genotoxic carcinogens, the tool will not calculate the MOE, but a HQ. The chronic exposure ranges from 0.0002 to 0.0218 μg/kg bw per day. At the first glance, we see that in the category of 'other children', the TDI is exceeded in the upper bound scenario of high consumers. As 36 of 38 samples were below LOQ, this scenario is very likely an overestimation. However, this is a good illustration of how the tool can identify potential risks related to chemicals in food. The risk characterisation step of the tool confirms a HQ >1 for high-consuming children between 3 and 9 years old at the upper bound scenario, indicating a potential risk by turning red (Table 3).

Table 2: Exposure to the sum of T-2 and HT-2 toxin through consumption of oat bran in Belgium. LB: lower bound scenario, UB: upper bound scenario

Population	Acute exposure				Chronic exposure			
	LB scenario		UB scenario		LB scenario		UB scenario	
	Average consumers (kg/kg bw per day)	High consumers (kg/kg bw per day)	Average consumers (kg/kg bw per day)	High consumers (kg/kg bw per day)	Average consumers (kg/kg bw per day)	High consumers (kg/kg bw per day)	Average consumers (kg/kg bw per day)	High consumers (kg/kg bw per day)
Other children	0.0010	0.0022	0.0148	0.0309	0.0007	0.0015	0.0093	0.0218
Adolescents	0.0004	0.0009	0.0057	0.0123	0.0002	0.0004	0.0030	0.0064
Adults	0.0003	0.0007	0.0049	0.0103	0.0002	0.0006	0.0033	0.0079

bw: body weight.

Table 3: Risk characterisation of the sum of T-2 and HT-2 toxin in oats in the Belgian population expressed as hazard quotient. For acute exposure, this means the acute exposure divided by the acute reference dose. For chronic exposure, the hazard quotient is calculated as the quotient of the chronic exposure and the group tolerable daily intake

Population	Acute Risk (hazard quotient)				Chronic exposure (hazard quotient)			
	LB scenario		UB scenario		LB scenario		UB scenario	
	Average consumers	High consumers	Average consumers	High consumers	Average consumers	High consumers	Average consumers	High consumers
Other children	0.0035	0.0073	0.0493	0.1030	0.0372	0.077	0.4633	1.0902
Adolescents	0.0013	0.0028	0.0189	0.0410	0.0107	0.0225	0.1518	0.3192
Adults	0.0012	0.0024	0.0163	0.0343	0.0115	0.0281	0.1635	0.3971

LB: lower bound scenario, UB: upper bound scenario.

2.2.2. Feedback of users of the risk assessment toolkit and further steps

The toolkit was presented to the FSOLab members and demonstrated to be very efficient to perform preliminary risk assessments for emerging chemical contaminants, even by non-experts. The received feedback was overwhelmingly positive, underlining its potential as a valuable resource for researchers, students and individuals engaged in food safety environments.

A noteworthy aspect of the toolkit is its alignment with the EFSA's tool for rapid assessment of contaminant exposure (RACE tool), which is specifically designed to be accessible to non-experts. The toolkit shares a similar rationale, extending its applicability to a broader audience. Notably, the developed toolkit goes beyond its counterparts by offering a comprehensive template for rapidly collecting essential analytical data. This feature not only facilitates the risk assessment process but also ensures that a robust foundation of data is available for accurate evaluations. Additionally, the toolkit's ability to calculate lower and upper bound scenarios based on the provided analytical results further enhances its utility. This feature aids in understanding the potential range of risks associated with the analysed contaminants, allowing for a more comprehensive evaluation. A key advantage of the toolkit lies in its user-friendly interface, which guides the user through the four fundamental steps of risk assessment. This streamlined approach facilitates a basic understanding of risk assessment

procedures, making it accessible to users with varying levels of expertise. Moreover, the toolkit promotes an insightful approach to risk assessment by encouraging users to seek and incorporate relevant reference values and consumption data. By doing so, users gain valuable insights into the underlying calculations and the basis for their risk assessments, enhancing the overall transparency and reliability of the results.

The toolkit is freely available on the Foodsafety4EU Platform (www.foodsafety4.eu) as a beta version, and will be further optimised based on user feedback. Besides the manual, further training videos will be provided to help users to efficiently apply the toolkit.

Lastly, it is intended to implement the toolkit in an upcoming European-African food safety project to spread awareness on the necessity of proper risk assessment methods. Many target groups such as students, food safety professionals, food business operators and policy makers will benefit from the toolkit and will have a basic understanding of chemical risk assessment.

2.2.3. Other activities during the EU-FORA fellowship

- **Side project on crisis communication**

Figure 1: Workshop invitation

While the EU-FORA project focused mainly on risk assessment, a few weeks of the programme were dedicated to risk communication, more specifically crisis communication. Ways to involve young students in the process of risk communication were explored. A workshop was organised at Ghent University (Figure 1), where students were challenged to come up with ideas on how to improve crisis communication, using *Salmonella* in chocolate crisis in Belgium as a model case (ECDC and EFSA, 2022). Three communication experts were also invited to the workshop and gave a short presentation on challenges they encountered during the *Salmonella* crisis. After the presentations, the students co-created solutions for communication challenges based on what they have heard during the talks of the experts. The results were presented by the fellow on the second pre-forum of FoodSafety4EU on 15 December in Brussels. These two activities led to a multi-actor policy brief entitled: 'Towards the EU Food Safety Forum: shaping together the new collaborative platform' FoodSafety4EU PRE-FORUM 2022 'The new sustainability regulation: how to integrate it into food safety?' (Ivanov et al., 2023).

- **Involvement in other FoodSafety4EU FSOLabs**
  The fellow was deeply involved in three other FSOLabs running during the FoodSafety4EU project, which were focused on preparing a strategic food safety research agenda, mapping food safety funding schemes in Europe and exploring ways for improved food safety communication to citizens.

- **Risk assessment training modules EU-FORA**
  The fellow attended all trainings modules organised within the EU-FORA training programme in September (Parma), November (online), March (online), June (Parma) and August (online).

- **EFSA webinars on the FoodEx2 classification system**
  The fellow followed all available webinars concerning the FoodEx2 classification system presented by EFSA to gain comprehensive knowledge on the use and implementation of the system.

- **Horizon Europe proposal writing including food safety and risk assessment strategies in African countries**
  The fellow was highly involved in writing a proposal for the call 'HORIZON-CL6-2023-FARM2FORK-01-20 – EU-Africa Union – food safety'. Both the hosting and sending institutes are main partners within this project proposal. As the project will focus on risk assessment and food policy, the expertise gained from the EU-FORA programme will be exploited substantially. The proposal was successful and is now in the grant agreement preparation phase.

- **Organisation of the EU Food Safety Forum 2023**
  On 28 and 29 November 2023, the European Food Safety Forum will be organised for the first time in Brussels. The fellow will chair a session involving young students dedicated to different aspects in the food safety field.

3. Conclusion

Solutions were provided for a selection of the needs in current risk assessment procedures identified by a multi-actor team in the FSOLab. The selected needs were (a) hindered analytical data collection for specific emerging contaminants and (b) need for rapid risk assessment of emerging contaminants using simple processes. The developed risk assessment toolkit addresses both needs, as it provides a template for rapid data collection based on the SSD2 format but without the need to read supplementary guidance, and a spreadsheet where risk assessment calculations can be performed rapidly. The toolkit does not replace the current risk assessment procedures of EFSA, but serves as a tool to quickly assess risks of emerging or unregulated chemical contaminants by non-experts.

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Abbreviations

ARfD	acute reference dose
$BMDL_{10}$	benchmark dose lower confidence limit
bw	body weight
CONTAM	Panel on Contaminants in the Food Chain
FSOLab	Food Safety Operational Lab
HBGV	health-based guidance value
HQ	hazard quotient
LOQ	limit of quantification
MOE	margin of exposure

P95	95th percentile
SSD2	Standard sample description version 2.0
TDI	tolerable daily intake

Appendix A – The risk assessment toolkit

A.1. Data collection format

This is a condensed version of the Excel spreadsheet for illustration purposes. In the normal version, all entry parameters are ranked next to each other, from column A to column R.

	A	B	C
1	**Sampler**	**Sample point**	**Sample country**
2	Industry/official/industry AND official/HACCP or other checks/private/public/other	Which point of food chain: manufacturing, farm, transport, retail...	Where was sample taken
3			
4			
5	**Region/province/department**	**Sampling year**	**Sample FoodEx code**
6		When was sample taken	Search for the correct FoodEx2 code for the food matrix in the EFSA catalogue browser
7			
8			
9	**Sample country of origin**	**LOT number**	**Expiration date**
10	Where did the sample originate from	if available	if available
11			
12			
13	**Year of analysis**	**Lab accreditation**	**Code analyte**
14	When was sample analysed	Accredited/third party assessment/none	Assign a code to the analyte
15			
16			
17	**Method of analysis**	**Result unit**	**Result LOD**
18	LC-MS; GC-MS; ELISA ...	µg/kg; ng/kg ...	specify LOD
19			
20			
21	**Result LOQ**	**Result value**	**Result type**
22	specify LOQ	Numeric value expressed in specified unit	<LOD; <LOQ; > LOQ
23			

A.2. Risk assessment tool

This is a screenshot of the Excel spreadsheet for risk assessment for illustration purposes.

Q30

	A	B	C	D	E	F	G	H	I
1	**1. HAZARD IDENTIFICATION**								
2	Briefly describe the potential hazards to humans that the presence of a substance in food may pose								
3	**2. HAZARD CHARACTERISATION**								
4	*** Genotoxic carcinogen?**	**RP in µg/kg bw/day**	**Type**	**References**					
5	Find genotoxicity evaluation in File **Genotoxicity_KJ** and specify reference point (RP) from File **ReferencePoints_KJ** on the *OpenFoodTox database* (NOAEL, $BMDL_{10}$...):		$BMDL_{10}$						
6	https://zenodo.org/record/3693783								
7	If no RP: Use TTC of 0.0025 µg/kg bw/day								
8	*** NOT genotoxic?**								
9	**ACUTE**	**ARfD in µg/kg bw/day**	**Type**	**References**					
10	Specify the acute reference dose (ARfD) from File **ReferenceValues_KJ** on *OpenFoodTox database*		ARfD						
11	https://zenodo.org/record/3693783								
12									
13	**CHRONIC**	**HGBV in µg/kg bw/day**	**Type**	**References**					
14	Specify health-based guidance value (HBGV) from File **ReferenceValues_KJ** on *OpenFoodTox database* (TTC, TDI, ADI, TWI...):								
15	https://zenodo.org/record/3693783								
16	If no RV: Specify Cramer Class using *Toxtree* . Class I: use TTC of 30 µg/kg bw/day; Class II-III: use TTC of 1,5 µg/kg								
17	https://apps.ideaconsult.net/data/ui/toxtree								

3. EXPOSURE ASSESSMENT

CONTAMINATION DATA

LB mean in µg/kg	UB mean in µg/kg
#DELING.DOOR.0!	#DELING.DOOR.0!

	ACUTE CONSUMPTION DATA		CHRONIC CONSUMPTION DATA	
	Average consumers in kg/kg bw/day	High consumers in kg/kg bw/day (95th percentile)	Average consumers in kg/kg bw/day	High consumers in kg/kg bw/day (95th percentile)
Infants 0-11 months				
Toddlers 12-35 months				
Other children 36 months-9 years				
Adolescents 10-17 years				
Adults 18-64 years				
Elderly 65-74years				
Very elderly 75 years and older				

	ACUTE EXPOSURE = CONTAMINATION X ACUTE CONSUMPTION				CHRONIC EXPOSURE = CONTAMINATION X CHRONIC CONSUMPTION			
	LB MEAN CONCENTRATION		UB mean CONCENTRATION		LB MEAN CONCENTRATION		UB mean CONCENTRATION	
	Average consumers (µg/kg bw/day)	High consumers (µg/kg bw/day) (95th percentile)	Average consumers (µg/kg bw/day)	High consumers (µg/kg bw/day) (95th percentile)	Average consumers (µg/kg bw/day)	High consumers (µg/kg bw/day) (95th percentile)	Average consumers (µg/kg bw/day)	High consumers (µg/kg bw/day) (95th percentile)
Infants 0-11 months								
Toddlers 12-35 months								
Other children 36 months-9 years								
Adolescents 10-17 years								
Adults 18-64 years								
Elderly 65-74years								
Very elderly 75 years and older								

4. RISK CHARACTERIZATION

Non-genotoxic ACUTE HQ = Exposure/ARfD

	LB MEAN CONCENTRATION		UB mean CONCENTRATION	
	Average consumers (µg/kg bw/day)	High consumers (µg/kg bw/day) (95th percentile)	Average consumers (µg/kg bw/day)	High consumers (µg/kg bw/day) (95th percentile)
Infants 0-11 months				
Toddlers 12-35 months				
Other children 36 months-9 years				
Adolescents 10-17 years				
Adults 18-64 years				
Elderly 65-74years				
Very elderly 75 years and older				

Genotoxic compounds MOE = RP/chronic exposure

	LB MEAN CONCENTRATION		UB MEAN CONCENTRATION	
	Average consumers	High consumers	Average consumers	High consumers
Infants 0-11 months				
Toddlers 12-35 months				
Other children 36 months-9 years				
Adolescents 10-17 years				
Adults 18-64 years				
Elderly 65-74years				
Very elderly 75 years and older				

Non-genotoxic compounds HQ = chronic exposure/HBGV

	LB MEAN CONCENTRATION		UB MEAN CONCENTRATION	
	Average consumers	High consumers	Average consumers	High consumers
Infants 0-11 months				
Toddlers 12-35 months				
Other children 36 months-9 years				
Adolescents 10-17 years				
Adults 18-64 years				
Elderly 65-74years				
Very elderly 75 years and older				

Instructions | Risk assessment tool | Contamination data | Consumption data

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Unveiling the potential of proteomics in addressing food and feed safety challenges

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Abstract

The food and feed sector in Europe is rapidly evolving to address contemporary challenges, striving for fairer, safer, greener and more sustainable food systems. This includes the exploration of new protein sources for human consumption and animal feed such as protein derived from insects, algae or novel plant-derived proteins, and the re-evaluation of existing sources like processed animal protein (PAP). To generate reliable data on the diverse array of emerging protein sources for future food and feed safety assessments, a growing demand for the development and implementation of advanced analytical techniques exists. New approach methodologies (NAMs) including, mass spectrometry (MS)-based proteomics methods have been emerging as valuable techniques which potentially can be implemented in regulatory laboratory settings to complement conventional approaches in this realm. These MS-driven strategies have already proven their utility in diverse applications, including the detection of prohibited substances in feed, identification of allergens, differentiation of fish species in complex mixtures for fraud detection and the verification of novel foods and alternative protein sources. This EU-FORA programme was focused on three core objectives namely: (i) the training of the fellow in utilising MS-based proteomics for food and feed safety analyses, (ii) the involvement of the fellow in the development of standardised operating procedures (SOP) for targeted and non-targeted proteomic MS-based workflows for species and tissues specific PAP identification in a national reference laboratory (NRL) and (iii) the transfer and implementation of MS-based approaches and standardised protocols for PAP analysis at the fellow's home institution. Altogether, this programme facilitates the broadening and diversification of use of MS-based proteomic methodologies for reinforcing their significance within the domains of food and feed safety research and regulatory science applications.

Keywords: proteomics, alternative protein sources, processed animal protein, species identification, tissue identification, mass spectrometry

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1. Introduction

With the currently occurring green shift in food and feed domains in Europe and the envisaged focus on value creation from protein derived from novel sources and circular bio-based economies, new data gaps and challenges arise which cannot any longer be addressed with classic analytical tools and regulatory paradigms (Belghit et al., 2021; Delgado et al., 2022). In addition to novel terrestrial feed ingredients, also the authentication of fish species has become is a major concern worldwide (Varunjikar et al., 2022b). In the context of food safety, the development of novel methodologies and analytical tools to authenticate both species and tissue specific composition and origin of proteic material has become key to create and evaluate data which is fit for purpose for future feed safety risk assessments needs. This EU-FORA programme was performed under the auspices of, and contributed to, national and international initiatives aimed at fortifying the capacity of national regulatory laboratories for swift safety interventions and the enhancement of current risk assessment practices. Given the transformative dynamics within the food and feed sectors in relation to the emergence of novel proteic constituents, the training of the fellow in omics-based feed and food authenticity analyses and fraud detection will contribute to a wider implementation and dissemination of the use of new approach methodologies (NAMs) such as proteomics, for addressing emerging and future food and feed safety challenges.

