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Foreword

I am very pleased to introduce the fifth collection of reports of EFSA's Fellowship Programme. EU-FORA started in 2016 as part of EFSA's ambition to address the increasingly growing need for qualified and trained food risk assessors. For the past six years, EFSA has been supporting the development of future generations of Europe's experts in food safety and risk assessment. Throughout that time the Programme has evolved to meet the challenges of the ever-changing landscape of food risk assessment.

The wide spectrum of scientific work and experiences described in this document illustrate the diversity of programmes, personalities and competencies that comprise the Programme. Furthermore, they serve as a staple for any future risk-assessor that is yet to come and mark a new beginning for the European Food Risk Assessment Fellowship Programme (EU-FORA 2.0).

The timely execution of the programme would not have been possible without the support of EFSA colleagues across different departments and the dedication of the fellows and hosting sites.

We hope that EFSA will continue to empower food safety professionals under EU-FORA 2.0 for years to come. We strive to ensure an interconnected community of experts at Member States and EU level towards our mutual quest of lasting common EU risk assessment community and culture.

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Lastly, we would like to express our thanks to all participating organisations, as well as our partners for the successful cycle. We applaud our newly graduated fellows and hope to see them soon enough in other prominent roles in the risk-assessment ecosystem.

Barbara Gallani

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Introduction

We are delighted to present the 5th special issue of the *EFSA Journal* dedicated to EFSA's Fellowship Programme (EU-FORA). It provides 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 cooperation and dedication of all the actors involved – fellows, institutions, organisation and other partners of the wider EU-FORA network.

We congratulate the Fellowship graduates and would like to acknowledge their professionalism, engagement and desire to become part of the EU-FORA community. In the past year, they have acquire new expertise and complimented their skillset with the 'toolbox' of a food risk assessor. We hope they continue to be invested in the Community as Alumni as well as under other functions in the risk-assessment ecosystem.

This year we also had the opportunity to begin a new chapter in FSA's Fellowship Programme (EU-FORA 2.0). The improved Fellowship has brought the participation of new organisations and expanded the outreach across Member States. We hope that the Programme continues to contribute and inspire the risk assessors of tomorrow.

Finally, we would like to acknowledge the prowess demonstrated by everyone involved, including the support of EFSA's Management, and the flexibility and excellence of fellows, hosting sites and our training consortium.

on behalf of the EU-FORA Programme

Victoria Villamar

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Training in tools to develop quantitative microbial risk assessment along the food chain of Spanish products

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Abstract

Food safety is a widespread challenge. Every year it is estimated that almost 1 in 10 people in the world fall ill after eating contaminated food resulting in over 400,000 deaths. The risk of outbreaks is higher when consuming ready-to-eat (RTE) products because they are eaten without a further cooking process that could inactivate pathogenic microorganisms. Hence, food processing is essential to increase the safety of RTE products. Microbiological risk assessment (MRA) integrates food science, microbiology and data science to provide a comprehensive understanding of the safety of the food system. MRA provides qualitative and/or quantitative information to decision makers, which might promote the adoption of better food practices. In this contest, this project aims to study and implement tools for quantitative microbial risk assessment (QMRA) of food products along the food chain. A common RTE product (cured ham) from Spain was used as a case study. Following, the exposure assessment model was implemented using mathematical models and statistical software to describe the microbial behaviour along the food chain. The study presents the possibility to identify the risk exposure in different scenarios (e.g. growth during different storage conditions, inactivation induced by traditional or innovative decontamination techniques), showing the flexibility of the predictive tools developed.

Keywords: QMRA, ready-to-eat, *Listeria monocytogenes*, supercritical CO_2, low-temperature pasteurisation

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1. Introduction

1.1. Food safety in ready-to-eat meat products

Food safety is a widespread challenge. Every year almost 1 in 10 people in the world fall ill after eating contaminated food resulting in over 400,000 deaths (WHO). The risks of contamination are relevant in every step from farm to fork and require prevention and control throughout the whole food chain. The consumption of food from animal origin is associated with a high risk for the population. Indeed, infectious disease can be spread between animals and people by pathogenic microorganisms such as bacteria, viruses and parasites. Specifically, meat contamination can occur at the farm (e.g. parasites that infect food-producing animals), at slaughter (e.g. contamination by intestinal contents), during processing (e.g. cross-contamination by handling/surface/food) and preparation (e.g. improper use of safe handling practices). In 2020, the consumption of foods of animal origin was responsible for 65.7% of outbreaks in the EU and campylobacteriosis and salmonellosis were the first and second most reported zoonoses in humans in 2020 (EFSA and ECDC, 2021), followed by *Yersiniosis*, Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes* infections. Among these pathogens, *L. monocytogenes*, a Gram-positive and non-spore-forming bacterium that can grow under anaerobic and aerobic conditions, has the highest reported deaths, with a 13% of fatality rate in the EU in 2020. Listeriosis is a serious infection caused by *L. monocytogenes* which are transmitted to humans by contaminated food (99% of cases). Listeriosis occurs in both invasive (maternal-neonatal and non-maternal-neonatal) and non-invasive (gastroenteric) forms. Invasive listeriosis is the more severe form of the disease and affects particularly certain high-risk groups of the population. These include pregnant women and their newborns, the elderly, and people with weakened immune systems (EURL Lm, 2021). *L. monocytogenes* infections are most commonly reported in the age group 'over 64 years' and particularly in the age group 'over 84 years'. Spain is the third country in the EU with the highest number of confirmed cases of listeriosis with 362 cases (EFSA and ECDC, 2021).

Among the category of meat products, higher risk for public health is associated with meat consumed as ready-to-eat (RTE) or without a proper cooking. Global RTE meat products market is expected to grow at a CAGR of 4.8% during the forecast period 2019–2026 (https://straitsresearch.com/report/ready-to-eat-meat-products-market/). RTE meat products comprise manufactured, processed and fermented meat for direct consumption and include products such as beef jerky, pepperoni, salami, smoked deli meats and cured ham. *L. monocytogenes* occurs frequently in fresh pork meat at low or moderate levels and the pathogen may become established in the processing environment and cross-contaminate meat products (Thévenot et al., 2006). The ability of this microorganism to grow at refrigerated temperatures (≥ -0.4°C), at a pH between 4 and 9.6 (Välimaa et al., 2015), and water activities ≥ 0.92 in additions to its moderately high tolerance to salt, makes it very dangerous in a wide variety of RTE meat products (Burnett et al., 2005; Kurpas et al., 2018). Considering that the probability of infection is correlated to the quantity of serving, Spain is considered a country at risk since the consumption of RTE meat products is one of the highest in the EU with almost 1 kg/month per person (Celada et al., 2016).

The RTE meat production chain is complex and there are different possible sources of *L. monocytogenes* contamination in each step. Reduction or elimination of the microorganisms during processing will be helpful in limiting the bacteria in the food at the time of consumption. The risk associated with RTE products depends mainly on the effectiveness of control measures implemented by food business operators (FBOps), including Good Agricultural Practices (GAPs) at primary production, Good Manufacturing Practices (GMPs) and HACCP programmes at processing and retail. However, the ability of this microorganism to survive under extremely different environmental conditions and the fact that *L. monocytogenes* can form biofilms (Lee et al., 2019) on the surfaces of food industries make its control very challenging. Microbiological criteria for RTE foods is defined by Regulation (EC) No 2073/2005 and it indicates for products supporting the growth of *L. monocytogenes* to satisfy that the product will not exceed the limit 100 CFU/g throughout the shelf-life. The infectious dose in invasive listeriosis is not well known (Swaminathan and Gerner-Smidt, 2007).

1.2. Conventional and innovative food processing for the inactivation of *L. monocytogenes* in RTE meat

Food processing technologies can be applied to reduce the probability of *L. monocytogenes* being present in RTE meat. Common thermal treatments, such as pasteurisation and sterilisation, are

effective but can be applied only in cooked meat, while raw-derived products, such as cured ham would be affected by undesirable changes in sensorial characteristics. On the contrary, irradiation might be applied for sterilisation with good results on the sensorial acceptance (Jin et al., 2012), but its use in the EU is limited only to a few product categories by Directive 1999/2/EC and 1999/3/EC. Moreover irradiation could pose concerns to consumers (Cardello et al., 2007). The use of food preservatives and/or antimicrobial additives are common approaches to control *L. monocytogenes* growth in RTE meat and extend shelf life. However, they are almost ineffective to kill *L. monocytogenes* and their use can not exceed some precaution level by law (Dussault et al., 2016). For all these reasons, new processes are highly desirable.

In the last decades, alternative mild and non-thermal processing technologies have been developed to increase food safety without altering the product's quality. Within these technologies, high hydrostatic processing (HHP) is shown to be effective against *L. monocytogenes* inoculated in cured ham (Morales et al., 2006) without altering the sensory characteristics. The process needs very high pressure (> 300 MPa) which might discourage its applications in small enterprises and retails due to the high cost of machinery. Alternatively, the use of carbon dioxide at supercritical state $ScCO_2$ has also been successfully applied to inactivate *L. monocytogenes* on the surface and on slices of cured ham (Ferrentino et al., 2013). $ScCO_2$ has a low critical point (304.1 K, 7.38 MPa) which allows handling processes at relatively low-pressure conditions in comparison to HHP, thus lower investment costs. CO_2 can penetrate inside the tissue and inactivate microorganisms at the surface and internally. CO_2 has been defined as GRAS (Generally Regarded As Safe) by FDA, it is obtained by waste production and is fully recycled. CO_2 is a relatively inert gas, not affecting the other food compounds, non-toxic, nonflammable and easily removable after the process without any contaminants or residues. Consequently, it is approved for food processing without declaration (Brunner, 2005). However, the adoption of novel technologies in the food industry requires strong scientific evidence to drive its acceptance at industrial level. In this context, risk assessment can be used to support the development and use of innovative processing and identify any scientific/regulatory gap, which might be needed for its commercialisation.

1.3. Quantitative microbial risk assessment

Quantitative microbial risk assessment (QMRA) is a science-based process, which estimates the risk of infection and illness when a population is exposed to microorganisms in food (Mataragas et al., 2010). QMRA follows four steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation. In hazard identification, scientists identify biological hazards (such as bacteria, viruses, parasites, fungi and moulds or their toxins) that could be present in food. Hazard characterisation studies the nature of the health effects of the hazard (e.g. pain) and, where possible, proposes a dose–response model. In the exposure assessment, there is an association of the hazard present in the food with the population and the amount of food eaten. In the risk characterisation, assessors draw conclusions on the likelihood of the health effect of the specific pathogens for consumers in general or specific groups. For QMRA implementation, it is necessary to develop mathematical models for the analysis of the behaviour of the risk along the whole food chain. The likelihood of the event is usually described using stochastic methods, which include uncertainty and variability in the analysis (Thompson, 2002). In the last few years, different methodologies have been proposed to conduct QMRA and models and software are available for these studies.

2. Description of work programme

2.1. Aims

The main objective of EU-FORA is to enhance cooperation among Europe's food safety organisations and between them and EFSA, thus contributing to the harmonisation of food risk assessment practices across Europe. Specifically, this EU-FORA project was focused on the development of training and research activities to perform QMRA and evaluate the risk associated with the consumption of RTE foods. Considering the background of the fellow in innovative food processing, the training included the integration between the existing knowledge of the hosting institution and the fellow within the QMRA field, aiming at fostering the transfer of knowledge on a well know based environment and establishing a long-term study in this field that can be continued at the end of the programme. A synergistic research activity was carried out between the Polytechnic University of

Cartagena (UPCT) in Spain and the University of Padua (UNIPD) in Italy to promote the transfer of knowledge during and after the programme.

2.2. Activities/Methods

The activity focused on training in QMRA methodologies to cover the four steps of risk assessment (hazard identification, hazard characterisation, exposure assessment and risk characterisation). As part of the training, a practical case study based on a common RTE Spanish product (cured ham) has been used for the implementation, and different scenarios were identified and discussed. Among them, the use of innovative food processing technologies as an additional decontamination step, specifically the use of $ScCO_2$ pasteurisation, was considered to increase the product's food safety. As a hazard, *L. monocytogenes* was considered the most relevant pathogen in RTE meat. The description of the main activities carried out during the programme is reported below.

2.2.1. Training on methodologies related to risk assessment

These activities have provided the theoretical background and basic skills to perform a QMRA, complementing the education and knowledge acquired during the training modules by EFSA. Thanks to the incorporation in a working team with proven expertise in the use of risk assessment tools, the fellow received training on specific topics such as:

i) Handling of available databases (EFSA, FAO, the group's database for microbial inactivation);
ii) Optimal experimental design (including the bioOED software developed in the group) applied to growth and inactivation experiments;
iii) Growth and inactivation modelling (Combase and Bioinactivation developed in the group);
iv) Statistical analysis using Monte Carlo and Bayesian methods and risk ranking methodologies;
v) Software tools specific for risk assessment (MicroHibro, FDA-iRISK, biorisk);
vi) Training on the use of the R programming language and Rstudio.

2.2.2. Collecting data from literature and laboratory work

This activity provided knowledge on methodologies for data collection to characterise the microbial response along the food chain. Data collection is a key fundamental phase in QMRA because it allows the assessors to understand the current state of the art and, if necessary, to drive specific experimental campaigns when key data are missing. The collection included both literature search from available databases, scientific papers, reports and laboratory activities. The data needed for QMRA include inactivation kinetics of the relevant microorganism in industrial processing, as well as the growth during storage conditions (e.g. transport, retails, domestic). The use of inactivation processing technologies was also included in the study considering both traditional heat treatments and innovative non-thermal or low-temperature pasteurisation technologies. During this task, the fellow was trained on the use of a thermoresistometer available at UPCT applying Optimal Experiment Design methodologies already developed in the group. A Mastia thermoresistometer was used to study the inactivation kinetic behaviour in isothermal or dynamic experimental conditions of a microbiological strain (Conesa et al., 2009). For the enumeration, standard plate count technique was used using selective or non selective media using a colony counter. The fellow was also trained on the use of the HHP machine available at UPCT, performing exploratory experiments with packaged products in modified atmosphere packaging (MAP).

2.2.3. Development of mathematical models

Mathematical modelling is a common tool in food science and technology. The microbial behaviour along the food chain (prevalence, growth, inactivation, acclimation, etc.) was described using both existing models reported in literature and *de novo* models implemented based on data gathered from literature and/or novel experimental data. The prevalence levels of *L. monocytogenes* in dry cured meat is highly dependent on the type of meat. In case of cured ham the overall occurrence of *L. monocytogenes* in retail varies from not detected to a prevalence up to 12% (Serra-Castelló et al., 2020). In Spain, the prevalence in dry-cured ham deboning and slicing areas was 9.16% (Alía et al., 2020). The growth of *L. monocytogenes* can be modelled depending on storage temperature and water activity though secondary polynomial models (Serra-Castelló et al., 2020). Inactivation by innovative processing was analysed using Bioinactivation software (Garre et al., 2017) which allows to

predict the inactivation process of a population of microorganisms after an inactivation process. Inactivation of *L. monocytogenes* in cured ham with $ScCO_2$ (Ferrentino et al., 2013) was fitted using the Peleg model (Peleg and Cole, 1998) and can achieve up to 6 logCFU/g inactivation. Reductions on *L. monocytogenes* in low water activity dry-cured ham after HHP process at 600 MPa for 5 min was found moderate (Pérez-Baltar et al., 2021), and not considered in further studies.

2.2.4. Estimation of the risk based on different scenarios

The exposure assessment model for risk characterisation was elaborated according to the methodology by Codex *alimentarius* for QMRA. An exponential dose–response model by FAO/WHO was selected. Exposure assessment was implemented using the statistical software R applying a new package 'biorisk' developed by the group at UPCT and available in GitHub (https://github.com/albgarre/biorisk/). Other existing web-based tools (MicroHibro, FDA iRisk) were also used during the training. The data gathered from literature, together with the kinetic models was implemented using probability distributions including the relevance of variability and uncertainty, according to the recommendations of international food safety agencies. The risk was calculated on the basis of a stochastic model and solved using Monte Carlo simulations. The exposure assessment provides an estimate of the occurrence and level of the pathogen in a specified portion of food at the time of consumption, aiming to calculate the probability of consuming a given dose of the pathogen of interest. The exposure assessment considered the most relevant stages of the food chain, and included processes and storage until the consumption. Different scenarios can be defined by changing the process stages or the initial concentration or the dose of serving, permitting to consider the adoption of better practices which can reduce the risk for the population.

2.3. Secondary activities

Apart from the mentioned tasks, additional training and other activities were carried out/provided in person and/or remotely. This helped the further improvement of the skills on risk assessment and permitted to carry out communication, dissemination and exploitations activities. Moreover, the transfer of knowledge on topics related to food safety has been provided during the whole time thanks to the involvement in co-supervision and mentoring of PhD students, undergraduate and graduate students in topics related to risk assessment, food processing and data analysis. The submission of national and international research grant proposals was also carried out on topics related to innovative food processing and risk assessment with the involvement of the supervisor in the research team. Following are some details of the most important activities presented.

2.3.1. Visit to AESAN

A few days visit in the headquarters of the Spanish Agency for Food Safety and Nutrition (AESAN) on 23–24 February 2022, in Madrid and on 25 at the 'Centro Nacional de Alimentación' (CNA) in Majadahonda was organised for the fellows placed in Spain. During this visit, there was the opportunity to present the EUFORA activities to the AESAN Scientific Committee and meet in person all the other fellows. Moreover, seminars were organised for the fellows to present the AESAN's work on risk assessment, management (biological risk, chemical risks, nutritional safety) and communication. It was also explained the official food control and alerts and the Spanish strategy for nutrition, physical activity and the prevention of obesity (NAOS). During the visit at the laboratory at CNA, the activities carried out on food contact materials, toxin and food processing contaminants, residues of veterinary drugs, biotechnology and microbiology and antimicrobial resistance were explained with practical examples.

2.3.2. Participation in conferences/webinars/meetings

1) Webinar - Horizon Europe Cluster 6 'Food, Bioeconomy, Natural Resources, Agriculture and Environment' Information Days organised by the European Research Executive Agency, 25–26 October 2021, online.
2) Webinar 'Beginner's Guide to Using Sequencing to Facilitate Microbial Research' organised by Novogene, 11 November 2021, online.
3) NETVAL Webinar Training Orbit Intelligence, 1 December 2021, online.
4) RIBMINS 2nd Scientific Conference 'Towards the future of meat safety assurance', 7–8 April (2022) hybrid conference in Córdoba (Spain), online participation.

5) VIII National Congress of Industrial Microbiology and Microbial Biotechnology, 1–4 June 2022, Valencia (Spain), hybrid event, participation in person with the presentation of the poster 'Tools for quantitative microbial risk assessment along the food chain' (Annex A).
6) ONE – Health, Environment, Society – Conference 2022, 21–24 June 2022 in Brussels and online (online participation).
7) Symposium on Aerogels for Food Applications within the 2nd International Conference on Aerogels for Biomedical and Environmental Applications, 29 June–1 July 2022, Athens, participation in person.

2.3.3. Lecture and seminars to students

A seminar entitled 'Supercritical CO_2 technologies for the pasteurization and drying of food' has been organised the 27 October 2021 at UPCT for the TAIDA DOCTORAL PROGRAMME (face to face and online) addressed to PhD and master's students and researchers. The research activity on QMRA and risk assessment has been presented to the students of the courses 'Separation unit operations and process simulation' (online, 11 January 2022) and 'Food and Bioprocess Technology' (face to face and online, 24 May 2022) enrolled to the master degree in Chemical Engineering and Industrial Processes at UNIPD. A lecture entitled '$HPCO_2$ pasteurization and drying of food product' has been presented to international PhD and master students the 9th July in Maribor (Slovenia) during the Green Engineering by High Pressure Technology (GEHTP) summer school (COST action GREENERING).

3. Conclusions

3.1. Scientific outcomes

The development of tools to allow the easy implementation of exposure assessment along the food chain is important to harmonise practices and support risk assessors and decision-makers on the recommendation of the future commercial food production. The risk of listeriosis due to the consumption of RTE meat could be slowed down by implementing better practices of handling and storage to control the cross-contamination and growth of listeria. Another solution might be the development of new gold standard processes to reduce the risk of cross-contamination by inactivating the pathogens before slicing without altering the product quality. In this regard, the use of $ScCO_2$ pasteurisation technologies could be a suitable innovative technology for RTE meat. Data on the use of low-temperature pasteurisation technologies showed a reduced risk at the time of consumption. However, the implementation of such technologies should also consider economic aspects and the return of investment. Their adoption might require financial support by EC to SMEs and retailers to promote their use as a standard decontamination process.

Even if the use of low-temperature pasteurisation might be useful to increase food safety, more and more scientific evidence is needed to support their development. Specifically in case of $ScCO_2$ pasteurisation, after reviewing the current state of the art, it has been highlighted that little is known about the inactivation mechanism on solid food, and information about the behaviour of pathogenic microorganisms as well as inactivation data which can be modelled and used for QMRA. A comprehensive review on the current state of the art of the technology for the increase of the safety of meat is underwriting.

3.2. Participation of the programme

The overall focus of the work programme was the training on food risk assessment, thanks to the collaboration with a competent research group in food safety. During the programme, specific training on topics related to food safety and risk assessment has been provided by EFSA and partners within the participation of five modules of lectures and workshops. The fellow became familiar with methodologies, terminologies and practices commonly used in food risk assessment. Thanks to the EU-FORA, the fellow had the opportunity to learn by practice and apply the concept learnt to the field of innovative food processing for pasteurisation and drying, such as inactivation models and tools for the prediction of microbial behaviour. The participation in the EU-FORA programme allowed to expand the scientific network and start building cooperation and collaboration across Europe, which will be fundamental to establish interdisciplinary research focused on the future food processing industry. After the participation of the programme, the fellow will transfer the new knowledge to his research group in Italy by training students and new fellows in the field of food safety and risk assessment. The

programme was an incredible opportunity for personal and professional growth having a strong impact in the future career.

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Abbreviations

AESAN	Spanish Agency for Food Safety and Nutrition
CAGR	compound annual growth rate
CFU	colony forming unit
CNA	Centro Nacional de Alimentación
CRAN	Comprehensive R Archive Network
EU-FORA	European Food Risk Assessment
FBOp	food business operator
FDA	Food and Drug Administration
GAP	Good Agricultural Practices
GMP	Good Manufacturing Practices
GRAS	Generally Regarded As Safe
HACCP	Hazard Analysis and Critical Control Points
HHP	high hydrostatic pressure
$HPCO_2$	high-pressure carbon dioxide
MAP	modified atmosphere packaging
NAOS	Spanish strategy for nutrition, physical activity and prevention of obesity
NETVAL	network for the enhancement of research
PhD	Doctor of Philosophy
QMRA	quantitative microbial risk assessment
RIBMINS	risk-based meat inspection and integrated meat safety assurance
RTE	ready-to-eat
$ScCO_2$	supercritical carbon dioxide
STEC	Shigatoxin-producing *Escherichia coli*
TAIDA	Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario
UNIPD	University of Padua
UPCT	University Polytechnic of Cartagena
WHO	World Health Organization

Annex A – Poster presented at the VIII National Congress of Industrial Microbiology and Microbial Biotechnology

TOOLS FOR QUANTITATIVE MICROBIAL RISK ASSESSMENT ALONG THE FOOD CHAIN

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ABSTRACT

The aim of this work is to present an example of implementing tools for Quantitative Microbial Risk Assessment (QMRA) of food products along the food chain. A common ready-to-eat (RTE) product from Spain was used as a case study to identify a possible microbiological hazard for the population. Following, the exposure assessment model was implemented using mathematical models and statistical software to describe the microbial behavior along the food chain. The study will present the possibility to identify the risk exposure in different scenarios (e.g. growth during different storage conditions, inactivation induced by decontamination techniques), showing the flexibility of the tools. The outcome of the QMRA is to estimate the probability of illness of the target population after the consumption of possible contaminated food.

1. INTRODUCTION

Quantitative Microbial Risk Assessment (QMRA) is a scientific based process, which estimate the risk of infection and illness when a population is exposed to microorganisms in food. QMRA follows four steps (Fig 1). For QMRA implementation, it is necessary to develop mathematical models for the analysis of the behavior of the risk along the whole food chain. Here an example of implementing QMRA is reported considering as case study the risk for *Listeria monocytogenes* in RTE cured meat in combination with the adoption of low temperature pasteurization technologies.

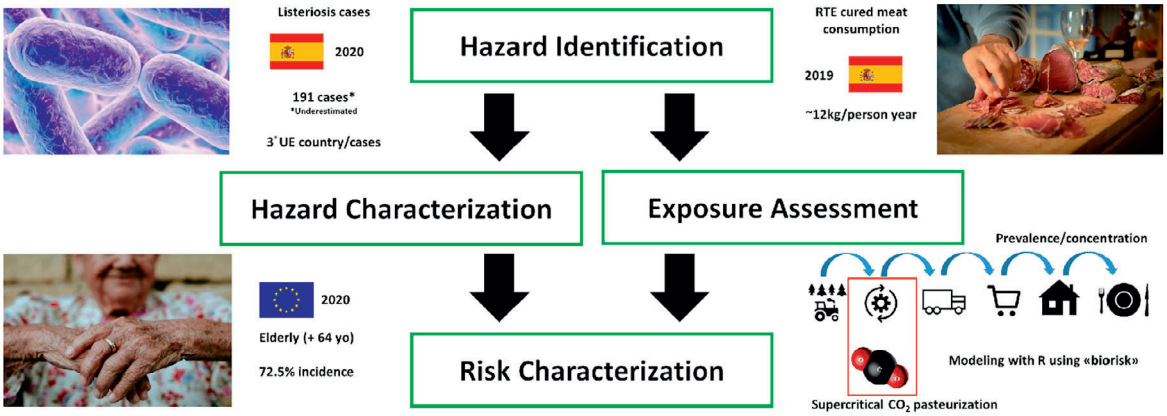

Figure 1. The four steps of risk assessment. Hazard characterization studies the nature of the health effects of the hazard (e.g. pain) and where possible propose a safe level of exposure for consumers. In the exposure assessment, there is an association of the hazard present in the food with the population and the amount of food eaten. In the risk characterization, assessors draw conclusions on the level of risk of the specific pathogens for consumers in general or specific groups

2. METHODS

The risk assessment was based on four separate stages in accordance with Codex Alimentarius. Hazard identification was performed collecting information from literature data on listeriosis and the behavior of *L. monocytogenes* in cured meat. For the hazard characterization the exponential dose-response model by FAO/WHO was selected. Growth and inactivation modelling were estimated using Combase and Bioinactivation [1]. Exposure assessment was implementing using the statistical software R and the package biorisk. The risk was characterized per number of serving.

3. RESULTS

The prevalence levels of *L. monocytogenes* in dry cured meat is highly dependent on the type of meat and post process operation, which is responsible of cross contamination. In case of dry cured ham the overall occurrence of *L. monocytogenes* in retail varies from not detected to a prevalence up to 12% [2]. *L. monocytogenes* can growth at low temperature during storage. The inactivation kinetic of *L. monocytogenes* by high pressure CO_2 in spiked dry ham at 8 and 12 MPa [3] was modeled using Weibull-Peleg inactivation model. Different pathways can be implemented for the exposure assessment, including growth at different stages (e.g transport, grocery, domestic) and including decontamination phases. Including data on consumption and threshold vales for specific group of people, it is possible to estimate the level of risk. The model permits to evaluate different case scenario in which the adoption of best practices can reduce the risk for the population.

4. CONCLUSIONS

L. monocytogenes is a harmful pathogens. In 2020, 27 MS reported 1,876 confirmed invasive human cases of *L. monocytogenes* that caused 780 hospitalizations and 167 deaths in the EU. Listeriosis was the fifth most commonly reported zoonosis in humans in the EU. *L. monocytogenes* infections were most commonly reported in the age group over 64 years' and particularly in the age group over 84 years'. The development of tools to easily implement exposure assessment along the food chain is important to harmonize practices and support risk assessors and decision-makers on the recommendation of the future commercial food production.

5. ACKNOWLEDGEMENTS

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Changes in terms of risks/benefits of shifting diets towards healthier and more sustainable dietary models

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Abstract

Food production and therefore human diet are identified as important sources of environmental impact. Unhealthy and unsustainable diets with high intake of meat and low intake of plant-based products are predominant worldwide. On the other hand, a balance between health and sustainability is necessary, since diets that are environmentally sustainable could lack essential macro- and micronutrients and result in nutrient deficiencies, non-communicable diseases and malnutrition. The aim of this project was to investigate the nutritional adequacy and environmental impact of adults' diet by comparing current dietary habits and new alternative dietary scenarios. First, a systematic review was conducted following PRISMA guidelines to assess the environmental impact of current diets and sustainable dietary scenarios such as vegan, vegetarian, pescatarian, omnivorous and optimised dietary patterns worldwide. In the second part of the project, an optimisation study was conducted, based on a multi-objective optimisation approach, with the purpose of creating the optimal diet based on nutritional and environmental constraints for the adult Italian population. The dietary scenarios of the optimised models were developed in accordance with the Italian food-based dietary guidelines. Food consumption data was extracted from the INHES study and used as baseline diet. A clear evaluation of the current environmental impact because of diet and potential sustainable dietary scenarios globally is challenging. Finally, national food-based guidelines should be adjusted by suggesting healthy and sustainable dietary recommendations in line with traditional eating habits and local food availability in order the desirable dietary shifts towards sustainability to be feasible.

Keywords: dietary scenarios, food sustainability, environmental impact, alternative diets, dietary patterns

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1. Introduction

Due to the technological innovations in agricultural sector, food availability and security have increased significantly. As a result, hunger, undernutrition, poverty and mortality have been reduced through the last years worldwide (Myers et al., 2017; Willett et al., 2019). However, diets with low vegetable, fruit, wholegrain and high in red and processed meat intake are dominant worldwide (Springmann et al., 2018; Kalmpourtzidou et al., 2020). As a result, high-calorie diets and animal-based protein sources have a negative impact both on health and environment (Heller et al., 2013; Willett et al., 2019; Grosso et al., 2020). Food production is responsible for the one-third of global greenhouse gas emissions (GHGEs) on the planet (Whitmee et al., 2015; Myers et al., 2017; Crippa et al., 2021). The nutritional and economic transition in low- and middle-income countries contributed largely to the double burden of malnutrition and high environmental impact of diets in these countries (Gill et al., 2015; Miller et al., 2016; Batal et al., 2018; Kalmpourtzidou et al., 2020). Therefore, a dietary shift to alternative, more sustainable choices is necessary.

According to Food Agriculture Organization (FAO), sustainable diets are defined as 'those diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future generations' (FAO, 2010). EAT-Lancet Commission defined global targets for sustainable diets and food systems taking into consideration environmental, nutritional and health factors (Willett et al., 2019). Even though the planetary health diet proposed by EAT-Lancet Commission could have a positive effect both on health and the environment, the eating habits, traditions and food availability of each country are taken into account, separately (Willett et al., 2019). Numerous studies in the last years have been conducted with the aim to evaluate the effect of the current diets and alternative diets on the environment and health. Vegetarian, Mediterranean and the New Nordic diet are some of the dietary patterns that could be considered sustainable (Aleksandrowicz et al., 2016).

Consequently, the main goal of the specific project, as part of the European Food Risk Assessment (EU-FORA) fellowship programme was initially to systematically review the environmental impact of the current diets and alternative diets worldwide. Based on these results, the current Italian diet was optimised by minimising its GHGEs and meeting the national food-based dietary guidelines (FBDGs) and the National Recommended Energy and Nutrient Intake Levels (LARN) proposed by the Italian Society of Human Nutrition (SINU).

2. Description of work programme

2.1. Aims

The aim of this project was to investigate the nutritional adequacy and environmental impact of adults' diet by comparing current dietary habits and new alternative dietary scenarios. To this purpose, firstly a systematic review and afterwards an optimisation study were conducted. The systematic review aimed to understand and analyse the sustainability of current diets worldwide in comparison to alternative diets in relation to their environmental impact. The objective of the optimisation study was to build optimal sustainable dietary models for the adult Italian population using the current Italian diet as baseline diet and taking into consideration the Italian FBDGs and LARN.

2.2. Activities/methods

2.2.1. Systematic review

In the first part of the project, a systematic review was conducted following PRISMA guidelines to assess the environmental impact of current population diets and alternative sustainable dietary scenarios such as vegan, vegetarian, pescatarian, flexitarian, including optimised dietary patterns worldwide. Scopus, Web of Sciences and Pubmed were searched for available data from October 2021 to January 2022. The key strings were adjusted based on the database searched. Only original studies, conducted and published from 2000 onwards, in English language, quantitatively assessing the nutritional and environmental impact of dietary patterns in healthy adult populations, were included.

2.2.2. Optimisation study

The analysis of health and environmental impact of more sustainable diets have been widely investigated in the last years in Europe (Laine et al., 2021). However, the attempts to optimise the Italian diet have been performed based on the last National Food consumption Survey INRAN-SCAI 2005–2006 (Leclercq et al., 2009), which does not represent the current dietary habits in Italy. Therefore, to allow a better reliability of the optimisation study, the use of food consumption data from the INHES study has been considered a preferable solution to better represent the most recent food consumption in Italian population (Pounis et al., 2017). In INHES, adults older than 18 years were recruited and 24-h recalls were collected for the food consumption assessment. The EFSA Food classification and description system for exposure assessment (FoodEx2) (EFSA, 2017) was applied for the extraction of food consumption data (Pounis et al., 2017).

The optimisation modelling was based on the multi-objective optimisation approach proposed by Donati et al. (2016). Specifically, a linear programming model was developed, and constraints related to the nutrients requirements per age and sex based on LARN (SINU, 2014), the frequency of food groups consumption based on the Italian FBDGs, alternative plant-based protein food products, acceptability and environmental impact indicators were applied. The nutritional constraints have been drawn up taking into account daily energy requirements distinguishing between men and women, and the different sources of energy. The model incorporated restrictions on energy and macronutrients according to LARN recommendations. The optimal combination of food products was obtained minimising the environmental impact and specifically the GHGEs of the diet. The objective functions were represented algebraically as follows:

1. Nutrients intake:

$$\sum_i \sum_j \text{food}_{i,j} a_{i,k} \geq \text{low}_k \ \forall k,$$

$$\sum_i \sum_j \text{food}_{i,j} a_{i,k} \leq \text{upp}_k \ \forall k,$$

where $\text{food}_{i,j}$: food item i of food category j; $a_{i,k}$: coefficients of energy and macronutrients k per gram of food i; low_k and upp_k: lower and upper level of nutrients, respectively.

2. FBDGs recommendations:

$$\sum_i \text{food}_{i,j} \leq \text{port}_j (1 + 0.10) \forall j,$$

where port_j: portion associated with each food category.

3. GHGEs minimisation:

$$\min_{\text{food}_{i,j} \geq 0} \text{MEM} = \sum_i \sum_j \text{food}_{i,j} \text{em}_i,$$

where MEM: minimise carbon dioxide with food item specific impact em_i (CO_2 emissions).