1.1. Applicability of proteomics in food and feed safety sectors

Proteomics, one of the so-called 'omics[1] methods and part of a suite of methods considered as NAMs for use in risk assessment (Marx-Stoelting et al., 2023), is an approach used to study proteins whereby the entire complement of proteins in a given sample (of tissue, cells or a biological fluid such as blood) is analysed simultaneously. Proteomic-based methods using liquid chromatography coupled to mass spectrometry (LC–MS) are increasingly recognised as promising tools to complement current standard techniques for food and feed quality assessments and traceability analysis (Rasinger et al., 2016).

As highlighted in EFSA Scientific Colloquium 24 – 'omics in risk assessment: state of the art and next steps', '*omics technologies are a valuable addition in some aspects of risk assessment of food and feed products and, if the current pitfalls associated with data collection, processing, interpretation and curation were resolved*, i.e. *by application of FAIR (findable, accessible, interoperable and reusable) data management principles, such methods could be routinely used in food and feed safety risk assessment*' (EFSA, 2018).

Originating in the last decades of the 20th century, the advent of MS-based proteomics owes its dawn to the ground-breaking efforts of John B. Fenn and Koichi Tanaka, who ushered in the era of soft desorption ionisation techniques for analysing biological macromolecules via MS (Patterson and Aebersold, 2003). While various forms of these techniques have witnessed extensive adoption across multiple facets of biological research, their integration into the sphere of food and feed safety research remained relatively modest. Nonetheless, several applications of proteomics have surfaced in the context of food and feed safety. These encompass the testing of proteinaceous materials to discern levels of adulteration and ensure traceability, the identification of food-borne pathogens, the detection of genetically modified organisms (GMO) and allergen identification. At the moment, the most foreseeable avenue for incorporating proteomics into routine food and feed testing is in the field of PAP. In 2019, the European Union Reference Laboratory for Animal Proteins in Feedingstuffs (EURL-AP) organised an inter-laboratory study on the detection of bovine PAP in feed by MS-based proteomics. The results of the study indicated that LC–MS was able to successfully identify the presence of various proteins of bovine origin in feed at an adulteration level of 1% (w/w), demonstrating that proteomics has matured enough to become a complementary method for the official control of the use of PAP in feedstuffs (Lecrenier et al., 2021). Furthermore, proteomics can provide a more comprehensive understanding of the molecular mechanisms of toxicity and, hence, be used to identify new biomarkers of exposure and toxicity, which can be used to develop new endpoints for chemical risk assessment (Li et al., 2023). A more exhaustive compilation of examples spotlighting the applicability of MS-based proteomics in the domain of food and feed safety is listed in Table 1, whereas a visual infographic is presented in Figure 1.

[1] As described in *Encyclopedia Britannica*, **omics** are any of several areas of biological study defined by the investigation of the entire complement of a specific type of biomolecule or the totality of a molecular process within an organism. Examples of well-established fields include genomics, transcriptomics, proteomics and metabolomics.

Table 1: Application examples of MS-based proteomics in food and feed safety as well as risk assessment

Subcategory	Application	Reference/–es
Allergens	Detection of known food allergens in food commodities by targeted proteomics	Monaci et al. (2020)
	Application of discovery untargeted proteomics to identify allergens in novel foods	López-Pedrouso et al. (2023), Barre et al. (2021), Bose et al. (2021)
Adulteration and traceability	Discovering protein indicators of freshness of refrigerated fish products via untargeted proteomics	Deng et al. (2019)
	Identification of species and the estimation of their respective relative abundances in a mixed samples for the purpose of authentication analysis of fish and meat	Varunjikar et al. (2022b), Stachniuk et al. (2021)
	Detection and identification of non-authorised proteic material (e.g. prohibited ruminant by-products) in processed animal protein (PAP) by targeted proteomics	Lecrenier et al. (2021)
	Species-specific discrimination of insect meals for aquafeeds via untargeted proteomics	Belghit et al. (2019), Varunjikar et al. (2022a)
	Identification of unique peptide markers for authentication of honey via untargeted proteomics	Bong et al. (2021)
	Detection of powdered milk in fresh cow's milk	Calvano et al. (2013)
Food-borne pathogens and contaminants	High-throughput diagnostic methods detection of parasites, fungi and food-borne bacteria in food and feed via MALDI-MS	Kästner et al. (2021), Pavlovic et al. (2013), Lima and Santos (2017)
	Detection and quantification of protein toxins, e.g. Botulinum toxin	Duracova et al. (2018)
	Identification of undergoing illicit veterinary treatments in livestock	Donna et al. (2009)
	Detection of Maillard reaction induced changes in thermally processed milk	Arena et al. (2017)
Risk assessment	Integration of proteomics in a multi-omics approach within a broader systems biology framework for augmenting current risk assessment practices of GMOs	Benevenuto et al. (2023)
	Applying standalone untargeted proteomics for the detection of transgenic events and evaluating proteome-level equivalence to bolster safety assessments of novel crop varieties destined for use in feed and food	Varunjikar et al. (2023)
	Integration of proteomics in a multi-omics toolbox as new approach methodology (NAM) to define biological responses for chemical safety assessment	Li et al. (2023)
	Application of proteomics along with other omics tools to study host-pathogen interactions for next generation microbiological risk assessment	Haddad et al. (2018)

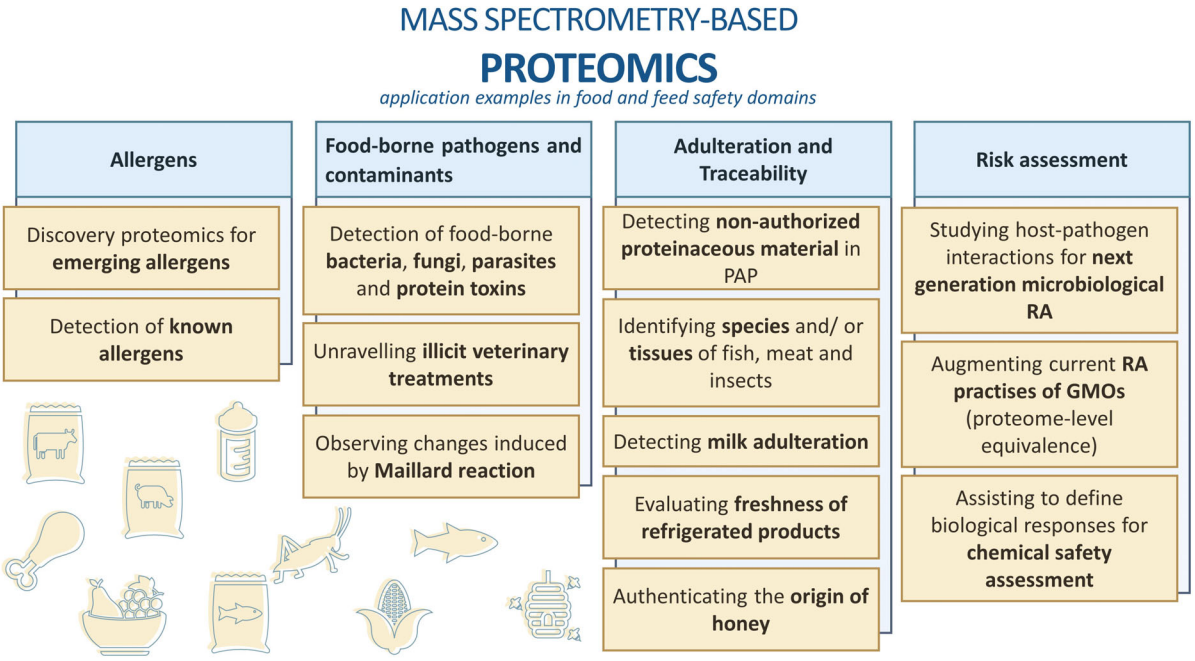

Figure 1: An infographic summarising the use of MS-based proteomics in food and feed safety and risk assessment

2. Description of work programme

The work programme was conducted within the framework of The European Food Risk Assessment (EU-FORA) Fellowship Programme at two institutions: the fellow's home institution, Institute of Food Safety, Animal Health and Environment 'BIOR' (BIOR, Latvia) and the hosting institution, Institute of Marine Research (IMR, Norway). Dr. Josef D. Rasinger, part of IMR's Marine Toxicology group, provided supervision. In essence, the work programme sought to prepare the fellow with practical training and theoretical insights, enabling confident use of MS-based proteomics techniques for use in feed and food safety assessments. Through knowledge sharing, expertise from the hosting site was transferred to the fellow's home institution. Thus, the work programme directly contributed to core EU-FORA objectives: enhancing risk analysis readiness and facilitating knowledge exchange for a unified EU risk assessment strategy.

2.1. Aims

While proteomics is not yet a widely used technique within the multi-omics toolbox applied in risk assessment, it is gaining recognition as one of the NAMs. Being prepared to address future challenges is essential, and proteomics has the potential to enhance risk assessment practices. Therefore, the work programme aimed to provide a thorough training experience, combining hands-on learning with strong theoretical foundations. Beyond technical expertise, the programme intended to acquaint the fellow with diverse application possibilities for proteomics. This strategy fostered versatility beyond a singular context and promoted a holistic view of the technique's possibilities.

2.2. Activities/methods

2.2.1. Laying the theoretical foundations of proteomics within the context of food and feed safety

Given the inaugural hands-on training in January 2023, it was vital to provide the fellow with solid theoretical grounding in MS-based proteomics prior to the arrival. Under IMR's guidance, the fellow conducted literature research, focusing on relevant EU documents regarding proteic materials in feed and novel foods, EURL-AP guidance documents and research papers from the past decade by IMR's Marine Toxicology group. The group has an extensive expertise in this field and has applied proteomics in various contexts; for instance, distinguishing insect meals for aquafeeds, detecting unauthorised proteic

material in PAP, the analysis of GM soy and the detection of mislabelled fish species in mixes (Rasinger et al., 2016; Belghit et al., 2019, 2021; Varunjikar et al., 2022b, 2023). The group also has ample experience in the use of omics for chemical risk assessment (Rasinger et al., 2014, 2017, 2018; Reffatto et al., 2018) and the generation and storage of data in line with FAIR principles (Pineda-Pampliega et al., 2022). Additionally, remote learning was pursued to deepen the fellow's technical knowledge and biological understanding of the field. Therefore, the fellow accessed complementary learning resources from two sources: (i) UC Davis Proteomics Online 2nd Short Course and (ii) the Proteomics Academy, a collaboration between the European Proteomics Association and the European Bioinformatics Community. In addition to that, the fellow also received training on the 'Application of omics in risk assessment', a 1-day online course held by the fellow's supervisor as part of the second training module of the EU-FORA course series. These endeavours, coupled with frequent guidance from the supervisor and other members of the IMR's Marine Toxicology group, ensured the fellow's readiness for practical training at the hosting facility.

2.2.2. Training in FASP and SP3 strategies for sample preparation in proteomics

Sample preparation strategies in contemporary proteomic studies differ considerably from previous gel-based approaches, because protein extraction and digestion prior to MS analysis is required. Commonly employed methods encompass filter-aided sample preparation (FASP), suspension-trapping (S-Trap), single-pot, solid-phase-enhanced sample preparation (SP3) and in-StageTip digestion (iST) (Sielaff et al., 2017; Araújo et al., 2021). Each laboratory typically leans towards a preferred approach, tailored to the specific proteomics application. IMR predominantly adopts the FASP technique, in which the fellow received training. To enrich the work programme's scope, the fellow also participated in SP3 protocol training at the University of Bergen's Proteomics Unit (PROBE), under the supervision of Olav Mjaavatten.

FASP, introduced by Manza et al. (2005), has gained widespread popularity due to its practicality. It effectively eliminates cellular debris, salts, lipids, chaotropes, detergents like sodium dodecyl sulphate (SDS) and other low-molecular weight contaminants through a straightforward centrifugation process using a molecular weight cut-off (MWCO) ultrafiltration device (Manza et al., 2005; Sielaff et al., 2017). This method retains proteins on a filter membrane, making them available for subsequent enzymatic digestion. Generated peptides, small enough to pass the filter, can be collected via centrifugation. After FASP-assisted digestion, roughly 50% of the initial material can be recovered, with no adverse impact on proteome coverage (Sielaff et al., 2017). This quality is particularly beneficial for applications in food and feed safety, where the initial sample amount of proteic material (e.g. insect-based aquafeed or PAP) is comparatively high, in contrast to mass-limited samples such as tissue biopsies from clinical research areas.

An in-depth description of the applied FASP protocol is available in research paper by Belghit et al. (2019). In brief, around 50–100 mg of protein containing sample (e.g. insects, animal tissue, PAP, etc.) is homogenised in lysis buffer (0.1 M Tris–HCl/4% SDS). Protein concentration in the extract is determined spectrophotometry (660 nm Protein Assay). Purification of protein extract is done via FASP, where disposable centrifugal ultrafiltration units allow for detergent depletion, protein digestion and isolation of peptides released by proteases from undigested material. Reduction of disulphide bonds and alkylation is done by dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Protein digestion is performed with trypsin (37°C for 16 h). Additional clean-up of the peptide extract is carried out by dispersed solid phase extraction methodology using C18 spin-columns. The final extract is evaporated and reconstituted in solvent mixture that matches the initial gradient conditions of the LC–MS gradient method (Belghit et al., 2019).

Unlike FASP, SP3 follows a paramagnetic bead approach, where ethanol-driven solvation captures proteins on hydrophilic beads functionalised with carboxylate groups. This mechanism effectively separates a broad range of biomolecules, ensuring unbiased recovery of proteins and peptides for bottom-up proteomics analysis. Additionally, SP3 stands out for its straightforward single-tube protocol, which is less labour-intensive than other methods, including FASP (Hughes et al., 2019). Nevertheless, both SP3 and FASP facilitate the removal of unwanted chemicals and biological components before digestion; the resulting peptide digest can be subjected to a further clean-up if needed. Detailed instructions for the complete SP3 protocol can be found in Hughes et al. (2019). A simplified scheme of the analytical pipeline of both SP3 and FASP protocols is given in Figure 2. During the work programme, the FASP protocol served as the principal sample preparation strategy, while SP3 was exclusively utilised for training purposes.

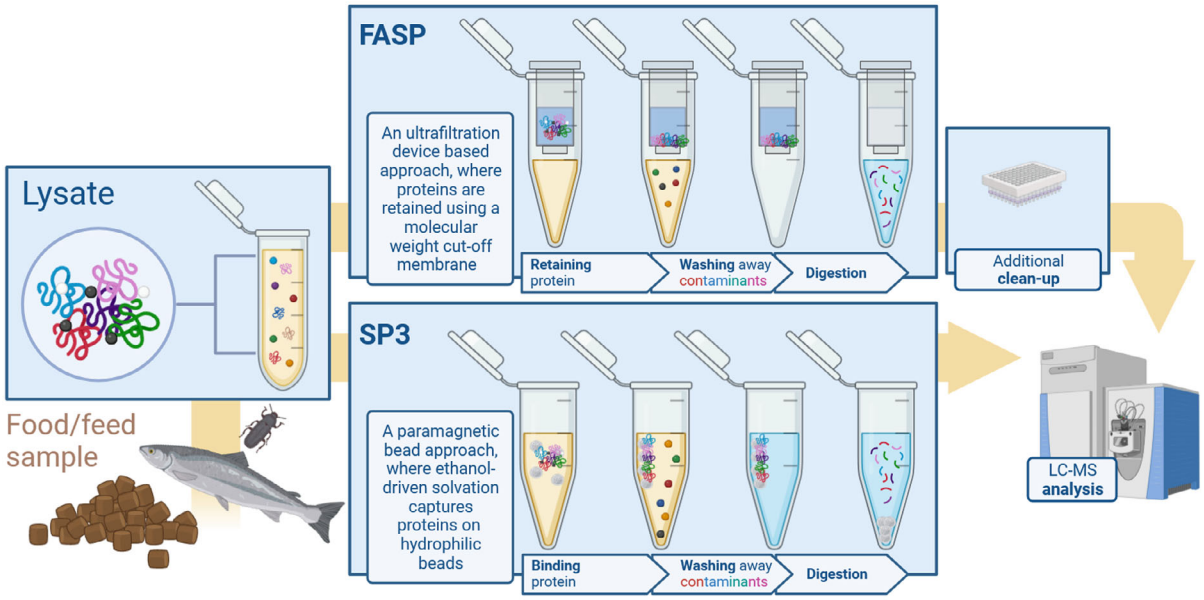

Figure 2: SP3 and FASP protocols: analytical workflow comparison (figure created with BioRender.com with licence number ZE25RV2OJQ)

2.2.3. LC–MS and interpretation of proteomics data utilising different tools

In this work program, a bottom-up proteomics method was employed. The sample extract comprises peptides generated through proteolytic digestion, which are subsequently analysed via LC–MS. Given the known digestion mechanism by a protease (trypsin in this case), the resulting peptide signals can be translated into amino acid sequences, resembling pieces of a puzzle that unveil the sample's proteome. There are different ways to implement instrumental part for bottom-up proteomics. For instance, nanoflow-LC (nano-LC) is used in combination with hybrid high-resolution MS systems, such as time-of-flight MS, Orbitrap-MS or even Fourier transform ion cyclotron resonance MS, to achieve the most out of the proteome analysis in non-target mode. On the other hand, simpler tandem mass spectrometry (MS/MS) systems combined with conventional high-performance liquid chromatography (HPLC) can be effectively used to perform targeted analyses for selected peptide markers (Lecrenier et al., 2021; Stachniuk et al., 2021). In this programme non-targeted approach was utilised. However, instead of using nano-LC, we opted to achieve peptide separation via HPLC with higher flow rates. This decision was rooted in the technical complexity and required expertise associated with nano-LC, despite its industry recognition as the gold standard. As a result, HPLC becomes a more feasible entry point for routine and research labs in the field of food and feed safety. This is particularly relevant for those less familiar with the intricate aspects of implementing proteomics protocols.