3. Conclusions

3.1. General conclusions of the project results

The results of the studies included in the systematic review vary within and in-between countries regarding the environmental impact of current and alternative diets. Ideally, common methodology regarding current food consumption and environmental impact assessment should be used to have comparable and harmonised results. Optimised dietary models have a lower CO_2 footprint, especially diets identified as sustainable or sustainable and healthy, than dietary scenarios based on literature. Comparing current diets to sustainable and health scenarios/models created per country, meat and dairy products consumption should decrease, while fruit, vegetable, fish and egg consumption should increase. National nutrition surveys instead of food supply/purchase or FAOSTAT food balance sheets should be used as baseline diets for the constructions of more sustainable and healthy scenarios to avoid the overestimation of current food consumption. Additionally, available data derived mainly from high-income countries, thus low- and middle-income countries are underrepresented. In order to have

a holistic overview of the environmental impact of the diets, further research is needed and should target low- and middle-income countries. Finally, the construction of optimal sustainable diets is complex. Multiple constraints need to be considered to propose diets being healthy, nutritious, acceptable, affordable, and environmentally sustainable for the general population.

3.2. Scientific activities of the fellowship

3.2.1. Optimisation course

The fellow followed the course 'Economical modelling and environmental policies', part of the MSc programme 'Sciences and technologies for environment and resources' of the University of Parma, taught by Professor Michele Donati. The knowledge of the construction of mathematical programming models and the use of related programming tools, such as the General Algebraic Modelling System (GAMS), helped the fellow to understand the optimisation modelling methodology for the construction of the sustainable optimal dietary models.

3.2.2. Poster presentation

The fellow made a poster presentation at the National Conference Italian Society of Human Nutrition 2022 that took place in Naples, Italy. Part of the results of the systematic review were presented. Current diets and alternative dietary scenarios and their environmental impacts in South Europe were the main focus.

3.2.3. Conferences and webinars (Table 1)

Table 1: Conferences and webinars followed by the fellow

Title	Type of activity	Organiser/Tutor	Date
Towards the Future of Food: the Cultured Meat between Food Safety, Sustainability and Public Perception	Webinar	University of Parma	27/9/2021, 10.00–13.00 CET
Sustainable and Healthy Diets Webinar 1: Introducing the concept and context of sustainable healthy diets Webinar 2: Current policies and global initiatives on sustainable and healthy diets Webinar 3: Revision of current Food Based Dietary Guidelines and policy required Webinar 4: The food cycle: from farming and sourcing to packaging and food waste Webinar 5: Applying Sustainability in Dietetic Practice	Webinar series	EFAD	16/9/2021–31/5/2022
Climate Change, biodiversity and nutrition: promoting healthy diets from efficient, inclusive, resilient and sustainable food systems	WHO Side Event at Pre-COP26	FAO	10 January 2021, 14:00–15.30 CEST
Alimentazione e tumori *(Nutrition and tumors)*	Conference	SINU	22/10/2021, 9.00–13.00 CET
Life Cycle Assessment for sustainability food systems: integrating nutritional and environmental assessment	Webinar	FAO	27/10/2021, 9.30–10.30 CEST
EAT-GlobeScan Consumer Research on a Sustainable Food System	Webinar	EAT	29/10/2021,
Achieving Healthy Diets from Sustainable Blue Food Systems in Small Island Developing States	WHO Side Event at COP26	WHO	11 February 2021, 18:00–19:00 CET
Changing Behaviour: From Policy to table. Moving the Dial towards Healthy Sustainable diets	E-symposium	Alpro Foundation	17/11/2021, 14:00–16:20 CET

Title	Type of activity	Organiser/Tutor	Date
Shifting our food systems to support sustainable eating for climate and public health	E-symposium	Oatly	12 September 2021, 14:00–18:15 CET
Vivi l'esperienza sostenibile *(Live the sustainable experience)*	Conference	SINU	14/12/2021, 9.00–17.30 CET
XLII National Conference Italian Society of Human Nutrition (SINU) 2022	Conference	SINU	04–4 June 2022
How can chefs help to save the planet? Discover the Life Climate Smart Chefs project	Webinar	Barilla Foundation	24/5/2022, 16:00–17:30 CET
Fruit and Vegetables for Sustainable Healthy Diets (FRESH)	Webinar	IFPRI	25/5/2022, 15:30–17:00 CET
Why consider soy alternatives to dairy and meat: Building a healthy, sustainable diet	Webinar	Alpro Foundation, Fuji Foundation for Protein research	14/6/2022, 9.00–11.30 CET
One Health Conference 2022	Conference	EFSA	21–24/06/2022

3.3. Additional information

Manuscripts reporting detailed results of the systematic review and the optimisation study are in preparation and will be submitted to peer-reviewed journals. Publications will follow after the end of the fellowship programme.

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Abbreviations

EFAD	European Federation of the Associations of Dietitians
EU-FORA	European Food Risk Assessment
FAO	Food Agriculture Organization
FBDGs	food-based dietary guidelines
GHGEs	greenhouse gas emissions
IFPRI	International Food Policy Research Institute
INHES	the Italian Nutrition and Health Survey
LARN	Livelli di Assunzione di Riferimento di Nutrienti ed energia (National Recommended Energy and Nutrient Intake Levels)
SINU	Società Italiana di Nutrizione Umana (Italian Society of Human Nutrition)
WHO	World Health Organization

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Implementation of food matrix effects into chemical food contaminant risk assessment

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Abstract

Food risk assessment plays an important role in protecting public health worldwide. Stakeholders involved in food risk assessment, such as national authorities, agencies, non-governmental organisations (NGOs), industry and consumers, need to properly understand the terminology of food risk assessment effectively. In this respect, the first part of the EU-FORA work programme (WP1) aimed to provide insights into the actual translation of two essential terms used in food risk assessment. 'Hazard' and 'risk' were first identified and compared between the English version of various food regulations and their equivalents in the national legislation of EU Member States. The comparison and critical evaluation revealed several inconsistencies. These inconsistencies could lead to misinterpretations, followed by errors in conducting risk assessments or communicating risks. We recommend that consistency is restored and maintained so that the message is properly communicated. The second part of the work programme (WP2) was focused on a specific area within chemical risk assessment (CRA). In this context, special attention was given to the impact of the food matrix on the bioaccessibility and bioavailability of heavy metals and metalloids. After collection and careful selection of data from scientific journals, a database with information on the bioaccessibility and bioavailability of cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As) in different food matrices was created for future statistical analyses related to dietary exposure.

Keywords: hazard, risk, food regulation, chemical contaminants, bioavailability, bioaccessibility, food matrix

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1. Introduction

In a constantly changing environment, the safety of food for human consumption remains a priority. For this purpose, science about food risk assessment aims to protect public health as much as possible. The steps used in food risk assessment are hazard identification, hazard characterisation, exposure assessment and risk characterisation. All these steps revolve around the terms 'hazard' and 'risk' (EUFIC, 2019). The use of these terms as synonyms or their interchange is a problem that has not yet been overcome, despite awareness raised by European food authorities and organisations (EFSA Scientific Committee, 2012; EFSA, 2016, 2021; EUFIC, 2017, 2019). It is essential for all food safety stakeholders to differentiate between 'hazard' and 'risk'. On the one hand, risk assessors, risk managers and the food industry need to clearly understand these terms, as they play an important role in preventing and controlling hazards and minimising risks. On the other hand, consumers should be able to discriminate between these two terms in risk communication to get an accurate information of the food safety level. Precise definitions of these terms can be found in the Codex Alimentarius, the General Food Law and some other food regulations (xxxx, 2015; xxxx, n.d.-a, n.d.-b). Hazard refers to any physical, chemical or biological agent or condition with the potential to cause an adverse health effect. Risk is a function of the probability of occurrence of an adverse effect due to exposure to a hazard. Furthermore, recognising the difference between 'hazard' and 'risk' is a basic requirement for any food risk assessment, whether it is microbiological or chemical. Chemical contamination of food can arise from chemical substances in the environment during production, processing, packing, transport and storage. In most cases, residues of metals and metalloids originate from human-related activities (e.g. agriculture, industry, etc.). Compounds such as Cd, Pb, Hg and As can enter the human body after food consumption and cause disease or adverse effects over time (EFSA, 2009; EFSA CONTAM Panel, 2009, 2010, 2012). Given the increasing variety of foods available to consumers, it is important to provide information on how effectively contaminants from digested foods are absorbed into the systemic circulation. Once ingested, digestion of foods in the gastrointestinal tract can positively or negatively affect the bioaccessibility and bioavailability of substances, depending on the food matrix.

2. Programme description

2.1. Aims and objectives

2.1.1. WP1

The aim of WP1 was to identify inconsistencies in the translation of 'hazard' and 'risk' in the national food legislation of the EU Member States. Understanding and using these terms correctly supports more accurate assessments and ensures effective communication between stakeholders such as assessors, managers and the general public. Interaction with the EU-FORA community and representatives of some EFSA focal points was maintained during this task to seek the opinion of native speakers of the official EU languages, who are also experts with background knowledge in food safety, on the correct translations. At the same time, this action served to network and build professional relationships between food safety experts.

2.1.2. WP2

The aim of WP2 was to find data on the bioaccessibility and bioavailability of Cd, Pb, Hg and As from different food matrices to build a database in a form that it will allow statistical analysis.

2.2. Activities and methods

2.2.1. WP1

First, a software tool was developed to identify inconsistencies in translation based on the comparison between national food regulations of EU Member States and their corresponding English version. With the help of this tool, we were able to speed up the process of comparing food regulations in English with their counterparts in each of the official EU languages: Bulgarian, Czech, Spanish, Danish, German, Estonian, Greek, French, Gaelic, Croatian, Italian, Latvian, Lithuanian, Hungarian, Maltese, Dutch, Polish, Portuguese, Romanian, Slovak, Slovenian, Finnish and Swedish.

Emphasis was placed on the Regulations where food risk assessment terminology occurs most frequently and on specific words such as 'hazard' and 'risk'. The old versions of the Regulations were reviewed in parallel with the new versions (Figure 1). All specific terms are defined in Regulation (EC) 178/2022 (Chapter I. Scope and Definitions) and Regulation (EC) 625/2017 (Title I. Article 3. Definitions) for each language. This served as a reference point to determine whether a translation is consistent or inconsistent with selected definitions of hazard or risk. Following the collection of inconsistencies in the selected regulations, a survey was prepared and different experts involved in food risk assessment who are also native speakers of the official EU languages were asked to participate. At least one native speaker of each language completed the survey. The results of the survey were expressed as marks from 1 (consistent) to 5 (inconsistent) (Figure 2). The marks were collected in pivot tables in Excel. The collected data was statistically analysed by different methods, e.g. clustering.

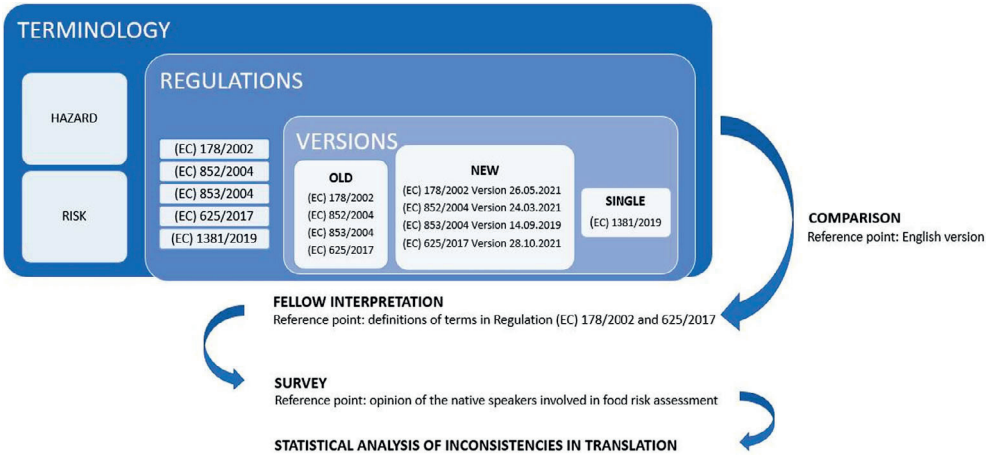

Figure 1: WP1 flow chart

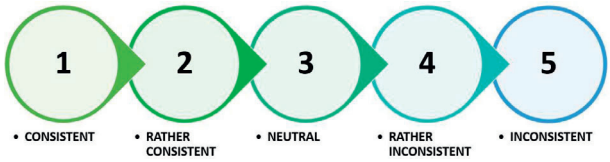

Figure 2: Marking system

2.2.2. WP2

The screening was conducted to collect data from scientific journals published between 2002 and 2022. The number of selected articles that were included into the database was 122 (see Figure 3 for the detailed process).

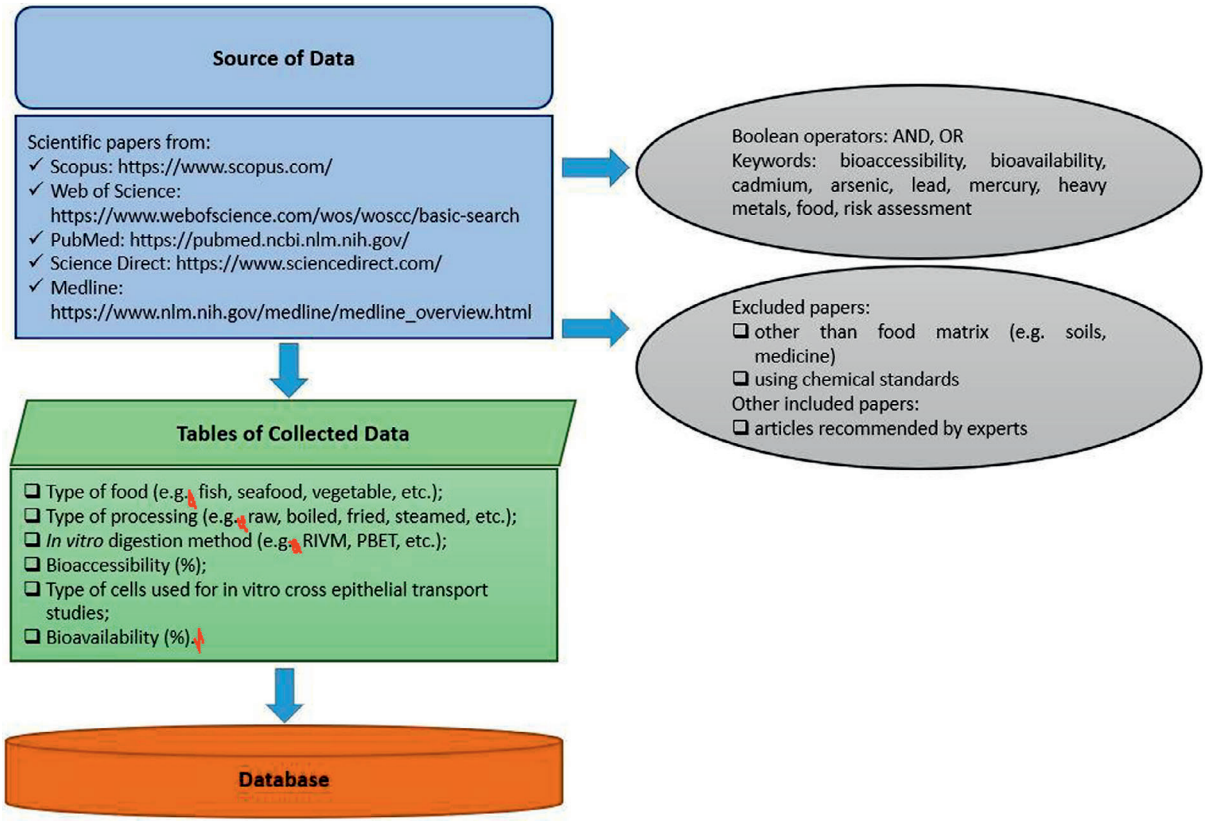

Figure 3: WP2 flow chart

3. Conclusions

3.1. WP1

The results showed inconsistencies in translation in all the regulations studied and for all official EU languages except Gaelic. Inconsistencies in translation include using the 'hazard' instead of 'risk' and vice versa, but also issues that are specific to some languages only. Specific problems include interchanging risk assessment with risk determination (Spanish), translating hazard to source of danger or risk (Greek) and translating hazard as risk factor (Greek, Slovenian and Lithuanian). In most cases, the native speakers confirmed our results, as seen in Figure 4. In very few cases where opinions diverged, we argued with the definitions of terms from Regulation (EC) 178/2022 and Regulation (EC) 625/2017, while the native speakers argued for linguistic freedom. In this case, linguistic freedom stems from the fine distinction between the meaning of 'hazard' and 'risk' in some languages and the degree of rigour in using technical terminology used by different experts in specific contexts. In the old versions of the regulations, the highest number of inconsistencies was found in Lithuanian (48). For the new versions of the regulations, the results showed that the Greek regulations have the highest number of inconsistencies, namely 36. Overall, the newer versions of the regulations (EC) 178/2002, 852/2004, 853/2004 and (EC) 625/2017 have a lower number of inconsistent translations than the old versions. However, revisions are still necessary. Regulation (EC) 1381/2019 has the lowest absolute number of problematic translations. In conclusion, we recommend correcting all regulations containing inconsistent translations of terms related to food risk assessment. Accurate translations should strictly follow the technical terms defined in Regulation (EC) 178/2022 and Regulation (EC) 625/2017. Any deviation from the definitions could lead to misinterpretations that complicate the understanding of 'hazard' and 'risk' for food safety stakeholders (Scheer et al., 2014).

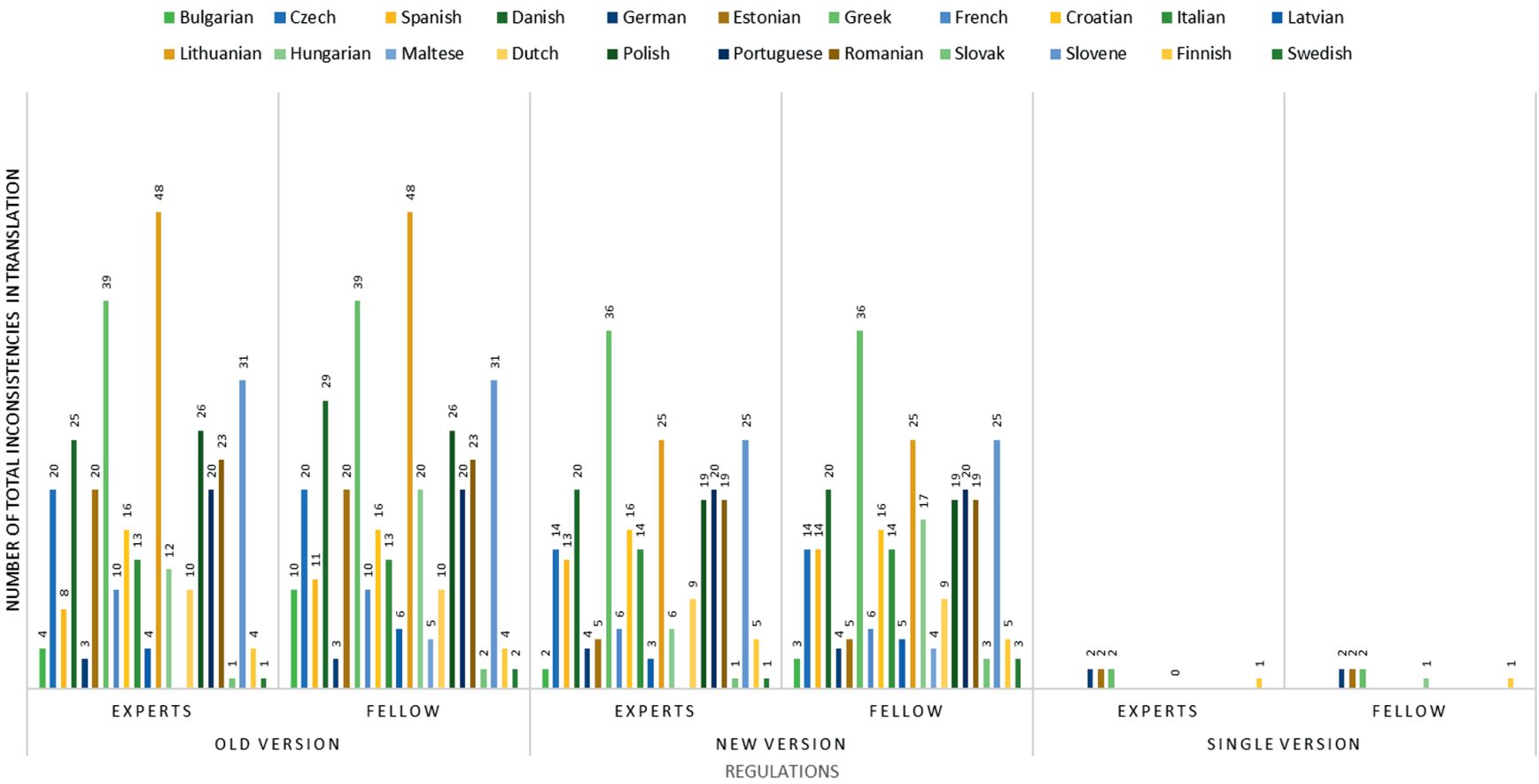

Figure 4: Comparison of total inconsistencies in translation. Old version – Regulations (EC) 178/2002, 852/2004, 853/2004 and 625/2017; New version – Regulations (EC) 178/2002, 852/2004, 853/2004 and 625/2017; Single version – Regulation (EC) 1381/2019.

3.2. WP2

The selected data included into our database contained information on bioaccessibility and bioavailability of heavy metals or metalloids in matrices such as fish, seafood, rice, vegetables, seaweed, mushrooms, etc. Studies on bioaccessibility were more commonly found than studies on bioavailability. Statistical processing of preliminary extracted data showed differences in the bioaccessibility of the individual chemical contaminants depending on the food matrix. The results presented in Figure 5 describe bioaccessibility in different foods, which was determined using average values from several *in vitro* studies. Although the data can be grouped in a more refined manner (e.g. groups for the exact type of fish, vegetables, mushrooms, etc.), the plots are sufficient to highlight the importance of the effects of the food matrix. In addition, the database provides access to some relevant information that could be considered in future research. It confirms the contribution of some factors to the bioaccessibility and bioavailability of heavy metals and metalloids. These are food processing factors (e.g. cooking, frying, grilling, steaming, etc.), factors related to *in vitro* analysis (e.g. *in vitro* method) or characteristics associated with the chemical element (e.g. speciation in the case of elements such as As, Hg). The existence of databases that collect knowledge in this area can improve chemical food risk assessment by incorporating the effects of the food matrix into the exposure assessment. This approach allows a more realistic understanding of the risks to human health.

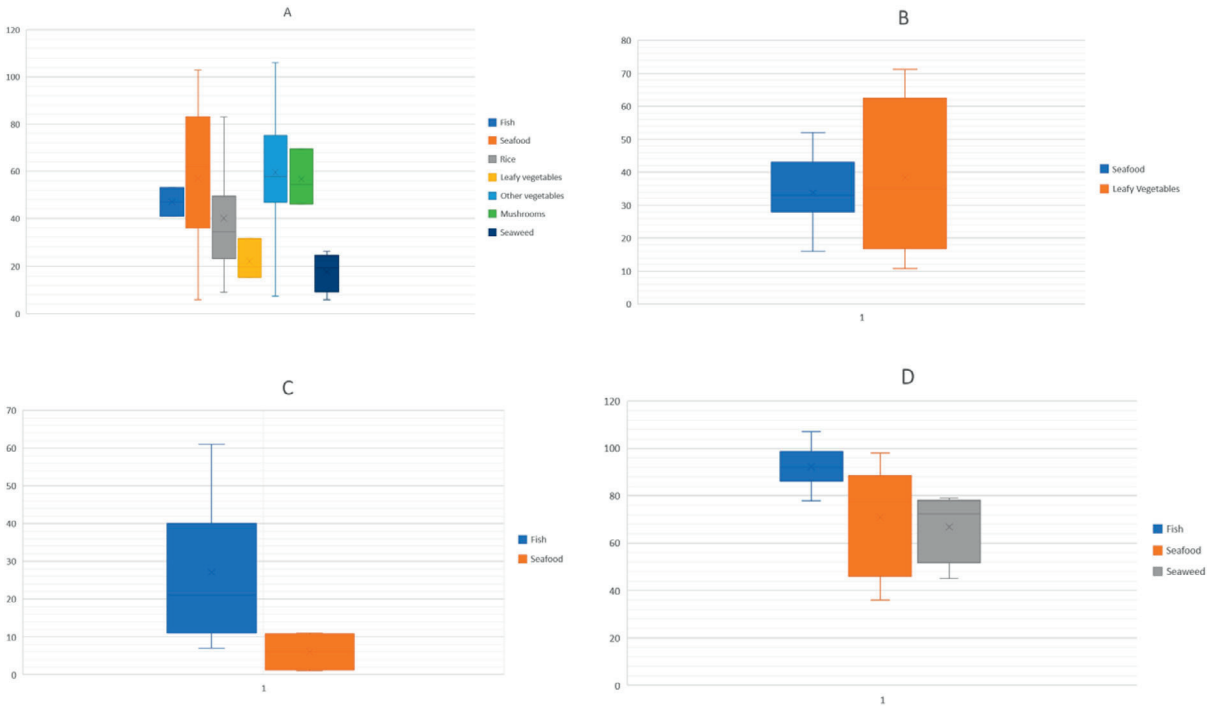

Figure 5: Cd (A), Pb (B), Hg (C) and As (D) bioaccessibility (%) in food

3.3. EU-FORA experience

It was a significant opportunity for the fellow to deepen her knowledge of food risk assessment terminology. The work programme enabled her to analyse and draw conclusions about the use of 'hazard' and 'risk' in oral and written communication. Understanding how these terms differ from each other was an important first step. After this clarification, the fellow was ready to expand her knowledge of food chemical risk assessment (CRA) and to address complex issues such as food matrix effects in bioaccessibility and bioavailability of heavy metals.

In addition to the individual research work, the fellow engaged EU-FORA fellows, Alumni and other relevant potential collaborators to complete her WP1 task. The achievement of interesting final results resulted from a combination of independent work and collaboration. This reinforced the idea that the EU-FORA programme provides a valuable context for building a professional network from the beginning of the fellowship.

In addition, the fellow participated in complementary activities to deepen the experience (described in Appendix A).

3.4. Disclaimer

More detailed results are excluded from this report to avoid specific copyright claims as they are intended for publication in scientific journals.

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Abbreviations

CRA	chemical risk assessment
NGOs	non-governmental organisations
PBET	physiologically based extraction technique
RIVM	Dutch National Institute for Public Health and the Environment
WP1	1st part of the work programme
WP2	2nd part of the work programme

Appendix A – Secondary activities

Additional relevant activities and learning opportunities completed by the fellow:

- Induction training of the European Food Risk Assessment Fellowship Programme' (EFSA) (30 August–17 September 2021 – virtual).
- Visit to 'Jožef Stefan Institute' in Ljubljana (23 September 2021) (www.ijs.si).
- SPSS Statistics lecture by coordinator Assist. Prof. Livija Tušar (14 October 2021, 8 November 2021 and 22 November 2021).
- 'Module 1 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (22–26 November 2021 – virtual).
- Visit to the renovated unit of the National Veterinary Institute (NVI) from Maribor (29 November 2021) (https://www.vf.uni-lj.si/en/area/nvi-maribor).
- 'Module 2 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (22–26 March 2022 – virtual).
- European Climate Pact Ambassador, DG CLIMA – European Commission (https://europa.eu/climate-pact/ambassadors/meet-our-ambassadors/ana-andreea-cioca_en#responsibilities) – volunteer (1 April 2022).
- SPSS Statistics lecture by coordinator Assist. Prof. Livija Tušar (30 May 2022).
- Field trip organised by the Department of Microbiology, Biochemistry, Molecular Biology and Biotechnology, University of Maribor for students of Food Safety Master Degree (23 May 2022):
  - Visit to 'Vinakoper', Koper, Slovenia – wine producing unit (https://www.vinakoper.si/sl/);
  - Visit to 'Laboratory of the Institute for Oliveculture', Koper, Slovenia – olive oil analysis unit (https://www.zrs-kp.si/index.php/en/institutes-units/lab-ifo/#1523593162108-9fa67f4a-f3ba).
- 'Module 3 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (6–10 June 2022, Athens, Greece – onsite).
- Team building Faculty of Agriculture and Life Science, University of Maribor (13–14 June 2022):
  - Visit to 'Konda' organic farm, Bela Krajina, Slovenia (sheep breeding and rearing, milk processing, Konda dairy products, http://www.kmetija-konda.si/sirarna/),
  - Visit to Vina Prus, Metlika, Slovenia – wine producing unit (https://www.vinaprus.si/).
- Conference Pesticides 2022, Ioannina, Greece, 23–26 June 2022 (online attendance). One abstract entitled 'Food Matrix Effects on Bioaccessibility and Bioavailability of Heavy Metals' was accepted. A poster was presented in POSTER SESSION III of the day 25 June 2022, between 15:10 and 16:00.
- Onsite attendance to lecture 'Post-Harvest Technologies in Agriculture' by Visiting Professor Elazar Fallik from the Volcani Institute, Rishon LeZiyyon Israel (16 June 2022).
- Regular meetings with supervisors (onsite and online).
- Online attendance at numerous scientific webinars and conferences:
  - RAFA 2021. 'Virtual event highlighting current Trends & Views', Recent advances in food analysis 2021 (3–4 November 2021);
  - EDQC 2021. European Dairy Quality Conference 2021 (16–18 November 2021);
  - eSymposium 'MILK THISTLE – PHYTOCHEMICAL, PHARMACOLOGICAL AND CLINICAL EVIDENCE', Herbal Medicinal Products Platform Austria (HMPPA) and the Society for Medicinal Plant and Natural Product Research (GA) (18 November 2021);
  - Taylor and Francis Group: The Impact of Publishing (22 November 2021);
  - Webinar on good practices in poultry production (23 November 2021);
  - Taller Internacional One Health y Cambio Climático 2021 (29 November–1 December 2021);
  - Future of Food Conference 2021 (30 November–1 December 2021);
  - OASPA Webinar: The Fully Open Access Agreement – an Essential Component of a Diverse, Open Access World (6 December 2021);
  - Evidence-based Toxicology Collaboration (EBTC) December Symposium (14 December 2021);
  - Food Safety 4 EU 'Sustainable food: how to keep it safe?' (15 December 2021);
  - Future of Food – Alternative Protein Industry and Our Diet (17–21 January 2022);

- Why you should consider Göttingen Minipigs as your large animal model (25 January 2022);
- CiFOOD Conference 2022 – MAJOR CHALLENGES OF FUTURE FOOD SYSTEMS (31 January–1 February 2022);
- A good start for calves (3 February 2022);
- Breakfast Bite: Food Safety Training – What you Need to Know (10 February 2022);
- Nutrition basics: Protein in pig feed (3 March 2022);
- SUSTAINABLE FOOD PACKAGING, ECO DESIGNS AND COMMUNICATION TO CONSUMERS, RESULTS FROM MARKET ANALYSES BY THE CIRCUL-A-BILITY NETWORK (21 April 2022).
- Solving critical gut challenges innovations contributing to farm profitability (11 May 2022);
- ONE Conference (21–22 June 2022).

The fellow will also attend the following events before the end of the EU-FORA programme:

- 'Module 4 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (22–25 August 2022);
- Conference FoodMicro2022, Athens, Greece, 28–31 August 2022 (online attendance with a poster accepted). Poster title: "Risk Analysis: Towards a Better Understanding of 'Hazard' and 'Risk'".

EU-FORA SERIES 5

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Impact of drinking water treatment processes on the residues of plant protection products for consumer and aquatic risk assessment: theoretical and experimental studies

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Abstract

Pesticides residues can occur in ground and surface waters, and potentially react with chemicals used for water disinfection treatments, such as chlorine. This can lead to the formation of unknown reaction products, which can be more toxic and/or persistent than the active substances themselves, and therefore become a potential risk for human health and environment. Thus, in the framework of the EU Regulation 1107/2009, the identification of these by-products and their potential risk should be assessed. Within the European Food Risk Assessment (EU-FORA) Fellowship Programme, the fellow studied the behaviour of herbicides belonging to the families of imidazolinones and sulfonylureas in waters treated with chlorine disinfectants. Due to their physicochemical properties, these herbicides are susceptible of reaching natural waters. In fact, some of them have been detected in water monitoring programmes. During the experimental part of the present work programme, reactions between the active substances and the most used chlorine disinfecting reactants (hypochlorite and chloramines) were performed. Degradation kinetic parameters such as half-lives and degradation constants were calculated. Results showed that herbicide degradation was both pH and chlorine/chloramines concentration dependent. In order to identify the degradation by-products, high-resolution mass spectrometry experiments were performed, and a possible route of formation of these compounds was proposed. Finally, their risk assessment was carried out by using tox/ecotoxicological properties determined by QSAR methodology and FOCUS modelling for hazard and exposure assessment, respectively. These results will contribute to the definition of a risk assessment scheme for pesticides by-products potentially occurring in drinking water.

Keywords: pesticides, drinking water, risk assessment, chlorine, degradation products, QSAR, FOCUS

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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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Table of contents

1. Introduction

The European Food Risk Assessment (EU-FORA) Fellowship Programme is a practical ('training by doing') programme that aims to increase the expertise of early to mid-career scientists in food safety risk assessment at both the European and national levels (Bronzwaer et al., 2016).

The fellow trainership was carried out at the Unit of Plant Protection Products of the National Institute for Agricultural and Food Research and Technology, INIA-CSIC, Madrid, Spain, with a work programme entitled: 'Impact of drinking water treatment processes on the residues of plant protection products for consumer risk assessment. Theoretical and experimental studies'.

The INIA is a public research institute in Spain accredited by the Spanish Ministry of Science and Innovation to perform the evaluation of the substances for which Spain is a Rapporteur Member State. The institute has been involved in the last 25 years in the implementation of the European legislation on plant protection products and has a large experience in the assessment of active substances and plant protection products. The main lines of research of the UPF are:

- Risk assessment of plant protection products in the framework of European Regulation (EC) No 1107/2009.
- Analysis of the strengths and weaknesses of the assessment process to improve the safe use of crop protection tools and to safeguard the competitiveness of European agriculture.
- The study of the environmental behaviour of active substances in terms of degradation studies in waters and soils, and the identification of possible reaction products formed as well as phyto-and eco- toxicological effects on target and non-target organism in laboratory.
- Validation of multiresidue methods for the analytical determination of pesticide residues, and development of computational methodologies for risk assessment of pesticides using statistical modelling methods, quantitative structure–activity relationships (QSARs) models and quantum chemistry.

Pesticide contamination of drinking water is considered a route of major concern. Contamination of tap water supplies by small amounts of pesticides may result in long-term exposure contaminants. However, clear links between individual pesticides and individual health effects can only be shown in animal studies, but the doses used in these studies are far higher than the enforced legally pesticide limits (Damalas and Eleftherohorinos, 2011; Harris and Gaston, 2004). Health risk, for prolonged exposure to very low levels of pesticide, is not always covered by routine toxicological tests performed with regulatory purposes. Therefore, regulation tends to be very conservative with respect to the permitted levels of contaminants in drinking water supplies. For example, EU water and drinking water directives provide that no individual pesticide may exceed 0.1 $\mu g\ L^{-1}$ and that the sum of all contaminants present in a drinking water sample may not exceed 0.5 $\mu g\ L^{-1}$ (European Commission, 2020).