Concurrently, mass spectra measurements were executed using a Q Exactive Orbitrap MS system. This decision stemmed from the shared presence of this equipment at both the hosting site (IMR) and the fellow's home institution (BIOR), streamlining the method transfer process in comparison to using distinct setups. The method's tune, acquisition and source parameters were directly adapted from Varunjikar et al. (2022a), where non-target bottom-up proteomics was successfully utilised for authentication, biological analyses and allergen detection in feed and food-grade insect species. These specific parameters can be located in the original research article (Varunjikar et al., 2022a).

The processing of proteomics data and its subsequent bioinformatics analysis are key for the translation of raw MS data into meaningful insights, necessary for delving into a sample's proteome. Amid the vast array of workflows that are routinely employed, a simplified strategy for handling bottom-up non-targeted proteomics data can be boiled down into the following steps (Chen et al., 2020; Deutsch et al., 2022):

1) Raw data pre-processing, e.g. conversion of the raw vendor format to mzML.
2) Peptide identification using peptide sequence search engine (usually from FASTA format), spectral library search engines or *de novo* search engines.

3) Validation of search engine results by assigning a probability (with or without the aid of a decoy database) to each peptide-spectrum match (PSM) to lower the false discovery rate (FDR).
4) Reconstruction of the peptide sequences into their original proteins, a process known as protein inference, where peptide assembly models normally adhere to the Occam's razor approach, which reports the smallest set of proteins that account for the detected peptides.
5) Quantifying the abundance of measured proteins based on the experimental workflow, label-free, isobaric labelling or isotopic labelling approaches.

In the course of this work programme, the participant became familiar with three data processing workflows employed by IMR researchers in the domain of food and feed safety. Each of these workflows is briefly summarised in Table 2.

Table 2: Summary of employed data processing workflows for the analysis of proteomics data in this EU-FORA work programme

Workflow	Summary
Direct spectral comparison using compareMS2 2.0	CompareMS2 2.0 is a versatile tool that enables molecular phylogenetics by aligning and matching tandem mass spectra features of peptides across different input datasets (Palmblad and Deelder, 2012; Marissen et al., 2023). This method has already been used in a range of applications, including food and feed species identification and has been recently updated with an improved user interface un functionalities providing even better applicability potential.
Spectral library building and matching with SpectraST in Trans-Proteomic Pipeline (TPP)	In this workflow spectral library is created from reference samples (Lam, 2011), for example, single-species samples of fish and insects. This library can subsequently be used to scrutinise unknown samples. By implementing a dot product threshold, matching spectra can be pinpointed and used as potential markers for species identification using the untargeted approach (Varunjikar et al., 2022a).
A standard bottom-up proteomics data analysis workflow with sequence search engine Comet in TPP	In this method, a traditional data analysis approach is employed. It involves using UniProt databases (FASTA files) to search for species-specific proteomes via the Comet sequence search engine in the TPP platform (Eng et al., 2013). Subsequently, pepXML files produced in this process are subjected to validation through PeptideProphet and ProteinProphet (Keller et al., 2002; Nesvizhskii et al., 2003), implementing a 1% FDR threshold to ensure accurate identification of markers in line with verified sequences. Despite its reliable nature, the scope of this approach remains somewhat limited beyond model species due to the scarcity of reviewed datasets within the UniProt database for species relevant to the food and feed sector.

Last but not the least, benefitting from work done in an earlier EU-FORA project performed at the hosting site (Pineda-Pampliega et al., 2022), the fellow gained insight into the FAIR (Findability, Accessibility, Interoperability and Reusability) data principles that govern the storage and dissemination of data. For the present project, this entailed familiarity with utilising ProteomeXchange resources, especially by the application of the MassIVE repository. In particular, datasets from two studies by Belghit et al. (2019) and Varunjikar et al. (2022a) were used throughout the programme with massIVE repository IDs MSV000083737 and MSV000088034, respectively.

2.2.4. Transitioning from knowledge to practical implementation of MS-based proteomics for food and feed safety

The introduction has set the stage for the multifaceted applications of MS-based proteomics in food and feed safety research. Across diverse applications intended for authentication and traceability of food and feed, a common objective prevails – capitalising on proteomic variations to distinguish taxa, species and notably tissues. The latter holds pivotal significance within proteomics. For instance, in authenticating PAP in regulatory laboratory settings, species identification predominantly relies on real-time polymerase chain reaction (qPCR), while tissue identification depends on light microscopy. Recent insights from the 2022 EURL-AP proficiency test result reveal that species identification through qPCR is far more accurate compared to light microscopy-based tissue analysis (Fumière et al., 2022). Thus, MS-based proteomics emerges as a potential complement to conventional light microscopy.

A meta-analysis conducted by Sudmant et al. (2015) showed that most transcriptomic studies have observed greater similarity in gene expression between homologous tissues from different vertebrate species than between diverse tissues of the same species (Sudmant et al., 2015). To some extent this holds true even at a level of amino acid profiles, where liver, kidney and brain are very much alike in amino acid composition but differ from stomach and lung in cystine, tryptophane, tyrosine and phenylalanine (Beach et al., 1943). As a result, tissue specification via proteomics emerges as one of the most attainable near-future goals within this domain. Within this context, proof-of-concept experiments were carried out, focusing on muscle tissues extracted from diverse species relevant to the food and feed sector (i.e. fish, pig, ruminant, poultry and crustacean). These were analysed alongside individual single-tissue samples sourced from salmon. This design aimed to gain insight whether non-target proteomics data could discern pronounced differences between tissues, enabling comparisons not only across species but also taxa. Detailed findings beyond the scope of this report have been omitted to prevent potential copyright conflicts, as these results are intended for publication in scientific journals.

With a solid foundation in the theoretical and practical aspects of MS-based proteomics, the fellow also embarked on the development of standard operating procedures (SOPs) at the hosting site. This involved devising SOPs for two distinct workflows: (i) a multi-purpose non-targeted bottom-up proteomics workflow intended for general application in food and feed samples and (ii) a targeted proteomics approach tailored for identifying prohibited materials in PAP. The former was curated by amalgamating methodologies from previous IMR research endeavours, while the latter was adapted from the inter-laboratory study on detecting adulterated PAP via MS-based proteomics, organised in 2019 by EURL AP, which included the participation of IMR among six laboratories (Lecrenier et al., 2021).

The final objective of this fellowship was the task of adapting the complete proteomics protocols implemented at the IMR for use at the fellow's home institution, BIOR. Despite BIOR's resourceful inventory of MS systems, the implementation of MS-based proteomics had not been previously undertaken in house. This transfer of protocols was executed in stages and followed a step-wise implementation described in (Varunjikar et al., 2022a,b).

Initially, the adapted method was evaluated based solely on instrumental capabilities, analysing varying amounts of HeLa digest. Data post-processing was performed against the UniProt human reference proteome (up000005640) using Comet search via TPP. Subsequent refinement of data through PeptideProphet and ProteinProphet was omitted. These results were then compared to analogous HeLa measurements conducted using the IMR's in-house method within the Varunjikar et al., 2022a study (massIVE ID: MSV000088034). Figure 3 (panel A) shows that the adapted instrumental method exhibited reduced sensitivity and required higher amount of HeLa to achieve a similar count of peptide and protein matches.

Moreover, additional experiments compared the instrumental method and the adapted sample preparation protocol through four scenarios. In scenarios 1 and 2, insect samples from various species (e.g. Black soldier fly, Yellow mealworm, Lesser mealworm) underwent FASP protocol at BIOR (scenario 1) and IMR (scenario 2). These extracts were analysed in BIOR and the data were processed by Comet search in TPP with reviewed Arthropoda sequences (accessed July 2023). Significant differences between samples prepared at both facilities were not observed (Figure 3, panel B). However, given that HeLa analysis indicated the BIOR's instrumental method is less sensitive than IMR's in-house method, it was essential to estimate the extent of this shortcoming. Thus, a batch of salmon tissue samples were prepared at the IMR by the fellow and the extracts were analysed on both LC–MS systems (scenario 3: BIOR, scenario 4: IMR). Data were processed in TPP via Comet search against Atlantic salmon proteome (UP000087266). Results confirmed that the LC–MS method at the IMR was superior yielding nearly double data compared to BIOR. Lastly, an independent insect dataset from Varunjikar et al., 2022a (massIVE ID: MSV000088034) was used to assess the overall performance of the transferred method. Similar proteomics post-processing pipeline was applied to the raw data as in scenarios 1 and 2. The number of features obtained from the study's data were around two times higher compared to data from insect samples that were independently prepared and analysed in BIOR by the fellow (Figure 3, panel B). However, the data fall within the same range as in scenario 4 and, hence, it becomes evident that sample preparation is not the key issue for the adapted method and the main shortcomings are related to the instrumental method. This could be partly attributed to the variance in column lengths (150 mm at BIOR vs. 250 mm at IMR). Future improvement of the method would entail fine-tuning the scanning and ionisation parameters of the Q Exactive Orbitrap MS system, and considering the possibility of increasing the protein load per injection.

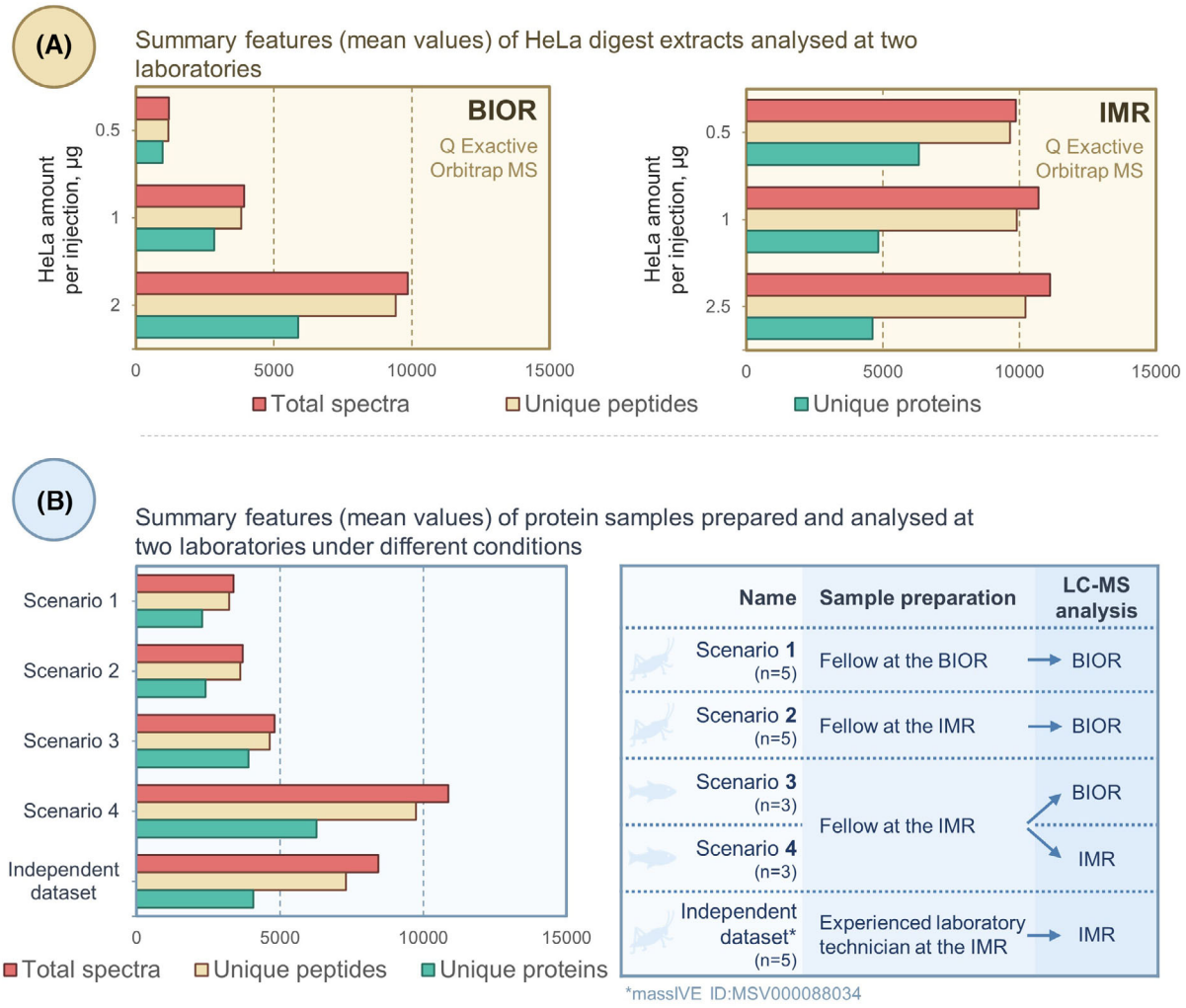

Figure 3: Comparative analysis of adapted instrumental method and sample preparation protocol (panel A – results from HeLa digest analysis, panel B – results from real sample analysis)

2.3. Secondary activities

In addition to the direct implementation of the work programme and participation in the five EU-FORA risk assessment training modules, the fellow engaged in extracurricular activities. On 10 March, 2023, the fellow attended Madhushri Shrikant Varunjikar's thesis defence, titled 'Proteomic Tools for Food and Feed Authentication', at the University of Bergen (Varunjikar, 2023). From 23 to 24 May, 2023, the fellow participated in a remote workshop organised by EURL-AP, which delved into MS-based proteomics approaches for use in regulatory settings for PAP detection. To further enhance practical skills, the fellow assisted in preparation of cod liver samples for proteomics analysis performed under the auspice of a ClimSeaFood research project (Norwegian Research Council; project number: 324374) at the IMR which focuses on the effects of climate change on marine eco-system and seafood safety. On 7 July, 2023, the fellow and the supervisor attended a brainstorming workshop for future joint project applications organised by the Unit Effect-based Analytics and Toxicogenomics of the German Federal Institute of Risk Assessment (BfR); the meeting focused on alternative protein sources, including the application of omics for tracing and risk assessment of novel protein sources.

3. Conclusions

Considering the evolving landscape of the European food and feed sectors, new avenues have emerged, such as the ascent of alternative protein sources as novel foods, shifts in PAP legislation, new aquafeed ingredients, GM ingredients and increased demand for aquaculture products. In all of these instances, proteins play a significant role within the risk assessment framework and, therefore,

the demand for advanced techniques to rigorously assess the safety, authenticity and traceability of protein materials has surged. Furthermore, these approaches must be fit for purpose for future feed safety risk assessments needs. Amidst this context, MS-based proteomics have surfaced as one key NAM and have already demonstrated their utility across various applications including, the detection of prohibited substances in feed, allergen identification and differentiation of species and tissues within intricate mixtures.