Environmental contamination of ground and surface natural waters is one of the main route of pesticides contamination. However, before entering the tap water net, water is submitted to physical and chemical treatments for disinfection. The main water disinfection treatments to remove microorganisms for drinking water purposes are chlorine in the form of sodium hypochlorite (NaClO) or chloramines. Chlorine is a potent oxidant that acts as a bactericide. At the same time, chlorine and the other agents are strong oxidants that may react with various organic functional groups of pesticide molecules to form reaction products (Sandín-España et al., 2005). Main degradation processes are hydrolysis, oxidation and chlorination among others (Duirk et al., 2009; Chamberlain et al., 2010). Scientific literature shows that these processes can lead to the formation of a variety of disinfection products (Brix et al., 2009; Quintana et al., 2014; Malato et al., 2014). Many of these are unknown compounds, and their rate of degradation may vary considerably (Sandín-España et al., 2005; Lerch et al., 2007; Vanderford et al., 2008). Information on degradation products and degradation pathways is of utmost importance in understanding the environmental fate of pesticides (Harir et al., 2007). In this regard, (European Commission, 2009) states that for residues (including metabolites, breakdown or reaction products) '(...) which are of toxicological, ecotoxicological, environmental or drinking water relevance, there shall be methods in general use for measuring them' as they can be a potential risk (Boxall et al., 2004; Chen and Young, 2008).

The main concern is that a reaction product 'is deemed relevant if there is a reason to assume that it has intrinsic properties comparable to the parent substance in terms of its biological target activity, or that it poses a higher or comparable risk to organisms than the parent substance or that it has certain toxicological properties that are considered unacceptable' (Regulation 1107/2009/EC). For this reason, the identification of the reaction products, and then the evaluation of their biological activities

and tox/ecotoxicological properties are of utmost importance since they might be 'relevant for the overall approval decision or for the definition of risk mitigation measures' (European Commission, 2009).

2. Description of work programme

2.1. Aims and objectives

The aim of this work programme was to investigate the behaviour of selected herbicides in chlorinated/chloraminated waters simulating drinking water treatments, and the study of their reaction products. After evaluating tox/ecotoxicological properties by QSAR methodology, and the exposure assessment by FOCUS modelling, the fellow received training in the aquatic and consumer risk assessment of these by-products.

The main disciplines of interest were analytical chemistry, environmental and agricultural sciences.

The major objectives of this work programme were the following:

Objective 1: general concepts regarding consumer risk assessment of drinking water and aquatic risk assessment.

- Review of EU Regulations and risk assessment guidance documents related to consumer risk assessment of drinking water and environmental exposure assessment.
- Review and selection of the potential active substances for the study.

Objective 2: experimental work.

- Laboratory simulation of the reaction between the selected active substances and different water disinfectants.
- Development of analytical methods.
- Determination of degradation curves.
- Identification of by-products.

Objective 3: theoretical work.

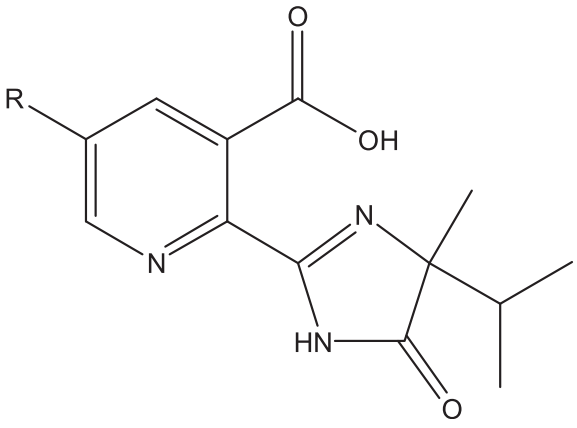
- Prediction of the most probable formation route of nicosulfuron by-products in chlorinated/ chloraminated waters.
- Determination of physicochemical and tox/ecotoxicological properties of the potential degradation products by using QSAR methodology.
- Estimation of predicted concentrations of these products in surface and groundwater.

Objective 4: conclusions

- Comparing of the experimental and theoretical results.
- Environmental and risk assessment of the degradation by-products.

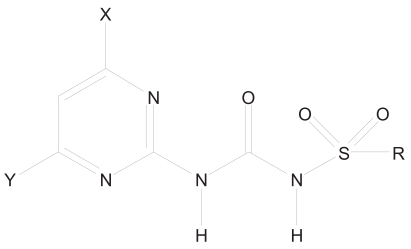
2.2. Activities/methods

In the frame of the present project, active substances considered susceptible to polluting surface and groundwater were selected on basis of their physical–chemical properties. At the same time, a bibliographic research on pesticides found in natural waters was carried out. According to this, five herbicides were selected: three belonging to the family of pyridine imidazolinones (imazamox, imazapyr and imazethapyr, Figure 1) and two to the family of sulfonylureas (azimsulfuron and nicosulfuron, Figure 2).



Active substance	R
Imazamox	$-CH_2-O-CH_3$
Imazapyr	-H
Imazethapyr	$-CH_2-CH_3$

Figure 1: Molecular structure of pyridine imidazolinones herbicides



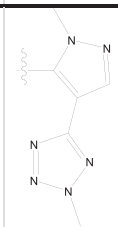
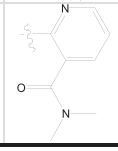
Active substance	R	X	Y
Azimsulfuron		$-OCH_3$	$-OCH_3$
Nicosulfuron		$-OCH_3$	$-OCH_3$

Figure 2: Molecular structure of sulfonylurea herbicides

2.2.1. Degradation kinetic experiments

In order to simulate the reactions between the selected herbicides and sodium hypochlorite (NaClO), different molar concentration ratios of active substance/disinfection chemical were used for the experiments at different pHs.

The exact concentration of NaClO in the stock solution was quantified using I 4500 Cl B – method, according to the iodometric titration standard method guidance (American Public Health Association APHA, 1999).

Chloramines solutions were prepared using different herbicide to disinfectant molar ratios. An appropriate mixture of sodium hypochlorite (NaClO)/ammonium chloride (NH_2Cl) was prepared for each pH. The hypochlorite solution was added drop by drop to ammonium chloride, and left stirring in

darkness. Finally, NH_2Cl solution was added to a solution of active substance (a.s.) at a known concentration.

For all the experiments (chlorination and chloramination), blank samples containing only the a.s. were used to ensure that no degradation or hydrolysis occurred. The reaction was quenched with sodium thiosulfate at scheduled time intervals.

A reversed-phase high-performance liquid chromatography with diode array detector (HPLC-DAD) procedure was developed to evaluate the kinetic evolution and the half-lives of the a.s. A 1260 Infinity HPLC model equipped with a diode array detector (Agilent Technologies, Inc., Palo Alto, CA, USA) was employed for analysis. For the a.s., a calibration plot was built for concentration ranging between 0.1 and 10 μg mL^{-1}. A good linearity was always observed. Pseudo–first-order kinetic was assumed in order to calculate the corresponding degradation rate constant (k):

$$C = C_0 \cdot e^{-kt},$$

where C_0 and C are the concentrations (mg L^{-1}) of the a.s. at t_0 and at a given time t, respectively, and k is the first-order degradation rate constant.

Half-life ($t_{1/2}$) was calculated from k using the following equation:

$$t_{1/2} = \ln 2/k.$$

Origin85 Software program was used to obtain non-linear least squares regression fit for the a.s. degradation data to the first-order model.

2.2.2. By-products identification and degradation fitting model

In order to identify the by-products deriving from the degradation of the a.s. in chlorinated/chloraminated water, liquid chromatography–electrospray-quadrupole time of flight-mass spectrometry (LC-ESI-QToF-MS) experiments were performed.

An HPLC coupled with a mass spectrometer equipped with a Q-ToF hybrid analyser model MAXIS II (Bruker) was used for the analysis. The chromatographic separations were achieved by 1100 HPLC model (Agilent Technologies, Inc., Palo Alto, CA, USA).

The analysis were carried out in positive ion mode by monitoring both $[M + H]^+$ and $[M + Na]^+$ ions, in a mass range of m/z: 50–3,000 a.m.u. A mass tolerance of maximum 5 ppm error was allowed.

CAKE Software (version 3.5) was used to estimate kinetic fits of the a.s. and their by-products, in order to determine their possible route of degradation in chlorinated/chloraminated water.

2.2.3. QSAR modelling

The key physicochemical, environmental and ecotoxicological properties of the by-products were estimated by computational-based models based on quantitative structure–activity/structure–property relationship (QSAR/QSPR) tools. To perform this task, Toxicity Estimation Software Tool (T.E.S.T.) and the EPI Suite™ software (United States Environmental Protection Agency – USEPA) were used. The models and the software were developed with the aim of regulatory use in line with strict quality criteria according to OECD guidelines (OECD, 2014).

2.2.4. Environmental exposure, aquatic and consumer risk assessment

Predicted environmental concentrations in surface water

The predicted environmental concentrations in surface water (PEC_{sw}) and sediment (PEC_{sed}) were calculated using the FOCUS simulation models Steps 1–2 v. 3.2, Focus SWASH v. 5.3 (PRZM v. 4.3.1, MACRO v. 5.5.4, TOXWA v. 5.5.3) and SWAN v. 5.0.1 (FOCUS, 2015) (Tables 1 and 3).

Table 1: Input parameters related to nicosulfuron application for $PEC_{sw/sed}$ calculations

	Input parameters
Crop	Maize
Application rate (g/ha)	62.0 g/ha
Application window	Steps 1–2: maize: Mar-May Steps 3–4: Default (post-emergence)
Application method	Steps 1–2: – Steps 3–4: ground spray
CAM (Chemical Application Method)	Steps 1–2: – Steps 3–4: CAM1
Soil depth (cm)	Steps 1–2: – Steps 3–4: 4 cm

Predicted environmental concentrations in groundwater

Predicted environmental concentrations in groundwater (PEC_{gw}) at a soil depth of 1 m, were calculated for the active substance nicosulfuron for its uses on maize, with simulation models FOCUS PELMO 6.6.4 and FOCUS PEARL 5.5.5 (FOCUS, 2021) (Tables 2 and 3).

Table 2: Application scenarios for nicosulfuron PEC_{gw} calculations

	Input parameters
Crop	Maize
Application rate (g/ha)	62.0 g/ha
Number of application	1
Crop interception (%)	0
Frequency of application	Annual
Application timing	From 2 to 4 leaves – BBCH 12

Table 3: Application generic nicosulfuron input parameters for $PEC_{sw/sed}$ calculations (STEP 1–4) and for PEC_{gw} leaching simulation (FOCUS PELMO/PEARL)

	Nicosulfuron	References
Molecular weight (g/mol)	410.4	EFSA Scientific Report 120 (2007)
Water solubility (mg/L)	9,500 (20°C)	EFSA Scientific Report 120 (2007)
Saturated water pressure (Pa)	8 E-10 (20°C)	EFSA Scientific Report 120 (2007)
DT_{50} in soil (day)	16.4	EFSA Scientific Report 120 (2007)
DT_{50} in water (day)	42.3	EFSA Scientific Report 120 (2007)
DT_{50} in sediment (day)	1,000	Worst case
DT_{50} in whole system (day)	42.3	EFSA Scientific Report 120 (2007)
Activation energy (KJ/mol)	54,5	FOCUS recommendation
K_{foc} (mL/g)/K_{fom}	15.34/8.9 (geomean)	EFSA Scientific Report 120 (2007)
Freundlich exponent (L/n)	0.94	EFSA Scientific Report 120 (2007)
Plant uptake factor	0	FOCUS recommendation
Diffusion coefficient in water (m^2/day)	Steps 1–2: – Steps 3–4: 4.3×10^{-5}	FOCUS recommendation
Diffusion coefficient in air (m^2/day)	Steps 1–2: – Steps 3–4: 0.43	FOCUS recommendation
Wash-off factor from crop (L/mm)	Steps 1–2: – Steps 3–4: 1×10^{-7} (MACRO) 0 (PRZM)	FOCUS recommendation

Aquatic risk assessment

The aquatic risk assessment was conducted by estimating the regulatory acceptable concentration in surface water ($RAC_{sw;ac}$) of nicosulfuron by-products, on the basis of ecotoxicological data for aquatic target organisms predicted by QSAR modelling. $RAC_{sw;ac}$ values were compared to PEC_{sw} calculated for each by-product by FOCUS surface water modelling.

Consumer risk assessment

The consumer risk assessment was conducted by estimating the maximum allowable concentration (MAC) of nicosulfuron by-products for infants, toddlers and adults. By-products were considered 100 times more toxic than the parent compound. Finally, MAC values were compared to PEC_{gw} calculated for each by-product by FOCUS groundwater modelling.

3. Conclusions

3.1. Conclusion regarding herbicides behaviour in disinfected water and risk assessment of their by-products

Within the EU-FORA Fellowship Programme, the fellow carried out the study of the behaviour of selected active substances in chlorinated/chloraminated waters. The subsequent formation of their reaction products under specific experimental conditions was evaluated. In particular, nicosulfuron degradation increased with the concentration of chlorine/chloramines following a first order kinetic equation, and it proved to be pH sensitive as well. In agreement with the stronger oxidising potential of hypochlorite vs. chloramines (Reckhow and Singer, 1990), degradation rate of nicosulfuron/chloramines was slower than nicosulfuron/hypochlorite. In addition, the chemical structures of the by-products generated by the reaction of nicosulfuron and the disinfectants were tentatively identified by LC-HR-MS. Their amount increased while that of nicosulfuron decreased over time. A possible route of degradation for these by-products was proposed by means of CAKE kinetic degradation fitting model.

In the last part of the work programme, the estimation of the RACs and the MAC of nicosulfuron residues was performed by means of FOCUS surface water and groundwater scenarios, respectively. The acute aquatic toxicity of the degradation products for *Daphnia magna* and fathead minnow, was predicted by QSAR models. These data were employed to estimate the RAC in surface water. As reported in the EFSA Scientific opinion on Plant Protection Products and their Residues (PPR) (EFSA PPR Panel, 2013), $RAC_{sw;ac}$ (RAC in surface water – SW – for adverse effects of pesticide exposure occurring within a relatively short period after exposure) is always compared with the $PEC_{sw;max}$ (the maximum PEC in surface water) derived from the predicted exposure profile of the active substance. In this study, in no cases the PEC_{sw} of nicosulfuron by-products was higher than the $RAC_{sw;ac,}$ thus indicating that they can be classified as a low risk. Regarding the environmental exposure risk assessment, the PEC of nicosulfuron in groundwater showed to be above the regulatory threshold of 0.1 μg/L at 1 m depth in three out of the eight scenarios considered in the FOCUS groundwater modelling. In no case, the PEC_{gw} of nicosulfuron by-products was higher than the MAC for adults, toddlers and infants in drinking waters. To the best of our knowledge, this is the first time that the behaviour of imazamox, imazapic and imazethapyr and nicosulfuron in chlorinated/chloraminated waters was studied. These results will contribute to supporting more complete risk assessment of the studied active substances, especially for nicosulfuron and its by-products potentially occurring in drinking water.

3.2. Conclusion regarding the participation to the EU-FORA programme

The hands-on training carried out during this year allowed the fellow to gain experience in the field of pesticide risk assessment. In particular, the fellow received training in generating data on pesticides residues and their degradation products, and in analysing these data in order to perform the environmental exposure assessment and risk assessment of the selected active substances and their by-products. Finally, participation in the EFSA EU-FORA work programme provided a valuable opportunity for the fellow to become part of a strong professional network that will provide basis for future collaborations in the field of food safety and risk assessment.

3.3. Additional scientific activities

During the EU-FORA programme, the fellow was presenting author of the poster entitled 'Behaviour of nicosulfuron herbicide in chlorinated drinking water. By-products identification and risk assessment', at the 11th European Conference on Pesticides and Related Organic Micropollulants in the Environment and the 17th Symposium on Chemistry and Fate of Modern Pesticides – Ioannina (Greece), June 23–26, 2022. (Annex A).

The fellow was invited by the Spanish Agency for Food Safety and Nutrition (AESAN) – Madrid (Spain) February 23–25, 2022, as speaker presenting her work programme, to attend seminars on AESAN's activities on risk assessment, management and communication, and, finally, to visit the laboratories of the National Centre for Food, located in Majadahonda (Madrid – Spain).

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Abbreviations

a.m.u.	atomic mass unit
a.s.	active substance
AESAN	Agencia Española de Seguridad Alimentaria y Nutrición
CSIC	Consejo Superior de Investigaciones Científicas
DT_{50}	degradation time 50
EPI Suite™	Estimation Programs Interface Suite™
EU-FORA	European Food Risk Assessment
FOCUS	FOrum for the Co-ordination of pesticide fate models and their USe
HPLC-DAD	high-performance liquid chromatography with diode array detector
HPLC–MS	high-performance liquid chromatography–mass spectrometry
INIA	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
LC–ESI-QToF-MS	liquid chromatography–electrospray-quadrupole time of flight-mass spectrometry
MAC	maximum allowable concentration
OECD	Organisation for Economic Co-operation and Development
PEARL	Pesticide Emission Assessment at Regional and Local scales
PEC	predicted environmental concentration
PELMO	PEsticide Leaching MOdel
PPP	Plant Protection Products
PPR	Plant Protection Residues
PRZM	pesticides root zone model
QSAR/QSPR	quantitative structure–activity/quantitative structure–property relationship
RAC_{sw}	regulatory acceptable concentration in surface water
SWAN	Surface Water Assessment eNabler
SWASH	Surface WAter Scenarios Help
T.E.S.T.	Toxicity Estimation Software Tool
TOXSWA	TOXic substances in Surface WAters
UPF	Unidad de Productos Fitosanitarios
USEPA	United States Environmental Protection Agency

Annex A – Abstract and certificate of attendance to the 11th European Conference on Pesticides and Related Organic Micropollutants in the Environment – Ioannina (Greece), June 23–26, 2022

11th European CONFERENCE on Pesticides and Related Organic Micropollutants in the Environment & 17th Symposium on Chemistry and Fate of Modern Pesticides

www.pesticides2022.gr

June 23-26 2022 Conference center "Karolos Papoulias" University of Ioannina, GREECE

CERTIFICATE OF ATTENDANCE

This is to certify that

MARI ANGELA

attended the
11th European Conference on Pesticides and Related Organic Micropollutants in the Environment
&
the 17th Symposium on Chemistry and Fate of Modern Pesticides
which was held in **Ioannina, Greece,** on **June 23-26, 2022.**

The Conference Chair

Prof. Triantafyllos A.D. Almpanis, Rector
Department of Chemistry, University of Ioannina,
Institute of Environment and Sustainable Development,
University Research Center of Ioannina (URCI)

Organized by:
DEPARTMENT OF CHEMISTRY SCHOOL OF SCIENCES UNIVERSITY OF IOANNINA
INSTITUTE OF ENVIRONMENT AND SUSTAINABLE DEVELOPMENT, UNIVERSITY RESEARCH CENTER OF IOANNINA (URCI)

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Innovative *in vitro* approaches to toxicological investigations of mycotoxins effects

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Abstract

Among the potential contaminants, mycotoxins are of particular concern due to the importance in terms of food and feed safety. The difficulty in establishing a diagnosis for mycotoxicosis relies in the fact that the effects are subclinical, and that multicontamination by various toxins is the most common scenario. The co-occurrence of these mycotoxins raises questions concerning both food safety and regulation. However, there is still limited knowledge on toxicity data on co-exposure. The current technical report will describe the activities performed by the fellow in the LUBEM-Brest University (France). In this context, the work programme offered by the hosting site consisted *in vitro* toxicological approaches to evaluate the toxicity of mycotoxin mixtures. The aim of this project was to assess human risk to the exposure of two main regulated mycotoxins (ochratoxin A and fumonisin B1) using different innovative cellular models (2D and 3D spheroids). In this framework, these mycotoxins were tested individually and as a combination on intestinal and hepatic cell lines alone or in co-cultures. Overall, our results show the outstanding potential of using more predictive and realistic approaches for the risk assessment (RA) of mycotoxins. It is of high importance to pursue further toxicological characterisations and exposure evaluations for mycotoxins, in order to determine a more detailed RA. This will serve as a reference to understand multicontamination mechanism of mycotoxins at the cell level and help authority to revise regulation.

Keyword: Food contaminants*in vitro* modelstoxicologymycotoxins

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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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Table of contents

1. Introduction

Globally, throughout our lives we may be exposed to multiple chemicals from a variety of sources (OECD, 2018). Many microorganisms including bacteria, yeasts and fungi are associated with cereals but the latter are of great importance as some species are toxinogenic (CAST, 2003). Over the years, it has been estimated that the overall global contamination of agricultural products to be around 25%. However, although several mycotoxins are individually regulated (ochratoxins, fumonisins, etc.), mycotoxins levels are characterised by being higher than the European limits. Therefore, there are important economic impacts resulting from the application of the European legislation regarding these mycotoxins (Eskola et al., 2020).

In this context, detection and quantification of mycotoxins are particularly important due to their human and animal health associated risks (Habschied et al., 2021; Kępińska-Pacelik and Biel, 2021). Recently, the European Food Safety Authority (EFSA) has undertaken to develop new methods to assess the risks associated with the complex issue of mixtures in the food chain and combined toxicity (EFSA Scientific Committee, 2019).

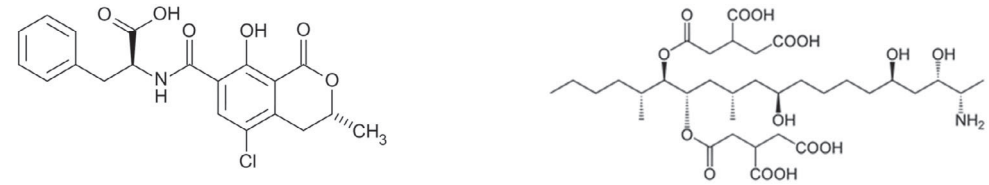
Presently, *in vitro* tests play a major role in obtaining information on toxicity mechanism with the perspective to be able to identify pathways of toxic responses by applying 'omics' techniques (Balmer et al., 2014). Physiology-based toxicokinetic modelling is using data from *in vitro* studies to build up a model for a specific compound. Information from both areas is incorporated into the risk assessment to derive compound-specific safety factors, which account for species differences and for the variability among the human population, including possible sensitive subpopulations. In link with the 3R concept, the question 'Why animal testing for safety evaluations have not yet been replaced?' can be raised (Smith et al., 2016).

1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by fungi, mainly of the genera *Aspergillus, Penicillium, Fusarium, Claviceps and Alternaria* genera, which can contaminate food and reach humans food and reach humans, causing toxic effects and damaging their health (Marin et al., 2013). It is a group of heterogeneous compounds that differ in their structures, their biochemical and physicochemical characteristics and their toxicological properties (Alshannaq and Yu, 2017). Exposure to mycotoxins produces different toxic effects, called mycotoxicosis (Streit et al., 2012), which can result in an acute mycotoxicosis (due to ingestion of high levels of toxins in a short period of time) or chronic mycotoxicosis (due to ingestion of low levels of toxins in a long period of time) (Ostry et al., 2017; Claeys et al., 2020).

More than 300 mycotoxins are known, but only 40 compounds are regulated and monitored by national or international food regulation authorities, up to now (European Commission, 2006). This may lead to an underestimation of actual food and feed contamination patterns, as well as the actual health risk for both humans and animals. In addition, co-exposure to multiple mycotoxins might generate additive, synergistic or antagonistic effects (Smith et al., 2016).

The International Agency for Research on Cancer (IARC) has studied some mycotoxins for their mycotoxins in terms of their potential carcinogenic risk. The studied mycotoxins for this project, ochratoxin A (OTA) and fumonisin B1 (FB1) (Figure 1), have been classified as 'possible carcinogens to human' (Group 2B) (IARC, 1987, 1993, 2012).



Ochratoxin A (OTA) Fumonisin B1 (FB1)

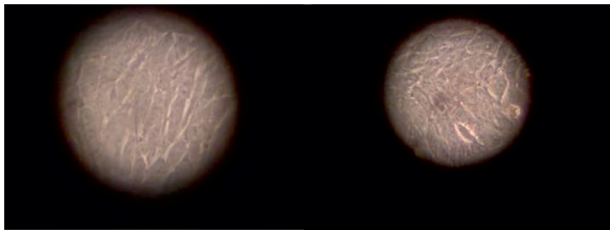
Figure 1: Chemical structures of the studied mycotoxins: OTA (left); FB1 (right)

1.2. Cell culture

1.2.1. HepaRG cell line

The liver being the reference detoxification organ of the organism, hepatocyte cells represent one of the most suitable *in vitro* study models for xenobiotic metabolism and for toxicological studies. Indeed, the functions of the liver are mainly performed by hepatocytes which represent about 80% of the total cell population. In addition, the alteration of liver functions is one of the main effects observed on animals following exposure to high doses of mycotoxins (Pitt, 2000). However, more than 50% of the drugs inducing liver damage in human clinical trials are not hepatotoxic in animals, which highlights the importance of using human hepatocytes to more accurately assess drug toxicity, or other bioactive molecules, *in vitro* in humans (Olson et al., 2000). Nevertheless, as for all primary cells, primary human hepatocytes are phenotypically unstable, have a limited lifespan and a high interdrug variability. On the other hand, hepatocyte lines of tumour origin or obtained by oncogenic immortalisation, such as HepG2 and C3A cells, lack some important liver-specific functions. In particular, these lines do not possess some major cytochromes of the P450 family involved in the metabolism of xenobiotics, and are therefore of limited interest for pharmaceutical and therapeutic studies (Guguen-guillouzo and Guillouzo, 2010). In contrast, the human hepatocyte cell line HepaRG, obtained from a liver tumour from a patient with hepatocarcinoma and hepatitis C infection (Gripon et al., 2002), seems to be a good substitute for primary hepatocytes for toxicology studies. Indeed, this line possesses both the metabolic performance of primary human hepatocytes and the growth capacities of hepatocyte lines (Guillouzo et al., 2007). In particular, HepaRG cells express many liver-specific functions such as the main cytochromes P450 and some nuclear receptors at levels comparable to those found in primary human hepatocytes.

Moreover, these cells can be maintained at confluence for several weeks while maintaining a stable metabolic activity, which makes them ideal model for the study of drug metabolism parameters as well as acute and chronic as well as the acute and chronic effects of xenobiotics on the human liver (Guillouzo et al., 2007; Anthérieu et al., 2012). On the other hand, the HepaRG cells have unique characteristics: when detached and reseeded at low density, they are able to dedifferentiate by reacquiring an undifferentiated morphology and dividing in a very active way to reach confluence quickly.



Undifferentiated HepaRG cells Differentiated HepaRG cells

Figure 2: Morphology of HepaRG cells in different states: undifferentiated (left); differentiated (right). Scale bar: 100 μm

As a result, two types of cell populations morphologically different can be distinguished (Figure 2): colonies formed by clusters of granular epithelial cells resembling hepatocytes, surrounded by more flattened cells. These cells can be differentiated respectively (with the addition of DMSO in the culture medium) into more granular cells having one or two nuclei and closely resembling adult primary hepatocytes for the former, and into biliary epithelial cells for the latter. The two cell states (undifferentiated and differentiated) from this line can be used and compared in toxicological studies (Guillouzo et al., 2007). HepaRG cells thus represent an innovative model for this type of toxicological study.

1.2.2. Caco-2 cell line

The intestinal barrier plays an important role in protecting the body against ingested toxic substances. It also represents one of the major sites of exposure to agents because of its large area of exposure and its physiological role in the transfer of nutrients from the lumen to the blood. The gastrointestinal tract, in addition to its role in the absorption of xenobiotics, is also actively in their

biotransformation (Vancamelbeke and Vermeire, 2017). The human tumour cell line Caco-2 of intestinal origin, obtained from colorectal adenocarcinoma, is very commonly used in *in vitro* toxicology studies as a model to mimic the intestinal barrier (De Angelis and Turco, 2011).

Despite the fact that reproducibility problems have been reported in the literature making it difficult to compare results between laboratories, the Caco-2 cell line has been widely used over the last 30 years in *in vitro* studies. These problems have been attributed to the intrinsic variability of the cells used in different laboratories, as well as to the different conditions related to the culture, such as the type of animal serum used, the supplements added to the culture medium, the number of passages and the origin of the clones (Natoli et al., 2012). In addition, these cells have the particularity of being able to differentiate spontaneously (Figure 3), leading to the formation of a monolayer of polarised intestinal cells expressing several morphological and functional characteristics of mature intestinal enterocytes (Sambuy et al., 2005).

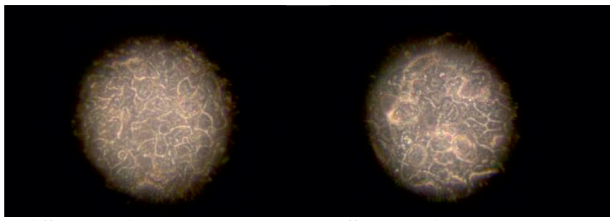


Figure 3: Morphology of Caco-2 cells in different states: Undifferentiated (left); differentiated (right). Scale bar: 100 μm.

1.3. Innovative *in vitro* systems

1.3.1. Co-culture models

In vitro cell culture models have proven to be extremely useful to understand the molecular mechanisms of transport, metabolism and toxicity of relevant molecules, such as mycotoxins. These systems allow to better mimic the *in vivo* situation by taking into account the interactions between different cell types, thus representing an interesting alternative to traditional systems (Smith et al., 2018).

However, one of their limitations is the need to culture individual types in isolation from other cells that are in constant and close physiological interaction *in vivo*. In an attempt to overcome these limitations, several interesting co-culture models have recently been unveiled, combining intestinal cells with cells of different origin, such as neural, liver, pancreatic or monocytic cells. These models better reproduce the cross-talk between tissues that occurs *in vivo* (Castell-Auví et al., 2010a,b). Differentiated enterocytes in the small intestine are responsible for the transport and first metabolism of ingested molecules. After crossing the intestinal barrier, metabolites come into contact with local cytotypes such as intraepithelial lymphocytes, monocytes, fibroblasts and enteric neuronal cells, or are transported to the liver and other organs via the hepatic portal circulation and the lymphatic system.

For this purpose, it is necessary to study cytotoxicity on a co-culture system to suggest the toxicological impact of mycotoxins on different cellular models. To reproduce this complex interaction *in vitro*, we used as an intestinal cell model the human adenocarcinoma cell line Caco-2 that spontaneously differentiates *in vitro*, expressing several morphological and functional characteristics of mature small intestinal enterocytes (Sambuy et al., 2005) with HepaRG cells.

For these co-culture experiments, Caco-2 cells on inserts were transferred to culture plates containing HepaRG cells, as shown in Figure 4.

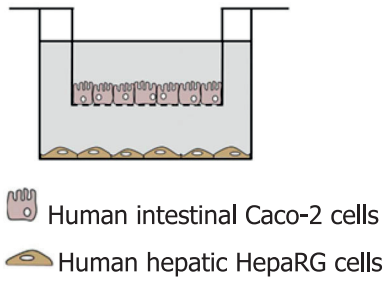


Figure 4: Schematic representation of the *in vitro* co-culture system

1.3.2. 3D models

Although co-cultures overcome some of the drawbacks of classical 2D systems (in particular, intercellular communication), they are still far from accurately reproducing all cellular functions observed in a tissue. 3D *in vitro* culture systems seem to be relevant models to try to approximate *in vivo* conditions (Hoarau-Véchot et al., 2018). In particular, spheroid systems have been developed that are now viable with high throughput (Kelm and Fussenegger, 2004). In such systems, the number of cells and thus the size of the spheroid can be adjusted (Fennema et al., 2013).

A study performed with HepaRG cells pointed out that 3D HepaRG spheroids had better functionality than conventional 2D HepaRG cultures and thus represented a good model for the study of drug toxicity, in particular for the study of drug metabolism (Mueller et al., 2014).

Therefore, there is a whole field of research possible today to mimic the *in vivo* context more precisely, and which could allow a more accurate assessment of the risk of mycotoxins to human and animal health.

2. Description of work programme

The fellow was hosted by the LUBEM (Laboratoire Universitaire de Biodiversité et Ecologie Microbienne), belonging to the Université de Bretagne Occidentale (UBO) in France. The hosting division has a long-standing experience in the risk assessment of innovative topics related to moulds (e.g. fungal diversity in the agri-food environment, predictive mycology, antifungal biopreservation), focuses mainly on secondary metabolites, in particular mycotoxins and their toxicological impact. Since 2022, the host laboratory team has been recognised as a participating institution of INRAE (National Research Institute for Agriculture, Food and the Environment).

In this field of research, the activities concern scientific and technological advances that can revolutionise the toxicological evaluation of these metabolites but also of various chemical compounds (e.g. pesticides). One of the main challenges in this field is the need to move away from animal testing and toward the use of *in vitro* methods, in agreement with the 3R concept. However, one of the main downfall of *in vitro* toxicological evaluation is the distance to the *in vivo* reality and thus the usability of the obtained data. In order to counteract this problem, we have developed 3D innovative new cellular models, like spheroids cell models, which are likely to have an impact on risk assessment procedures. Moreover, these models can also help to better take into account the reality of mycotoxin (or other compounds) exposure through multi-contamination and/or chronic exposure studies. The proposed project is directly linked to these scientific questions and was split into two different phases to better answer the different aspects.

2.1. Aims

The lack of regulatory consideration of multi-contaminations to mycotoxins is mainly due to the scarcity of toxicological data. Moreover, the effects of the combinations of mycotoxins on the cellular mechanisms are mechanisms are insufficiently known. In this context, the present work programme aimed to determine a 3D cell model relevant for *in vitro* toxicological evaluation using mycotoxin as an example.

More precisely, the aim was to study the cytotoxic effects of OTA and FB1 combinations and to identify the cellular mechanisms involved in the toxicity of these mixtures via toxicological approaches using different human cell lines (intestinal and hepatic) representative of the defence barriers of the organism and of the detoxification organ, privileged targets of mycotoxins.

The following specific tasks, articulated in two parts, arise from this general objective:

Task.1: Comparison of 2D and 3D hepatocyte models on mycotoxins toxicity.

Mycotoxins correspond to a critical food safety, in particular for cereal crops, posing significant health risk to humans and livestock. These fungal metabolites also have a significant economic impact that embraces yield losses, product recalls, costs linked to management, prevention and mitigation. Moreover, some are regulated at the European level and have been extensively studied in both *in vivo* and *in vitro* conditions. In this context, the goal of this task was to compare *in vitro* toxicological data obtained with the same cell line (human hepatoma) HepaRG but with two different experimental conditions, namely in 2D (classical cell culture) and 3D (spheroid). These models were used to compare the toxicological impact of two mycotoxins (OTA and FB1) in acute conditions.

Task.2: Comparison of 2D and 3D system cells to evaluate mycotoxins mixture effects.

As the reality of mycotoxin exposure corresponds to multi-contamination, toxicity of mixtures at actual concentrations encountered in cereals was assessed using cell viability and compared to control (toxicity of each mycotoxin alone). Mycotoxin interactions were analysed by the isobologram-combination index method and a toxicology predictive model was developed.

2.2. Activities and methodology of the project

The fellow was involved in the different steps of evaluation of mycotoxin *in vitro* toxicity from the beginning of the project. To gather contamination data, literature searches were included to further study about the topic and to select the tested mycotoxin concentrations.

In order to follow this work, the fellow was trained in cell culture methodology, data analysis and in vitro approaches. In this framework, to achieve the main goal of the project, toxicological effects of mycotoxins were assessed on different parameters to compare 2D and 3D approaches.