Within the framework of this EU-FORA programme, three central objectives were addressed. Firstly, the programme equipped the fellow with the skills to employ MS-based proteomics for food and feed safety analyses. Secondly, it contributed to SOP development for both targeted and non-targeted proteomic workflows that greatly benefitted the implementation of these techniques in both hosting site and home institution. The latter method was successfully transferred to the fellow's home institution, with the intent of evaluating the origin of proteinaceous material in food and feed. This directly aligns with the EU FORA programme's goals – facilitating knowledge exchange for a unified EU risk assessment strategy. Lastly, the programme endeavoured to conceptualise and implement a proof-of-concept strategy, applying MS-based proteomics to discern tissue and species-specific attributes within samples of animal origin. Collectively, this programme expands the realm of MS-based proteomic methodologies, enhancing their relevance and availability in the domains of food and feed safety.

4. Disclaimer

Detailed results obtained from the sample analysis are not included in this report to avoid certain copyright claims, as these results are intended for subsequent publication in peer-reviewed articles.

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Abbreviations

BIOR	Institute of Food Safety, Animal Health and Environment 'BIOR', Latvia
DTT	dithiothreitol
EU-FORA	The European Food Risk Assessment Fellowship Programme
EURL AP	European Reference Laboratory for Animal proteins in feedingstuffs
FAIR	an acronym for data which meet principles of findability, accessibility, interoperability and reusability
FASP	filter-aided sample preparation
FDR	false discovery rate
GMO	genetically modified organism

HPLC	high-performance liquid chromatography
IAA	iodoacetamide
IMR	Institute of Marine Research, Norway
LC–MS	liquid chromatography coupled to mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MWCO	molecular weight cut-off
NAM	new approach methodology
nano-LC	nanoflow liquid chromatography
PAP	processed animal protein
PSM	peptide-spectrum match
qPCR	real-time polymerase chain reaction
RA	risk assessment
SDS	sodium dodecyl sulphate
SOP	standard operating procedure
SP3	single-pot, solid-phase-enhanced sample preparation

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

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Abstract

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

Keywords: QMRA, R programme, Salmonella, heat elimination

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Summary

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

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1. Introduction

1.1. Fellow's personal background

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

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

1.2. Heat elimination of different *Salmonella* strains

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

1.3. Food safety of 'smoothies'

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

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

1.4. Quantitative microbiological risk assessment

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

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

2. Description of work programme

2.1. Practical work in the laboratory

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

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

2.1.1. Participation in ongoing projects at the host institution

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

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

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

2.1.2. Own research

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

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

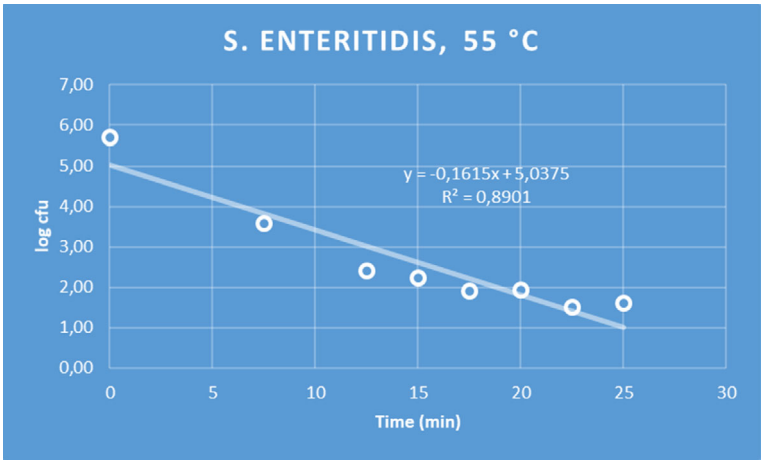

Figure 1: Heat inactivation of *S*. Enteritidis at 55°C

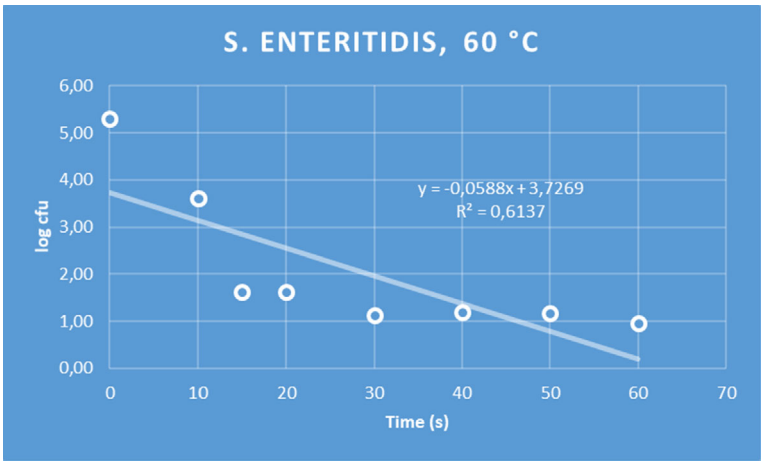

Figure 2: Heat inactivation of *S*. Enteritidis at 60°C

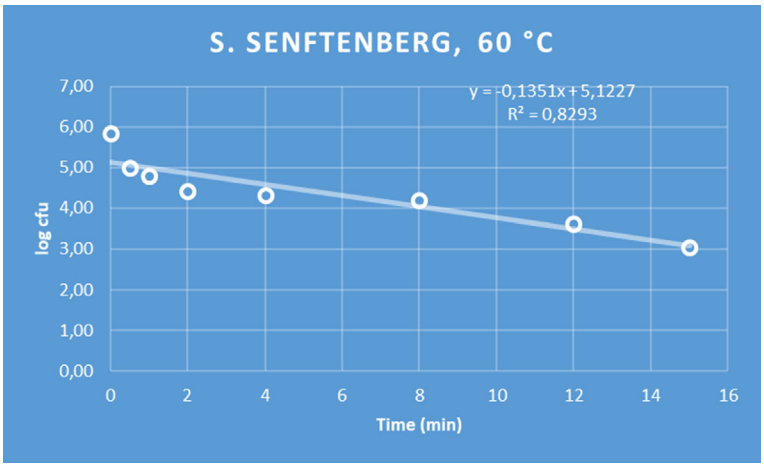

Figure 3: Heat inactivation of *S*. Senftenberg at 60°C

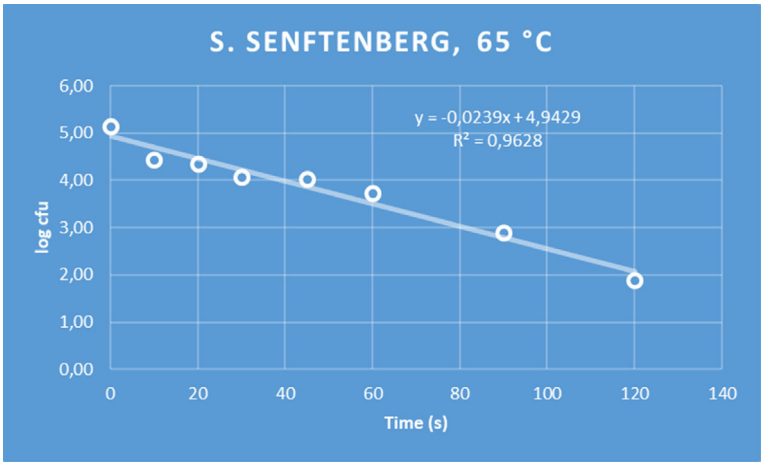

Figure 4: Heat inactivation of *S*. Senftenberg at 65°C

2.2. Theoretical work

2.2.1. Analysis of the inactivation data obtained

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

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

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

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

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

2.3. Extra activities

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

17 January

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

18 January

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

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

3. Conclusion

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

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

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

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Abbreviations

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

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Risk assessment of food contact materials

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Abstract

Bisphenol A (BPA) is authorised for use as a chemical compound for the production of plastic food contact materials (FCMs) under Regulation (EU) No 10/2011. But according to requirements of the Regulation (EU) No 2018/213, BPA has been banned in the manufacture of polycarbonate drinking cups or feeding bottles intended for infants and young children. Food has been identified as the main source of human exposure to BPA, followed by dermal absorption, air and dust inhalation, revealing ubiquitous and continuous contact with BPA. Considering that BPA is able to enter the food chain through the migration from food packaging into foodstuffs, assessment of dietary exposure is necessary for accurate estimations and identification of potential exposure from food sources. In 2015, EFSA set a temporary tolerable daily intake (TDI) for BPA of 4 μg/kg body weight (bw) per day and concluded that no health concern from BPA exposure for any age group was to be expected. In 2023, EFSA has re-evaluated BPA safety and the new TDI was reduced by a factor of 20,000 resulting in a TDI of 0.2 ng/kg bw per day. In this case, the CEP Panel concluded that there is a health concern from dietary exposure to BPA. Amongst others, the BfR identified several points of criticism which, in the opinion of the BfR, call into question the risk assessment carried out by EFSA. The BfR derived a TDI of 200 ng/kg bw per day and suggests taking this into account for risk assessment. In the proposed EU-FORA programme, the fellow had the opportunity to gain experience in the exposure assessment and then integrate the data together with the BfR hazard assessment to perform a comprehensive risk assessment. As second objective of the work programme, the fellow was in charge of performing a toxicokinetic analysis in an attempt to correlate external exposure with urinary BPA levels.

Keywords: bisphenol A, chemical exposure assessment, food consumption, food contact materials

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1. Introduction

Bisphenol A (BPA) is a chemical compound produced in high amounts by the plastic manufacturing industries since 1950 (Akash et al., 2020). BPA is widely used as a basic component for production of polycarbonate plastics and epoxy resins, which are found in a broad range of daily consumer products such as food storage containers, personal care products, kitchenware, toys, thermal paper, dental composites and electronic devices, as well as in inner coating of canned products and jar caps (Hartle et al., 2016; Ramírez et al., 2021). It reveals the ubiquitous and continuous human exposure to BPA.

Food has been identified as the main contributor to BPA exposure of humans, followed by dermal absorption, air and dust inhalation (Rubin et al., 2019). The overall BPA exposure results in quantifiable levels in biological samples including urine, saliva, blood, placenta, breast milk and umbilical cord serum (Berge et al., 2017; Lee et al., 2018). BPA is able to migrate from food contact materials (FCMs) into foodstuffs, and EFSA identified FCM as the main source for BPA entering the food chain (EFSA CEF Panel, 2015).

Evidence from animal and human observational studies has linked BPA exposure to several adverse effects, including reproductive, developmental, cardiovascular, metabolic, immuno, respiratory, renal and hepatic toxicities (Ma et al., 2019). Therefore, BPA is a multitarget compound displaying multiorgan system effects, but the underlying biological mechanisms by which BPA predisposes to disease development remain uncertain in humans. Endocrine disruption has been shown to play an important rule for some of the effects (e.g., reproductive toxicity). BPA analogues (BPS, BPF, BPB, BPE and BPAF) are being utilised as BPA alternatives, but they are structurally similar to BPA and have been found to also show endocrine disruption based on in vivo and/or in vitro studies (Barboza et al., 2020; Heindel et al., 2022). However, there is much less data on these compounds compared to BPA. So far, tolerable daily intake (TDI) has only been established for BPA and there are limited data on exposure to BPA analogues. Future efforts to sample for BPA and its analogues are required to gain a better understanding of current status of overall exposure to bisphenols in all population groups.

In 2015, EFSA set a temporary TDI for BPA of 4 μg/kg bw per day and concluded that no health concern from BPA exposure for any age group was to be expected (EFSA CEF Panel, 2015). Nonetheless, EFSA has recently published a re-evaluation of BPA safety in which the TDI was reduced by a factor of 20,000 resulting in a TDI of 0.2 ng/kg bw per day. No current exposure estimation was performed. However, with respect to the low value, the new TDI is expected to be exceeded by all age groups, and the CEP Panel concluded that there is a health concern from dietary exposure to BPA (EFSA, 2023). Amongst others, the BfR identified several points of criticism which, in the opinion of the BfR, call into question the risk assessment carried out by EFSA (BfR, 2022). Therefore, the BfR derived a TDI of 200 ng/kg bw per day (20-fold lower than the former value of EFSA, 2015) and suggests taking this into account for risk assessment (BfR, 2023).

The main objective of this technical report within the EU-FORA work programme 'Risk Assessment of Food Contact Materials' was the estimation of the daily dietary intake of total BPA for Spanish children, adolescents and adults, and the comparing it to the TDI derived by the BfR in 2023.

Likewise, after oral intake in humans, BPA is rapidly transformed into highly hydrophilic BPA-glucuronide by the liver and excreted mainly via urine (Ramírez et al., 2021). The biological half-life of BPA is less than 6 h, and it is totally eliminated from the body in 24 h. Therefore, total urinary BPA excretion (free or unconjugated plus conjugated BPA) can be used as a biomarker tool to reflect the daily dietary BPA exposure (EFSA CEF Panel, 2015; Peng et al., 2019). As second part of the work programme, the fellow was in charge of performing a toxicokinetic analysis in an attempt to correlate external exposure with BPA levels measured in urine.

2. Data and methodologies

2.1. Description of work programme

As part of the EU-FORA fellowship, this study was focused on acquiring knowledge on how to perform a risk assessment for a compound related to FCM based on existing exposure estimates. The experimental work of this project was developed within Institute of Nutrition and Food Technology (INYTA) of the University of Granada (Spain); and the data processing and statistical treatments were carried out at the Department of Chemicals and Product Safety of the BfR with the support of experts in risk and toxicological assessment.

In this way, the fellow was involved in all the activities and methods required to collect, process, and analyse datasets (Figure 1). In the first place, the fellow participated in the recruitment of children; collection of anthropometric measurements and food surveys; and biological sampling. Later on, the fellow was involved in the design of databases containing the food consumption of each participant, chemical determination of BPA in different food matrices and estimation of individual dietary exposure to BPA from each food consumption questionnaire. Besides exposure assessment, the fellow was engaged to address the agreement between urine levels of BPA and external exposure through the dietary intake.

The activities performed and methods applied during the programme are listed below:

1) Recruitment of children collecting all the necessary information: sociodemographic characteristics, dietary recalls, anthropometric data and questions related to different sources of exposure to BPA at home.
2) Preparation of a database reporting the food consumption and concentrations of BPA in each food needed to perform the exposure assessment in children, adolescents and adults.
3) Urine sampling collection, treatment and determination of BPA.
4) Software tools required to data processing and interpretation.