- Cell growth and viability effect were analysed in 2D and 3D system at the same time on intestinal cell line (Caco-2), hepatocyte line (HepaRG). On cell lines, the proliferation was measured by common cytotoxicity tests based on the analysis of mitochondrial activity (MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium assay) test or ATP assay for 3D models) after 48 h of exposure with mycotoxins alone or in cocktail.
- Barrier function. When intestinal epithelial cells (Caco-2) are grown on inserts 'transwell system', they form a monolayer and differentiate into mature intestinal epithelium (expression of apical villi, formation of tight junctions, etc.). It is then possible to assess the integrity of this monolayer by measuring the transepithelial electrical resistance (TEER) established between the basal and the apical compartments (Bouhet, 2004). We investigated the effects of OTA and FB1 alone and in cocktail at no cytotoxic concentrations on the monolayer TEER during 48 h.
- For mycotoxin assay, the optimisation of mycotoxin extraction methods (suitable for cell lines and targeted mycotoxins) was carried out and their quantification was made by LC-Q-TOF in extra and intracellular systems.

According to the main Task 1, the following deliverables were obtained: results of cell viability study on each cell model exposed to mycotoxin alone or in cocktail, determination of interaction type (additive, synergic, potentiate or antagonist), results of mycotoxins effect on barrier function (Comparison of results in 2D and 3D systems) and quantification of OTA, FB1 in the co-culture system in apical, basolateral and intracellular fraction after 48 h of exposure.

To better characterise the impact of combined mycotoxin exposure (Task 2): Human system cells were used to evaluate mycotoxins mixture effects after exposure. Comparison of cellular models and effects of OTA and FB1 on systems were performed.

2.3. *In vitro* cell culture systems for the assessment of toxicity of mycotoxins

Following an ethical approach aimed at reducing animal experimentation (European Commission, 2010), many toxicology studies are now conducted *in vitro* toxicology studies are now conducted on *in vitro* systems. In particular, since the introduction of the '3Rs' rule (reduce, refine, replace), major efforts have been made to improve these have been made to improve these cell culture models so that they can replace animal experiments (Spielmann et al., 2008).

The use of an accurate *in vitro* model for toxicity studies should identify the cellular pathways and mechanisms affected while reflecting the *in vivo* situation (Committee on Toxicity Testing and Assessment of Environmental Agents and National Research Council, 2007). Such a system must also allow for long-term functional culture of the cells. However, most of these studies are performed in classical 2D culture systems using a single type of cell, and therefore do not reflect the complexity of a 3D organ, leading to discrepancies between *in vitro* and *in vivo* experimental data (Antoni et al., 2015). In particular, this type of culture does not take into account some important factors that allow to accurately reproduce the physiology of cells and tissues, such as the communication between the cell and its matrix as well as the communication between cells, but also the consecutive exposure of several cell types during the ingestion of a toxin.

2.4. Additional scientific activities

Besides the specific activities in the risk assessment work programme (EU-FORA training), the fellow participated in a full range of activities of the research unit, which is indeed a group young and committed, taking part in the exciting and numerous activities of the unit: contributed to teaching and learning conference (Master of Health Biology in Brest University).

In order to maximise knowledge transfer, the fellow was actively engaged through numerous events, including meetings and international conferences (The World Mycotoxin Forum and Mycotoxin Research) concerning mycotoxins, food contaminants, microbiology or toxicology, to present the results of these new innovative cell culture models. All these activities completed during the fellowship period are reported in Appendix A.

3. Conclusions

The work programme at the LUBEM (Laboratoire Universitaire de Biodiversité et Ecologie Microbienne)-Université de Bretagne Occidentale (UBO) provided the fellow the opportunity to develop important skills within critical aspects of the risk assessment framework, mainly focused on toxicological aspects.

The fellowship programme, beyond the personal enrichment that an experience living abroad always brings, has provided the opportunity to gain expertise in different areas of toxicological risk assessment, especially with regard to cell culture, data analysis and toxicology predictive approaches. The fellow has also considered this a valuable experience to consolidate her background in terms of mycotoxin knowledge, especially in the context of impact on risk assessment.

Working at the LUBEM has been such an exciting opportunity to further develop research skills and complement education by learning new techniques in the field of metabolism and toxicological effects. The proposed work was a perfect fit with the experience and expectations of the fellow.

During this year, thanks to the participation in different scientific conferences and the EU-FORA trainings (covering in-depth all over the different areas of food safety risk assessment, risk management and risk communication), the fellow has gained autonomy in decision-making, collaboration with other researchers and also a great capacity of interaction to communicate science. Attendance to these complementary 'learning-by-doing' courses has allowed the trainee to work in groups and to make quick decisions through the evaluation of high-level scientific articles and case studies. The working methodology applied by EFSA in risk assessment and the integration in such a multidisciplinary research group has provided a great environment to build a strong network of professional and personal experiences, opening the door to future collaborations and maximising knowledge transfer.

The fellow and the supervisor have valued the EU-FORA Fellowship programme as a positive professional experience to exchange and cooperate with advanced knowledge in a specific research area. In this framework, it is expected that the general risk assessment methodology applied for this particular project will be extended and applied by the fellow and the group in future positions, to expand knowledge and collaborations in the toxicological evaluation of mycotoxins.

3.1. Disclaimer

Detailed results obtained from the method development, sample analysis and risk assessment during the research study are not included in this report to avoid certain copyright claims, as the final deliverables and complete work will be subsequently published in other scientific journals.

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Abbreviations

AGES	Austrian Agency for Health and Food Safety
BfR	German Federal Institute for Risk Assessment
EFET	Hellenic Food Authority
EU-FORA	European Food Risk Assessment Fellowship Programme
FB1	fumonisin B1
IARC	International Agency for Research on Cancer
LC-Q-TOF	liquid chromatography coupled to quadrupole time-of-flight
LUBEM	Laboratoire Universitaire de Biodiversité et Ecologie Microbienne
MS	mass spectrometry
OTA	ochratoxin A
RA	risk assessment
TEER	transepithelial electrical resistance
UBO	Université de Bretagne Occidentale
WMF	World Mycotoxin Forum

Appendix A – Training activities

Table A.1: Training activities during the EU-FORA Fellowship Programme

Type of event	Event	Contribution	Location	Date
Meeting	Challenges in Public Health Protection in the 21st Century: New Methods, Omics and Novel Concepts in Toxicology	Attendance	Online	15–17 November 2021
Webinar	'Mycotoxins in the food chain'	Attendance	Online	7–8 October 2021
	'Toxicity and authenticity testing of foods with trace elemental and stable isotope analysis'	Attendance	Online	4 November 2021
Conference	Human Exposome WMF 2022	Oral Communication. 'Biomonitoring and human exposure to multiple mycotoxins'	Online	12 October 2021
	Virtual ICFC 2021	Oral Communication. 'Exposure to mycotoxins in Spanish children through the analysis of their levels in plasma samples'	Online	27–28 September 2021
	43rd Mycotoxin Workshop Toulouse	Written Communication. 'Toxicological effects of ochratoxin A and fumonisin B1 on human cells using innovative 2D and 3D *in vitro* models'	Toulouse (France)	30 May–1 June 2022
	The World Mycotoxin Forum (WMF) 2022	Written Communication. 'Innovative 2D and 3D *in vitro* models to evaluate toxicological effects of ochratoxin A and fumonisin B1 on human cells'	Parma (Italy)	16–18 May 2022
	'ONE – Health, Environment, Society – Conference 2022'	Attendance	Online	21–24 June 2022
	WMF 2022. Pre-Conference on Analysis. Development and trends in (multi)mycotoxin detection	Attendance	Online	1 February 2022
	WMF 2022. Pre-Conference on Animal Health. Mycotoxins-ongoing issues of animal health and productivity	Attendance	Online	30 November 2021
	RAFA 2021	Attendance	Online	3–4 November 2021
EU-FORA Training Courses	Induction Training	EFSA Training	Online	30 August–17 September 2021
	Module 1. Novel Foods (AGES–EFSA)		Online	22–26 November 2021
	Module 2. Risk Communication (BfR–EFSA)		Online	21–25 March 2022
	Module 3. Emerging Risks (EFET –EFSA)		Athens (Greece)	6–10 June 2022
	Module 4. Data Collection and Reporting (EFSA)		Online	21–25 August 2022

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The use of NAMs and omics data in risk assessment

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Abstract

The animal-centric approach so far predominantly employed in risk assessment has been questioned in recent years due to a number of shortcomings regarding performance, consistency, transferability of results, sustainability, costs and ethical reasons. Alternatives to animal testing, collectively termed NAMs, may have the potential to deliver sound, cost-effective, prompt and reliable information, but their regulatory acceptance has not been established yet. The main reasons behind this are mostly related to actual methodological obstacles, with particular reference to addressing complex endpoints such as repeated-dose toxicity, the issue of translating the concept of adversity to NAMs, and doubts of stakeholders about the level of chemical safety ensured by NAMs. With the aim of providing an updated view on major conceptual and methodological developments in the field of toxicology, a symposium and a workshop were organised by the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BfR) and Helmholtz Centre for Environmental Research on 15–17 November 2021 in Berlin. The conference, entitled 'Challenges in Public Health Protection in the 21st Century: New Methods, Omics and Novel Concepts in Toxicology' brought together eminent scientists with representatives from industry and regulatory authorities. The organisation, day-to-day operations and the reporting of the event main outcomes in a position paper were the main focus of the present EFSA EU-FORA work programme. Tasks pertaining to 'The use of NAMs and omics data in risk assessment' were implemented under the shared supervision of units 'Testing and Assessment Strategies Pesticides' and 'Effect-based Analytics and Toxicogenomics' of the German Federal Institute for Risk Assessment.

Keywords: New Approach Methods, Animal experiment, Omics, Risk assessment, 3Rs principle, Workshop

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1. Introduction

New approach methodologies (NAMs) are defined as any non-animal technology, methodology, approach or combination thereof that can be used to provide information on chemical hazard and human risk assessment (Dent et al., 2018). NAMs can be used as alternative or complementary methods to traditional animal testing, and comprise mostly *in silico* (e.g. QSAR, PBK models, machine learning models and artificial intelligence) (Hartung and Tsatsakis, 2021; Thompson et al., 2021), *in vitro* (cell cultures, organoids and other microphysiological systems) (Marx et al., 2020; Pamies et al., 2022), *ex vivo* (e.g. the large field of omics applications, which are anyway also applicable to entirely *in vitro* investigations) (Buesen et al., 2017; Mav et al., 2018; Gwinn et al., 2020; Malinowska et al., 2022), and *in chemico* (i.e. a term for generally referring to the use of abiotic methods aimed at identifying chemical reactivity) (Gerberick et al., 2008) approaches. When information related to human adverse effects and exposure are used, NAMs represent an enhancement of traditional animal testing with regard to risk assessment aimed at ensuring chemical safety and human health protection.

Alternative approaches are commonly utilised by industry and regulatory agencies, at least internally, for preliminary prioritisation and read-across purposes (Ball et al., 2020; van der Stel et al., 2021).

Over the last 15 years, several European projects have explored the regulatory capacity and acceptance of NAMs, and the just-started 7-year long PARC project (Horizon Europe 2021–2027 framework, 200 partners, 28 countries, 400 million € budget) aims at designing a next generation chemical risk assessment (NGRA) by leveraging on one of the largest scale and innovative partnerships of its kind.

One of EFSA's vision is to routinely make use of NAMs (e.g. omics and associated bioinformatic approaches) in relevant RAs by 2030 (EFSA, 2021) to ultimately support the transition into a NGRA that is actually exposure-led, hypothesis-driven, truly data-centric and AOP-based.

At last, the traditional animal testing approach currently in place notoriously suffers from many shortcomings, the most severe of which are related to reproducibility issues, interspecies concordance, poor sustainability of toxicological data generation resource- and time-wise, and ethical concerns (Luechtefeld et al., 2018; Smirnova et al., 2018; Karmaus et al., 2022).

Despite the potential of several NAMs was demonstrated, their regulatory acceptance has mostly not been established yet, mainly due to the difficulty of addressing complex end-points (e.g. repeated-dose toxicity), the issue of translating the concept of adversity to NAMs, doubts of stakeholders about the level of chemical safety ensured by NAMs, and lack of internationally harmonised guidance on the interpretation of NAM-derived data.

The present EFSA EU-FORA work programme was entitled 'The use of NAMs and omics data in risk assessment' and was implemented at the German Federal Institute for Risk Assessment under the shared supervision of Prof. Dr. Braeuning (Unit 51, 'Effect-based Analytics and Toxicogenomics') and Dr. Marx-Stoelting (Unit 66, 'Testing and Assessment Strategies Pesticides'). Its overarching aims were (i) to provide the most up-to-date picture on the potential of NAMs in risk assessment, (ii) to clarify the reasons of the slow progress in regulatory uptake as well as (iii) to suggest possible ways forward in terms of testing strategies, standardisation, harmonisation and validation procedures, knowledge transfer and confidence building, and the required changes in regulation for facilitating the integration of NAM into risk assessment.

2. Description of work programme

2.1. Aims

The implementation of the work programme was centred on the international conference and workshop entitled 'Challenges in Public Health Protection in the 21st Century: New Methods, Omics and Novel Concepts in Toxicology', organised by the hosting site in partnership with UFZ on 15–17 November 2021. Knowledge in the current state of integration of NAM and omics techniques in toxicological risk assessment of food contaminants, pesticides and other areas, as well as into technical limitations and steps needed for further implementation was generated.

2.2. Activities/Methods

The work programme activities of the fellow can be classified into three main categories, i.e. pre-, during and post-conference.

2.2.1. Pre-conference

The pre-conference activities revolved around (i) the acquaintance of the fellow to the subject of NAMs and omics in risk assessment, (ii) the refinement of the conference schedule, (iii) the screening of the responses to a preliminary questionnaire sent to invited workshop participants up to 4 weeks prior to the conference start and (iv) the preparation of the material to be presented at the workshop for moderation purposes. In particular, activity (i) was implemented via relevant literature searches of latest scientific (2009–2022 time frame), institutional (e.g. JRC's Reference Laboratory for alternatives to animal testing, Scientific Committee on Consumer Safety, US EPA, US FDA) and intergovernmental (OECD) publications/reports, as well as with an in-depth analysis of 17 breakthrough projects funded by the EU both within the Horizon 2020 and Horizon Europe frameworks (i.e. EURION cluster, ASPIS cluster, PARC) and US federal funds (e.g. Tox21). Such information later served in the preparation of one of the position paper tables, providing a comparative outlook with regards to project aims, methods, *in vivo* and *in vitro* test models, *in silico* and high-throughput approaches, toxicological targets, investigated toxicity and interaction with regulatory agencies.

2.2.2. Conference

The activities performed during the conference were mainly related to the day-to-day operations and the moderation of the workshop, during which a synthesis of all questionnaire responses received was presented, interpreted and used for stimulating discussion.

2.2.3. Post-conference

The activities performed following the conference pertained to the compilation of the input from all symposium sections and the subsequent drafting of a position paper on the use of NAM and omics in regulatory toxicology based on the conference contributions, panel discussion and invite-only workshop discussion and visions.

3. Conclusions

3.1. New Approach Methodologies in human regulatory toxicology

Invited workshop participants were asked to fill a questionnaire within 4 weeks prior to their attendance to the workshop. The questionnaire was composed of six sections, namely (i) Current uses of NAMs, (ii) Regulatory toxicology areas currently covered by NAMs, (iii) Regulatory toxicology areas currently not covered by NAMs, (iv) Challenges for the implementation of NAMs, (v) Possible ways forward and (vi) Approach to exploit the full potential of NAMs in the regulatory field. Based on a synthesis of the stakeholders' responses, the fellow prepared the material to be presented for moderating the workshop and stimulating the discussion.

The conference 'Challenges in Public Health Protection in the 21st Century: New Methods, Omics and Novel Concepts in Toxicology' took place at the Langenbeck-Virchow-Haus venue in Berlin on 15–17 November 2021, and encompassed 4 sessions, namely 'Recent advances in the application of NAM' (6 presentations), 'Use of omics techniques in experimental toxicology' (7 presentations), 'Use of omics techniques in regulatory toxicology' (5 presentations), 'Current applications – a regulatory authority perspective' (6 presentations, 1 panel discussion, 3 position talks). An invite-only workshop entitled 'Future concepts and strategies', to which representatives from relevant stakeholders participated, was conducted at the end of the 3-day programme. Contributions from academia, industry and regulatory authorities were presented and stimulated the discussion both in presence and remotely. The full programme of the event, together with select presentation and streaming recording, is available at https://www.bfr-akademie.de/english/archive/2021/omics.html.

A total of 485 people (54 in presence, 431 remotely) attended the event. The event was advertised through the following networks: EUROTOX (Federation of European Toxicologists & European Societies of Toxicology), German Toxicology Society, European Partnership for Alternative Approaches to Animal Testing (EPAA), EU-ToxRisk project, UFZ, hosting site's LinkedIn announcement and webpage. A high level of satisfaction with the conference was perceived from the follow-up questionnaire sent to all participants, which was compiled by 113 people (23.3% of the total participants, 17 in presence and 96 remotely). The most valued features of the event were the level of expertise of contributors (score 4.7/5), the support provided during the event (4.9/5), and future practical applicability of the knowledge obtained (4.2/5).

A position paper was produced based on the main outcomes of the event. The manuscript explores the potential of NAMs, which alone promise faster and more efficient hazard and exposure assessments, to transform today's regulatory work, ultimately allowing for a more human-relevant decision-making, as well as the obstacles still hampering the expansion of regulatory applications of NAMs. A conceptual perspective was also included as to how NAMs can be gradually integrated into chemical risk assessment, until an animal-free NGRA is achieved (Figure 1).

The first draft of the position paper was circulated internally already by December 2021. The second draft was later circulated among all co-authors in May 2022 after a second internal revision and is currently (July 2022) at a consolidated version. The tentative title of the manuscript is 'New Approach Methodologies in human regulatory toxicology – not an if question, but how and when!'. The manuscript will be expectedly submitted to a high-rank scientific journal within August 2022. Shared first authorship of the manuscript was assigned to the fellow and to Dr. Sebastian Schmeisser (BfR), whose guidance and support throughout the pre- and post-conference activities proved precious.

The list of co-authors and corresponding affiliations is included in Table 1.

Table 1: Names and affiliations of tentative authors of the position paper on NAMs in regulatory toxicology

Name of co-author	Affiliation
Von Bergen M	UFZ
Berggren E	European Commission's Joint Research Centre
Braeuning A	BfR
Busch W	UFZ
Desaintes C	European Commission's Directorate General for Research and Innovation
Gourmelon A	OECD, Environment Directorate
Grafstoem R	Karolinska Institute
Harrill J	Center for Computational Toxicology and Exposure, U.S. EPA
Hartung T	Johns Hopkins Center for Alternatives to Animal Testing
Herzler M	BfR
Kass G	EFSA
Kleinstreuer N	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
Leist M	Johns Hopkins Center for Alternatives to Animal Testing - Europe
Marx-Stoelting P	BfR
Poetz O	Natural and Medical Science Institute, University of Tübingen; SIGNATOPE GmbH
van Ravenzwaay B	Wageningen University and Research
Roggeband R	European Partnership for Alternative Approaches to Animal Testing; Procter and Gamble Services Company NV/SA
Rogiers V	Scientific Committee on Consumer Safety; Vrije Universiteit Brussel
Roth A	F. Hoffmann-La Roche Ltd
Sanders P	French Agency for Food, Environmental and Occupational Health & Safety
Vinggaard AM	National Food Institute, Technical University of Denmark
Vinken M	Vrije Universiteit Brussel
van de Water B	Leiden University
Luch A	BfR
Tewes T	BfR

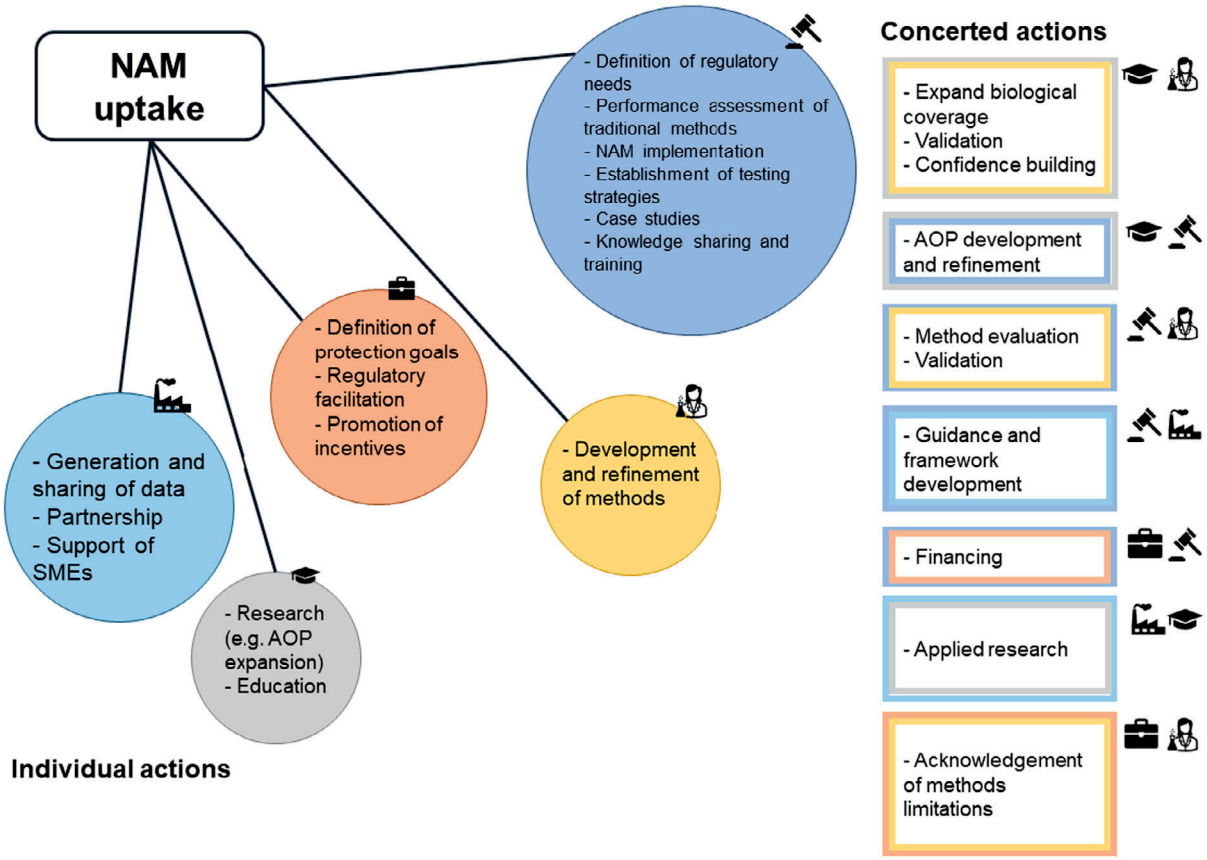

Figure 1: Responsibilities of the different actors involved in a transition towards NAM implementation in regulatory risk assessment. Light blue: Industry; Orange: Politics; Violet: Regulatory community; Yellow: Developers; Grey: Academia.

3.2. Additional activities

In addition to the work programme-specific goals, the fellow engaged in extracurricular laboratory- and desktop-based activities, as follows:

i) Within the October 2021–August 2022 time frame, the fellow participated remotely to the following symposia and workshops, listed in chronological order:

   a) April 2022 – EURION cluster – 'Cross-omics discovery of adverse outcome pathways linked to exposure to endocrine disrupting compounds'.
   b) February 2022 – American Society for Cellular and Computational Toxicology (ASCCT) – 'Serum-Free Cultures: Why and How?', presented by Dr. Barbara Jozef (Swiss Federal Institute of Aquatic Science and Technology) and Dr. Aline Chary (Luxembourg Institute of Science and Technology).
   c) November 2021 – EU-ToxRisk – 'The final outcomes and legacy of EU-ToxRisk, including case studies and a comprehensive collection of learnings and gaps in the application of NAM-enhanced read-across'.
   d) November 2021 – ASCCT – 'How to Control and to Maintain the Quality of Cell Cultures', presented by Dr. Oliver Wehmeier, acCELLerate GmbH.
   e) October 2021 – 'Next Generation Approaches for identifying Endocrine Disruptors'.
   f) October 2021 – EU-ToxRisk – 'Application of a science-driven approach to solve the needs of regulatory and industry communities in the implementation of NAM-supported read-across in regulatory dossiers'.

ii) October 2021 – ASCCT – 'Practical applications of new tools in toxicology'.
iii) The fellow participated in presence to bi-weekly hosting site's Unit 51 seminars and presented an oral contribution on 1 March 2022. The presentation reported on the main

outcomes of the conference 'Challenges in Public Health Protection in the 21st Century: New Methods, Omics and Novel Concepts in Toxicology' as well as introduced further involvement of the fellow in hosting site activities.

iv) On 9 November 2021 the fellow attended a whole-day course held by the hosting site entitled 'Risk assessment and risk management of Genetically Modified Organisms'.

v) The fellow engaged actively in laboratory work since 30 January 2022, within the framework of the project 'Analysis of key functional events in cell culture models', run jointly by the hosting site, the Natural and Medical Science Institute of the University of Tübingen, and SIGNATOPE GmbH (http://www.signatope.com/). The aim of the project is the investigation of cytotoxicity and transcriptomic/proteomic signatures of two human hepatic and renal cells lines caused by the single exposure to six pesticidal active compounds and five food contaminants in a multifactorial design concentration- and time-wise. In some cases, available cytotoxicity information was used as starting point for dose selection in the current project. Of note, the substances have been characterised with regards to toxicological effects by means of traditional *in vivo* experiments (Table 2). The significance of the project, therefore, lies in the verification or identification of the MoA by means of NAMs, and will ultimately contribute to progressing in the slow but steady establishment of NAMs in regulatory risk assessment. Results will be expectedly published in relevant peer-reviewed scientific journals.

Table 2: Known *in vivo* toxicological effects of *in vitro* test substances

Substance	Usage	Known *in vivo* toxicity
Cyproconazole	Azole fungicide	Liver: Hypertrophy, Steatosis
Fluxapyroxad	Pyrazole anilide fungicide	Liver: Hepatocellular necrosis, Steatosis
Azoxystrobin	Strobilurin fungicide	Liver: Hepatocellular cell death, Hypertrophy
Chlorotoluron	Urea herbicide	Kidney: Tumours, Tubular hyperplasia
Thiabendazole	Benzimidazole fungicide	Liver: Lesions of biliary epithelium Kidney: Pelvis and tubular hyperplasia
2-Phenylphenol	Pesticides, etc.	Kidney: Cell death due to oxidative stress
PFOS	PFAS	Liver: Steatosis, Cholestasis
PFOA	PFAS	Liver: Steatosis, Cholestasis
Aflatoxin B1	Mycotoxin	Liver: Tumours
Cadmium	Heavy metal	Kidney: Tubular toxicity
Lasiocarpine	Pyrrolizidine alkaloid	Liver: Hepatic veno-occlusive disease, Tumours

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Abbreviations

AOP	adverse outcome pathway
BfR	German Federal Institute for Risk Assessment
JRC	European Commission's Joint Research Centre
MoA	mode of action
NAM	new approach method
NGRA	next generation risk assessment
OECD	Organisation for Economic Cooperation and Development
PARC	Partnership for the Assessment of Risks from Chemicals
PBK	physiologically-based kinetic models
QSAR	quantitative structure–activity relationship
RA	risk assessment
SME	small- and medium-sized enterprise
US EPA	Environmental Protection Agency
US FDA	Food and Drug Administration
UFZ	Helmholtz Centre for Environmental Research

EU-FORA SERIES 5

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Insects in food and their relevance regarding allergenicity assessment

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Abstract

Within the European Green Deal, the 'Farm-to-Fork' strategy aims to accelerate the transition to a sustainable food system and to make food systems fair, healthy and environmentally friendly. Insects contribute to the circularity of agriculture, and are ideal candidates to complement traditional sources of protein. In this context, a working programme within the European Food Risk Assessment (EU-FORA) Fellowship Programme framework was developed at the German Federal Institute for Risk Assessment in collaboration with the Spanish National Research Council. The purpose of this technical report is to describe the activities in which the fellow was involved. As part of the training, the fellow performed a literature search regarding insects as food and allergenicity resulting in 493 hits. Out of the literature search a comprehensive scientific database with 200 publications has been built using the application 'EndNote'. Furthermore, an extensive scientific review with the title 'Sustainable food systems: EU regulatory framework and contribution of insects to the Farm-to-Fork strategy' approaching several important issues regarding insects (Regulatory frame, Market situation, Labelling and Control, Application as food/feed, Consumer acceptance and Allergenicity risk assessment) has been drafted and sent for publication in a peer reviewed journal. In order to analyse the impact of food processing on the allergenicity of insects, different food samples were prepared and artificially digested using a protocol simulating the gastrointestinal tract. Further laboratory work to analyse the readouts, including enzyme-linked immunosorbent assay (ELISA), has been discussed and proposed, scheduled for the end of July. In conclusion, the present working programme, together with additional activities and training provided by different institutions, enabled the fellow to gain a broader perspective in food safety, particularly concerning insects as novel foods and their safety assessment.

Keywords: allergen, allergenicity risk assessment, entomophagy, food safety, insects, novel food, tropomyosin

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1. Introduction

1.1. European food risk assessment fellowship programme

The European Food Risk Assessment (EU-FORA) Fellowship Programme is a key initiative organised by the European Food Safety Authority (EFSA) to ensure preparedness for future risk analysis needs. The aim of the programme is to increase the pool of food safety risk assessment experts in Europe and stimulate the involvement of Member States in risk assessment, all with the ultimate objective of building a common EU culture for risk assessment.

The EU-FORA fellow participated in the general work programme entitled 'Insects in food and their relevance regarding allergenicity assessment'. This project brings together the expertise from the German Federal Institute for Risk Assessment (BfR) Unit Effect-based Analytics and Toxicogenomics (Unit 51) and from the Spanish National Research Council (CSIC) to generate knowledge on the allergenic risk using insects as food.

The work programme comprised the elaboration of a detailed scientific review covering different aspects of the use of insects as food and feed, highlighting its regulatory framework in the European Union. Also, the implementation of different protein-based techniques to be used in the analysis of *in vitro* (mainly, proteolysis studies) tools for the identification and assessment of potentially allergenic foodstuff derived from insects.

In addition, the programme proposed the exploration of the usefulness of more refined and advanced *in silico* approaches to assess the safety assessment of proteins. These novel approaches are not currently implemented by regulatory authorities but there are ongoing projects addressing these new tools with the aim of moving further the field of allergenicity risk assessment of novel proteins. Concretely, improved *in silico* analysis approaches based on more advanced bioinformatics tools that might better predict the risk of a novel protein to trigger toxic or allergic reactions.

1.2. General information on the project

The Farm-to-Fork strategy is a crucial part of the European Green Deal regarding the agri-food chain. By implementing the objectives of the strategy, insects as food and feed can significantly contribute to the improvement of the sustainability from Farm to Fork. A significant aspect is the promotion of the production and usage of new protein sources that can help to relieve pressure on agricultural land. Insect farming can reduce the need of importing some food and feed commodities by expanding the spectrum of products with high content of protein available in the EU, while shortening the agri-food chain. Globally more than 2,000 insect species have been documented in the literature as edible. The most commonly eaten insects are members of the Coleoptera (beetles), Lepidoptera (Caterpillars of butterflies and moths), Flymenoptera (bees, wasps, ants), Orthoptera (grasshoppers, locusts, crickets, termites), Hemiptera (cicadas, leaf and plant hoppers, true bugs, scale insects), Odonata (dragonflies) and Diptera (flies) families (Van Huis et al., 2013; Jongema, 2017). At present, several dossiers for different insect species are waiting the safety assessment carried out by EFSA and the market authorisation as novel foods (Regulation (EU) No 2015/2283) and six insect preparations from *Tenebrio molitor*, *Acheta domesticus*, *Locusta migratoria* and *Alphitobius diaperinus* have already been approved by the European Commission. However, there can be hazards associated with insects for use in food and feed depending on species, feed, production and processing conditions (Schlüter et al., 2017; Testa et al., 2017) and, in its scientific opinions, EFSA concluded that further research on the allergenicity of insects is needed.

To date, little information is publicly available about the hazards associated with insects for use in food and feed. However, the scarce information available indicates that insect proteins can cause allergic reactions in humans and animals with allergy to other arthropods, such as crustaceans (e.g. shrimp, prawn and crab) and house dust mites, due to allergen cross-reactivity (de Gier and Verhoeckx, 2018; Premrov et al., 2021). This is also reflected in the scientific opinions on insect species as food by the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) published so far (Safety of frozen and dried formulations from whole yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283; Safety of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283; Safety of frozen and dried formulations from migratory locust (*Locusta migratoria*) as a novel food pursuant to Regulation (EU) 2015/2283; Safety of frozen and dried formulations from whole house crickets (*Acheta domesticus*) as a Novel food pursuant to Regulation (EU) 2015/2283; Safety of partially defatted house cricket (*Acheta*

domesticus) powder as a novel food pursuant to Regulation (EU) 2015/2283; Safety of frozen and freeze-dried formulations of the lesser mealworm (*Alphitobius diaperinus* larva) as a Novel food pursuant to Regulation (EU) 2015/2283.

Cross-reactivity can be explained because many proteins occurring in currently consumed edible insects are phylogenetically related to allergens widely dispersed in the different groups of arthropods (crustaceans, insects, mites) and molluscs, as it is the case of pan-allergens such as tropomyosin, enolase, alpha-actin or arginine kinase, among others (Barre et al., 2014). Besides pan-allergens, some proteins belonging to distinct protein families, including apolipophorin III, the chemosensensory protein, the cockroach allergen-like protein, hexamerin, the larval cuticle protein, the receptor for activated protein kinase and the odorant-binding protein, among others, have arisen as proteins very specific for edible insects (Barre et al., 2021; He et al., 2021). These proteins, lacking or much less represented in other groups of arthropods, molluscs or nematodes, share well- conserved amino acid sequences and highly similar quaternary structures. Consequently, they have been recently proposed as probes for the specific detection of insect proteins added either intentionally as food ingredients or involuntarily as hidden allergens to food products in complement to other detection methods (Gautreau et al., 2017). Therefore, there is a need to assess their food safety, especially for people allergic to edible insects. Likewise, recently several anaphylactic reactions have been reported in Europe and America after consumption of certain insect species (i.e. yellow mealworm *T. molitor* and silkworm pupae *B. mori*) from individuals whose clinical history consisted exclusively of allergy to house dust mites (Beaumont et al., 2019) without any previous reported allergy (Gautreau et al., 2017), indicating that the consumption of insects may also induce primary sensitisation in previously non-allergic individuals (Garino et al., 2020). This was confirmed in professional mealworm breeders sensitised after occupational exposure and showing allergic symptoms upon in food challenges with *T. molitor* (Broekman et al., 2017).

Consequently, there is a need to undertake an assessment of the allergenic potential of insects intended for human consumption (EFSA NDA Panel, 2021) in particularly because only limited or no history of human consumption at all of insects has been demonstrated so far in the EU. Risk assessment includes an examination of the protein content of insects; an evaluation of the degree of sequence homology of the protein components with known allergens, and immunological assays to identify potential allergenic components.

As part of a comprehensive assessment, the *in vitro* digestion of potential allergenic proteins is considered a useful test to support the weight-of-evidence approach applied today for allergen risk assessment. Recently, the GMO Panel has assessed the usefulness on *in vitro* protein digestibility tests in allergenicity and protein safety assessment (EFSA GMO Panel, 2021).

2. Description of work programme

2.1. Aims

A literature search will be conducted to identify the number of insects and insect material already consumed in the EU and all aspects regarding the safe consumption of insect material. The final goal will be the compilation of all relevant research data regarding the safety of insects as food or food ingredients and its potential consumption in the EU.