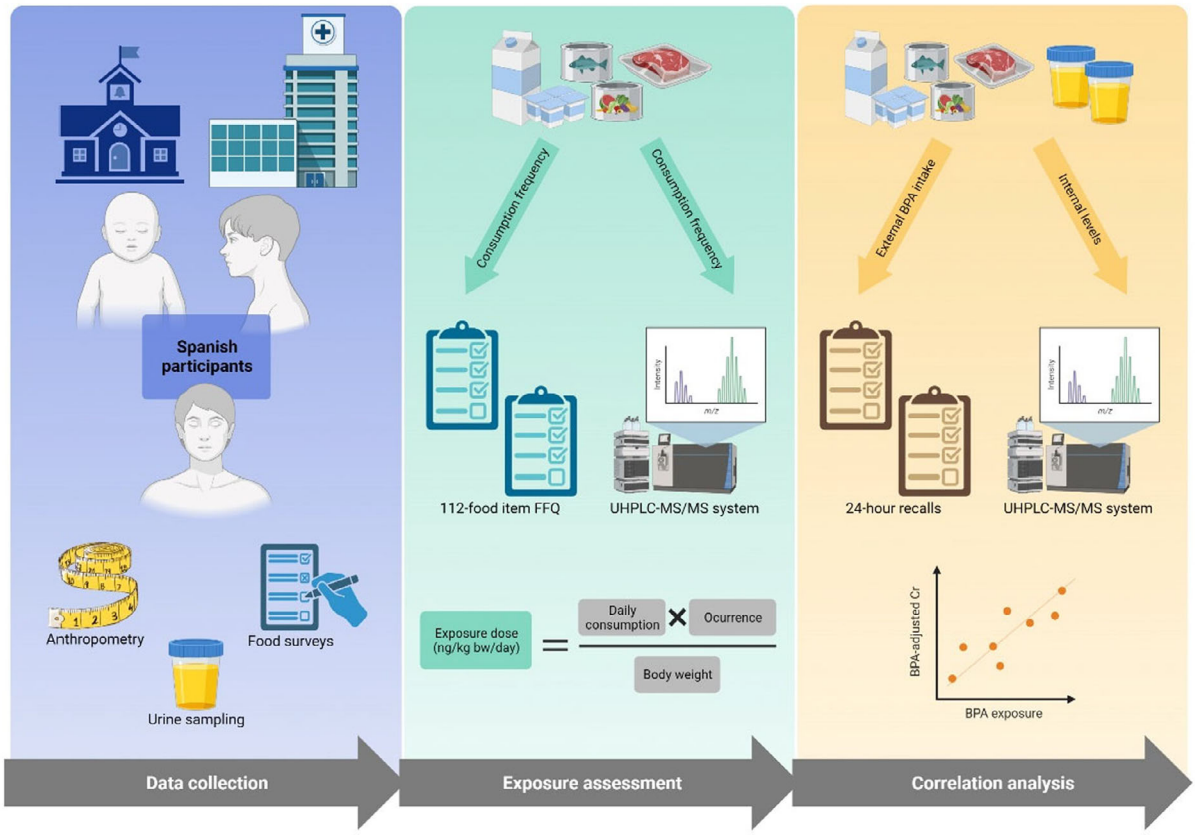

Figure 1: Work programme workflow

2.2. Materials and methods

2.2.1. Study subjects

The study populations included in this report formed part of different previous projects awarded to the research group (GP/EFSA/ENCO/2018/03, PI20/01278 and PE-0250-2019). Children were recruited from different elementary schools and primary care centres in Granada (Spain) between 2020 and 2023. Adolescents and adults from Toledo (Spain) were recruited in 2017–2018. Both projects were approved by Ethics Committee of Provincial Biomedical Research of Granada (CEI).

Then, participants were selected according to the availability of data on weight, height and records on dietary exposure to BPA. The age ranges for children, adolescents and adults were established

following the Spanish Dietary Datasets ENALIA 1 (National Dietary Survey on Children and Adolescents) and ENALIA 2 (National Food Survey on Adults, the Elderly and Pregnant women) that have been included within EFSA Comprehensive European Consumption Database. In this way, approximately 500 participants aged 3 to 39 years were selected for the present exposure assessment. The anthropometric measurements were taken at each follow-up visit using calibrated electronic scales and a wall-mounted stadiometer.

2.2.2. Exposure assessment

Total dietary exposure to BPA (ng/kg of body weight (bw) per day) was calculated on an individual basis, following the next steps: (1) estimation of daily intake of different foods (g/day), (2) determination of mean BPA concentration found in these foods (ng/g of food), (3) calculation of daily BPA intake through different food items (ng/day), and (4) calculation of individual overall daily BPA exposure.

Estimation of daily food intake

A semi-quantitative food frequency questionnaire (FFQ) was completed by each participant thorough a face-to-face interview. The FFQ has shown to be the most convenient dietary assessment tool for estimating exposure to food contaminants in epidemiology (Notario-Barandiaran et al., 2020). Considering geographical, cultural and age differences, the FFQ should be validated for each specific population. In our case, 24-h dietary recalls (24H-R) were previously used as validation method (Robles-Aguilera et al., 2021). The FFQ was designed to ask about 112 food items classified into 13 categories, e.g. dairy products, meat and meat products, vegetables and cereals.

Consumption frequency was categorised as never or hardly ever, once a week, 2–4 times per week, 5–6 times per week, once a day, 2–3 times per day, 4–6 times per day and more than 6 times per day. The type of food packaging (plastic, glass, metal or cardboard) was also recorded. Then, mean daily intake (g/day) was obtained by multiplying the consumption frequency of each item (servings/day) by its corresponding portion size (g/serving). The recommended amounts of each food group are established for Spanish children, adolescents and adults (Monteagudo et al., 2021).

Determination of BPA concentration found in food

Based on FFQ responses, the foods most frequently consumed by the whole population were identified by stepwise regression model. This analysis shown those foods providing more than 95% of daily energy intake; consequently, they were purchased from different national and local supermarkets and their BPA content was chemically analysed via ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) system, according to the methodology previously described Galvez-Ontiveros et al. (2021). For left-censored data, i.e. samples with concentrations below the LOD or LOQ, the lower bound (LB) and upper bound (UB) approaches were used.

Calculation of daily BPA intake through different food items

BPA content (ng/g) after applying both substitution methods was multiplied by the daily food intake (g/day) for all individual study participants. This resulting daily BPA intake per food item (ng/day) was divided by body weight (kg) obtaining the daily dietary exposure to BPA for all food items (ng/kg bw per day) for each participant. Afterwards, food items were grouped into different food categories according to EFSA Food Classification (Level 1 of exposure hierarchy) (EFSA, 2015) in order to identify the greatest contributor to the total exposure to BPA.

Calculation of overall BPA exposure for all study participants

For all individuals, total exposure dose was estimated by summing up BPA exposure from all food items. Afterwards, statistical analysis was performed for the different age groups in order to compare the outcome between different age groups and to the BfR TDI.

In addition, first steps to include the exposure data in the risk assessment, both by deterministic and probabilistic approaches, were undertaken.

2.2.3. Correlation analysis

The goal of the second part of the work programme was to evaluate the correlation between the dietary exposure and the urinary BPA levels on an individual basis. For this analysis, all children aged 3–13 years with available urinary BPA and creatinine levels, dietary and anthropometric records were

selected. External exposure through dietary intake was estimated from the 24H-R following the same procedure as for the FFQs. On the other hand, a urine sample from each participant's first morning void was collected in a sterile polyethylene container and stored at −80°C until analysis. Total BPA was extracted as previously described by Moscoso-Ruiz et al. (2022). Each sample was analysed in duplicate, with and without glucuronidase pre-treatment in order to receive values for total and free BPA. In the non-enzymatic process, 4 mL of urine, 4 mL of NaCl aqueous solution (10%, w/v) and 100 μL of HCl (6 N) were mixed to pH 2. The dispersive liquid–liquid microextraction was followed. A mixture of 400 μL of acetone plus 600 μL of chloroform was rapidly injected into the urine sample. The low phase was collected after vortexing and centrifugation. This step was repeated four times, and the final organic layer was evaporated to dryness. The solid residue was reconstituted in ultrapure water/MeOH mixture (80:20; v/v), centrifuged and directly injected into the UHPLC–MS/MS system. For the enzymatic treatment, 4 mL of urine was incubated with 25 μL of β-glucuronidase/sulfatase and 100 μL of β-glucuronidase for 24 h at 37°C. Then, the protocol follows as the free form.

Urinary BPA concentrations were adjusted by creatinine content and body weight (μg/g Cr per kg of body weight). Creatinine in urine was determined by Ángel Méndez Soto Clinical Analysis Laboratory (Granada, Spain). After this adjustment, correlations of external exposure to BPA and internal adjusted levels were tested by Spearman's rank correlation.

3. Conclusion

The EU-FORA programme was a great opportunity for the fellow to go deep into chemical exposure assessment of FCM. The fellow acquired theoretical and practical knowledge from expert assessors on FCM safety. This programme offered the fellow the opportunity to learn about the different methods for assessing dietary exposure to food contaminants and how to analyse the datasets by putting her data science related knowledge into practice.

Importantly, the programme has not only allowed the fellow to deal in real exposure data, but has also been a special stay to build connections with outstanding professionals in the field of Food Risk Assessment, leading to promising improvements on the analyses in the near future.

In addition to the work at INYTA and BfR, the fellow attended to the five training modules organised by EFSA, AGES and BfR. The additional scientific activities developed during the fellowship are detailed in Appendix A.

4. Disclaimer

The results of the exposure assessment and correlation study 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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Abbreviations

24H-R	24-h dietary recall
AESAN	Spanish Agency for Food Safety and Nutrition
AGES	Agency for Health and Food Safety
BfR	Bundesinstitut für Risikobewertung
BPA	bisphenol A
BPAF	bisphenol AF
BPB	bisphenol B
BPE	bisphenol E
BPF	bisphenol F
BPS	bisphenol S
bw	body weight
CEI	Ethics Committee of Provincial Biomedical Research of Granada
Cr	creatinine
D	detected
ENALIA 1	National Dietary Survey on Children and Adolescents
ENALIA 2	National Food Survey on Adults, the Elderly and Pregnant women
FCM	food contact material
FFQ	food frequency questionnaire
HCl	hydrochloric acid
INYTA	Institute of Nutrition and Food Technology
LB	lower bound
LDR	linear dynamic range
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
NaCl	sodium chloride
ND	not detected
TDI	tolerable dietary intake

UB	upper bound
UHPLC–MS/MS	ultra-high-performance liquid chromatography–tandem mass spectrometry system
WHO	World Health Organization

Appendix A – Scientific activities

Event	Title	Contribution	Location	Date
Congress	XL Congress of the Spanish Society of Physiological Sciences. Joint meeting between Spanish and Portuguese physiologists	Poster	Badajoz, Spain	19–22 September 2022
Congress	XXI Scientific Meeting of the Spanish Society of Chromatography and Related Techniques	Oral communication	Almería, Spain	25–27 October 2022
Congress	'XIV Congreso Español de Toxicología y VIII Iberoamericano'	Poster	Córdoba, Spain	11 November 2022
Congress	'III Jornadas de Jóvenes Investigadores'	Poster	Granada, Spain	17–18 November 2022
Meeting	Spanish Agency for Food Safety & Nutrition (AESAN) Meeting	Attendance	Madrid, Spain	17–18 January 2023
Congress	'XXVII Jornadas Internacionales de Nutrición Práctica/XVI Congreso Internacional de SEDCA'	Poster	Madrid, Spain	15–16 March 2023
Congress	'V Jornada de Avances en Investigación en Epidemiología y Salud Pública'	Poster	Granada, Spain	23 June 2023

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Quantitative tools in microbial and chemical risk assessment

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Abstract

The EU-FORA programme 'Quantitative tools in microbial and chemical risk assessment' was dedicated to training on predictive microbiology fundamentals, implementation of different modelling strategies, design of experiments and software tools such as MATLAB, GInaFiT and DMFit. The fellow performed MATLAB training on maximum specific growth rate (μ_{max}) determination according to the Ratkowsky model. GInaFiT training on different models for bacterial inactivation and DMFit training on growth parameters of *Vibrio parahaemolyticus* were also carried out. Optical density measurements of *V. parahaemolyticus* bacterial cultures were performed. The obtained kinetics of optical density measurements were used to estimate μ_{max}. Hereafter, Minimum inhibitory concentrations and non-inhibitory concentrations of aminoglycoside antibiotics were estimated based on the quantification of the fractional areas of the optical density vs time. It can be concluded that the results of the quantitative characterisation of *V. parahaemolyticus* are reliable and can be used for exposure assessments. Also, the turbidimetric assay can be applied for successful estimation of minimum inhibitory concentrations and non-inhibitory concentrations.

Keywords: predictive microbiology, risk assessment, software tools, maximum specific growth rate, minimum inhibitory concentrations, non-inhibitory concentrations, *Vibrio parahaemolyticus*

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Summary

The final report of the EU-FORA fellowship programme 'Quantitative tools in microbial and chemical risk assessment' hosted by the National and Kapodistrian University of Athens, and supervised by Prof. Vasilis P. Valdramidis and Prof. Charalampos Proestos is dedicated to summarising the activities of the fellow Deyan Stratev during programme implementation. The fellow is currently working as an associate professor in food safety and his scientific background is related to seafood microbiology. The introduction describes an outline of the working programme and its aims which included training in predictive microbiology fundamentals, the implementation of different modelling strategies, the design of experiments, training with the use of three different software tools (MATLAB, GInaFiT and DMFit) selected according to fellow's research topic and provided with an overall understanding of the main differences between modelling growth or inactivation models. Conclusions provide the main achievements as a result of EU-FORA programme training while some recommendations are also suggested.

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1. Introduction

1.1. Description of the work programme

The EU-FORA training programme 'Quantitative tools in microbial and chemical risk assessment' was designed to train fellow Deyan Stratev in the use and development of predictive models for assessing the bacterial responses for survival, growth and the probability of microbial recovery. The assessments included microbial responses under different food processing and preservation scenarios. Applications were performed by programming in MATLAB while analysis and interpretation of data were considered as inputs in microbial and chemical risk assessment studies. By the end of the activity, the trainee attained a fundamental understanding of the substantial body of applied modelling, statistics and recent developments in the field of predictive modelling and quantitative risk assessment of foods in the area of microbiology and chemistry. The trainee has also exercised personal responsibility and autonomous initiative in solving complex food safety, spoilage and chemical problems that are solved in a rigorous and sound approach. He has exploited available software packages and quantitative approaches for enriching current studies in the field in order to communicate results and innovations of research to peers.

1.2. Aims

The overall aim of the EU-FORA training programme 'Quantitative tools in microbial and chemical risk assessment' was to:

i) develop trainee's capacity to design and generate informative experimental data;
ii) understand model structure development and selection to describe quantitatively chemical, and microbiological phenomena and develop capabilities for quantifying accurately the sources of stochasticity;
iii) make trainee familiar with optimisation software and model simulation tools that can be exploited for developing decision-making and quantitative risk assessment in food microbiology and chemistry.

1.3. Additional activities for network widening

The fellow participated in various events to improve his multidisciplinary work and research activities. He also participated in several secondary activities to meet these goals during the EU-FORA programme, which are described in Appendix A.

2. Training

2.1. Training on software

There are two possible ways to organise data processing in scientific research. One of them is MATLAB software, which has a pre-made code and can be tailored to the needs of the project. Another option is to use Excel and possibly some additional features such as GInaFiT or DMFit. These give better support if users are very familiar with the predicative microbiology topic.

2.1.1. MATLAB

MATLAB is a versatile tool used in numerous scientific fields, e.g. engineering, data analysis, algorithm development, etc. With it, one can analyse data, visualise it and create scripts. When used with a pre-written code, MATLAB can simulate different scenarios in real cases. An example of MATLAB implementation was how to determine the maximum specific growth rate (μ_{max}) according to the Ratkowsky model (Figure 1).