The practical training programme consists of:

i) insect sample management.
ii) selecting appropriate insect's allergens.
iii) preparation of an *in vitro* model that allow studying protein digestion.
iv) analysing readouts.
v) preparing a report and contributing to a publication.

Special emphasis will be put on internationally accepted *in vitro* digestion models to be used so that the scientific output can be put in comparison with already existing systems. In this way, the project spans the bridge between bioinformatics, basic research, practical experience and law enforcement requirements so that the fellow will gain insights into all functionalities and aspects of these modules. At the end, the fellow will gain practical experience using different modern laboratory techniques and get trained in conducting a scientific literature search applied to a specific research question on insects' consumption and allergy risk assessment. The preparation of scientific publications will be supported

and accompanied by experienced mentors. Finally, a publication shall have a positive impact on the fellows' personal career.

The research proposal consists of two interacting parts, a literature review regarding the consumption of insects and related aspects; and the practical research using insect proteins in *in vitro* digestion systems aiming to generate new data on the risk assessment of insect as a potential allergic food.

2.2. Activities/Methods

2.2.1. Literature review and establishment of a database about insects as food and feed

During the first 3 months of the fellowship, a literature search regarding insects as food and allergenicity was created and conducted resulting in 493 hits in the following databases: Science Direct, Scopus, PubMed, Web of Science and Google Scholar. Out of the literature search a comprehensive scientific database with 200 publications has been built using the application 'EndNote'. A system of classification into several thematic groups (EU legislation, General issues, Feed, Cross Reactivity, Processing, Bioinformatics, Nutrition, Risk Assessment, Allergenicity, Tropomyosin, Arginine-Kinase) has been used to classify the publications and facilitate the user-friendly navigation through the database.

2.2.2. Preparation of a scientific review regarding insects and its regulatory framework

The European Green Deal is a set of policy initiatives of the EU that aim to make Europe the first climate-neutral continent by 2050. Within this Deal, the 'Farm-to-Fork' strategy aims to accelerate the transition to a sustainable food system and to make food systems fair, healthy and environmentally friendly. Insects contribute to the circularity of agriculture, and are ideal candidates to complement traditional sources of protein. The placement of insects on the EU food market needs to be authorised by the Commission following a risk assessment. To date, three insect species have been approved for their commercialisation, while the use of insect proteins in feed is strictly regulated. Insect farming is an expanding industry in Europe, and more consumers are willing to try insect-based foods. To consolidate the insect market, it is also very important to assure the safety of eating insects. EFSA is the body in charge of assessing the microbiological and chemical risks related to the production and consumption of insects as food. Aside from the risks posed by contaminants or pathogens, insects consumed as food may be a threat for certain allergic consumers, who might develop an adverse reaction mainly due to a cross-reactivity to crustaceans and/or house dust mites. The European insect's market is also analysed, with a special emphasis on the regulatory aspects and on the safety assessment. Furthermore, an updated overview on the consumer acceptance is presented. Overall, the entry of insects into the EU market represents a great opportunity for the economic and ecological growth of the Community; however, consumers need to be exhaustively informed and protected from the hazards that these novel products might cause.

2.2.3. Experimental work on the effect of food processing on the allergenicity of insect proteins

Ten different processed samples containing *Tenebrio molitor* larvae were prepared:

Grounded Dust

1) 5A-Brot 5% Extruding A + 95% wheat flour (5% extruded insect +95% flour)
2) 5C-Brot 5% Whole +95% wheat flour (5% whole insect +95% flour)
3) 20A-Brot 20% Extruding A + 80% wheat flour (20% extruded insect +80% flour)
4) 20C-Brot 20% Whole A + 80% wheat flour (20% whole insect +80% flour)

Canned Meat

1) B10% 80°C (10% insect treated at 80°C)
2) B10% 80°C (10% insect treated at 100°C)
3) B10% 80°C (10% insect treated at 121°C)

Processed Foods containing the insect

1) Krispies
2) Chips
3) Tortillas

After that, the samples were carried to the BAM (Federal Institute for Materials Research and Testing) in Berlin and artificially digested following the protocol reported by Sieg et al. (2020).

At the end of the procedure, three different samples corresponding to three stages of the digestion (salivary phase, gastric phase and intestinal phase) were collected and after enzyme inactivation, digested samples were freeze until further treatment.

To analyse the readouts of the artificial digestion, the fellow visited the CIAL (Research Institute for Food Sciences) in Madrid. Two samples (20A-Brot 20% Extrudant A +80% Weizenmeht (20% extruded insect +80% flour and 2)) and B10% 80°C (10% insect treated at 100°C) out of the 10 samples artificially digested were selected to perform pilot research. The main purpose was to assess the degradation of tropomyosin, the main allergenic protein in insects, and the impact in its immunoreactive properties. Therefore, an enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the ability of the method to recognise insect tropomyosin. It is important to highlight at this point that the ELISA test was developed for the determination of crustacean tropomyosin and not insect tropomyosin. However, as no specific kit for insects exist so far, and due to the high degree of sequence similarity between the tropomyosin of insects and crustaceans, it was decided as the more promising option.

Furthermore, two predictive bioinformatics tools have been used to complete the allergenicity analysis of *Tenebrio molitor*'s tropomyosin. AllergenOnline is an online tool where the protein sequence of interest can be compared with well-known allergens in their database, if the identity matches greater than 50% indicates possible cross-reactivity. A precautionary search using a sliding window of 80 amino acid segments of each protein can also be used to find identities greater than 35%, what according to CODEX Alimentarius guidelines 2003 means that IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility (FAO, 2009; EFSA GMO Panel, 2017). The second tool is PeptideCutter from Expasy, that allows the prediction of potential cleavage sites cleaved by proteases or chemicals in a given protein sequence.

The analysis of results is not included in this report to avoid certain copyright claims, as the authors aim to publish them in other scientific journals. Further research on the results as well as new experimental work, including western blotting, will be performed before the end of the fellowship.

2.2.4. EU-FORA fellowship supporting programme

At the starting of the fellowship, the fellow was introduced to the activities performed at the Department of Food Safety of the BfR and at the hosting unit (Unit of Effect-based Analytics and Toxicogenomics). The fellow obtained experience in the area of insects as novel foods by performing an initial literature review followed by a complete scientific review on the EU regulatory framework of insects and their contribution to the Farm-to-Fork strategy.

The fellow also gained experience *in vitro* digestion models and further processing of readouts to assess allergenicity of insect's proteins.

The above work was completed under thorough guidance and effective supervision provided through fortnightly meetings conducted *in situ* or online. Apart from the practical work at the BfR during the fellowship year, the fellow participated in the following activities described in Table 1.

Table 1: Supporting activities during the EU-FORA fellowship programme

Trainings	Dates
Training Modules provided by EFSA	
Induction training: Microbiological and Chemical Risk Assessment, EFSA (Virtual)	30 August–17 September 2021
Training Module 1: Risk Assessment of other Food Areas, AGES (Virtual)	22–26 November 2021
Training Module 2: Risk Communication, BfR (Virtual)	21–25 March 2022
Training Module 3: Emerging Risks, EFET (On site)	6–10 June 2022
Training Module 4: Data Collection and Reporting, EFSA (Virtual)	22–25 August 2022

Trainings	Dates
Other trainings/Workshops	
EFSA Summer: School Food Safety Aspects of Integrated Food Systems	28–30 September 2021
Workshop: Risk Assessment and Risk Management of Genetically Modified Organisms (GMO), BfR	9 November 2021
DISH Cluster event: Towards healthy and safe diet	6 April 2022
EFSA Online Stakeholder workshop on small particles and nanoparticles in food	31 March–1 April 2022
Conferences	
EIT Future of Food Conference 2021 (Virtual)	30 November–1 December 2021
One Health Conference, Brussels	21–24 June 2022
Other activities	
Regular meetings with supervisors (virtual and on site)	Fortnightly
Regular seminars organised by the Department of Food Safety of the BfR (virtual and on site)	Fortnightly
Presentation by the fellow's work at the department seminar (BfR)	1 February 2022

3. Conclusions

3.1. Conclusions regarding insects in food and their relevance regarding allergenicity risk assessment

When known allergenic structures, such as insect allergens, are already present in the novel food, a comparative assessment can be performed, and the scope of cross-reactivity explored. Among the key parameters to be evaluated are:

- the amino acid primary sequence homology to known allergens (through bioinformatics analysis);
- the secondary and tertiary structure (looking for conformational epitopes);
- the ability to bind specific IgEs (through *in vitro* assays such as ELISA or immunoblot) and to elicit an immune response (checking the biological activity through *in vitro* or *in vivo* tests).

For a comprehensive assessment, other parameters like thermal and chemical stability to food processing, as well as resistance to gastric and duodenal digestion need to be carefully evaluated. All together will contribute to the 'weight-of-evidence' of the allergenicity risk assessment that might be result in further analysis.

Furthermore, the EFSA Genetically Modified Organisms (GMO) Panel has recently published a scientific opinion addressing the identification of specific research needs to improve the allergenicity risk assessment of new proteins (EFSA GMO Panel, 2022) This document has highlighted some potential gaps on allergenicity prediction which could indicate that some of the methods included in the current weight-of-evidence approach for the allergenicity risk assessment could not be easily applicable to complex mixtures of proteins that often make up whole foods, such as insects. Among other issues, the EFSA GMO Panel has recommended the consideration of the clinical relevance, route of exposure and potential threshold values of food allergens, as well as the use of improved *in silico* analysis approaches based on more advanced bioinformatics tools that may better predict the risk of a novel protein to trigger allergic reactions. Likewise, the draft of a roadmap that (re)defines the allergenicity safety objectives and risk assessment needs will be required to address a series of key questions for risk assessors and risk managers.

3.2. Conclusions regarding the participation in the EU-FORA fellowship

During the year of the EU-FORA fellowship programme, the fellow learned general principles of risk assessment and gained experience in the risk assessment of novel foods, especially insects, improving also her skills in the systematic extraction, structuring and evaluation of relevant scientific data. Furthermore, the participation in the EU-FORA programme provided an exceptional opportunity for the fellow to become part of an international experts' network that is expected to be a valuable source of future professional support and to provide a basis for further collaboration.

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Abbreviations

AGES	Austrian Agency for Health and Food Safety
BAM	Federal Institute for Materials Research and Testing
BfR	Bundesinstitut für Risikobewertung (German Federal Institute for Risk Assessment)
CIAL	Research Institute for Food Sciences
CSIC	Spanish Research Council
EFET	Hellenic Food Authority
ELISA	enzyme-linked immunosorbent assay
EU-FORA	European Food Risk Assessment Fellowship Programme
GMO	genetically modified organism
IgE	immunoglobulin E

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Novel foods: allergenicity assessment of insect proteins

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Abstract

Insects represent a promising source of proteins and have been reported as a great potential for being used as novel food and feed proteins. This makes them a valuable source of nutrients to face the increasing demand of food necessitated by the growing global population. The current European food legislation on novel food (EU Reg. 2015/2283), which entered into force in 2018, provides the provisions that should be considered in the applications for the authorisation of novel foods in the European market. Insects, intended as an alternative source of food proteins for human consumption, are considered novel foods. Since food allergens are mostly proteins, the analysis and identification of the potential allergenicity of novel proteins should be a fundamental activity that enables the applicants to fulfil the requirements for the application and authorisation to bring a novel food into the European market and ensures a high level of food safety for the European consumers. The main aims of the work of the EU-FORA fellow were to: (i) Review, assess and identify gaps in the current strategies for predicting allergenicity of novel foods and new alternative protein sources; and (ii) Familiarise, understand and perform an allergenicity assessment of a novel food protein source by: (a) Working on an allergenicity assessment case study of insect proteins from black soldier fly larva (*Hermetia Illucens*); and (b) Taking into consideration other risk assessment aspects of insects as novel food, including toxicological, nutritional and microbial risks. The project contributed to the continuous learning of the fellow on practical assays and methodologies for the *in silico*, *in vitro* and *in vivo* analysis principles and complemented personal skills related to the food risk assessment requirement for the preparation and submission of an application for authorisation of a novel food.

Keywords: novel food, alternative proteins, insects, allergenicity assessment, *Hermetia Illucens*, black soldier fly

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1. Introduction

The global population is growing, thus, the demand for food is expected to increase. To meet the future demand for food, alternative protein sources are being studied. Insects represent a promising source of proteins, and the potential of using insects as a novel source of food and feed proteins has been reported in several studies (FAO, 2009a; Rumpold and Schlüter, 2013; Van Huis, 2013; Akhtar and Isman, 2018; Kim et al., 2019; de Carvalho et al., 2020).

The possibility of using insects for food and feed has been investigated by some food authorities, including the Food and Agriculture Organization of the United Nations (FAO) and the European Food Safety Authority (EFSA), and the risk assessments related to insects as food or feed have been performed in some European Union (EU) Member States.

There are currently nine novel food applications for insects undergoing safety evaluation by EFSA and there have been already three insects for which EFSA has finished their safety evaluations (European Commission, 2022). These are: '*Tenebrio molitor*' larva – mealworm in frozen, dried and powder form (EFSA NDA Panel, 2021a,b); '*Locusta migratoria*' – migratory locust, grasshopper (EFSA NDA Panel, 2021); and '*Acheta domesticus*' – house cricket (EFSA NDA Panel, 2021d). These three insects have all been authorised by the European Commission to be placed on the EU market as a novel food (EU, 2021/882, 2021a; EU, 2022/169, 2022a; EU, 2021/1975, 2021b; EU, 2022/188, 2022b).

Currently, the European food legislation on novel food (EU Reg. 2015/2283) demands, among other information, a comprehensive assessment of nutritional, microbial and toxicological risks and evidence that the novel food is unlikely to trigger allergic reactions, before authorising the introduction of the novel food/protein source on the EU market. Well establish methods and standards are available for the assessment of nutritional, microbial and toxicological risks. On the contrary, the risk assessment of allergenic potential of novel proteins is still challenging (Verhoeckx et al., 2020). As several pieces of information or conduction of experimental methods are needed for the allergenicity assessment to provide sufficient evidence to predict allergenicity, which are currently not available or not yet validated, such as, among other, potential threshold doses of food allergens, integration and standardisation for *in vitro*/*in vivo* tests and protocols, and modernisation of the *in silico* tools and databases, as well as clinical data, a careful allergenicity assessment is difficult (EFSA GMO Panel, 2022).

Bearing this in mind, in the current 'learning by doing' education EU-FORA working programme, the fellow was engaged in analysing and discussing strategies for predicting allergenicity of a novel source of food protein using the insect black soldier fly larva as a case study.

2. Description of work programme

In the course of the work programme, the fellow, was hosted at the Research Group for Food Allergy at the National Food Institute, Technical University of Denmark (DTU Food) under the supervision of Head of Research Group. Here the fellow was introduced to various aspects of allergenicity assessment and involved in practical laboratory test and analysis steps, from extraction of proteins from a novel biomass to *in silico*, physicochemical, *in vitro* and *in vivo* assessment of the allergenic potential of the novel proteins, by carrying out a case study.

Through such supervised case study, the fellow experienced the challenges met in the whole process of performing an allergenicity risk assessment, which improved the fellow's understanding of the allergenicity risk assessment.

2.1. Aims

The main activities related to the project consisted of:

- Reviewing, assessing and identifying gaps in the current strategies for predicting allergenicity of novel foods and new alternative protein sources.
- Familiarise, understand and perform an allergenicity assessment of a novel food by:
    - Working on an allergenicity assessment case study of the insect black soldier fly larva (*Hermetia Illucens*) as a potential future source for alternative food proteins
    - Taking into consideration other risk assessment aspects of insects as a novel food, including toxicological, nutritional and microbial risks.

2.2. Activities/methods

2.2.1. Current strategies for predicting allergenicity

A literature search and review of the state of the art of current available methodology and approaches for performing allergenicity risk assessment of novel food or food proteins were performed by the fellow. Outcomes showed that at present there are no validated approaches for allergenicity risk assessment of novel protein (EFSA NDA Panel, 2014; Verhoeckx et al., 2020; EFSA GMO Panel, 2021, 2022). The findings were discussed and analysed in ad hoc monthly meetings with Senior Researchers in the Research Group for Food Allergy.

The aspects discussed included questions regarding:

- The existing knowledge about (high) allergenic vs. (low) non-allergenic foods and food proteins;
- The approaches and the tools currently available to predict allergenicity of new foods and food proteins;
- The need for efficient prediction of allergenicity and the purpose of the allergenicity risk assessment; and
- Which test and parameters could be included and what are the gaps that need to be filled in order to provide an improved allergenicity risk assessment procedure for novel food and food proteins.

Among the aspects that came to light, notably to consider were the limited options to assess the hazard and potential risks of new proteins causing *de novo* sensitisation (EFSA GMO Panel, 2017, 2022; Mazzucchelli et al., 2018; Remington et al., 2018), as there is currently no single test or parameter available that may provide sufficient evidence to predict *de novo* sensitisation. On the other hand, for cross-reactivity aspect, meaning for the assessment of the risk of inducing reactions in already allergic patients, tools and methods are available and these may well predict the potential cross-reactivity with a reasonable level of confidence (EFSA GMO Panel, 2010, 2022; Verhoeckx et al., 2020). Therefore, there is not a huge and urgent need for improving tools and methods, for assessing the risk of inducing reactions in already allergic patients due to cross-reactivity with known allergens. The large gap, and where there is a great need for improving and developing new predictive tools, is for the evaluation of *de novo* sensitisation for assessing the potential risk of inducing a new allergy of similar severity as peanut allergy.

Key elements of knowledge and methods are moreover lacking, which includes among other: integration and standardisation for *in vitro* and *in vivo* tests and protocols, as well as modernisation of the *in silico* tools and databases; data regarding the allergenic potency of other allergenic foods than those recently published FAO/WHO for which a reference doses has been proposed; the consensus on the clinically relevant allergens and clinical data on threshold doses for individual allergenic protein molecules (EFSA GMO Panel, 2022; FAO and WHO, 2021).

Therefore, the 'weight-of-evidence' approach, currently in use, remains the most suitable method applicable for allergenicity assessment. Still, a better clarity on the use of the 'weight-of-evidence' approach is needed, since the results might differ depending on whether a food product is derived from genetically modified organisms, from new biotechnological food or true novel food is being assessed.

For this task, a draft document on reviewing and discussing strategies for predicting allergenicity has been initiated. The document is an ongoing work aiming at a potential manuscript for publication in a peer-reviewed journal.

2.2.2. Allergenicity assessment of novel food – a case study

At present, the allergenicity assessment, as part of the risk assessment of novel food, evaluates the allergenic risk that a new protein or whole food pose to the existing allergic population identified by the immunoglobulin E (IgE) cross-reactivity (EFSA Guidance, 2021; EFSA GMO Panel, 2022). Current methodology is suitable mainly for assessing the allergenic potential of new proteins for the cross-reactivity, whereas methodologies for assessing allergenic potential of new proteins due to *de novo* sensitisation are limited. Food allergens are mostly protein; therefore, the analysis of the potential allergenic proteins becomes a fundamental aspect to evaluate the potential allergenic risk in the context of the allergenicity assessment of novel food (EFSA Guidance, 2021).

Taking into account that black soldier fly larva is considered a potential alternative protein source (EFSA Scientific Opinion, 2015) it is, therefore, important to investigate and characterise the allergenic potential, for both cross-reaction and *de novo* sensitisation, of black soldier fly larva proteins.

As a preliminary step, a literature search was performed. This was carried out to identify studies on the risk assessment and findings of insects as a future source for alternative proteins and on the allergenicity assessment of black soldier fly larva. Further, a literature search with a focus on gathering information and reviewing studies related to the black soldier fly larva proteins was performed, by searching whether there were any proteins already identified and characterised and which method was applied for their characterisation (Figure 1 – I and V).

The next step of the allergenicity assessment consisted of the extraction and preparation of the material for characterisation of the composition and for use in further analyses. The original material, of the black soldier fly larva, was kindly provided by ENORM BioFactory A/S –Denmark in powder form and was afterwards treated by a defatting process. The defatted material was further processed for protein extraction to provide the material for use in further analyses. The protein concentration of the extract was determined by the bicinchoninic acid protein (BCA) protein assay and amino acid analysis. The amino acid analysis also provided the amino acid composition of the extracted proteins. Further, the extract was characterised by gel electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis – SDS–PAGE) for protein separation and protein size profiling as well as by tandem mass spectrometry (MS/MS) analysis in order to determine the presence of specific proteins (Figure 1 – II).

Then a computer/database-based analysis was performed where the amino acid sequences of black soldier fly larva were retrieved and grouped according to the allergenic protein super-families (NCBI-Protein, 1988; Radauer et al., 2014; Finn et al., 2016; Goodman et al., 2016; The UniProt Consortium, 2021). The degree of protein sequence homology between the studied proteins and known allergens was then determined by bioinformatic tools to evaluate the potential allergenicity risk due to cross-reactivity as recommended in the Codex Alimentarius Commission (FAO, 2009b) (Figure 1 – IV).

To identify the sensitising capacity of black soldier fly larva an animal study was carried out with Brown Norway rats. The immunogenicity and sensitising capacity of black soldier fly larva proteins were evaluated in comparison to known (high) allergenic and known (low) non-allergenic food proteins by means of several enzyme-linked immunosorbent assays (ELISA) analyses. All the tests were carried out by developing and applying in-house protocols generated at Research Group for Food Allergy in the National Food Institute.

To evaluate the digestibility, an *in vitro* simulated gastro and gastroduodenal digestion of black soldier fly larva was performed in simulated gastric fluid and simulated duodenal fluid solutions, respectively. Aliquots were taken for further analysis by SDS–PAGE and immunoblotting (Figure 1 – III).

The fellow was introduced to the above-mentioned practical laboratory assays and methodologies. Nevertheless, most laboratory work was supported and carried out with the help of the lab technicians.

A manuscript, currently under preparation, will present a full description of the allergenicity risk assessment of the black soldier fly larva, including protein chemical analysis, *in silico* analysis, the *in vitro* analysis and the *in vivo* analysis (animal studies). This manuscript will be submitted for publication in a peer-reviewed journal.

An overview of the activities carried out during the EU-FORA fellowship are presented in the Figure 1 below.

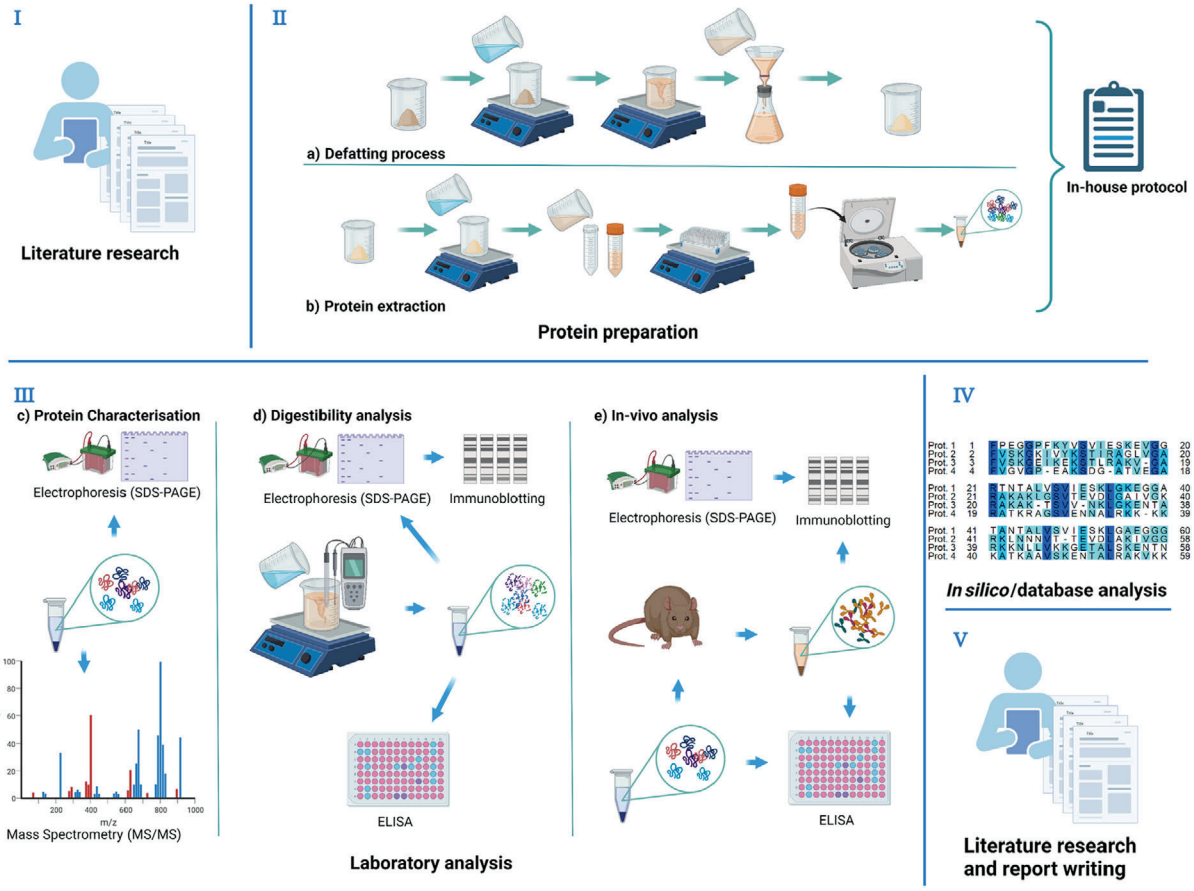

Figure 1: Analytical approaches carried out within the case study, including literature review, material preparation (protein defatting and protein extraction), laboratory analysis (protein characterisation - Electrophoresis, Mass Spectrometry, ELISA; *in vitro* analysis; *in vivo*/ animal studies), and *in silico* analysis. Figures in I, II, III, V were created in BioRender.com

2.3. Secondary activities

Besides being introduced to the aspects of the allergenicity risk assessment approach, the fellow was introduced to food enzyme allergenicity assessments conducted by the Research Group for Food Allergy to authorities, particularly on *in silico* allergenicity assessment cases.

In addition to the regular weekly meetings with the main supervisor and the research group meetings, the fellow took part of other activities:

- Attended the 9th BfR-Summer Academy-Lecture Series on food safety and appropriate risk assessment, organised by the German Federal Institute for Risk Assessment (BfR-Bundesinstitut für Risikobewertung).
- Attended the Fundamentals of Immunology course on Innate Immunity and B-Cell Function. Offered by Rice University. Rice University in Houston, Texas, via Coursera online courses.
- Participated in the online Stakeholder workshop on small particles and nanoparticles in food. Organised by EFSA Working Group on Nanotechnologies (WG Nano).
- Participated in the ONE – Health, Environment, Society – Conference 2022. Organised by EFSA in partnership with the European Centre for Disease Prevention and Control (ECDC), the European Chemicals Agency (ECHA), the European Environment Agency (EEA), the European Medicines Agency (EMA), and the European Commission's Joint Research Centre. 21–24 June 2022, Brussels.

3. Conclusions

In the course of the EU-FORA fellowship programme, the fellow, hosted at the DTU Food, was introduced to the food allergenicity assessment and familiarised with the role of the allergenicity assessment on the risk assessment of novel foods.

In this process, the fellow learned practical assays and methodologies for the *in silico*, *in vitro* and *in vivo* analysis principles and complemented personal skills related to the allergenicity risk assessment requirement for the preparation and submission of an application for authorisation of a novel food.

The EU-FORA fellowship project at DTU Food contributed to the process of learning new skills and knowledge of the fellow on the food risk assessment methodologies, on the understanding of laboratory assays and tools, and on the use of *in silico* analysis and bioinformatics tools. The EU-FORA programme also provided a rich social experience both during the training modules and the placement at the DTU Food.

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Abbreviations

BCA	bicinchoninic acid
EFSA GMO Panel	EFSA Panel on Genetically Modified Organisms
EFSA NDA Panel	EFSA Panel on Dietetic Products, Nutrition and Allergies
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
GMO	genetically modified organisms
IgE	immunoglobulin type E
MS/MS	tandem Mass Spectrometry
SDF	simulated duodenal fluid
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SGF	simulated gastric fluid

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Dietary exposure and risk characterisation of multiple chemical contaminants in rye-wheat bread marketed in Poland

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Abstract

The aim of this work was to collect occurrence data on chemical contaminants in rye-wheat bread marketed in Poland and subsequently estimate the risk due to dietary exposure to those chemicals for different population age groups in Poland. The choice of the food matrix has been driven by the fact that bread is an important component of the diet of Poles. Therefore, the collection of data on this staple food might be of interest form a public health perspective. The analytes under investigation belong to different categories: process contaminants (acrylamide), mycotoxins (deoxynivalenol, deoxynivalenol-3-glucoside, nivalenol and nivalenol-3-glucoside) and environmental contaminants (aluminium, arsenic, cadmium, chromium, lead and nickel). The activities presented in this technical report have been carried out in the frame of the European Food Risk Assessment (EU-FORA) Fellowship Programme. The objective of the fellowship is to provide middle-early career scientists with new skills and knowledge on food risk assessment. This goal is pursued through a hands-on experience in a competent organisation active in fields within EFSA's mission. In this case, the work programme has been implemented at the Department of Food Safety and Chemical Analysis of the State Research Institute of Agriculture and Food Biotechnology Prof. Wacław Dąbrowski (IBPRS-PIB), located in Warsaw, Poland. A significant part of the research work consisted in the implementation of different analytical techniques to collect occurrence data that were subsequently combined with consumption data retrieved from the EFSA Comprehensive European Food Consumption Database to assess the exposure and characterise the risk. Finally, an attempt has been made to identify uncertainties and evaluate their impact on results.

Keywords: EU-FORA, food risk assessment, chemical contaminants, bread and rolls

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1. Introduction

The activities described in this report have been carried out in the frame of the European Food Risk Assessment (EU-FORA) Fellowship Programme cycle 2021–2022. The main purpose of this initiative, established by the European Food Safety Authority (EFSA) in 2016, is to increase the pool of food safety experts and strengthen the food risk assessment network across Europe with the ultimate objective of building a common culture for risk assessment in the European Union. This initiative fulfils Article 23 (b) of EFSA's Founding Regulation (Reg. EC no 178/2002) that appoints EFSA to be responsible for promoting and coordinating the development of uniform risk assessment methodologies in the fields falling within its mission. During the 12-month EU-FORA fellowship, participants are entitled to carry out risk assessment activities with a learning-by-doing approach at the premises of one of the competent organisations adopted by EFSA Management Board according to Article 36 of Regulation (EC) no 178/2002. The hands-on experience was complemented by 7 weeks of training divided in 3 weeks of induction training and four specific 1-week modules held throughout the 12-month placement period.

2. Description of work programme

2.1. Aims

The main goal of the project was to estimate the risk to Polish population related to the dietary exposure to chemical contaminants with special focus on the consumption of rye-wheat bread. Contamination levels and dietary habits are constantly changing. On this regard, the work was conducted with the purposes of contributing to a better understanding and to the monitoring of the adequacy and safety of the diet of Poles. Gathering up-to-date data is key to reach this intent. The regular collection of accurate and reliable data is a prerequisite for informed risk assessment and risk management.

2.2. Activities/Methods

Food matrix and sampling strategy

Bread is a staple food. It is consumed every day by many people, from children to elderly, in many countries. For this reason, investigating the risks associated to the consumption of this product could be interesting from a public health perspective. Since the most common type of bread in Poland is the one produced with rye-wheat flour (Mojska et al., 2010), the research has been focused on the category 'Rye-wheat bread and rolls, refined flour'. The 51 loaves composing the sample subject to the study have been randomly collected in different popular supermarkets in Warsaw, Poland. The implementation of this non-targeted sampling strategy at the market place aimed to reflect Poles' consumption habits and to estimate the contaminants' concentration as close to the time and real conditions of consumption as possible. This matrix is labelled with the code A005N and described in the EFSA's food classification and description system MTX (FoodEx2 Matrix) catalogue version 13.1. as 'any type of bread and rolls containing considerable proportions of wheat and rye refined flours (i.e. containing very little or no bran). The part consumed/analysed is by default the whole or a portion of it representing the observed heterogeneity'. A representative portion of each loaf has been lyophilised, and grinded to a powder. This ensured prolonged storage stability thanks to the removal of water. Slices were weighted before and after lyophilisation to allow the expression of results as fresh product (as it is actually consumed).

Bread consumption

Since 1989, Poland experienced profound political and economic amendments. The switch from a centrally planned to the market economy system leaded to considerable changes in the lifestyle of Poles. Therefore, assessing the nutritional situation of the population became of great interest. Given the lack of representative, countrywide, individual dietary surveys in Poland, the government requested technical assistance from the United Nations Food and Agriculture Organization (FAO) to enrich the existing nutrition information system. This leaded to the implementation of a project that examined the diets and anthropometric characteristics of a nationally representative sample of Polish households surveyed from September to November 2000. Data have been collected through household budget

surveys (HBS) and individual nutrition surveys (INS) based on single 24-h recall with the support of an album of colour photographs representing 201 food items, dishes, soft drinks and alcoholic beverages commonly consumed in Poland in three different portion sizes. Body weight have been measured using digital electronic personal weighing scales. Individuals were aged between 0 and 96 years. It is worth noticing that food eaten away from home was excluded from the analysis to facilitate the comparison between the two methodologies. The results obtained from the two survey methodologies showed poor agreement between purchasing and consumption of bread and rolls. Specifically, the acquisition was 24–30% greater than consumption (Szponar et al., 2001, 2003; Sekula et al., 2004). This shows that a proportion of purchased bread and rolls is likely to be wasted (Sekula et al., 2004). When intra-individual variation is small relative to interindividual variation, population intakes can be reliably estimated with consumption data from a smaller number of days than should be obtained if both types of variation are large (Petersen, 2000). Therefore, 24-h recall surveys can be considered quite accurate for the assessment of dietary exposure to contaminants present in commonly consumed products such as bread. For these reasons, the current risk assessment has been based on the data obtained with this survey. The results of this 24-h recall are present in the EFSA Comprehensive European Food Consumption Database (code: IZZ FAO 2000).

Acrylamide

Acrylamide (AA) is a low molecular weight, highly water soluble, organic compound. It forms in foods containing asparagine and reducing sugars prepared at temperatures above 120°C and low moisture. Therefore, it is known to be present in numerous baked or fried carbohydrate-rich foods, including French fries, potato crisps, breads, biscuits and coffee. This chemical has also been found in cigarette smoke. Different types of grains have shown different levels of asparagine. Typically, asparagine levels are the highest in rye and in descending order lower in oats, wheat, maize and with the lowest levels in rice (Reg. EC no 2158/2017). The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) listed 'Soft bread' among the main contributors to the total dietary exposure to AA of toddlers, other children, adolescents, adults, elderly and very elderly (EFSA, 2015). Moreover, scientists of the Polish National Food and Nutrition Institute – State Research Institute (NIZP PZH – PIB), identified bread as the main food source of AA in the Polish diet contributing to the 31% of the total dietary exposure to AA of 1–6 age children and to the 49% exposure of the adult population. According to the authors, this is due to the frequency and amount of consumption of this staple more than its contamination level (Mojska et al., 2010). Benchmark levels for the presence of AA in foodstuffs have been set in Regulation (EU) no 2158/2017. In case of soft bread other than wheat-based bread, the benchmark level is 100 μg/kg.

Occurrence

The levels of AA in the analytical samples were determined with the use of a gas chromatograph coupled to single quadrupole mass spectrometer. The analytical method has been implemented adding an internal standard solution to the analytical samples and using solid-phase extraction (SPE) cartridges to extract the analyte from the matrix. The method was based on an in-house validated procedure performed with few adjustments (Roszko et al., 2019).