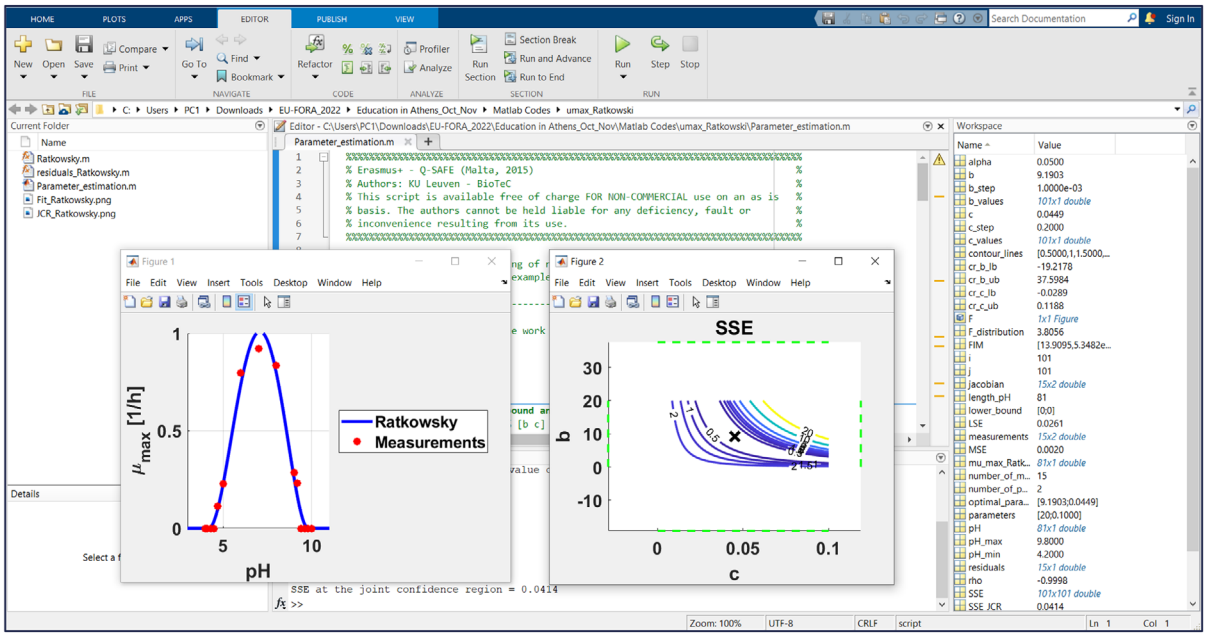


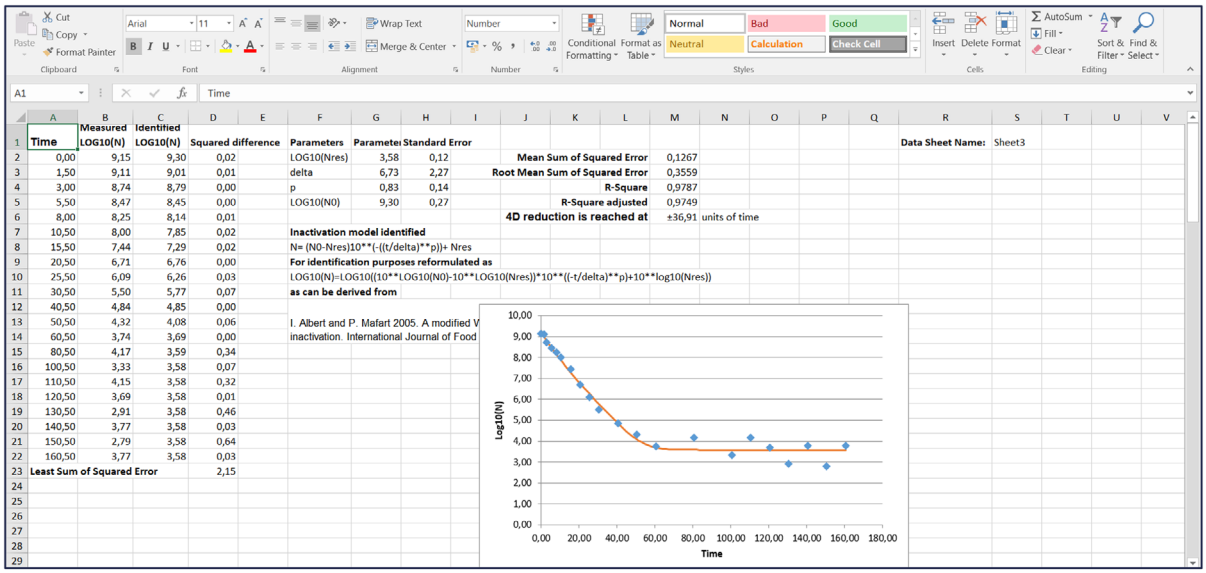
Figure 1: MATLAB training example on maximum specific growth rate (μ_{max}) determination according to the Ratkowsky model by the use of Q-Safe book (Valdramidis et al., 2017)

2.1.2. Microsoft® Excel add-ins – GInaFiT

Excel can help with many calculations and GInaFiT can help to simplify some of the calculations and even prevent typical mistakes in data processing (Figure 2). The tool is efficient in testing 10 types of microbial survival models relating to the growth of the microbial population with time (Geeraerd et al., 2005). These are:

1) classical log-linear curves,
2) curves displaying a shoulder before a log-linear decrease,
3) curves displaying a tail after a log-linear decrease,
4) survival curves displaying both shoulder and tail,
5) concave curves,
6) convex curves,
7) convex/concave curves followed by tailing,
8) biphasic inactivation kinetics,
9) biphasic inactivation kinetics preceded by a shoulder,
10) curves with a double concave/convex shape.

The models were originally published by Bigelow and Esty (1920), Cerf (1977), Geeraerd et al. (2000, 2005), Mafart et al. (2002), Albert and Mafart (2005), and Coroller et al. (2006). Along with the obtained parameter values, some statistical measures are automatically given: standard errors of the parameter values, the sum of squared errors, the (root) mean sum of squared errors, the R2 and the adjusted R2. In addition, t4D, the time needed for a 4 log reduction of the initial microbial population, as originally proposed by Buchanan et al. (1993), is also automatically reported (for data sets covering at least 4 decimal reductions).



Time	Measured LOG10(N)	Identified LOG10(N)	Squared difference
0,00	9,15	9,30	0,02
1,50	9,11	9,01	0,01
3,00	8,74	8,79	0,00
5,50	8,47	8,45	0,00
8,00	8,25	8,14	0,01
10,50	8,00	7,85	0,02
15,50	7,44	7,29	0,02
20,50	6,71	6,76	0,00
25,50	6,09	6,26	0,03
30,50	5,50	5,77	0,07
40,50	4,84	4,85	0,00
50,50	4,32	4,08	0,06
60,50	3,74	3,69	0,00
80,50	4,17	3,59	0,34
100,50	3,33	3,58	0,07
110,50	4,15	3,58	0,32
120,50	3,69	3,58	0,01
130,50	2,91	3,58	0,46
140,50	3,77	3,58	0,03
150,50	2,79	3,58	0,64
160,50	3,77	3,58	0,03
Least Sum of Squared Error			2,15

Parameters	Parameter	Standard Error
LOG10(Nres)	3,58	0,12
delta	6,73	2,27
p	0,83	0,14
LOG10(N0)	9,30	0,27

Mean Sum of Squared Error	0,1267
Root Mean Sum of Squared Error	0,3559
R-Square	0,9787
R-Square adjusted	0,9749
4D reduction is reached at	±36,91 units of time

Inactivation model identified
N= (N0-Nres)10**(-((t/delta)**p))+ Nres
For identification purposes reformulated as
LOG10(N)=LOG10((10**LOG10(N0)-10**LOG10(Nres))*10**((-t/delta)**p)+10**log10(Nres))
as can be derived from

I. Albert and P. Mafart 2005. A modified V
inactivation. International Journal of Food

Data Sheet Name: Sheet3

Figure 2: GInaFiT example on a modified Weibull model for bacterial inactivation

There are two approaches in using the tool depending on whether users have or do not have an idea of the general shape of their survival curves. The time for a 4-decimal reduction can be useful to summarise the information present in a data set, for example if a common survivor curve shape cannot be selected for a range of different conditions tested (Geeraerd et al., 2000).

The tool is also equipped with some built-in features testing for misuse, e.g. when the number of data points (observations) used is too limited in comparison with the number of parameters in the model type chosen (the number of parameters ranges from 2 to 5 for the 10 model types available).

2.1.3. Microsoft® Excel add-ins – DMFit

DMFit is a tool that can be found online at https://browser.combase.cc/DMFit.aspx or added as a Microsoft® Excel add-in. The web edition is a web-based application compliant with bacterial curves. In it, a linear phase is preceded and followed by a stationary phase. The desktop version is used to model the time variation of the logarithm of cell concentrations of bacterial batch cultures. DMFit can be used to present in a visual mode growth or survival data, obtain parameters estimates from data consistent with growth or survival models, calculate growth or death rate, lag phase time, initial or final cell count and estimate standard errors on these parameters (Figure 3).

Data input is fairly easy since it is compatible with Excel spreadsheets or text files (in online and desktop versions). Data can be inserted in two different types of models:

a) Model of Baranyi and Roberts (1994) – describes a sigmoid bacterial curve. This model, unlike other sigmoid curves, has very close to linear mid-phase. It has four main parameters (initial value, lag, maximum rate and final value) and two curvature parameters: mCurv and nCurv which describe the curvature of the sigmoid curve, respectively, at the beginning and at the end of the growth phase. This tool allows to describe only the growth or death at the stationary phase, if mCurv parameter is set to zero and 'no lag' model option is selected. The model can also describe only the lag phase and growth or death phase if nCurv is set to zero and 'no asymtot' model option is selected.
b) Trilinear, biphasic or linear models. The trilinear model describes a bacterial growth curve with three straight lines: the lag phase and the stationary phase are presented by two horizontal straight lines. The slope of the third straight line describing the growth/death phase is called the 'maximum rate'. The biphasic model must be used when a data-based curve has no lag phase or no stationary phase. The linear model can be used in case bacterial counts describe only growth or death phase.

In the tool time is given in hours, bacteria count in $\log_{10}$ cfu/g and maximum growth or death rate is given as $\log_{10}$ cfu/g/h. After loading data, it must be ensured that units are aligned accordingly.

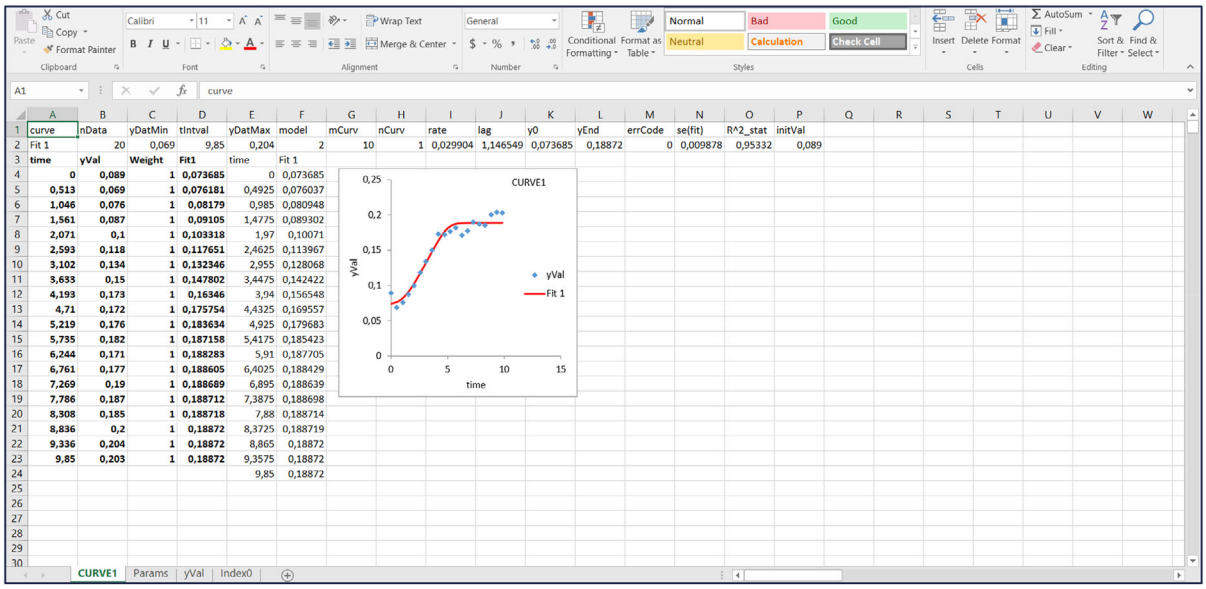


Figure 3: DMFit training example on growth parameters of *V. parahaemolyticus*

3. Case study

3.1. Data collection

OD measurements of *Vibrio parahaemolyticus* bacterial cultures were performed by means of a microplate reader at 630 nm. The obtained kinetics of OD measurements were used to estimate the growth rate of the cells, i.e. μ_{max}. Hereafter, MIC and NIC of aminoglycoside antibiotics (i.e. gentamicin and tobramycin) for the slowest and highest growers were estimated based on the quantification of the fractional areas of the optical density versus time.

3.2. Quantitative evaluations

The μ_{max} (h^{-1}) of *V. parahaemolyticus* isolated from Mediterranean mussels, veined rapa whelk, Mediterranean horse mackerel, oysters, Gilthead bream, Atlantic salmon, bluefish, European seabass ranged from 0.73 to 2.33. The highest grower was isolated from Atlantic salmon while the slowest one was recovered from Mediterranean mussels (Figure 4).

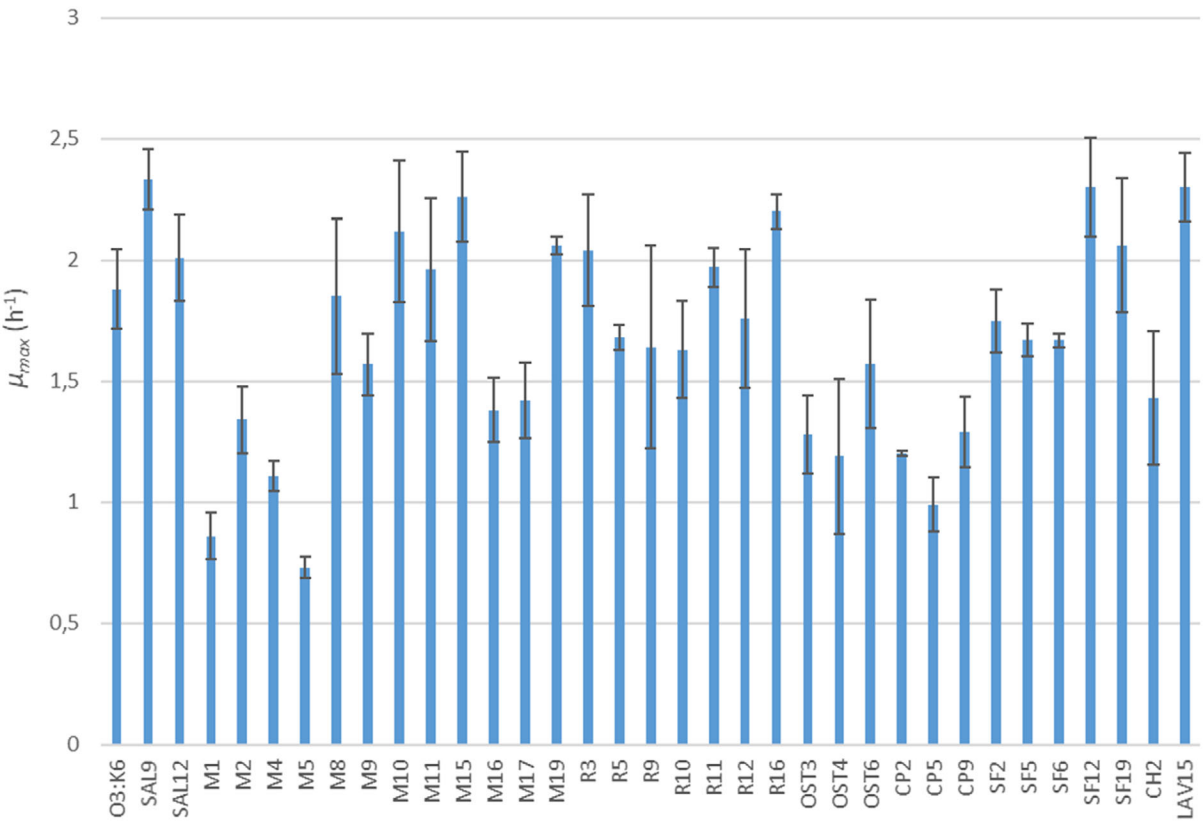


Figure 4: Growth variability of *Vibrio parahaemolyticus* strains isolated from seafood

MIC and NIC of the slowest grower of *V. parahaemolyticus* for gentamicin were found to be below 35 mg/mL, while for tobramycin they were found to be below 0.10 mg/mL. The highest grower showed a lower value of MIC for gentamicin, but a higher value for tobramycin. Also, the highest grower showed lower values of NIC for both gentamicin and tobramycin.

4. Conclusion

It can be concluded that all planned activities of the EU-FORA programme were carried out successfully. The hosting site provided the required material basis and full implementation of the fellow to the current work of the Department of Food Chemistry. The fellow developed skills to design and generate experimental data and apply software tools related to quantitative risk assessment and food microbiology. The hosting and sending organisations have already developed a collaboration and they are going to continue future work by staff exchange and training. Moreover, the methods for determination of maximum specific growth rate (μ_{max}) and MIC and NIC for aminoglycoside antibiotics were fully implemented in the microbiological laboratory of the sending organisation. It is worth mentioning that the staff of this sending laboratory has already applied these methods that the fellow transferred to them. The completed case study provided useful information for the quantitative characterisation of *V. parahaemolyticus* growth, which can be a main input for microbial exposure assessments as part of risk analysis of food-borne pathogens. It was demonstrated that the turbidimetric assay was useful for the successful estimation of MIC and NIC for antibiotics. This method can be applied in order to obtain reliable results for MIC and NIC of *V. parahaemolyticus* and address current needs for identifying the antibiotic resistance of food-borne pathogens.