Results show that the mean (40.73 μg/kg) is bigger than the median (38.10 μg/kg) and skewness is positive (1.10). Other values of interest are Standard Deviation (17.11 μg/kg) and maximum value (94.67 μg/kg). Considering those values, it is assumed that data follow a log-normal distribution. This is in line with what stated in literature: in the field of food contaminants, the log-normality of the distribution is generally assumed (Kroes et al., 2002). Since the log of the measurements is normally distributed, it is more accurate to work with the logarithms of the measurements (Miller and Miller, 2010) and calculate the geometric mean (37.60 μg/kg) and its 95% confidence interval (lower bound (LB) 33.58 μg/kg and upper bound (UB) 42.10 μg/kg).

Exposure assessment

Chronic exposure to AA was estimated for each population group by multiplying the 95th percentile consumption data (g/kg bw per day) with the upper level of the 95% confidence interval of the geometric mean (0.042 μg/g). Assuming that that bread contributes for 31% of the total dietary exposure to AA in 1–6 age children, for 40% in children and adolescents (7–8 years) and for 49% in adults from 19 to 96 (Mojska et al., 2010), the total dietary exposure to AA for each age group have been calculated.

Risk characterisation

The EFSA CONTAM Panel considered inappropriate to establish a tolerable daily intake (TDI) since AA is of concern with respect to genotoxicity. Therefore, the reference point for risk characterisation of the non-neoplastic effects of AA derived by the CONTAM Panel is the $BMDL_{10}$ value of 0.43 mg/kg bw per day and the one for risk characterisation of the neoplastic effects is $BMDL_{10}$ of 0.17 mg/kg bw per day. Both $BMDL_{10}$ values were selected based on experimental animal evidence since data from human studies were inadequate for dose–response assessment. Usually, for non-genotoxic compounds, a margin of exposure (MOE) of 100 is considered sufficient to conclude that there is no health concern. This MOE covers uncertainties and variability with regard to differences between experimental animals and humans (factor $4 \times 2.5 = 10$), and within the human population (factor $3.2 \times 3.2 = 10$). However, the CONTAM Panel considered appropriate to use a compound-specific adjustment factor for risk characterisation of the non-neoplastic effects of AA. This results in an adjusted MOE of 125. The CONTAM Panel deemed correct the use of the MOE approach for compounds that are both genotoxic and carcinogenic for risk characterisation of the neoplastic effects. In this case, an MOE of 10,000 or higher, based on a $BMDL_{10}$ from an animal study, would be of low concern from a public health point of view (EFSA, 2015).

MOE values for non-neoplastic and neoplastic effects related to the estimated total dietary AA were calculated for the 95th percentile exposure for each population group. Results show that MOEs for non-neoplastic effects across age groups are not of concern (MOE $\geq$ 125). Whereas, MOEs for neoplastic effects are of concern from a public health point of view across all age groups (MOE $<$ 10,000). It is worth noticing that MOEs for neoplastic effects across all age groups are substantially below the safe value even when considering only bread consumption. These results are in line with what stated by EFSA in the last Scientific Opinion on AA in food related to the European population (EFSA, 2015) and in a previous study on risk evaluation of AA intake with bread in Poland (Roszko et al., 2019).

Mycotoxins

Mycotoxins are secondary fungal metabolites, toxic to human and animals. Fungi producing mycotoxins can infect living plants in the field and/or colonise crops during storage. Deoxynivalenol (DON) and nivalenol (NIV) are produced by various species of *Fusarium* genus. Plants use detoxification mechanisms as a defensive strategy against pathogens. This process leads to the transformation of the chemical structure of mycotoxins into modified forms such as deoxynivalenol-3-glucoside (DON-3-glucoside) and nivalenol-3-glucoside (NIV-3-glucoside). Those transformed molecules are called masked mycotoxins (Berthiller et al., 2013; Ksieniewicz-Woźniak et al., 2019). All these compounds belong to the chemical group of type B trichothecenes. DON is chemically stable and to some extent resistant to thermal processing. As a result, it is found in cereal-based foods such as bread. EFSA assessed the risks to human and animal health related to the presence of NIV, DON and its modified forms in food and feed. In both cases, grains and grain-based foods were the main contributors as dietary sources to the exposure for humans (EFSA, 2013, 2017). The maximum level (ML) of DON in bread have been set to 500 μg/kg in Regulation (EC) No 1881/2006.

Occurrence

The analytical method used in this study was aimed at determining the co-occurrence of DON, DON-3-glucoside, NIV and NIV-3-glucoside. Considering their chemical similarities, the use of immunoaffinity columns containing antibodies specific both to DON and NIV facilitated the simultaneous extraction of all the compounds from the matrix. After extraction and clean-up steps, analytes were determined with high-performance liquid chromatography (HPLC) coupled to UV detector. The results of the analysis show that NIV, NIV-3-glucoside and DON-3-glucoside were not found and DON have been detected in 48 out of 51 loaves analysed. Descriptive statistics of DON's analytical data show that the mean (49.50 μg/kg) is bigger than the median (37.63 μg/kg) and skewness is positive (0.98). Other values of interest are Standard Deviation (26.84 μg/kg) and maximum value (109.11 μg/kg). Considering those values, it is assumed that data follow a log-normal distribution. In this case, it is more accurate to work with the logarithms of the measurements (Miller and Miller, 2010) and calculate the geometric mean (43.46 μg/kg) and its 95% confidence interval (LB 37.53 μg/kg and UB 50.32 μg/kg).

Exposure assessment

Chronic exposure to DON was estimated for each population group by multiplying the 95th percentile consumption data (g/kg bw per day) with the upper level of the 95% confidence interval of the geometric mean (0.050 μg/g). According to the EFSA opinion, the category 'bread and rolls' can be responsible for up to 50% of the total dietary exposure in the population groups from infants to adults and exceed 50% for elderly and very elderly (EFSA, 2017). In case of elderly and very elderly a contribution of 65% has been assumed. Based on these considerations and on the occurrence data obtained from the analysis, the total dietary chronic exposure to DON has been calculated for each population group.

Risk characterisation

The CONTAM Panel established a group-TDI of 1 μg/kg bw per day for the sum of DON and its modified forms. Moreover, a group acute reference dose (ARfD) of 8 μg/kg bw per eating occasion has been established based on human data on vomiting and gastrointestinal effects, collected in a number of epidemiological studies on the outbreaks of acute mycotoxicosis in Asia. According to the results of this work, the estimate of chronic dietary exposure to DON for other children is above the TDI set by EFSA and just slightly below it for toddlers and adolescents. This indicates that a potential health concern for those three population groups cannot be excluded.

Heavy metals

Maximum levels of heavy metals in food have been set in the European legislation. However, not all of them have been regulated. A summary is presented in Table 1.

Table 1: Maximum levels of heavy metals in food as per European legislation

Analyte	Food matrix	ML [mg/kg] (wet weight)	Regulation
Arsenic	Rice waffles, rice wafers, rice crackers and rice cakes	0.3	Commission Regulation (EU) 2015/1006 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of inorganic arsenic in foodstuff
Cadmium	Rye and barley	0.05	Commission Regulation (EU) 2021/1323 of 10 August 2021 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in certain foodstuffs
Lead	Cereals and pulses	0.2	Commission Regulation (EU) 2021/1317 of 9 August 2021 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs

Occurrence

The analytical determination has been carried out according the European standard EN 15763:2009 (*European Committee for Standardization*, 2009). The technique is based on inductively coupled plasma mass spectrometry (ICP-MS) after pressure digestion. The implementation of this multielemental method of high sensitivity makes possible to determine several trace elements simultaneously.

Results of analytical measurement show that the mean is bigger than the median and skewness is positive. Therefore, it is assumed that data follow a log-normal distribution. This is in line with what stated in literature: in the field of food contaminants, the log-normality of the distribution is generally assumed (Kroes et al., 2002). Since the log of the measurements is normally distributed, it is more accurate to work with the logarithms of the measurements (Miller and Miller, 2010) and calculate the geometric mean UB. The value of the geometric mean UB is below the ML for every metal analysed. Results are summarised in Table 2.

Table 2: Mean [μg/kg], median [μg/kg], standard deviation [μg/kg] and geometric mean UB [mg/kg] of heavy metals occurrence in rye-wheat bread

Analyte	Mean [μg/kg]	Median [μg/kg]	Standard deviation [μg/kg]	Geometric mean UB [mg/kg]
Aluminium	4054.00	3919.00	1251.00	4.11
Arsenic	5.62	5.10	2.39	0.01
Cadmium	13.93	13.21	3.60	0.01
Chromium	134.61	106.17	84.5	0.13
Lead	82.39	72.50	54.33	0.08
Nickel	260.82	234.39	101.97	0.26

UB: upper bound.

Exposure assessment and risk characterisation

Chronic exposure to heavy metals was estimated for each population group by multiplying the 95th percentile consumption data (g/kg bw per day or week) with the upper level of the 95% confidence interval of the geometric mean.

Aluminium (Al). Food additives are reported to be the greatest contributors to intake of aluminium from food, but other sources also contribute to the overall intake, e.g. aluminium naturally present in plant products and migration from food contact materials. Plants can take up aluminium from the soil and from water in which aluminium (the third most abundant element, constituting approximately 8% of the earth's crust) is present. Therefore, the relatively high occurrence levels found in bread can be due to the widespread environmental occurrence of aluminium. From studies in the UK and France, cereals and cereal products, vegetables, and beverages appeared to be the main contributors (> 10%) to the dietary aluminium exposure. As also assessed by EFSA, cereals and cereal products, vegetables, beverages and certain infant formulae appear to be the main contributors to the dietary aluminium exposure (EFSA, 2008). In view of the cumulative nature of aluminium in the organism, the EFSA Panel considered it more appropriate to establish a tolerable weekly intake (TWI) of 1 mg/kg bw per week rather than a TDI (EFSA, 2008). The estimated weekly dietary exposure to aluminium exceeded the TWI for all populations groups. This is in line with the results reported by the EFSA Panel in the Scientific Opinion on Safety of aluminium from dietary intake where the TWI is likely to be exceeded in a significant part of the European population.

Arsenic (As). The largest contributors to overall total arsenic exposure seem to be 'fish and seafood' and 'cereals and cereal products'. It should be noted that within the 'cereal and cereal products' food category, due to its high total arsenic amount, rice is one of the major contributors to the inorganic arsenic forms (EFSA, 2009a). The contribution (%) of rye-wheat bread consumed in Poland to the total As dietary exposure have been calculated considering the occurrence data obtained from the analysis, and the inorganic arsenic input from 'cereals and cereal products' and the total arsenic dietary exposure of 95th percentile in different population groups reported in the EFSA opinion. The CONTAM Panel considered appropriate to set a range of $BMDL_{01}$ values to be used in the assessment instead of a single reference point (0.3–8 μg/kg bw per day) (EFSA, 2009a). The estimated total dietary exposures to arsenic for toddlers and other children are within the range of the $BMDL_{01}$ values identified by the CONTAM Panel for lung and bladder cancer and for dermal lesions. Therefore, the possibility of a risk to these population groups cannot be excluded. However, this does not necessarily indicate that children are at greater risk because the effects are due to long term exposure (EFSA, 2009a). For the others, the estimated dietary exposures are below 0.3 μg/kg bw per day. Therefore, the risk can be considered low for these population groups.

Cadmium (Cd). Cadmium absorption after dietary exposure in humans is relatively low (3–5%) but it is efficiently retained in the kidney and liver, with a very long biological half-life ranging from 10 to 30 years. Cadmium is primarily toxic to the kidney, especially to the proximal tubular cells where it accumulates over time and may cause renal dysfunction. The CONTAM Panel identified the beta-2-microglobulin (B2M), a low molecular weight protein, as the most useful biomarker in relation to tubular effects. The food group that contributes to the major part of the dietary cadmium exposure, primarily because of the high consumption, is 'cereals and cereal products' (EFSA, 2009b). The total dietary Cd exposure has been calculated based on the data reported in EFSA Opinion and the occurrence data obtained from the analysis. Calculations have been based on the estimated average consumer exposure to cadmium by different food groups. It must be noticed that data on the

estimated average consumer exposure to cadmium by different food groups reported in EFSA Opinion refers to the adult population. Therefore, calculations for toddlers and other children encompass a higher degree of uncertainty. The CONTAM Panel identified a benchmark dose lower confidence limit for a 5% increase of the prevalence of elevated B2M ($BMDL_{05}$) of 1.0 μg Cd/g creatinine. In order to remain below 1 μg Cd/g creatinine in urine in 95% of the population by age 50, the average daily dietary cadmium intake should not exceed 0.36 μg Cd/kg bw, corresponding to a weekly dietary intake of 2.52 μg Cd/kg bw (TWI). The dietary exposure to cadmium exceeds the TWI set by the EFSA Panel for the groups toddlers and other children. This might be primarily due to the greater amount of food consumed in relation to body weight.

Chromium (Cr). The EFSA CONTAM Panel decided to consider all the reported analytical results in food as Cr(III). In infants and toddlers the main contributors to the chronic exposure to Cr(III) were 'foods for infants and small children', followed by 'milk and dairy products' and 'bread and rolls'. In the other age classes, the main contributors to the chronic exposure to Cr(III) were the food categories 'milk and dairy products' and 'bread and rolls'. The total dietary Cd exposure has been calculated based on the data related to the contribution of the 'bread and rolls' category to the total dietary Cr exposure reported in EFSA Opinion for each population group (EFSA, 2014) and on the occurrence data obtained from the analysis. The CONTAM Panel derived a TDI of 300 μg Cr(III)/kg bw per day. Dietary exposure across all age groups are well below the TDI. Therefore, data indicates that dietary exposure to Cr(III) does not raise concern from a public health point of view.

Lead (Pb). Cereal products contribute most to dietary lead exposure. The total dietary exposure to Pb have been calculated considering the contribution of bread consumption to total dietary Pb exposure (%) corresponding to Poland reported in the EFSA Opinion on lead in food (EFSA, 2010) and the occurrence data obtained from the analysis. It must be noticed that the value of the relative contribution reported in the EFSA Opinion refers to the broad category 'Cereals and cereal products' and to the general population. Therefore, this information carries a quite high level of uncertainty. The CONTAM Panel identified developmental neurotoxicity in young children and cardiovascular effects and nephrotoxicity in adults as potential critical adverse effects of lead on which to base the risk assessment. A $BMDL_{01}$ intake level of 0.50 μg/kg bw per day for neurodevelopmental effects have been established for children. Whereas, BMDL dietary lead intake values in adults of 1.50 μg/kg bw per day and 0.63 μg/kg bw per day were derived for the cardiovascular and kidney effects, respectively. As there was no evidence for a threshold for a number of critical endpoints, the CONTAM Panel does consider it appropriate to calculate margins of exposure to support the risk characterisation. An MOE of 10 or greater would be sufficient to ensure that there was no appreciable risk of a clinically significant effect on neurodevelopmental, cardiovascular and kidney effects. Even at MOEs of greater than 1.0 the risk would be very low (EFSA, 2010). In all cases, the MOEs estimated in this work are below 1. Therefore, there is indication of health concern. This reflects the relatively high levels of Pb founded in the bread (geometric mean UB: 0.076 mg/kg). This is higher than the mean UB of Pb in 'cereals and cereal products' reported in the EFSA Opinion (0.044 mg/kg). However, the ML set for 'cereals and pulses' (0.2 mg/kg) has not been exceeded.

Nickel (Ni). According to the EFSA Opinion on Nickel in food and drinking water the food categories mainly contributing to the mean LB chronic dietary exposure to nickel across all age classes is 'grains and grain-based products' with contributions reaching up to 49% in infants and toddlers. 'Bread and rolls' has the highest contribution among the food subcategories belonging to 'grains and grain-based products'. The mean nickel concentration levels for 'bread and rolls' are not particularly high; therefore, it is likely that their high contribution is driven by high consumption rather than the presence of nickel. The total dietary Ni exposure has been calculated based on the data related to the contribution of the 'bread and rolls' category to the total dietary Ni exposure reported in EFSA Opinion on Nickel in food and drinking water for each population group (EFSA, 2020) and on the occurrence data obtained from the analysis. An increased incidence of post-implantation loss in rats was identified as the critical effect for the risk characterisation of chronic oral exposure and a $BMDL_{10}$ of 1.3 mg Ni/kg body weight per day was selected as the reference point for the establishment of a TDI of 13 μg/kg bw (EFSA, 2020). Mean chronic dietary exposure was the highest for toddlers. In this case the TDI is exceeded and it might indicate a concern.

3. Conclusions

3.1. The work programme

Results of the occurrence investigation work showed that rye-wheat bread marketed in Poland has generally low levels of the chemical contaminants analysed. However, if the total dietary exposure to those contaminants is derived as described in the report, a public health concern cannot be excluded in some cases. A summary of the situations of higher risk is presented in Table 3.

Table 3: Chemical contaminants rising a public health concern

Contaminant	Public health concern
Acrylamide	MOEs for neoplastic effects are far below the safe level across all age groups.
Deoxynivalenol	The estimate of chronic dietary exposure to DON for other children is above the TDI and just slightly below it for toddlers and adolescents.
Aluminium	The estimated weekly dietary exposure to aluminium exceeded the TWI for all populations groups.
Arsenic	The estimated dietary exposures to arsenic for toddlers and other children are within the range of the $BMDL_{01}$ values.
Cadmium	The dietary exposure to cadmium exceeds the TWI for the groups toddlers and other children.
Lead	The MOEs are below the safe level across all population groups.
Nickel	The estimated dietary exposure to nickel exceeds the TDI for toddlers.

The exposure to AA and aluminium is of concern for all population groups. This is in line with what calculated by EFSA Panels (EFSA, 2008, 2015). The calculated exposure to lead rises concerns across all the population groups too. However, in this case results differs from what stated by the Panel in the Opinion (EFSA, 2010). The calculated high level of exposure is due to the relatively high concentration of Pb founded in the analysed bread in comparison with literature. In the other cases (deoxynivalenol, arsenic, cadmium and nickel), the most vulnerable groups are mainly toddlers and other children. This might be primarily due to the greater amount of food consumed in relation to body weight.

It is important to stress that the outcomes of scientific risk assessments should be evaluated along with uncertainties (EFSA, 2018b). On this regard, please see Appendix A.

It must be noticed that the type of bread analysed was made with refined flour. Health recommendations and dietary trends promote the consumption of wholemeal cereals. Since the outer hull of the grain has a higher concentration of contaminants such as AA (EFSA, 2015) and mycotoxins (Bergamini et al., 2010; EFSA, 2017), it might be valuable to assess the exposure of high consumers of wholemeal products.

Moreover, although the levels of the contaminants investigated in this study are below the MLs, it might be interesting to investigate eventual health consequences due to the cooccurrence of multiple chemicals in the same food matrix.

3.2. EU-FORA

All in all, EU-FORA is a key opportunity for early-mid career food specialists and scientists. It fosters professional development through a comprehensive and multidisciplinary hands-on experience in food safety risk assessment. Among the other benefits, it facilitates international collaborations and active involvement in the network of professionals in the area of food safety. The new skills and knowledge contribute to the development of solid scientific bases to better understand current and emerging food-related issues. The gained expertise can be an asset in academia, public health bodies and food companies. Details regarding additional activities performed during the programme are reported in Appendix B.

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Abbreviations

AA	acrylamide
ARfD	acute reference dose
B2M	beta-2-microglobulin
BMDL	benchmark dose lower confidence limit; 95%-confidence lower bound
$BMDL_{01}$	95% lower confidence limit for the benchmark dose response of 1%
$BMDL_{05}$	95% lower confidence limit for the benchmark dose response of 5%
$BMDL_{10}$	95% lower confidence limit for the benchmark dose response of 10%
BVL	Federal Office of Consumer Protection and Food Safety
bw	body weight
CEN	European Committee for Standardization
CONTAM Panel	Panel on Contaminants in the Food Chain
DON	DEOXYNIVALENOL
DON-3-glucoside	Deoxynivalenol-3-glucoside
EU-FORA	European food risk assessment
FAO	Food and Agriculture Organization
HBS	household budget survey
HPLC	igh-performance liquid chromatography
IAFP	International Association for Food Protection
IBPRS -PIB	Prof. Wacław Dąbrowski Institute of Agriculture and Food Biotechnology
ICP-MS	inductively coupled plasma mass spectrometry
INS	individual nutrition survey
LB	lower bound
ML	maximum level
MOE	margin of exposure
MRI	Max rubner-institut
MTB	methyl *tert*-butyl ether
NIV	nivalenol
NIV-3-glucoside	nivalenol-3-glucoside
NIZP PZH – PIB	National Food and Nutrition Institute – State Research Institute
RAFA	Recent Advances in Food Analysis
SD	standard deviation
SPE	solid-phase extraction
TDI	tolerable daily intake
TWI	tolerable weekly intake
UB	upper bound

Appendix A – Uncertainties

Uncertainty analysis is the process of identifying limitations in scientific knowledge and evaluating their implications for scientific conclusions. In other words, uncertainty is used as a general term referring to all types of limitations in available knowledge that affect the range and probability of possible answers to an assessment question (EFSA, 2018a). The evaluation of the inherent uncertainties in the assessment has been mainly performed following the EFSA guidance related to Uncertainties in dietary exposure assessment and the guidance on uncertainty analysis in scientific assessments (EFSA, 2006, 2018a). Moreover, it is necessary to consider the uncertainties expressed by the EFSA Panels in the Scientific Opinions on each contaminant.

An attempt was made to provide a summary of the most important uncertainties and the relative magnitude and direction of their influence on the assessment output (Table A.1). The estimate of whether the source of uncertainty might have led to an over- or underestimation of the dietary exposure or the resulting risk has been performed with plus and/or minus signs. Plus signs for an uncertainty indicate that it could have caused small (+), medium (++) or large (+++) over-estimation, minus signs that it could have caused small (−), medium (− −) or large (− − −) under-estimation. Some uncertainties are evaluated as potentially causing either over- or under-estimation (e.g. +/−).

Table A.1: Summary of the most important uncertainties and the relative magnitude and direction of their influence on the assessment output

	Source of uncertainty	Direction and magnitude
Sample		
Sampling strategy	Bread have been collected only in supermarkets	−/+
Exposure assessment		
Food category	The food matrix analysed was 'rye-wheat bread and rolls, refined flour'. However, the consumption data present in the survey used as reference and in the EFSA Comprehensive European Food Consumption Database for Poland refer to the category 'bread and similar products'	+
24-h recall dietary survey	Some misreporting of consumption by individuals also occur (Kroes et al., 2002; Sekula et al., 2004)	−
	Food eaten away from home was excluded from the analysis (Sekula et al., 2004)	−
	Food consumption estimates are likely to be mutually dependent when subjects from the same household are interviewed, thus leading to a reduced variability in terms of dietary pattern observed (Food and Authority, 2011)	−/+
Acrylamide		
Occurrence	Bread have been collected only in supermarkets	−
	Analytical analysis has been performed on bread after purchase. Therefore, home-cooking habits (e.g. toasting) have not been considered	−
Exposure assessment	The subdivision of the population in age groups performed by the Polish National Food and Nutrition Institute (Mojska et al., 2010) does not perfectly correspond to the one commonly used by EFSA	−/+
Mycotoxins		
Sampling strategy	Bread have been collected in a single sampling occasion. Mycotoxins level can vary between seasons and different years (Kroes et al., 2002; Bryła et al., 2018; Bryla et al., 2019; Ksieniewicz-Woźniak et al., 2019)	−−/++
Heavy metals		
	The total dietary intake of metals for the Polish population was calculated assuming that the contribution of bread consumption (%) to the total dietary intake (%) is the same as for the general European population.	−
	The food matrix analysed was 'rye-wheat bread and rolls, refined flour'. However, the data present in the EFSA's Opinions often refer to broader food categories.	−/+

Overall, the impact of the identified uncertainties is deemed moderate. In addition, it is important to notice that the impact of uncertainty elements can differ when considering different population groups.

Appendix B – Additional activities

Besides the work carried out at the hosting site, the participation to international events, conferences and trainings fostered the learning process and offered the stimulating opportunity get cutting-edge insights on the latest developments and techniques in food science and safety, meet international experts working in industry, government, and academia and have a glimpse of the pivotal issues currently under the spot light of EFSA and the scientific community. Special mention deserves the participation as a speaker at the European Symposium on Food Safety 2022 organised by the International Association for Food Protection (IAFP) presenting in the symposium 'New Hazards and Old Threats; Foodborne Viruses and Risk Assessment in Food Safety' with the collaboration of other EU-FORA colleagues (Kevin Hunt, Monika Trzaskowska and Constantine Richard Stefanou). Relevant event and conferences have been listed in Table B.1.

Table B.1: Relevant events and conferences attended during the fellowship

Event/Conference	Date	Avenue
EFSA International Workshop on Risk Assessment of Combined Exposure to Multiple Chemicals	18, 19, 20 October 2021	Virtual
RAFA 2021 – Recent Advances in Food Analysis	3–4 November 2021	Virtual
EFSA Scientific Colloquium 26 on risk–benefit assessment of combined exposure to nutrients and contaminants through food	15, 16, 17 February 2022	Virtual
International Training for Safer Food - 4th Federal Office of Consumer Protection and Food Safety (BVL)/Max Rubner-Institut (MRI) Course on Food Safety, Food Authenticity and Risk Management	14, 15, 16 March 2022	Virtual
International Association for Food Protection (IAFP) – European Symposium on Food Safety	4, 5, 6 May 2022	Munich, Germany

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Risk assessment of honey bee stressors based on *in silico* analysis of molecular interactions

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Abstract

A global decline of the honey bee *Apis mellifera* has been observed in the last decades. This pollinator plays a fundamental role in food production and the economy in Europe. The decline of honey bee colonies is linked to several stressors, including pesticides. The current pesticide risk assessment of honey bees in Europe focuses on lethal effects and lacks reflection on sublethal effects. A better understanding of the consequences that exposure to these chemicals has on honey bees is still needed. In this context, the aim of this European Food Risk Assessment Fellowship Programme fellowship project has been to use *in silico* methodologies, such as virtual screening, as a first step to identify possible interactions at the molecular level between *A. mellifera* proteins and pesticide ligands. For this purpose, a docking study of the proteins from *A. mellifera* and pesticide ligands extracted from online databases has been performed by using the software Autodock Vina. The results obtained were a ranking based on the predicted affinity of the pesticides for specific and non-specific binding sites on bee macromolecules. These results were compared with data obtained from the literature and linked to potential sublethal effects. Finally, a risk assessment analysis of the identified molecular stressors of honey bees was performed. The results of this study are considered a starting point to identify new sources of possible stress for honey bees and thereby contribute to the overall understanding of the honey bee decline.

Keywords: honey bee, *Apis mellifera*, pesticide, risk assessment, virtual screening, docking, NAMs

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1. Introduction

1.1. European Food Risk Assessment Fellowship Programme

The present work is part of the European Food Risk Assessment Fellowship Programme (EU-FORA). This programme is an initiative of the European Food Safety Authority (EFSA) created to provide practical training to scientists, increasing their knowledge in food safety risk assessment and knowledge community. The fellow was hosted by the Institute of Biochemistry and Cell Biology at the Italian National Research Council (CNR). The project focused on the use of the *in silico* approach using docking analyses to identify possible adverse interactions between *Apis mellifera* proteins and pesticide ligands. This approach could enhance the protection of honey bees in Europe.

1.2. Virtual docking in risk assessment of *Apis mellifera*

There is an observed global decline of the honey bee *A. mellifera* (Di Noi et al., 2021). This pollinator plays a fundamental socio-economic role in food production because of its pollination services and bee products such as honey (Gallai et al., 2009). The use of pesticides is one of the main stressors linked to the observed decline of this species (Sanchez-Bayo and Goka, 2014; Chmiel et al., 2020). In the European Union (EU), there are currently 1,461 active substances registered, of which 454 are approved (EU Pesticides Database, 2021). In addition, non-approved active substances, such as the neonicotinoid imidacloprid, are still used under exceptional circumstances laid in Article 53 Emergency authorisation of Regulation (EC) No 1107/2009.

The current bee risk assessment of pesticides in Europe for approval of pesticides focuses on lethal effects (Sgolastra et al., 2020). However, sublethal endpoints such as effects in immunity, behaviour, or sensor ability can affect honey bee performance and reduce populations. Studies aiming to describe the non-lethal effects of pesticides in *A. mellifera* are available in the literature (Chmiel et al., 2020; Di Noi et al., 2021). Nevertheless, most of these studies are performed with insecticides, mainly from the neonicotinoid family. Other pesticide classes, such as fungicides and herbicides, are not well represented and evidence indicates that they could also affect the honey bee's health. In addition, certain types of effects particularly within enzymatic and molecular responses are not well characterised (Di Noi et al., 2021). Thus, a better understanding of the consequences that exposure to pesticides has on honey bees is still needed, but this is a challenging task considering the number of available pesticides and possible endpoints. A quick and cost-effective screening assay that identifies chemicals and pathways of concern could be beneficial.

Virtual screening is commonly used in drug discovery to predict the binding free energy of libraries of small molecules to a target protein structure and the bound conformation (Forli et al., 2016; Naqvi et al., 2018). This technique could also be a promising tool for chemical toxicity screening at a molecular level (Goldsmith et al., 2014). Proteins are essential components of living organisms with different functions such as enzymes, signalling components and transport/storage constituents. They usually bind to other molecules (ligands) with high specificity and affinity to perform their respective task. The binding is determined by the physicochemical properties of the amino acid residues and the shape of the binding pocket. Alteration in the protein–ligand interaction can affect protein functions and molecular pathways which could ultimately be linked to sublethal effects such as immunity dysfunctions or communication (Eder et al., 2007; Li, 2007). Currently, dozens of 3D structures of proteins from *A. mellifera* are present in online databases including odorant-binding proteins, pheromone binding proteins and proteins from the immune system. The objective of this fellowship was to verify if these proteins could be affected by toxic compounds, such as pesticides, using virtual screening and docking approaches. Furthermore, the *in silico* results were compared with literature data.

2. Description of work programme

2.1. Aims

The main aim of this project was to investigate *in silico* interactions of bee proteins and pesticides to produce a risk assessment analysis that can enhance the protection of honey bees in Europe. To achieve this goal, the fellow prepared data sets of proteins from *A. mellifera* and pesticides for the docking analysis. Subsequently to the virtual screening analysis, the data were processed to obtain a ranking of affinity and possible interference of pesticides in the protein functions and organism

pathways. These results were compared with data obtained from the literature. The last activity of the present work was dedicated to the risk assessment analysis of the effects of pesticide stressors on honey bees.

2.2. Activities/methods

2.2.1. Proteins and ligands selection and preparation

All the different steps performed in this section are summarised in Figure 1. In the first part of the program, the fellow accomplished a search in databases of protein 3D structures (RCSB PDB) from *A. mellifera*. Once identified, the structure files were retrieved and stored in a pdb format. The pesticide molecules were selected based on the list of approved active substances from the EU pesticides database. Subsequently, the 3D structures were downloaded from PubChem. If the 3D structure was disallowed (e.g. mixtures, salts or too large compounds), the pesticide was not included in the final ligand data set. In addition, ligands were classified by pesticide group and mode of action (MoA). The protein and ligand structures were optimised and converted to pdbqt format using the program MGLTools (http://mgltools.scripps.edu/). This step was essential as the program used for docking, Autodock Vina (http://autodock.scripps.edu/), requires as inputs molecular pdbqt files. The preparation included removing water molecules and any residual non-standard ligands and adding polar hydrogen atoms and Gasteiger charges. Finally, grid boxes with a spacing of 1 Å were created to scan the protein surface. The boxes could incorporate the entire protein, in the case of blind docking or only the binding pocket in the case of a more targeted docking (see Figure 2).

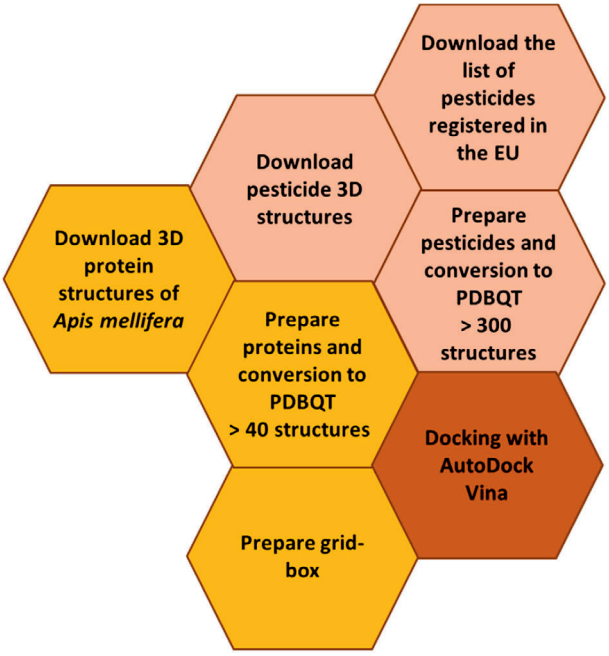

Figure 1: Different preparation steps followed to perform the virtual screening. The molecular docking analysis was performed with Autodock vina. The program requires as inputs a pdbqt molecular files of the 3D structure of the protein and the ligand as well as a grid-box to scan the protein surface. The analysis returns an affinity score (kcal/mol) of the protein-ligand affinity

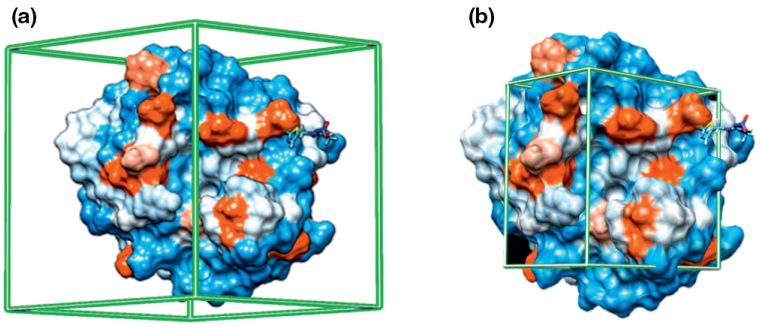

Figure 2: Preparation example of the grid-box, which defines the space around the protein used to perform the docking analysis. The box can incorporate the entire protein (a) or just the binding pocket (b)

2.2.2. Virtual docking and data processing

The fellow performed the molecular docking analysis using AutoDock Vina (Trott and Olson, 2009). The exhaustiveness was set to 16 and the maximum number of simultaneous threads was set to 9. The analysis was performed with an I7 Intel workstation equipped with 32 GB RAM, 2 SATA HD of 2 TB each for data storage, 1 SSD with the operative system (linux OS debian based), a dual monitor, online connection with the server for remote control. Shell and python scripts were developed to automatise some steps. The best affinity scores of each protein–ligand complex were examined with a hierarchical heatmap. Good affinity scores were considered for interactions ≤ -6 kcal/mol. Visual inspection of the complex and predicted possess was performed with Pymol, Charmm-GUI and MGLTools.

2.2.3. Literature search

A more in-depth bibliographic search on the principal bibliographic sources, such as PubMed or Web of Science, was performed to collect as much as possible data on molecular stressors for honey bees. The fellow compared the data obtained from literature with the data set obtained in the *in silico* analysis to identify a new source of possible stress for honey bees.

2.2.4. Risk assessment analysis

The final part of the work programme concerned the risk assessment analysis of the identified molecular stressors in the investigated area of honey bees' increased mortality. The fellow identified the molecular hazards which could negatively impact relevant honey bees' pathways and ultimately jeopardise their survival. These results were compared with the results obtained from the literature.