5. Recommendations

EU-FORA is an already recognised programme in training fellows in food risk assessment based on its 6 years of experience. It would be excellent if the programme continues training fellows and increases the pool of experts in food risk assessment.

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Abbreviations

EU-FORA	The European Food Risk Assessment Fellowship Programme
MIC	minimum inhibitory concentrations
NIC	non-inhibitory concentrations
OD	optical density

Appendix A – Secondary activities

1) Lecture sessions provided by the hosting site
    - Lecture – 'A History of Predictive Microbiology'
    - Lecture – 'Predictive Microbiology Fundamentals'
    - Lecture – 'Predictive Microbiology Fundamentals'
    - Lecture – 'Experimental Design 1'
    - Lecture – 'Kinetic Models'
    - Lecture – 'Experimental Design 2'
    - Lecture – 'QMRA Model - Scenario Analysis'
    - Lecture – 'Parameter Estimation'
    - Lecture – 'Applied Modelling'
    - Lecture – 'Non-Linear Regression in Excel'
    - Lecture – 'Predicting Microbial Behaviour during Food Storage in Risk Assessment'
    - Lecture – 'Binomial Distribution'
    - Lecture – 'Risk Assessment'
    - Lecture – 'MATLAB - Getting Started'
    - Lecture – 'MATLAB - Parameter Estimation'
    - Lecture – 'MATLAB - Simulations'
    - Lecture – 'Life Cycle Assessment'
    - Lecture – 'Integrating Process Modelling approaches in Microbial Modelling'
    - Lecture – 'Modelling Effect of Process and Formulation on Microbial Level in Food'
    - Lecture – 'Thermal Inactivation: Application of Models'
    - Lecture – 'Partitioning'
    - Lecture – 'Implementation of Microbial Risk Assessment Model'
    - Lecture – 'Building a Microbial Risk Assessment Model'
    - Lecture – 'Basics of Multiscale Modelling in Predictive Microbiology'
2) Participation in IAFP's European Symposium on Food Safety with poster 'Growth variability of *Vibrio parahaemolyticus* strains isolated from seafood', Aberdeen, 3–5 May 2023.
3) 'FunShielf4Med' seminar 'Identification of Mycotoxins', Institute of Technology of Agricultural Products, Athens, 16 May 2023.
4) Visit at Laboratory of Food Quality Control and Hygiene of Agricultural University of Athens, 18 May 2023.
5) Participation in the 14th edition of the International Congress on Engineering and Food (ICEF14) with poster 'Quantitative assessments to characterise the antibiotic resistance of seafood microbial isolates', 19–23 June, Nantes, France.
6) Participation in working group meetings with PhD students of the National and Kapodistrian University of Athens and the research groups of Prof. Vasilis Valdramidis.

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Quantitative tools in microbial and chemical risk assessment

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Abstract

The popularity of biological origin food protection substances is driven by demands from consumers for natural and clean label product, increasing various food-related safety and health concerns and sustainability issues. Lactic acid bacteria (LAB) are most promising because they are a large group of beneficial microorganisms commonly used in food protection due to their ability to inhibit the growth of pathogenic bacteria and enhance food safety. Extensive scientific research has been conducted to understand the mechanisms by which LAB exert their protective effects in various food systems. Even though LAB activity against various food pathogens and spoilers is distinguished, use of cell-free supernatant (CFS) is still under investigation. This report is dedicated to present how qualitative measures can elaborate in new bacteria-origin food additive investigation. As part of the EU-FORA programme, the fellow was involved in the risk assessment tasks and projects which include gaining basic knowledge in predicative microbiology fundamentals, including different types of modelling strategies; delivering essential understanding about experimental design, knowledge in three specific software tools (MATLAB, GInaFiT and DMFit) and gained overall understanding what are the main differences while modelling growth or inactivation models. Secondary activities were included as a way to expand competences beyond qualitative measures to overall all activities done regarding risk assessment and build a strong network of food safety experts and professionals to continue engaging in risk assessment beyond fellowship programme.

Keywords: quantitative tools, predicative modelling, Lactiplantibacillus plantarum, secondary metabolites, inhibitory activity

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Summary

The final report of EU-FORA fellowship programme 'Quantitative tools in microbial and chemical risk assessment' hosted by National and Kapodistrian University of Athens (NKUA) supervised by Vasilis P. Valdramidis and Charalampos Proestos is dedicated to introducing fellow activities during programme implementation. Fellow is a junior scientist, first year PhD student in chemical engineering, with focus on bacteria-based antimicrobial substance investigation. Introduction part provides brief background about lactic acid bacteria (LAB) on how and why they are used in food protection with special importance to implement usage of not only whole bacteria, but also concentrated cell-free supernatants (CFS) containing metabolites and none live cells. In the work programme description aims are outlined such as the introduction to predicative microbiology fundamentals, including different types of modelling strategies, essential understanding about experimental design, training on three specific tools (MATLAB, GInaFiT and DMFit). The latter are selected according to fellow research topic and provided with overall understanding what are the main differences between modelling microbial growth, survival and inactivation. Furthermore, there descriptions on the development of different informative experimental designs were discussed. Only minor part of obtained data is presented in report, due to fact that research is ongoing even after the course and a separate scientific publication will be written with this data. Preliminary results of *L. plantarum* CFS concentrated by lyophilisation inhibitory activity were discussed. In addition, EFSA scientific opinions, concerning *Lactiplantibacillus plantarum* safety and efficacy as feed additive for various animal species and updates of the list of qualified presumption of safety recommended microbiological agents intentionally added to food or feed, released in EFSA Journal during past few years were revised, to get better understanding, what issues can occur while transiting from whole bacteria usage to use of only concentrated metabolites. Lastly, conclusions provide more insights of situation now and future prospects.

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1. Introduction

Predictive modelling is a mathematical way to predict future outcomes by analysing patterns in input data. This type of analysis gives a good idea of what kind of events are more likely to occur and provide statistical possibilities, with a defined certainty that one or another event under given conditions may or may not appear. Predicative modelling can be used in many areas, but this programme was dedicated to its use for assessing microbial populations in various food matrices and situations, concerning food safety.

Predictive modelling is used to get knowledge for a better understanding the different processing technologies act on microorganisms and their behaviour (Stavropoulou and Bezirtzoglou, 2019). To develop an accurate predicative model input data must be of sufficient quality, which leads to the need of having adequate experimental design, providing enough data to develop and validate the model. This also requires that researchers, working in risk assessment would be well trained not only to produce laboratory work but also be capable to interpret the data correctly, select best possible models which could accurately describe particular process.

Growing consumer demand for natural and clean label products, various food-related safety and health concerns, sustainability issues, regulatory and labelling requirements are forcing science and industry to elaborate on creating and widely using biological origin food protection substances. One of the most promising options can be the lactic acid bacteria (LAB), which are a group of beneficial microorganisms commonly used in food protection due to their ability to inhibit the growth of pathogenic bacteria and enhance food safety. Extensive scientific research has been conducted to understand the mechanisms by which LAB exert their protective effects in various food systems. LAB are characterised by their ability to convert sugars into lactic acid through fermentation. The production of lactic acid creates an acidic environment in the food, lowering the pH and inhibiting the growth of pathogenic bacteria (Holzapfel et al., 1998; Zapaśnik et al., 2022). These bacteria are known to produce various antimicrobial compounds, such as bacteriocins, hydrogen peroxide and organic acids, which can inhibit the growth of pathogenic bacteria. These compounds act by disrupting the cell membranes or metabolic processes of the target bacteria (De Vuyst and Vandamme, 1994; Chen et al., 2022).

LAB can also compete with pathogenic bacteria for nutrients and adhesion sites in the food matrix, thereby preventing the attachment and colonisation of harmful bacteria. This competitive exclusion mechanism helps maintain the safety and quality of the food product (Jacobsen, 1997; Siedler et al., 2020). Additionally, LAB have been shown to modulate the immune response in the human gut, enhancing the body's defence mechanisms against pathogens. This immunomodulatory effect contributes to the overall protection against foodborne infections (Vinderola and Ouwehand, 2018).

Alongside the development of methods for predictive microbiology, genome sequencing used to identify microorganism species and the analysis and identification of their produced metabolites, there is a growing interest in the activity of not only live bacteria, but also secondary metabolites isolated from them and used against food pathogens and spoilers. LAB are capable to produce various organic acids, such as lactic acid, acetic acid, and propionic acid, which contribute to the preservation of food by significantly reducing pH and creating an acidic environment unfavourable for the growth of most pathogens and spoilers (Li et al., 2017). Other peptides or proteins, that can disrupt the membrane integrity of target bacteria or interfere with their cellular processes, leading to growth inhibition are called bacteriocins. Several LAB bacteriocins, such as nisin and pediocin, have been extensively studied for their application in food preservation (Cotter et al., 2005).

This training programme was dedicated to instructing the fellow on how predictive modelling can expand knowledge about bacterial and fungal responses to substances (in this case – secondary cell-free metabolites from unique type of LAB – *L. plantarum*) used to supress their growth and even to examine the probability of microbial recovery after treatment. During this research activity, *L. plantarum* from KTU bacteria collection was selected as reference strain to investigate whether cell-free secondary metabolites can be as active as live bacteria. *L. plantarum* is well known for producing bacteriocin plantaricin – it is an antimicrobial substance that exhibit inhibitory activity against various bacteria, including pathogenic and spoilage organisms.

1.1. Description of work programme

The EU-FORA training programme 'Quantitative tools in microbial and chemical risk assessment' was designed to provide training in the use and development of predictive models for assessing the bacterial/ fungal responses for survival, growth and the probability of microbial recovery. Assessments include but not

limited to microbial responses under different food processing/preservation scenarios. Primarily, use and applications were planned to be performed by programming in MATLAB. After taking into consideration fellow's basic knowledge in statistics, mathematics, programming and other skills, as well as additional simplified tools were also introduced in the training course. This was done in order to provide useful and purposeful knowledge, that the fellow would be able to implement independently after the course. Analysis and interpretation of data could be used as inputs in microbial/chemical risk assessments studies.

1.2. Aims

The aims of EU-FORA training programme 'Quantitative tools in microbial and chemical risk assessment' was:

- to introduce fellow to predicative microbiology fundamentals, including different types of modelling strategies;
- to deliver essential understanding about experimental design;
- to provide basic knowledge on how to use some specific tools, selected according to fellow research topic;
- to give overall understanding what are the main differences while modelling growth or inactivation models.

As attending fellow was not very familiar with predicative modelling weekly meetings, organised and supervised by Vasilis P. Valdramidis, were held to ensure that all occurring questions would be addressed and clarified during periods while fellow was at sending organisation and daily meetings were held while staying at hosting site.

1.3. Additional activities for network widening

Mobility programs, such as EU-FORA is a great opportunity to introduce fellows to different work groups, participate in various events to improve multidisciplinary co-working and co-research activities. To meet these goals during fellowship programme, fellow was introduced to several secondary activities, described in Appendix A.

2. Data and methodologies

2.1. Data

Input data used to test the functionalities of specific tools was provided by supervisor Vasilis Valdramidis' research group. These data were used to understand the development of the experimental design, generation and processing results by doing practical exercises with real case data, received by methods included in work programme. Additional data for analysis were obtained from planned experiments at the fellow's sending organisation. The report presents only partial data, as all these outcomes are planned to be included in a scientific publication based on the data obtained.

2.2. Investigation of inhibitory activity

Preparation of *Lactiplantibacillus plantaru*m metabolites:

bacteria were grown at three different temperatures (+25, 37 and 45°C) for 72 h at two different initial pH (3.5 and 7.0). After incubation cell-free supernatant (CFS) was collected and lyophilised.

PDA media was enriched with different quantities (5% and 10%) of a concentrated metabolites mixture. On the hardened medium, mycelial discs (10 mm in diameter) of each isolate, taken from the edge of cultures of fungi isolates grown for 7 days, were placed mycelial downwards in the centre of each Petri dish. The plates were incubated in a thermostat at 25 ± 2°C for 7 days. Every 24 h, the growth of the isolate was measured with a ruler in two perpendicular directions until the growth of the test isolate in the control plate reaches the edge of the plate. The suppression coefficient (SC) was calculated according to the formula.

$$sc = (a-b)/a \times 100\%, \quad (1)$$

a – diameter of isolate in Petri plate on the control media (mm), b – diameter of isolate in Petri plate on media with CFS (mm).

Suppression coefficient sensitivity is divided into three main levels, given in Figure 1.

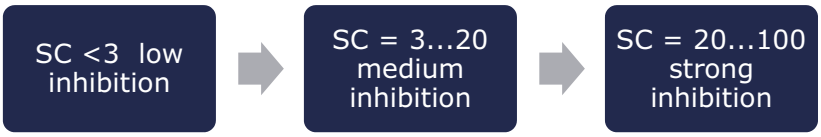

Figure 1: Suppression coefficient sensitivity levels

2.3. Methodologies on certain software usage

Two data processing strategies were used while implementing work programme. First strategy was to use MATLAB software with premade code, adjusting it to fit the needs and purposes of the research. Second strategy was to use Excel with additional add-ins like GInaFiT or DMFit, which allows to have better support for users, not yet very familiar with the predicative microbiology topic.

2.3.1. MATLAB

MATLAB is incredibly versatile tool used in many different scientific fields, such as engineering, data analysis, algorithm development and others. Specifically in microbiology this software can be used to analyse data, visualise it (as given in Figure 2) or create scripts. For this programme implementation MATLAB was used with prewritten code, allowing to simulate different scenarios in real cases. One of real-case analysis was to simulate and determine growth or no growth of certain bacteria inoculum size under certain concentration of nanoparticles.

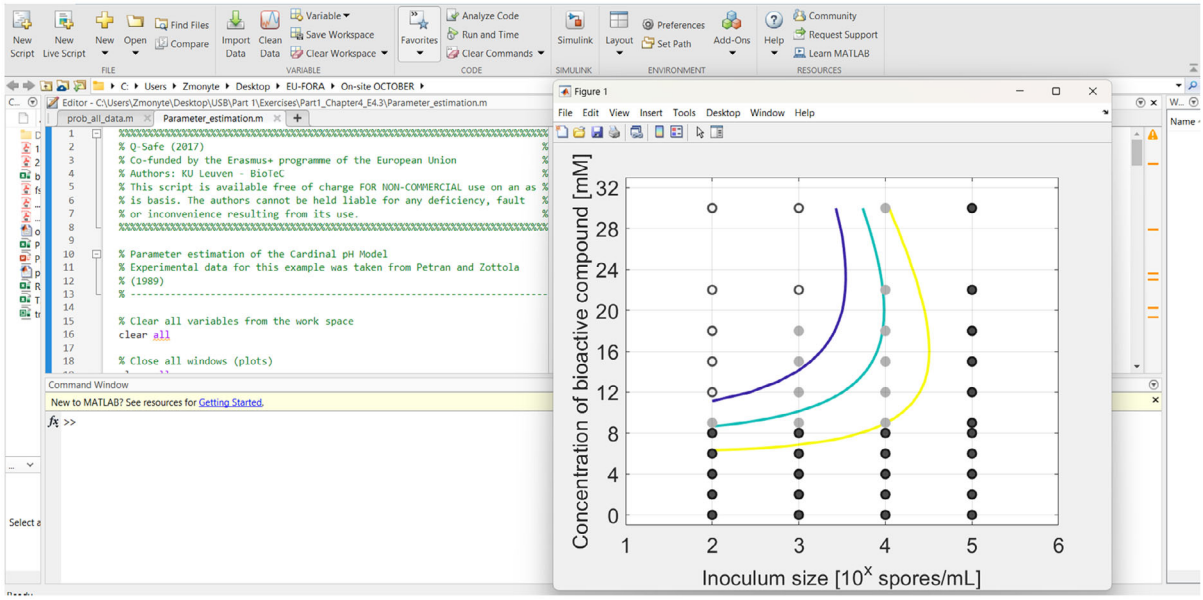

Figure 2: Hands-on MATLAB training example

As shown in Figure 2, hands-on trainings with MATLAB were carried out by fellow and supervisor. This type of research can provide valuable information about efficacy of any chemical or non-chemical treatment to survival rate of certain microorganism to certain treatment conditions.