3. Conclusions

3.1. Overall conclusion

In the present work, *in silico* results obtained from the virtual docking analysis were compared with information collected from the literature.

In total, more than 12,000 protein–ligand pairs, corresponding to > 300 pesticides and > 40 protein structures analysed, were evaluated. In Figure 3, a hierarchical heatmap of the affinity scores obtained from a screening search was reported. The highest affinity score was −12.5 kcal/mol and the lowest −0.6. Good affinity scores were considered for interactions ≤ -6 kcal/mol. According to the heatmap, the pesticide structures can be clustered into three main groups. The first group indicated that most of the tested pesticides do not target honey bee protein (binding affinity ≥ -6 kcal/mol) or only interact with a few of them such as pheromone-binding proteins. The second group revealed the multi-target behaviour of a large number of pesticides that exhibited good affinity scores (≤ -6 kcal/mol). Interestingly, not only insecticides but also herbicides and fungicides were among the pesticides with the highest affinities. This result suggests that herbicides and fungicides can also affect honey bees. The last cluster belongs to a smaller group of pesticides that did not interact with any of the protein structures.

The results from the literature search showed that pesticides affect cognition, immunity and reproduction among other sublethal effects. Effects on proteins could be linked to some of these responses, for instance, Li and collaborators suggested that olfactory recognition could be affected as imidacloprid decreases the binding affinity of odorant-binding proteins to floral volatiles (Li et al., 2015). Moreover, neonicotinoids can affect the Toll and Imd immune pathways, which are essential in triggering the innate immune response in insects (Di Prisco et al., 2013; Chmiel et al., 2019). In accordance with literature data, we found an interaction between some proteins involved in the innate immune response with some pesticides including neonicotinoids.

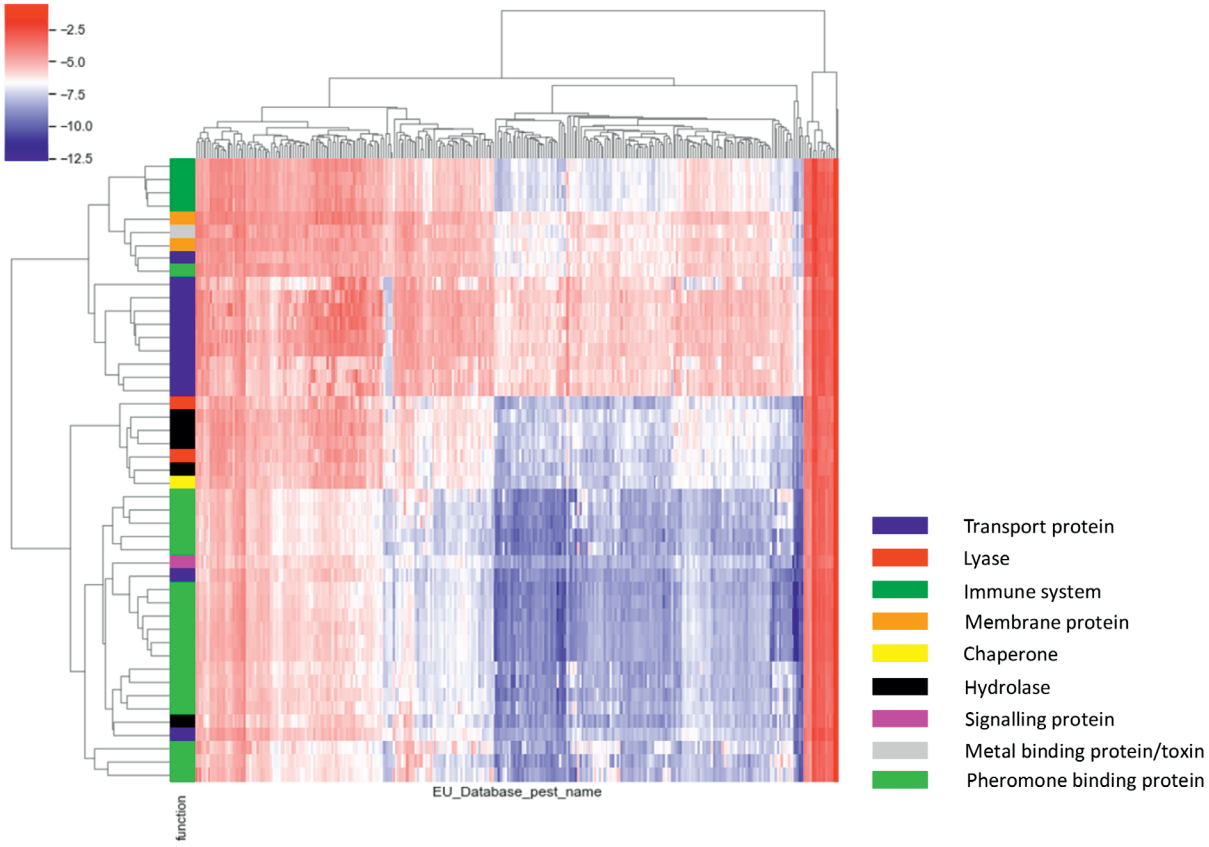

Figure 3: Hierarchical heatmap of the molecular docking affinity scores obtained in the virtual docking. The right and upper dendrogram correspond to the pesticide and protein molecules respectively. *Apis mellifera* protein structures are coloured by functions. The protein–ligand pairs with the strongest binding affinities are represented in blue and the ones with lower affinity in red

In conclusion, this research contributes to understanding how certain pesticides could interact with honey bee proteins with a high theoretical affinity. Some studies have linked sublethal exposure to pesticides with adverse effects on honey bees. However, these studies have mostly been performed on insecticides (mainly neonicotinoids). Other types of pesticides are underrepresented. Furthermore, a recent review by Chmiel et al. (2020) also highlighted that knowledge gaps exist in certain responses, particularly within enzymatic and molecular levels, such as those regarding the immune system and genotoxicity. Therefore, the protein–ligand interactions presented in this study could be used as guidance for the experimental testing of pesticides and in this way contribute to the overall protection of honey bees in Europe.

3.2. Additional scientific activities

During the EU-FORA fellowship programme, the fellow was involved in extracurricular activities. These included the participation in the SETAC Europe 32nd Annual Meeting Conference held 15–19 May 2022 in Copenhagen. The fellow had the opportunity to expose a poster titled 'Risk assessment of honey bee stressors based on *in silico* analysis of molecular interactions' at the SETAC Europe 32nd Annual Meeting

Conference 15–19 May 2022 in the Bella Center Copenhagen. Moreover, the fellow attended the ONE – Health, Environment, Society – Conference, 21–24 June 2022. In addition, the fellow attended a meeting organised by the Italian focal point with researchers working on bees and other pollinators. Finally, the fellow collaborated with the university group at the Department of Science and Technology University of Naples Parthenope (Italy). Currently, two students of this university are performing their bachelor thesis on informatics about this project. The fellow had the opportunity to help define the objectives of the thesis and to supervise the students. The writing of two manuscripts is in progress.

3.3. Disclaimer

Detailed results obtained from the method development, results and risk assessment are not included in this report to avoid certain copyright claims, as these results will be subsequently published in other scientific journals.

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Abbreviations

CNR	National Research Council
EU-FORA	European Food Risk Assessment Fellowship Programme
MoA	mode of action
SETAC	Society of Environmental Toxicology and Chemistry

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Emerging risk identification by applying data analytical tools

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Abstract

The working programme 'Emerging risk identification by applying data analytical tools' was delivered by the Digital Food Chain Education, Research, Development, and Innovation Institute (Digital Food Institute, DFI) on the field of emerging risks at the University of Veterinary Medicine Budapest, Hungary. The Institute is the University's research and education unit that provides data analysis and research along the whole food chain and takes networking in this area to a new level. The Fellow joined the hub of experts and researchers in the field of food chain safety data analysis, responsible for protecting public health concerning food in Hungary. The programme consisted of several different activities to provide an overview of the different tools that can be employed in the emerging risk identification process and prepare various stakeholders for new food chain safety issues. The programme was split into four modules to run over the one-year fellowship covering different areas of data analysis and emerging risk identification. The aim was to be fully integrated with the organisation's work experience, increase knowledge of scientific aspects relevant in the field of data analysis and visualisation tools in the emerging risk identification area, and implement the results into various EU stakeholders' environments assessments.

Keywords: data analysis, visualisation tool, emerging risk identification, text mining

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1. Introduction

The working programme 'Emerging risk identification by applying data analytical tools' was proposed for the EUFORA fellowship programme by the Digital Food Institute. DFI is a centre for risk assessment, data analysis, and research, and provides practical support and development of digitalisation possibilities to the actors of the agricultural and food economy, and ensures complex veterinary training based on digital technology with a food chain approach. As such, the Institute is actively involved in the promotion of digitalisation efforts in Hungary, including the Digital Success Programme (DSP) and the Digital Food Strategy (DFS). The main objective of the DFS is to promote the digitalisation potential of data in the food industry, thereby helping the sector to operate more efficiently. This requires the processing of the large volumes of data available in the food industry, the use of artificial intelligence, and the development of human resources. The Institute also works closely with the Hungarian National Food Chain Safety Authority (NÉBIH) in several areas, including the identification of emerging risks. The most important foreign partner of the Institute is the European Food Safety Authority (EFSA). DFI staff represent Hungary in several working groups and networks of EFSA. The Fellow was part of the DFI group throughout the fellowship. She collaborated with the staff as well as attended several events such as presentations, training courses, conferences, EFSA meetings and project-related meetings. The working programme covered different data analysis tools that can be applied in a wider range of food safety risk assessment areas and were organised into four different modules.

2. Description of work programme

The EU-FORA work programme was comprised of four modules. The fellow had the opportunity to implement the emerging risk identification (ERI) results in various stakeholders' environments. She had an overview of the different data analysis tools that can be applied also in a wider range of food safety risk assessment areas, to acquire skills and competencies that can be used in her future career. The fellow worked within our expert teams attending Teams and Unit meetings, as well as all associated social events. In addition, as an integral member of the EFSA, the fellow followed a wide range of continuous internal risk assessment training programmes, workshops, presentations and webinars.

2.1. Aims

The working programme prepared for the fellow covered different areas of data analysis and ERI. The main goals of the fellowship project were:

- Learning best practices on data analysis principles (including transparency, validation and documentation).
- Getting insight into tasks of the ERI, the various procedures and possible outcomes.
- Getting familiar with software tools for data mining and data visualisation (Knime, InfraNodus).

2.2. Activities/Methods

Module 1: **Application of data science in food safety: data science basics, overview of data science tools**

Under the first module, the fellow got an overview of data science and data science tools in food science, including data collection, preparation, modelling (linear and non-linear methods, network analysis, etc.), limitations and interpretation. The fellow learned how to see food chain safety problems with a data analytical-focused perspective, define the problem, structure it, and select and apply the possible and appropriate data analytical tools to solve them. She participated in the 'Application possibilities of data analysis in the field of food safety' course organised by the Digital Food Institute. The course focused on the evaluation of the safety of the food chain, risk assessment, and in many cases risk management and computation-intensive analyses and methods. The general objective was to acquaint the main computational methods applied in the field of food chain safety, their basics, application possibilities and limitations.

Module 2: **Emerging Risk Identification: basic concept, procedures, overview of ERI tools**

In the second module, the fellow got an overview of the concept of ERI workflow, the various procedures and possible outcomes (Figure 1). An emerging risk is a risk resulting from a newly identified hazard to which a significant exposure may occur, or from an unexpected new or increased

significant exposure and/or susceptibility to a known hazard (EFSA, 2018). The successful identification of emerging risks is at the heart of protecting public health and the environment. By identifying emerging risks in the food chain early, risks are anticipated and allow to take effective and timely prevention measures to protect consumers, animals, plants and the environment. Identifying emerging risks also helps to meet future risk assessment challenges, for example by mining new sources of data, developing new analytical tools and methods, and broadening networks of scientific knowledge. A large number of data (e.g. scientific literature, news, patents, etc.) is arising continuously, and it is a real challenge to derive meaningful information from this huge noise regarding emerging risks. Early and prompt identification of emerging risks enables better preparedness and policymaking in the food safety decision-making system. The fellow was deeply involved in the weekly identification of potential emerging risks through the help of data analysis tools for screening and prioritising large data sets to capture dependent changes in the food system. By analysing graphs and topics, she identified potential emerging issues. Then, a multi-step selection procedure was conducted by the fellow and our expert group to select the emerging risks that need further action to mitigate the adverse effects. In particular, the criteria considered are novelty, soundness, imminence and scale based on limited data and expert knowledge with high levels of uncertainty and low reproducibility. Additional information regarding the nature of the hazard identified, or associated drivers and trends are also included. Important objectives for the procedure for identification of emerging risks are to raise awareness of risk managers for emerging risks and improve preparedness for risk assessors. Indeed, the emerging risk that needed further measures were selected and forwarded to the relevant stakeholders such as the industry, the authorities and the research community, depending on the issues and depending on the risks themselves identified. In particular, the Fellow identified more than 100 potential issues (Table 1), from which more than 70 were uploaded to our management tool that helps us in tracking and monitoring emerging issues. She was also involved in preparing the whole documentation for the identified emerging issues and risks to be published on our DFI website.

Module 3: **Data analytic and visualisation methods for ERI: theory, method development and practical application**

In the third module, the fellow acquired knowledge on the use of data science tools in ERI. She got useful experiences in data analytic and visualisation methods, to show their results to various stakeholders. Indeed, in our Emerging Risk Identification system, we retrieved food safety news collected by Europe Media Monitor, which is a freely accessible news database with an RSS feed that can be used as an input for data analysis purposes (Meijer et al., 2020). Then, a text mining application software such as Knime or InfraNodus is used. The steps of data preparation and analysis which help us to find clusters of words and/or topics are:

- *Data retrieval*: The news feed is downloaded, parsed, and transformed into documents through the node RSS feed reader.
- *Text preprocessing* (e.g. stop word filtering and punctuation erasure): Preprocessing component uses extremely fast text processing to remove/filter specific types of characters from a string column.
- *Creating bag of words*: This node creates a bag of words which consisting of one column containing the terms occurring in the corresponding document and copied to the output table.
- *Co-occurrence analysis*: The node counts the number of co-occurrences for the given list of terms within the selected parts, e.g. sentence, paragraph, section and title of the corresponding document.
- *Network analysis with clustering algorithms*: This component counts incoming/outgoing edges and the edge with the highest value is the most central node. The clustering algorithm includes the neighbourhood's analysis of the node and the group's identification in the data.

The resulted graph (Figure 2) is based on the co-occurrence network of words and identifies different topics with the Latent Dirichlet Allocation algorithm. By analysing the graph and selecting topics (Figure 3), potential emerging issues can be identified. The fellow was involved in the entire emerging risk identification process, where she assisted the process to derive meaningful information from the huge noise regarding emerging risks. Communication to stakeholders involved the collaboration with the EFSA Emerging Risk Exchange Network (EREN), a very successful network for the sharing, analysis, and dissemination of emerging issues via a strong and committed membership including EFSA and the other Member States. The fellow attended the 26th and 27th EREN meetings. She was also involved in developing preparatory materials (short issues, briefing notes and presentations). One of the outcomes of her daily work of identification and analysis of news and data

brought to the development of a briefing note for the 27th EREN meeting. Indeed, she found a relevant article about Palmitic acid that stimulates metastasis in a long-term stable manner (Pascual et al., 2021). She prepared the briefing note to be presented to EFSA and Member states. After further analysis and evaluation, the topic was evaluated as an emerging issue because new sound evidence was found, and it was proposed to initiate a new risk assessment. The fellow presented her results also at the Agrostat 2022 Conference in Lyon, France, as a poster titled 'Applying a text mining software for emerging risk identification in the food chain'.[1]

Module 4: **Practical applications: early warning systems in various stakeholders 'environment.**

Our institution has ongoing projects with both governmental and industrial partners, so the fellow had a great opportunity to join these projects and see the role of result implementation in practical applications, and how ERI or other data analysis-based systems can be turned into practical applications.

Drug repositioning Data lake project

One of the data analysis-based projects the fellow joined, was developing a data science-assisted veterinary drug repositioning platform to combat antimicrobial resistance, where different data analytical pipelines will be used to suggest drug candidate molecules already used in other indications which potentiate the effects of antimicrobials in poultry. With the help of the newly forming platform, the goal is to nominate several drug candidates for repositioning and to test them in vitro and in vivo. The platform could serve as a base for the establishment of a future food chain safety Data lake to be developed which would be valuable for the integration and complex analysis of animal health, veterinary public health, food safety and human health data from different sources. The fellow supported the project by creating a list of potential databases useful for the creation of this platform. She went through several data sources to establish which database was a potential candidate and created a detailed list of more than 70 databases with information about the data owner, the public availability, how much data was stored, data format, and other information shown in Table 2. These databases collected are now being evaluated by the project consortium and will be used to develop a data model. The project provides an opportunity to develop a cross-sectoral data storage strategy that will later provide for the complete analysis of the food chain and an integrated approach to risk assessment. The resulting data lake can serve as a model for other countries as well as for the common European veterinary public health profession, proving great market opportunities of the project.

RASFF project

Another task is linked to collecting short-term, early warning signs from the Rapid Alert System for Food and Feed (RASFF). RASFF has been put in place by the European Commission to provide food and feed control authorities with an effective tool to exchange information about measures taken to respond to serious risks detected concerning food or feed. This exchange of information helps EU countries to act more rapidly and in a coordinated manner in response to a health threat caused by food or feed. RASFF tool is for these reasons an important portal to be connected to our emerging risk identification. In our internal project, the aim was to connect the RASFF data to our emerging risk data, making it possible to analyse risk profile from an emerging risk perspective combined with the RASFF perspective. To enable this analysis, an alignment of RASFF data structure with FoodEx2 (a comprehensive EFSA food classification and description system) and EFSA parameter codes is necessary. For this reason, the project idea was to align food and hazard categories through applying an easy and convenient own categorisation system. The fellow was involved in the creation of a workflow in Knime software to make automated category mapping as a part of data preprocessing. Knime is an open-source data analytics, reporting, and integration platform that allows assembly of nodes blending different data sources, including preprocessing for modelling, data analysis and visualisation without, or with only minimal, programming. With the help of this tool, she created the workflow of the product (food, feed and live animals and relative subgroups) and hazards (physical, chemicals and biological and relative subgroups) classification.

[1] https://agrostat2022.sciencesconf.org/data/pages/book_agrostat2022_en_posters_.pdf

3. Conclusions

The working programme 'Emerging risk identification by applying data analytical tools' offered the fellow an opportunity to familiarise herself with emerging risk identification, data analysis and data visualisation. The four modules included activities that successfully trained the EU-FORA fellow such as attending the meetings of several scientific national advisory committees that ensured the fellow's understanding of the interaction between different international organisations. The fellow was successfully integrated into the day-by-day workflow of Digital Food Institute, gaining first-hand experience in a scientific and interdisciplinary context. This experience provided the fellow the opportunity to develop her data science-related skills, which will benefit her professional development as a data analyst. Moreover, the fellow gained an overview of several topics related to food safety risk assessment by attending the EU-FORA training modules.

Table 1: Selection of the emerging issues identified by the Fellow

Description	Link of the issue	Date of the issue	Action
High levels of toxins found in more baby food brands	https://www.cbsnews.com/news/baby-food-toxins-government-report/	2021.9.29	New card in the tool Trello. To be monitored
Alcohol content of fermented beverages	https://www.longfordleader.ie/news/national-news/668712/on-the-kombucha-craze-new-alert-issued-over-alcohol-content-problem.html	2021.9.23	Attached to 'home fermentation' card
Another gene edited tomato	https://www.bullfrag.com/tomatoes-that-reduce-stress-and-lower-blood-pressure-go-on-sale-in-japan-life/	2021.9.30	Attached to 'GM tomato' card
Palmitic acid promotes a prometastatic memory	https://www.nature.com/articles/s41586-021-04075-0	2021.11.10	Communicated to EFSA and shared in our website
Bacteria-killing, biodegradable food packaging material	https://www.straitstimes.com/singapore/environment/spore-us-scientists-create-bacteria-killing-biodegradable-food-packaging-material	2021.12.29	New card in the tool Trello
Pseudomonas aeruginosa in liquid probiotics	https://kswfoodmicro.com/2021/12/02/usa-livia-global-announces-voluntary-recall-of-two-lots-of-its-liviaone-liquid-probiotics-because-of-the-potential-for-contamination-with-pseudomonas-aeruginosa/	2021.12.2	New card in the tool Trello. To be monitored
Demand for crocodile meat doubles following rise in pork prices	https://www.laprensalatina.com/thais-turn-to-crocodile-meat-as-price-of-pork-soars/	2022.1.22	Communicated in LinkedIn
Cadmium and risk of diabetes	https://www.sciencedirect.com/science/article/pii/S0160412021005456	2022.1.26	New card and to be monitored
Artificial sweeteners and cancer risk	https://journals.plos.org/plosmedicine/article?id=10.1371/journal.pmed.1003950	2022.3.24	Communicated in LinkedIn
Dietary intake with urinary melamine	https://www.mdpi.com/1660-4601/19/9/4964/htm	2022.4.19	New card and to be monitored

Table 2: Selection of the data sources assessed for the Drug repositioning Data lake project

Domain	Database	Technology (data access)	Refresh methodology	Data size	Access link
Microbiology	Food-borne pathogens	XLSX, PDF, CSV	Frequently updated	4,588 results	https://zenodo.org/search?page=1&size=20&q=foodborne%20pathogens

Domain	Database	Technology (data access)	Refresh methodology	Data size	Access link
Veterinary drugs	Veterinary drug residues	XLSX, PDF, CSV	Frequently updated	420 results	https://zenodo.org/communities/efsa-kj/search?page=1&size=20&q=veterinary%20drugs%20residues
Chemicals	REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals Regulation	PDF, XML, CSV, XLSX	Frequently updated	32 current consultations	https://echa.europa.eu/regulations/reach/understanding-reach
Medicines	European public assessment reports (EPAR)	PDF, XLSX, HTML	2 weeks after EC decision	1,601 reports	https://www.ema.europa.eu/en/medicines/field_ema_web_categories%253Aname_field/Human/ema_group_types/ema_medicine
Chemical structure data	ChEMBL	TXT, CSV, JSON, XLSX, MySQL, TSV, SDF	Every 3–4 months	more than 65,000 journal articles	https://www.ebi.ac.uk/chembl/

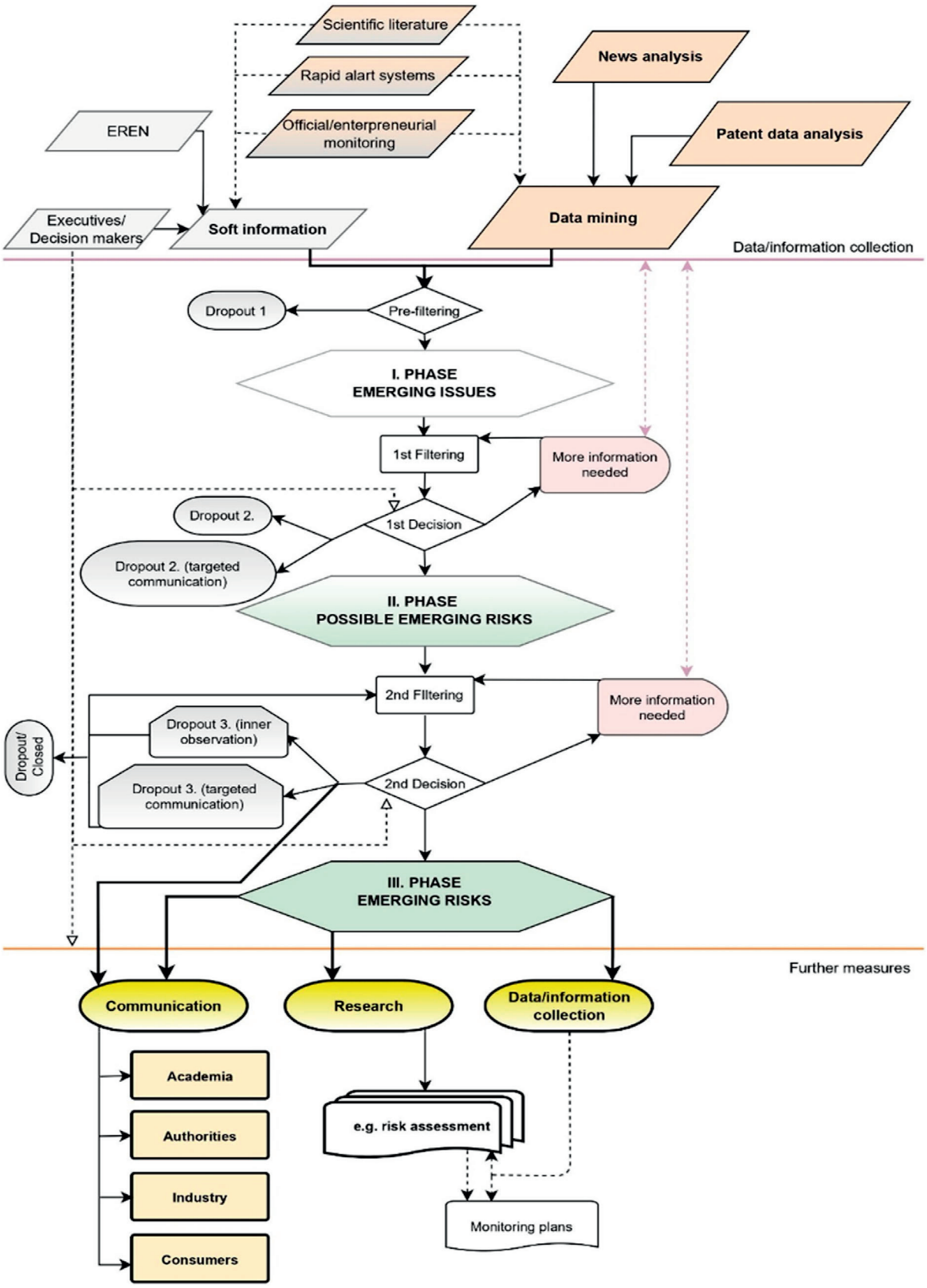

Figure 1: Emerging issue identification (ERI) workflow

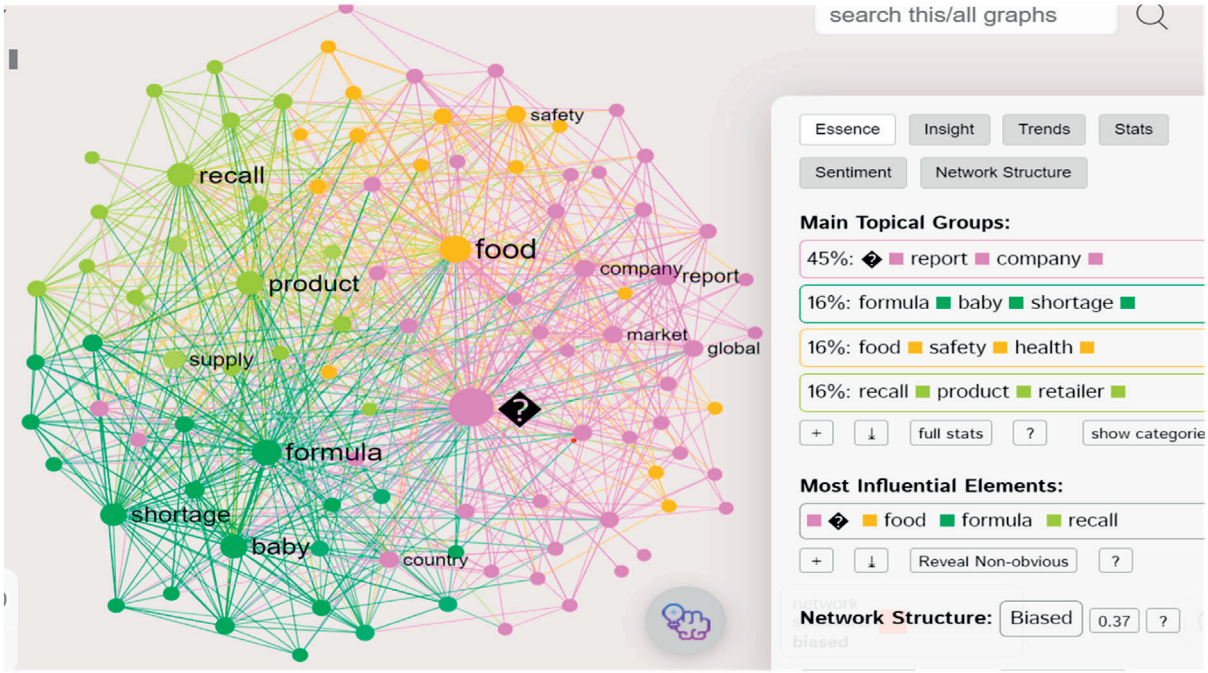

Figure 2: Topic detection and visualisation with InfraNodus software

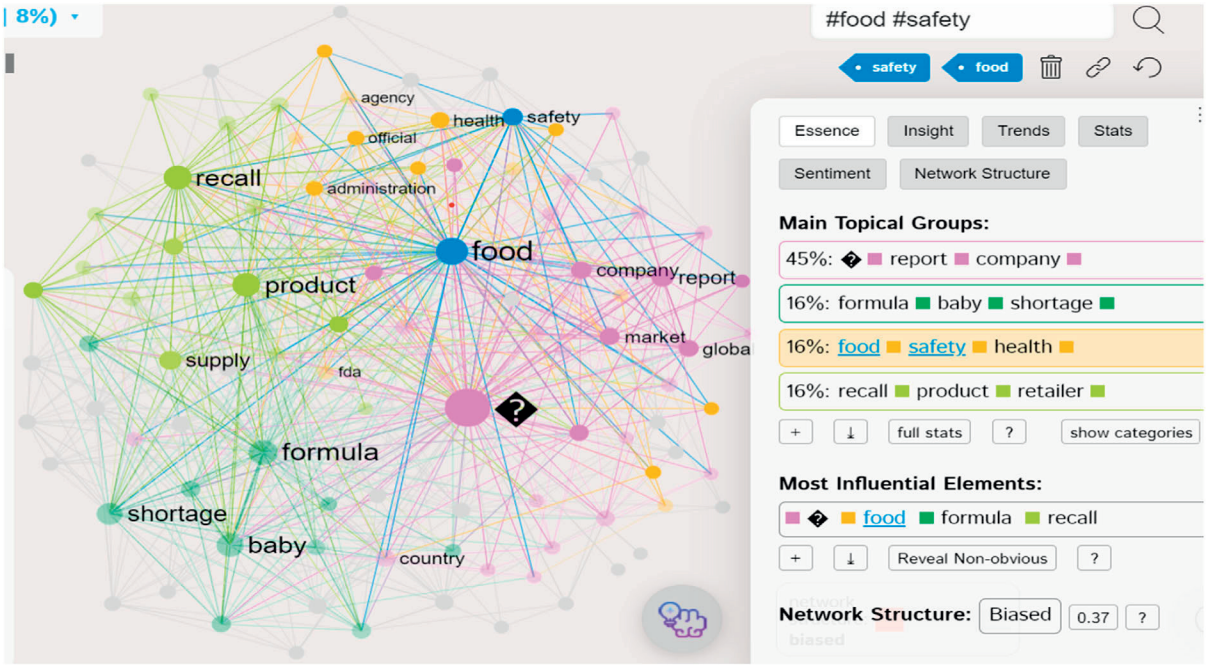

Figure 3: Topic detection and visualisation with InfraNodus software after the selection of the topic 'food safety'

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Abbreviations

DFI	Digital Food Institute
DFS	Digital Food Strategy
ERI	emerging risk identification
EU-FORA	European Union Food Risk Assessment
NÉBIH	National Food Chain Safety Authority
RASFF	Rapid Alert System for Food and Feed

Appendix A – Trainings and activities

Type	Title	Date
Course training	Data analysis and computational science in general. Defining computational methods, general applications, timeliness, benefits and limitations.	2022.10.20
	Modelling basics. Problems solvable by modelling and the limitations of modelling. Linear models.	2022.10.20
	Modelling. Markov models, game theory.	2022.10.20
	Modelling. Non-linear models, complexity science, their role and importance in food chain safety.	2022.11.3
	Network analysis. Basics of network analysis and application possibilities in the field of food chain safety.	2022.11.3
	Network analysis. Microbial metabolic pathways as networks. Epidemies and foodborne incidents as networks.	2022.11.3
	Epidemiological modelling. Diffusion, compartment, agent-based, spatio-temporal and network models.	2022.11.17
	Applications: KNIME, R, Python	2022.11.17
	Applications: Gephi, STEM, GleamViz	2022.11.17
	Data mining, text mining. Basics and application possibilities. Case study: identifying emerging risks with text mining.	2022.12.1
	Predictive microbiology. Basics and application possibilities from industrial, policy and research perspective.	2022.12.1
	Traceability. Role of data and IT in traceability systems. Investigation of foodborne outbreaks with Food Chain Lab. Blockchain-based traceability systems.	2022.12.1
	Food chain data analysis, driver analysis. Process mining, Bayesian network analysis. Case study: milk production chain automated driver analysis and alert system.	2022.12.1
	Decision support. Data visualization, interpretation and communication of results. Communicating limitations and uncertainties. Ethical considerations. Decision making processes	2022.12.15
	The future food data scientist: challenges and new areas. Big data and artificial intelligence (AI) application possibilities and limitations.	2022.12.15
	Case study: literature review with the help of AI.	2022.12.15
	Data analysis lecture on KNIME and text mining with practical exercises	2022.12.15
Seminars and conferences	26th EREN meeting	2021.10.21–10-22
	The Bilateral symposia serials with Huazhong Agricultural University	2022.2.16–2.17
	27th EREN meeting	2022.5.11–5.12
	Agrostat 2022 – presentation of the poster 'Applying a text mining software for emerging risk identification in the food chain'	2022.6.16–6.17
	One Health Conference 2022	2022.6.21–6.24

EU-FORA SERIES 5

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FoodSafety4EU: paving the way for the food safety system of the future

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Abstract

Food safety is a top priority for the European Commission, which policies aim at sustaining a high level of protection of human health and consumers' interests, while ensuring an effective functioning of the internal EU market. Under the New Transparency Regulation (EU/2019/138), the 3-year EU-funded FoodSafety4EU project (FS4EU) kicked off in January 2021, represents a significant step for the European Union (EU) food safety system (FSS), towards more transparency, better engagement, and closer cooperation. This Horizon 2020 Project, coordinated by CNR-ISPA (Italy), focuses on building a multi-stakeholder platform for the future EU FSS. The FoodSafety4EU Network currently consists of 23 consortium partners and around 50 stakeholders: Food Safety Authorities (FSA), consumer associations, academia, research centres and networks, food industries and sector associations, thinktanks, etc. Through a structured, digitally supported, participatory process, the platform hosts the co-design of future strategic research and innovation agenda (SRIA), as well as risk communication models tailored to the specificities of various target groups. Among the goals: providing scientific advice and technical support for EU food safety policies, by enabling actors to access, share and exchange scientific knowledge, resources, and data more efficiently, to better synchronise food safety research and policies, and to contribute to a more transparent communication through the FSS. Overall, the FS4EU project underpins the EFSA missions in risk assessment, including risk communication, with the ambition to be a basis for a Knowledge Centre for Food Safety in Europe. Accordingly, the EU-FORA 2021–2022 fellow (based in CNR-ISPA under the Work Programme RECIPE: Risk AssEssment/Risk CommunIcation: understanding the context and addressing Priorities of the futurE – a learning-by-doing approach) committed to multiple Work Packages, actively participated to various activities, thus successfully contributing to the FS4EU project meeting its objectives by the end of 2023.