2.3.2. Microsoft® Excel add-ins – GInaFiT

Excel itself can help with many calculations needed to create predicative models and freeware as GInaFiT can help to fasten and simplify some of the calculations and even prevent typical mistakes in data processing. GInaFiT aims to bridge the gap between people developing predictive modelling approaches and end-users in the food industry or research groups not disposing of advanced non-linear regression analysis tools.

The tool is useful for testing 10 different types of microbial survival models on user-specific experimental data relating the evolution of the microbial population with time. The 10 model types are:

1) classical log-linear curves,
2) curves displaying a so-called shoulder before a log-linear decrease is apparent,
3) curves displaying a so-called tail after a log-linear decrease,
4) survival curves displaying both shoulder and tailing behaviour,
5) concave curves,
6) convex curves,
7) convex/concave curves followed by tailing,
8) biphasic inactivation kinetics,
9) biphasic inactivation kinetics preceded by a shoulder,
10) curves with a double concave/convex shape.

The models were originally published as Bigelow and Esty (1920), Cerf (1977), Geeraerd et al. (2000), Mafart et al. (2002), Albert and Mafart (2005), Geeraerd et al. (2005) and Coroller et al. (2006). Next to the obtained parameter values, the following statistical measures are automatically reported: standard errors of the parameter values, the sum of squared errors, the (root) mean sum of squared errors, the R2 and the adjusted R2. In addition, t4D, the time needed for a 4 log reduction of the initial microbial population, as originally proposed by Buchanan et al. (1993), is also automatically reported (for data sets covering at least four decimal reductions).

The tool can be used in two ways. On one hand, for end-users having already a qualitative idea of the general shape of their survival curves, the choice for one of the model types is obvious. On the other hand, if the end-user does not have a clear idea yet, two or more of the different model types available can be tested and compared. The time for a four decimal reduction can be useful to summarise the information present in a data set, for example, if a common survivor curve shape cannot be selected for a range of different conditions tested (Geeraerd et al., 2000).

Additionally, the tool has some built-in features testing for misuse, for example, when trying to identify a model with tailing on data not having a tail or when using a too limited number of data points (observations) in comparison with the number of parameters in the model type chosen (the number of parameters ranges from 2 to 5 for the ten model types available).

2.3.3. Microsoft® Excel add-ins – DMFit

DMFit is another free tool, that can be accessed either online (https://browser.combase.cc/DMFit.aspx) or added as Microsoft® Excel add-in. Web edition is a web-based application to fit bacterial curves where a linear phase is preceded and followed by a stationary phase. The desktop version is part of the system used in-house at the Institute of Food Research to model the time-variation of the logarithm of cell concentrations of bacterial batch cultures. DMFit can be used to visualise growth or survival data, obtain parameters estimates from data fitted to growth or survival models, calculate maximum growth or death rate, lag phase time, initial or final cell count and estimate standard errors on these parameters.

Data input is user-friendly because it is compatible with Excel spreadsheets or textfiles (both online and desktop versions). Data can be fitted to two different types of models:

a) Model of Baranyi and Roberts (1994), which describes a sigmoid bacterial curve. This model, unlike other sigmoid curves, has very close to linear mid-phase. This model has four main parameters (Initial Value, lag, maximum rate, Final Value) and two curvature parameters: mCurv and nCurv which describe the curvature of the sigmoid curve, respectively, at the beginning and at the end of the growth phase. This tool allow to describe only the growth or death at the stationary phase, if mCurv parameter is set to zero and 'no lag' model option is selected. Model can also describe only the lag phase and growth or death phase if nCurv is set to zero and 'no asymtot' model option is selected.
b) Trilinear, biphasic or linear models. Trilinear model describes a bacterial growth curve with three straight lines: the lag phase and the stationary phase are described by two horizontal straight lines. The slope of the third straight line describing the growth/death phase is called the 'maximum rate'. Biphasic model must be used when data-based curve has no lag phase or no stationary phase. Linear model can be used in case bacterial counts describe only growth or death phase.

Even though tool use most common units, users must pay attention that by default time is given in hours, bacteria counts in log10 cfu/g and that leads to maximum growth or death rate given as log10cfu/g per h. User after loading data must ensure, that units are aligned accordingly.

3. Inhibitory activity and main points in risk assessment

3.1. CFS inhibitory activity results

Lactiplantibacillus plantarum MI-LPI recently has shown promising activity by inhibiting growth of various food pathogens, such as bacteria or yeast with specific inhibition effectiveness on Candida albicans (Riešuté et al., 2022). But mentioned activity was determined by using live lactic acid bacteria in model systems. *L. plantarum* from unique Kaunas University of Technology collection is known for producing plantaricin, which has potential antifungal properties. The aim of this investigation was to use CFS to investigate excreted metabolites activity. In order to have higher concentration of excreted metabolites CFS was lyophilised and dry powders were used for experiments. The yeast species used in the study were selected based on the highest potential results in the live bacteria study and the moulds were isolated from organic wheat grown in Lithuania.

CFS inhibitory activity was tested on three different yeast (*Candida albicans, Saccharomyces cerevisiae, Rhodotorula rubra*) and six fungi (identified from organically grown wheat) (*Penicillium commune, Penicillium roqueforti, Penicillium griseofulvum, Fusarium oxysporum, Fusarium solani, Aspergillus cristatus*) and nine bacteria (*Bacillus cereus, Bacillus subtilis subsp. Spizizenii, Enterococcus faecalis, Escherichia coli, Klebsiella aerogenes, Listeria monocytogenes (serotype 4B), Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella enterica subsp. Enterica serovar* Typhimirium*)*.

Metabolites from cultivation on neutral pH media showed no measurable inhibition, and samples cultivated in acidic pH at 45°C also showed no measurable inhibition. Inhibitory activity on different fungi was detected, but SC value was lower than 3. Medium and strong inhibition was identified on yeast samples. The highest inhibition was identified in *Candida albicans* when treated with a 10% metabolite additive in media. This result supports the idea, that CFS with *L. plantarum* metabolites can be considered as yeast growth inhibitor. As neutral pH samples did not show measurable inhibition, further investigation and determination of active metabolites is needed to eliminate hypothesis, that activity is based only on organic acids influencing the acidic pH. Research on CFS inhibitory activity on nine selected bacteria are being continued and newly gained knowledge on tools are being used to analyse data obtained. After completion of additional experiments scientific publication will be released.

3.2. Safety concerns and risk assessment done by EFSA

Just as in other industries, food sector experiences influence of different trends. For instance, usage of various bacteria for food safety or other technological purposes is one of them. Safety questions and concerns about *Lactiplantibacillus plantarum* use in food and feed is already circulating in EFSA opinions.

EFSA periodically releases updated list on generic pre-evaluation of the safety of microorganisms, intended to use in food and feed chains (EFSA BIOHAZ Panel, 2023). These statements are based on an assessment of published data for each agent, with respect to its taxonomic identity, the body of relevant knowledge and safety concerns. Safety concerns identified for a taxonomic unit (TU) are, where possible, confirmed at the species/strain or product level and reflected by 'qualifications'. These continuing publications provide updated and current knowledge not only to The Panels in EFSA, but also researchers and industry.

During past few years several opinions were dedicated to investigating safety of *Lactiplantibacillus plantarum* in various forms and terms of use. Also, EFSA opinions support one health approach and consider safety in the aspect of animal, human and environment health. Summarised opinions are given below.

In September of 2022 scientific opinion provided conclusions on the efficacy of two technological additives to improve ensiling of forages consisting of *Lactiplantibacillus plantarum* strains ATCC 55058 and ATCC 55942, respectively, for all animal species (EFSA FEEDAP Panel, 2022a). Request confirmed, that both additives were intended to be used for all forages and for all animal species and minimum bacteria concentration was given at 5×10^6 CFU/kg forage. In previous scientific opinions The Panel could not conclude decision on safety and efficacy due to insufficient data. But in this case, applicant provided supplementary information, which included enough evidence, that the addition of *L. plantarum* strains ATCC 55058 or ATCC 55942, have the potential to improve the ensiling process by reducing protein degradation in all type of forages as indicated by the reduction of ammonia production.

Similar scientific opinion was released in September 2021. This was also requested from European Commission, but in this case concentration and strain was different. In scientific opinion safety and efficacy of *Lactiplantibacillus plantarum* when used as a technological additive intended to improve ensiling of forage intended to use in all types of forage and for all animal species with minimum concentration of 1×10^8 CFU/kg (EFSA FEEDAP Panel, 2021a). In this case, strain identity was also clearly established with no signs of antimicrobial resistance. The Panel concluded that use of the strain as additive can be considered to be safe for animals, humans and environment. Furthermore, any signs of skin or eye sensitisation, but mentioned as possible respiratory sensitiser. Similar conclusions were given in two scientific opinions released in June of 2021 (EFSA FEEDAP Panel, 2021b,c).

Upon request from European Commission scientific opinion was released by EFSA in March 2023 (EFSA FEEDAP Panel, 2023a). In this case, *L. plantarum* was used as acidity regulator incorporated in oat-derived products, carrot root-derived products and coconut flesh-derived products at minimum inclusion level of 8.0×10^{10} CFU/kg with intention to use mentioned products in feed for horses, dogs, cats and pet rabbits. The reference strain has no signs of resistance to certain antibiotics used in humans or in veterinary. The Panel concluded that in this particular use this particular strain is considered as safe for target species, consumers of horse meat and environment. Regarding user safety, no evidence of skin or eye irritation was found, but taking into consideration the proteinaceous nature of additive, special precautions for respiratory sensibilisation were given.

This year the scientific opinion was published in EFSA Journal. This was done by following request from European Commission to deliver scientific opinion on the feed additive consisting of *L. plantarum* and *L. reuteri* (AQ02) as a zootechnical feed additive for suckling piglets (EFSA FEEDAP Panel, 2023b). Even though in previous opinion FEEDAP concluded that mentioned additive was considered safe for target species, consumers and the environment, new conclusion was not so straightforward. The Panel drawn attention to possible respiratory irritations and insufficient data to conclude on skin or eye irritation potential. Based on provided data The Panel could not conclude on the efficacy of the additive.

Another opinion, released in May 2023 considering the assessment of the application for renewal of *Lactiplantibacillus plantarum* DSM 23375, a technological additive to improve ensiling of fresh material for all animal species was requested (EFSA FEEDAP Panel, 2023c). In this case, the applicant provided evidence, that additive is already on the market and complies existing conditions of authorisation. The Panel concluded, that *L. plantarum* DSM 23375 remains safe for all animal species, consumers and environment under authorised conditions of use. It is important to notice, that This Panel also could not provide conclusions on user safety and the skin sensitisation potential of the additive. Similar conclusions in similar situation when renewal of authorisation was needed was also released earlier in EFSA Journal on January of 2022 (EFSA FEEDAP Panel, 2022b).

4. Conclusion

Exploration and implementation of novel microorganism-based food protection substances require to focus effort in well planned and funded research. Predictive microbiology can be an incredibly useful tool used to optimise laboratory research and providing more knowledge about growth or death rates which leads to process optimisation towards best possible results. Even though *L. plantarum* demonstrated tremendous potential in food and feed applications, further investigations are still needed. In past few years EFSA concluded several opinions on *L. plantarum* usage in feed, but usage possibilities in food are still under investigation. Furthermore, the transition from live bacteria to concentrate of metabolites cannot be done without adequate safety measures.

Fellow will continue research on *L. plantarum* metabolites inhibitory activity at the sending organisation. Next steps will aim at better understanding the mechanism and efficacy of metabolites was *in silico* investigation of possible compounds and will be followed by identification of these compounds in CFS. Furthermore, regarding the shown inhibitory activity against spoilers, investigation of metabolites efficacy in model food matrix will be done.

Qualitative models cannot always provide sufficient data for proper risk assessment so quantitative tools are essential part of adequate risk assessment of any kind. In this work programme microbial and chemical risk assessment became very close one to another. The main target was CFS which already was without any living bacteria cells, but this cannot be taken into consideration as only chemical risk source as the substances were obtained directly from certain bacteria species (*L. plantarum*). During fellowship implementation all aims were addressed. Fellow was introduced to predicative microbiology fundamentals by participating in lectures, workshops with supervisor and self-

learning activities. Delivered understanding about experimental design was used for laboratory research planning and implementation. Furthermore, three specific tools were combined to better understand differences between different models, data fitness for purpose and differences between growth and inactivation models in general.

5. Recommendations

As fellowship programme is not only about gaining specific knowledge on various steps in risk assessment but also expanding fellows circle of contacts, follow up meetings, organised in any convenient way for both – fellow and supervisor, might be a good practice to maintain created working groups in different organisations.

6. Documentation provided to EFSA

1) Quantitative tools in microbial and chemical risk assessment. July 2023. KTU & NKUA

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Abbreviations

CFS	cell-free supernatant
DM	dynamic modelling
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
KTU	Kaunas University of technology
LAB	lactic acid bacteria
NKUA	National and Kapodistrian University of Athens
QPS	qualified presumption of safety
SC	suppression coefficient

Appendix A – Secondary activities

Additional relevant activities and learning opportunities completed by fellow:

1) 'Induction training of the European Food Risk Assessment Fellowship Programme', EFSA premises, 5–23 of September 2022
2) Lecture sessions provided by hosting site, NKUA premises, October – November, 2022
   Lecture - 'A History of Predictive Microbiology'
   Lecture - Predictive Microbiology Fundamentals'
   Lecture - 'Predictive Microbiology Fundamentals'
   Lecture - 'Experimental Design 1'
   Lecture - 'Kinetic Models'
   Lecture - 'Experimental Design 2'
   Lecture - 'QMRA Model - Scenario Analysis'
   Lecture - 'Parameter Estimation'
   Lecture - 'Applied Modelling'
   Lecture - 'Non-Linear Regression in Excel'
   Lecture - 'Predicting Microbial Behaviour during Food Storage in Risk Assessment'
   Lecture - 'Binomial Distribution'
   Lecture - 'Risk Assessment'
   Lecture - 'MATLAB - Getting Started'
   Lecture - 'MATLAB - Parameter Estimation'
   Lecture - 'MATLAB - Simulations'
   Lecture - 'Life Cycle Assessment'
   Lecture - 'Integrating Process Modelling approaches in Microbial Modelling'
   Lecture - 'Modelling Effect of Process and Formulation on Microbial Level in Food'
   Lecture - 'Thermal Inactivation: Application of Models'
   Lecture - 'Partitioning'
   Lecture - 'Implementation of Microbial Risk Assessment Model'
   Lecture - 'Building a Microbial Risk Assessment Model'
   Lecture - 'Basics of Multiscale Modelling in Predictive Microbiology'
3) 'Module 1 training of the European Food Risk Assessment Fellowship Programme', held online, 28th of November – 2nd of December 2022
4) 'Risk Assessment Research Assembly', organised by EFSA in Berlin, 7th of December 2022
5) Participating in organisational committee of 5th congress of Baltic microbiologists, September 2022 – October 2023 (conference will be held in 11–13th of October in Vilnius)
6) Course in English 'Science communication to society' 1 ECTS, Kaunas, October-December 2022
7) Participation in course in national language (Lithuanian) 'Introduction to variance analysis with SPSS', Kaunas, 18th and 31st of January 2023
8) Course in English 'Principles of academic and scientific writing in English' 1 ECTS, Kaunas, March-April 2022
9) Course in English 'Digital tools for scientific writing and publishing' 1 ECTS, Kaunas, March-April 2022
10) Participation in course in national language (Lithuanian) 'Validation and verification of research methods', Kaunas, 2nd of March 2023
11) 'Module 2 training of the European Food Risk Assessment Fellowship Programme', held online, 20–27th March 2023
12) Participation in IAFP's European Symposium on Food Safety with poster 'Investigation antifungal properties of L. plantarum metabolites concentrated by lyophilisation', Aberdeen, 3–5th of May, 2023
13) 'FunShielf4Med' seminar 'Identification of Mycotoxins', Institute of Technology of Agricultural Products, Athens, 16th of May 2023
14) Visit at laboratory of Food Quality Control and Hygiene of Agricultural University of Athens, 18th of May 2023
15) 'Module 3 training of the European Food Risk Assessment Fellowship Programme', EFSA premises, 12-16th of June 2023

16) Training School 'Modelling the effects of low pH and other stresses on foodborne micro-organisms to improve food and drink quality' of the COST Action CA18113 EuroMicropH, 'Understanding and exploiting the impacts of low pH on micro-organisms' NKUA, Athens, 28-30th of June 2023

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