Keywords: food safety, food safety system, multi-stakeholder platform, SRIA, risk assessment, risk communication

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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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Table of contents

1. Introducing FoodSafety4EU (Lattanzio, 2022; Lattanzio et al., 2022)

1.1. FoodSafety4EU strives for an adaptive, iterative, proactive Food Safety System

Food safety is imperative for health, social and economic reasons. Currently, the EU food safety system (FSS) regulatory framework relies on evidence-based risk assessment and data analysis, at times proving not quick enough to adapt and undertake emerging issues arising from our modern high-paced food sector dynamics as well as caused by the climate change-driven unstable agricultural supplies, recently resulting in crisis of various motives and amplitudes. Consequently, consumers are often only likely to hear about and share a concern for food safety in times of crisis, precisely when the risks of misinformation and the loss of citizen trust are on the rise. Those issues are constituent to our current FSS: as a matter of fact, researchers, FSAs, policy makers, food business operators do not collaborate enough through integrated mechanisms and spaces to be able to nurture a continuous and trusted interaction.

FoodSafety4EU (FS4EU) aims to tackle these challenges, co-designing new collaborative, multi-stakeholder schemes and experimenting innovative ways to promote food safety information among the large public, to progressively advance the EU FSS from a reactive system to a collaborative, proactive one.

1.2. What is the FoodSafety4EU platform?

This platform is intended to drive the transition of the existing European FSS towards a comprehensive and constantly updated framework, enabling the FSS actors to access efficiently resources and data, share and exchange scientific knowledge and contributions, interact and network, create new partnerships and joint activities.

On top of the digital dimension, the platform fosters 'physical' interactions, guaranteeing the participatory process through four layers of interaction between all the FSS stakeholders.

Layer 1 – Food Safety Hubs

As the first level of interaction, **four Food Safety Hubs** (Figure 1) work as decentralised nodes to **catalyse the main experts of the respective geographical areas and create the basis to feed a multi-actor discussion**. The reason behind this geographical division is that each of these four areas faces similar issues in the local FSSs and has specific food production chains, market dynamics and innovation/technological levels. Inside each Food Safety Hub, representatives of policy makers, researchers, food business operators and consumers are actively involved in the participatory process.

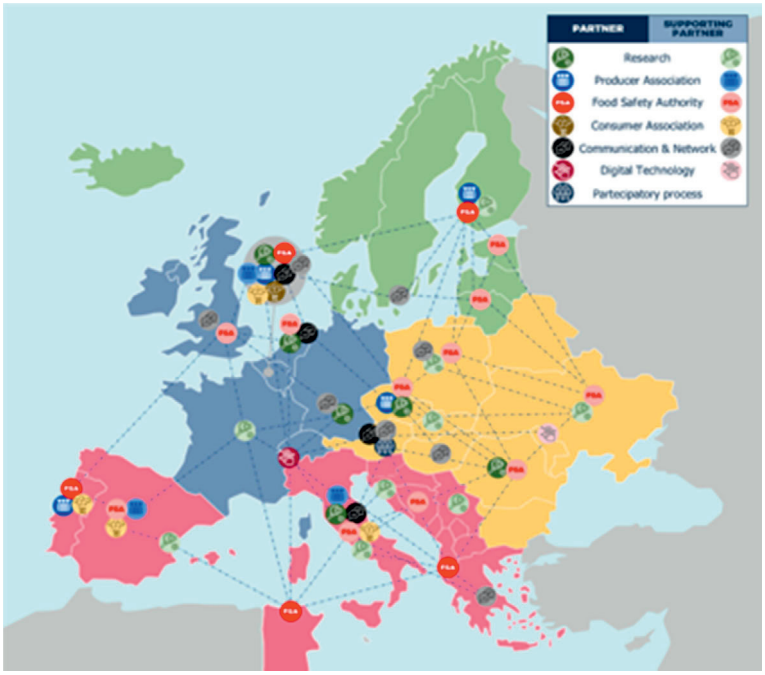

Figure 1: The 4 FoodSafety4EU Hubs

This ecosystem was already set up before the beginning of the project, through a mapping and the involvement of FS4EU Supporting Partners – stakeholders committed to contribute to the definition of the FSS of the future. Currently, the project consists of 50 Supporting Partners (in addition to the 23 beneficiaries), but the numbers are still increasing, as the project stays open to new collaborations.

Layer 2 – Food Safety Operational Labs

As a second layer of the participatory process**, FSS actors interact through four Food Safety Operational Labs (FSOLabs), 'rooms' for social experimentation addressing complex food safety related topics on a systemic level** (Figure 2). They involve experts from various Hubs, representing different FSS actors in a cycle of interactive meetings, in which they can discuss and find new ways to tackle the selected four challenges:

- FSO Lab 1: **Harmonisation and integration of the future FSS**, to be supported through pilot actions and recommendations.
- FSO Lab 2: **Alignment of research priorities and funding cycles**, within and among EU Member States.
- FSO Lab 3: **Development of a Strategic Research and Innovation Agenda** (SRIA) on Food Safety.
- FSO Lab 4: Definition of **Innovative approaches and models to reach inform and involve the civil society.**

The experts were involved by the project beneficiaries, Supporting Partners and other stakeholders in the FSS, including EFSA – the European Food Safety Authority, participating in all FSOLabs.

After a preliminary challenge mapping and analysis exercise to set up each FSOLab, the experts met and progressively co-created possible solutions to address the identified channels, developing also pilot activities to be tested in real-life scenarios: the process will run until the end of 2022, then each FSOLab will assess and analyse the results obtained, translating them in new outputs to be adopted by FSS stakeholders, such as FSAs, policy makers and business operators.

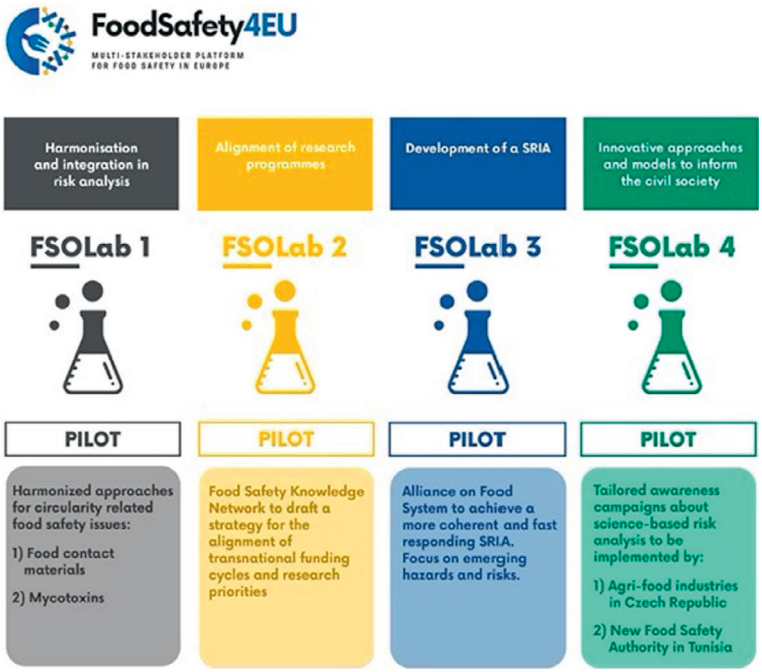

Figure 2: The 4 FSOLabs and their pilots

FSOLab 1 focuses on the **need for harmonisation and integration**, specifically **linked to risk analysis of emerging hazards in a circular economy**: the increasing call for sustainability comes from the desire to reduce our environmental impact. However, this raises new potential issues concerning food safety. For example, recycled materials can contain contaminants from previous (mis-) uses. These issues are unlikely to be predicted through conventional risk assessment. As well, FSAs do not already have the necessary amount and quality of data to perform a regulatory-grade risk assessment. This FSOLab attempts to increase the ability of the FSS to deal with the environmental transition safely, particularly within two pilot action topics: **Food Contact Materials** and **Mycotoxins**.

FSOLab 2 aims to propose **strategies for a better alignment of research programmes and funding opportunities**, both at national and EU levels. Given the large inequalities in the distribution of economic resources and research funds among individual countries, there can be major implications for a country's performance in terms of Food Safety progress. FSOLab2 pilots a **Food Safety Knowledge Network** to draft a strategy for the alignment of transnational funding cycles and research priorities, as a fundamental step towards safe and sustainable food systems.

FSOLab 3 is committed to developing a proposal for a **SRIA**. Its mission is to build up a common **strategy to prioritise and address emerging food risks and hazards**. Specifically, FSOLab3 is developing a process for achieving a more coherent SRIA by piloting an **Alliance on Food Systems**, thus contributing to reinforcing the role of Europe as a global leader in food safety standards and to ensuring ever more efficiently adequate levels of public safety.

FSOLab 4 is focused on **innovative and novel communication methods** to reach **and inform civil society about food safety science and the risk assessment process**. Two tailored, cartoon-based communication campaigns have been designed to increase awareness about Food Safety and the science-based risk analysis processes in young adults from two countries with different stakes and issues regarding the FSS. Those campaigns are now being implemented:

- in the Czech Republic, by national, innovative agri-food aquaponics industries;
- in Tunisia, by the new-born Food Safety Authority, INSSPA.

Layer 3 – digital platform and tools

Besides the four Food Safety Hubs and the four FSO Labs, the third layer of interaction relies on the implementation of a **digital platform with apps** that allows all actors within the FSS to interact fluidly and facilitates their access to a variety of resources related to Food Safety issues. Ultimately, its

goal is to become a **central platform as a single-entry point for EU FSS actors** in the near future.

The platform already allows users to get information about running FSO Labs, including results and options to give feedback. The platform also allows to look for specific experts, projects and topics, including their contact persons. In addition, the platform provides catalogues for data sets, apps, services and other materials allowing users to search resources of their interest in food safety. Another functionality is a file storage system which can be used not only by FSO Labs but also by users of the platform to exchange or collaborate on certain documents. The platform also hosts news and agenda sections for the forthcoming activities (i.e. events, webinars) and is used as one of the main communication channels inside the FSS actors' community.

Layer 4 – EU Food Safety Forum

The fourth level of interaction among the FSS actors is based on the participatory and networking process – progressively turning the FS4EU platform into this reliable, **fluid interface for long-term science-policy-society cooperation** within the EU FSS, the **EU Food Safety Forum**. As a main output of the project, the Forum will be built upon the interactions of all FSS actors during the project timespan and through the three previous layers.

Thanks to the vibrant ecosystem already established during the first year, the first milestone of the path towards a long-term and self-sustainable forum took place in December 2021, with the first edition of the pre-Forum, attended by more than 120 participants – representing Supporting Partners and other stakeholders. After inspirational pitches, this online conference turned into a participatory session of learnings and discussion, the audience asking both free and guided questions to the experts, expressing opinions and sharing ideas via real-time polls. Gathered and analysed, these insights will guide FS4EU in the definition of the 2nd Pre-Forum (December 2022).

2. Involvement of the EU-FORA fellow

2.1. Global and technical support to the FoodSafety4EU platform

Besides specific tasks written in the work programme, the fellow brought day-to-day support to the FS4EU coordination team in CNR-ISPA. Below are listed (non-exhaustively) various tasks and actions undertaken by the fellow all along the year, on demand or proactively, for the benefit of the FS4EU project.

Updating the FS4EU website

- Writing articles on recent events, FSOLab workshops and milestones, new FS4EU partners agreements.
- Updating and remodelling sections of the website, such as the FS4EU community of supporting partners, the agenda/calendar.

Participating in, designing and hosting online FS4EU events

- Taking part in FS4EU FSOLab workshops and Work Package meetings, as attendee or coordinator.
- Animating online meetings organised by FS4EU (1st pre-Forum, H2020 project 'DISH cluster' meetings, etc.): designing interactive sessions of questions on Mentimeter, hosting those sessions live, analysing and communicating the results.

Contributing to FS4EU global communication and awareness

- Writing content for both internal and social media communication campaigns.
- Writing and reviewing scientific publications about FS4EU.
- Preparing and presenting FS4EU communication documents at the Mycokey Conference 2022.
- Contributing to the presentation of a poster (VMT et al., 2022) at ONE conference 2022 (*V. Lattanzio* et al. *'Towards a more engaged and co-operative Food Safety System in Europe*: *the contribution by FoodSafety4EU'*).
- Designing and presenting a scientific poster (Bayer et al., 2022) about FS4EU at the World Mycotoxin Forum 2022 (*F. Bayer* et al. *'FoodSafety4EU*: *a step towards improved engagement, cooperation and transparency in the European Food Safety System'*).

2.2. Project management for FSOLab 4

Early on, the fellow could contribute to this scientific outreach FSOLab by moderating and animating the 2nd FSOLab4 workshop held on 8–9 November 2021, presenting his own experience, ideas and examples of cartoon-based communication campaigns with Food Safety purposes. The workgroup was composed of actors from all the European FSS: FSA representatives (including Tunisia INSSPA), researchers, consumer associations representatives as well as communication consultants.

Two separate pilot actions were then designed, each one bearing the voice of **a specific source**, with **respective objectives**:

- **Food Safety Agency pilot** (Tunisia)
  - carried out by **INSSPA**, newly formed **Tunisian** Food Safety Authority;
  - informing and educating about domestic safe food handling and practices;
  - raising awareness about national institutions ensuring and enforcing food safety.
- **Industry pilot** (the Czech Republic)
  - carried out by **Aquaponics producers in the Czech Republic**;
  - raising awareness about **practitioners' safe handling of food**;
  - appealing to **consumers' social and environmental responsibility** concerning safe food production.

In reference to the shared pilot idea, for both pilot actions, it was decided to **design cartoon strips for further dissemination, both online (via social media) and print (via leaflets and posters),** according to the most relevant channels in those two countries to reach the agreed **target – namely young adults and their families**.

With the fellow coordinating the discussions, the FSOLab participants agreed to implement the **two national communication campaigns** based on this generic pilot concept: **science-based reliable information on food safety and the risk assessment process should be provided by funny, human characters**.

Those characters features (look and feel, missions, psychology, etc.) were then co-designed during the workshop, aligning cultural expectations and habits of all representants of this FSO Lab. Consensus was found around **three characters**:

- **a relatable main character** representing the target group (young adults) to be used universally. The main character is a young adult who is ignorant or has little knowledge not only about food safety in general but also about hygiene, cooking practices, etc.
- **a relatable secondary character,** usually a parent, who is a bit less ignorant about FS, but still can make mistakes and have unsourced, biased opinions about the FSS.
- **a team of actors representing the FSS** (FSA scientist, food producer, etc.) that revolves around the main characters in realistic situations, providing expertise and the necessary knowledge to reassure, educate and improve awareness about food safety.

See **Annex A** for the characters final graphic representations as well as **Annex B** for the first sketches of the Tunisian cartoon strips.

All in all, throughout the year, the fellow was involved in:

- co-designing the pilot actions, characters features and dissemination plan;
- creating, editing, and co-validating the scenarios for each pilot action;
- designing the storyboards and creating the dialogues;
- coordinating the project with the professional drawer.

2.3. Development of a scientific communication module

One of the objectives of FS4EU is to come up **with innovative ways of communicating food risks and food safety**, for the future EU FSS to be even more open, transparent and resilient. Complementary to finding new channels of communication as well as updated, targeted messages, from food scientists towards society, food system stakeholders and policy makers alike, the fellow has piloted and provided a **toolbox for food scientists themselves to better enjoy communicating their work**.

One of the main features of this toolbox is to stay funny, interactive, participative and overall accessible, **allowing any motivated, communication-sensitive food scientist to lead workshop sessions of media training in his/her lab**. The overall objective is for the trainees to improve their abilities and skills in:

- Pitching a scientific study.
- Writing media headlines and articles.
- Facing a journalist in an online interview setting.

For each trainer, and each session, **up to six trainees** can participate to the workshop. A larger group would imply fewer individual feedbacks and interactions for trainees with the trainer and each other, which would result in a less impactful training.

The workshop is composed of three **consecutive sessions** (ideally to be held on the same week):

- **Day 1 session: How to write**. *Duration 3 h30 (plenary meeting)*.
- **Homework session: Preparing for an interview**. *Duration 2 h (on their own)*.
- **Day 2 session: How to speak**. *Duration 20mn (Interview) + 2 h (plenary meeting)*.

The fellow himself successfully **hosted the pilot workshop** in June, for five food scientists involved in FS4EU Labs, then asked and received feedbacks for further improvement of the toolbox. See **Annex C** for images of this pilot workshop.

All in all, the fellow was involved in:

- Designing the science-media training workshop and making it entirely ownable through a turnkey toolbox.
- Preparing the educational material: Presentations, Mentimeter quiz, Miro Board templates for collaborative work, examples of communications, Criteria for evaluation of interviews.
- Organising and hosting the pilot workshop within FS4EU: animating the presentation, collaborative work on Miro as well as commenting on Mentimeter quizzes, reviewing press communicates written by trainees, interviewing trainees in an online setting, giving feedbacks on those interviews.

The training module will be available on the project website by the end of 2022 to be replicated within and outside the project.

3. Summary of the project and EU-FORA fellow work programme completion

FoodSafety4EU contributes to enhancing common efforts and building synergies within the EU FSS through the co-design of an integrated and multi-stakeholder platform. This enables a network of FSS actors at the national, European and international levels to access resources efficiently, synchronise food safety research strategies, share and exchange scientific knowledge.

Through a structured, digitally supported, participatory process, the platform hosts the co-design of future research and innovation strategies, as well as risk communication models tailored to the specificities of various target groups. For example, through so-called Food Safety Operational Labs, multi-actors' workgroups find new ways to tackle selected food safety challenges – e.g. food contact materials, mycotoxins, consumer practices education, perception of the role of industries in food sustainability, etc.– following a continuous process, from co-designing to implementing and evaluating relevant pilot actions.

Furthermore, by engaging EU citizens in these structured dialogues, FS4EU supports a clearer understanding of the scientific evidence at the base of any risk management decision, thus enhancing public confidence in the European FSS.

The FS4EU Network currently consists of 23 consortium partners and around 50 stakeholders – Food Safety Authorities, consumer associations, academia, research centres and networks, food industries and sector associations, thinktanks, etc. This community is expected to grow into a collaborative European Food Safety Forum by the end of the project, late 2023.

Concerning the EU-FORA fellow, hosted in CNR-ISPA from September 2021 to August 2022, the work programme (RECIPE_Risk AssEssment/Risk CommunIcation: understanding the context and addressing Priorities of the futurE – a learning-by-doing approach) has been completed according to the foreseen timeframe. The work programme was drafted in relation to the research activities ongoing at the hosting site that are in alignment with the overall objectives of the EU-FORA

programme. Specifically, in the framework of the FS4EU project, coordinated by CNR-ISPA, fellowship activities were designed in order to provide the fellow with a comprehensive overview of the risk assessment process and expected challenges for the incoming years, with emphasis on risk communication aspects.

Specifically, the fellow has been involved in activities relevant to the harmonisation of enforcement practices as well as to innovative approaches and models to inform civil society about the risk assessment process. Both topics included a focus on circularity-related food safety issues. It is worth to point out that the fellowship period matched with a crucial phase of the FSOLab process, i.e. the co-creation, discussion and implementation of pilot actions, thus the fellow concretely experienced the interdisciplinary and multi-actor approach, that can be applied in future projects to complement with research activities. On the other side, the fellow sharing with the supervisor the skills acquired in EU FORA training modules was an acknowledged added value of this programme for the hosting site.

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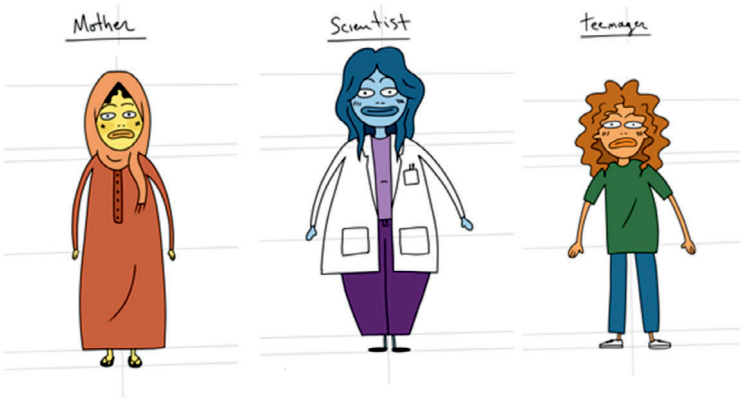
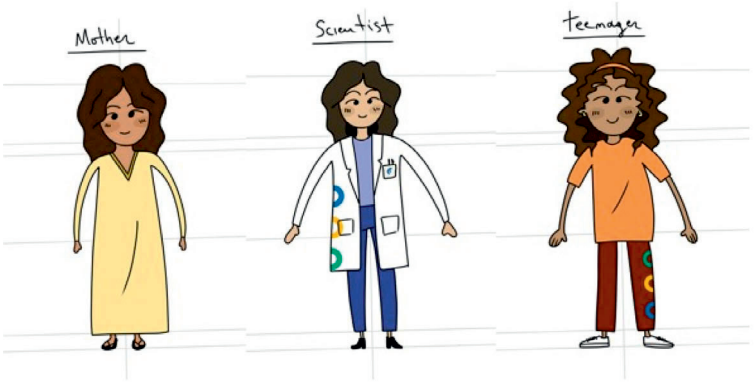
Abbreviations

FS	food safety
FSA	Food Safety Agency
FSOLab	Food Safety Operational Lab
FSS	food safety system
FS4EU	FoodSafety4EU
SRIA	Strategic Research and Innovation Agenda

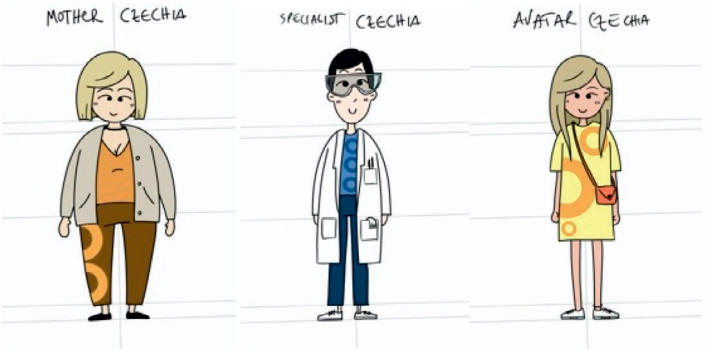
Annex A – FSOLab 4 characters



Tunisia first characters
(Rejected for being too "cartoonish" and not "serious" enough to represent the voice of a newly created FSA)



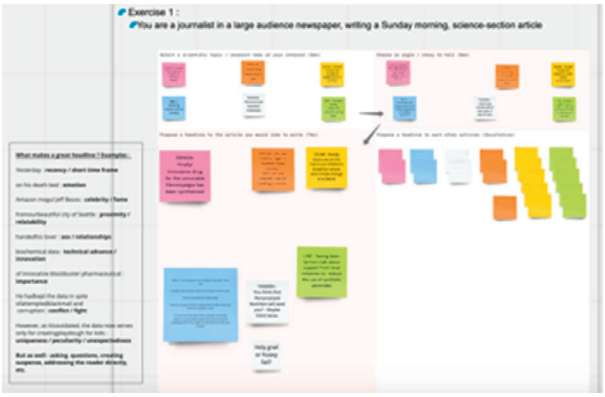
Tunisia final characters



Czech Republic final characters

Annex B – Early drafts of cartoon strips for the Tunisia Pilot

Annex C – Images of the ScienceMediaTraining pilot workshop



Miro board of the first exercise (writing scientific headlines)

Example of Mentimeter live quiz question

Footage of the pilot session interviews

EU-FORA SERIES 5

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Microbiota analysis for risk assessment of xenobiotics: toxicomicrobiomics, incorporating the gut microbiome in the risk assessment of xenobiotics and identifying beneficial components for One Health

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Abstract

This work explores three areas of relevance to the gut microbiome in the context of One Health; the incorporation of the microbiome in food safety risk assessment of xenobiotics; the identification and application of beneficial microbial components to various areas under One Health, and specifically, in the context of antimicrobial resistance. We conclude that, although challenging, focusing on the microbiota resilience, function and active components, are critical for advancing the incorporation of the gut microbiome in the risk assessment of xenobiotics. Moreover, research technologies, such as toxicomicrobiomics, culturomics and genomics, especially in combination, have revealed that the human microbiota may be a promising source of beneficial taxa or other components, with the potential to metabolise and biodegrade xenobiotics. These may have possible applications in several health areas, including in animals or plants for detoxification or in the environment for bioremediation. This approach would be of particular interest for antimicrobials, with the potential to ameliorate antimicrobial resistance development. Finally, we propose that the concept of resistance to xenobiotics in the context of the gut microbiome may deserve further investigation in the pursuit of holistically elucidating their involvement in the balance between health and disease.

Keywords: One Health, gut microbiome, xenobiotics, microbiota-disrupting chemicals, next-generation risk assessment, antimicrobial resistance, next-generation probiotics

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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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1. Introduction

1.1. Microbiome and One Health

The human microbiome, a characteristic microbial community occupying a complex, but reasonably well-defined habitat with distinct physio-chemical properties, encompasses the microorganisms involved (microbiota), as well as their structural and molecular elements (e.g. nucleic acids), metabolites and surrounding environmental conditions (Berg et al., 2020). One Health (OH) is the holistic methodology of transdisciplinary cooperation to improve human, animal, plant and environmental health simultaneously, and its adoption is continuously expanding (CDC, 2020; Bronzwaer et al., 2021). Due to their functionalities and physiological potential and considering their known associations with a range of diseases, microbiomes are key elements in the OH framework (CNBBSV, 2019; Merten et al., 2020). Their significance is partly due to pathogenic and commensal microbial transfer between humans, animals and the environment, and the human microbiome stands out with regard to its interactions with environmental and dietary chemicals that affect human health outcomes (Trinh et al., 2018). Of particular interest are the contact and mutual influence between the human gut microbiome (GM) and exogenous toxic chemicals, xenobiotics, focusing on their fate, metabolism and toxicity (NASEM, 2018; Abdelsalam et al., 2020).

1.2. Microbiota-disrupting chemicals and the gut microbiome

Among xenobiotics, endocrine-disrupting chemicals (EDCs) are especially important and they have been associated with metabolic disorders, such as obesity, as well as with changes in the GM (Gálvez-Ontiveros et al., 2020; Aguilera et al., 2021). Recently the concept of microbiota-disrupting chemicals (MDCs) has been proposed, which comprise EDCs and other xenobiotics with potential to alter the gut microbiota's composition and metabolism (Aguilera et al., 2020) via dietary exposure, e.g. bisphenols, parabens (Andújar et al., 2019; Monteagudo et al., 2021; Robles-Aguilera et al., 2021).

The interactions between MDCs and the GM are complex. This is partly because multiple general mechanisms are involved, including; direct effects of the MDC on the microbiome; altered epithelial-barrier functions (affecting uptake or excretion of MDCs); direct chemical transformations of MDCs; secondary transformation of host-generated metabolites (e.g. deconjugation by β-glucuronidases); and altered expression of host-tissue metabolic enzymes and pathways (e.g. in the liver via microbial signalling molecules) (Ulluwishewa et al., 2011; Patterson and Turnbaugh, 2014; Peterson and Artis, 2014; Kelly et al., 2015; Selwyn et al., 2015, 2016; Claus et al., 2016; Spanogiannopoulos et al., 2016; NASEM, 2018). Although these interactions can decrease MDC exposure and toxicity effects, they can also increase them. For example, several bacterial phyla in the human GM can produce azoreductases, which have been shown to reduce azo dyes that are common in foods into mutagenic and carcinogenic aromatic amines (Rafii et al., 1990; Xu et al., 2007). Overall, the role of these complex interactions in modifying human susceptibility to MDCs is beginning to be elucidated.

1.3. Risk assessment of xenobiotics and the gut microbiome

Risk assessment (RA) is the science-based component of the food safety risk analysis framework, alongside risk management and risk communication. RA comprising; hazard identification; hazard characterisation; exposure assessment; and risk characterisation (CAC, 1999; European Commission, 2002). Traditionally, xenobiotic RA relies on data from animal experiments, human trials and/or human observational/epidemiological studies. Importantly, the extrapolation of this data across species or studied populations is not without challenge, partially due to GM variability (e.g. in homogeneous populations, such as healthy adults) and variation (e.g. between species or life stages) and the complexity of MDC/GM interactions (NASEM, 2018). Thus, the need for the incorporation of the GM in food safety RA of xenobiotics is well-justified (Merten et al., 2020) and by extension to MDCs.

1.4. Seeking beneficial taxa/components in the gut microbiome

Another area of relevance to the GM in the context of OH is the identification of beneficial taxa and derived components (e.g. enzymes and biocompounds) in the GM and their potential application. In this context, toxicomicrobiomics, which study the aforementioned microbiome-xenobiotic/MDC interactions, along with culturomics, which aim to cultivate components of the human GM through the use of optimised selective and/or enrichment culture conditions coupled with metagenomic taxa

identification, can shed light on the microbiome's capacity to metabolise xenobiotics (Aziz, 2018; Lagier et al., 2018; CNBBSV, 2019; Abdelsalam et al., 2020; López-Moreno et al., 2022) and by extension MDCs. Thus, these approaches can help identify GM components with beneficial effects under OH, for example detoxification activity (López-Moreno et al., 2021b) or next-generation probiotics (NGPs) (López-Moreno et al., 2021a).

1.5. Antimicrobial resistance and the gut microbiome

A third area of relevance is antimicrobial resistance (AMR). Undoubtedly, AMR is an important OH issue, with the major contributor being the misuse of antibiotics (WHO, 2015, 2021; O'Neill, 2016). Moreover, the GM has previously been considered as a reservoir for antibiotic resistance genes (Gibson et al., 2015; Anthony et al., 2021). Moreover, non-antibiotic antimicrobials, including MDCs triclosan and parabens, commonly used as preservatives in foods, food contact materials (FCMs) and personal care products (Soni et al., 2001, 2002, 2005; CIR, 2008; Halden et al., 2017), may also contribute to AMR (SCCS, 2010). This is because some resistance mechanisms are common to both biocidal MDCs and antibiotics, for example, the former may; exert selective stress leading to the expression of bacterial resistance mechanisms and their dissemination; and/or maintain mobile genetic elements carrying genes involved in antibiotic cross-resistance (SCENIHR, 2009). Therefore, detoxification potential from specific GM taxa become of particular interest in the context of such antimicrobials.

2. Work programme

2.1. Aims

This work builds upon a previous EU-FORA project (Cerk and Aguilera-Gomez, 2022) and aims to explore three areas of relevance to the microbiome in the context of OH. Firstly, the incorporation of the GM in RA of xenobiotics was explored. Secondly, the potential application of beneficial GM taxa (or their bioactive compounds), identified via toxicomicrobiomics and culturomics approaches was considered. Finally, a specific aspect of this application was further investigated in the context of AMR, and xenobiotic resistance was also considered.

2.2. Additional activities

Additional activities and training opportunities were identified during this EU-FORA fellowship project, based on the fellow's background and professional interests. These are listed in Appendix A.

2.3. Outputs

The three focal areas of this work and their links to each-other, OH and the GM are summarised in Figure 1. The first area, i.e. the incorporation of the GM in RA of xenobiotics, relates primarily to human health. However, depending on the output of the RA and the antimicrobial or not nature of the xenobiotic, the other two areas, i.e. the application of beneficial GM taxa/compounds generally or specifically in the context of AMR, could be highly relevant to holistic xenobiotic risk management.

The following sections present the outputs for each of these three areas. These have been previously published in a scientific journal (Ampatzoglou et al., 2022) and presented at scientific conferences, during the fellowship programme. Further detail is available in Annex A.

2.3.1. Moving towards the gut microbiome's incorporation in risk assessment of xenobiotics

2.3.1.1. The need – current challenges to address

GM variation and variability adds layers of complexity to the already intricate interactions between MDCs and health. The observed differences in the composition, gene content and function of the GM has been attributed to multiple factors including age (Yatsunenko et al., 2012), antibiotic use (Dethlefsen and Relman, 2011), diet (Yatsunenko et al., 2012), disease state (Mar et al., 2016), environmental exposures (NASEM, 2018), exercise (O'Sullivan et al., 2015), genetics (Goodrich et al., 2014), geography (Yatsunenko et al., 2012), pregnancy status (Koren et al., 2012), sex (Markle et al., 2013), socioeconomic status (Levin et al., 2016) and surgical interventions (Tremaroli et al., 2015). Moreover, these factors may only explain a small fraction of the total GM variation

(Falony et al., 2016; CNBBSV, 2019). Importantly, due to this variability, observations of microbiome-influenced toxicities in a studied population might have little relevance to other populations with substantially different GM composition and function (Rodricks et al., 2019).

In addition, there is considerable variation between the GMs of humans and animals, due to anatomical, physiological, functional, immunological and compositional differences. Some of these have been partially overcome via the use of 'humanised' animals in toxicological studies (Sonnenburg and Bäckhed, 2016). Nevertheless, extrapolation from such studies to humans still carries considerable uncertainty (Rodricks et al., 2019) and, along with the intraspecies variability, necessitates the use of uncertainty/safety factors, frequently reaching two orders of magnitude (Dorne and Renwick, 2005; Benford et al., 2018). Based on these factors, traditional RAs may overestimate or underestimate the risk associated with exposure to an MDC, partially because they do not account for its interactions with the microbiome (NASEM, 2018; Merten et al., 2020). Consequent risk management decisions may place considerable pressure on the industry. For example, EFSA's recent proposal to considerably reduce the tolerable daily intake for bisphenol A (BPA) (EFSA, 2021), may further increase the use of bisphenol analogues in FCMs, which may also trigger dysbiosis and obesogenic phenotypes (Andújar et al., 2019; Monteagudo et al., 2021).

Although the need is clear to incorporate the GM in the RA of xenobiotics, there are additional hurdles, i.e. the fundamental requirements to; establish causation and molecular mechanisms linking phenotypes, e.g. obesity, with microbiota profiles (Fischbach, 2018); and define what constitutes a healthy GM, which still remains elusive (Merten et al., 2020). Considering that these tasks require significant resources, it might be a useful first step to establish principles on how to evaluate the potential of xenobiotics to alter the GM.

2.3.1.2. Assessing the potential of xenobiotics to alter the gut microbiome

Interestingly, a three-tier framework has recently been proposed by the Unilever Safety and Environmental Assurance Centre for assessing the potential of personal care formulations to perturb the skin and oral microbiomes (Métris et al., 2021). The following sections briefly present this framework and suggest amendments which could make it suitable for application to the GM in the context of xenobiotic RA.

First tier – xenobiotic cross-reference

The first tier benchmarks new formulations against ones regarded as safe because of a long 'history of safe use' (HoSU). However, this approach cannot apply directly to xenobiotics, for reasons such as their nature as contaminants or that they may not be intended to be ingested (e.g., if used in FCMs). Moreover, it is challenging to establish robust links between GM, cumulative exposure and resulting adverse effects (Gruszecka-Kosowska et al., 2022; Ortiz et al., 2022). Nevertheless, evidence has been compiling in recent years on the impact of several contaminants and groups of xenobiotics, including pesticides, bisphenols, phthalates, metals, triclosan, parabens and polybrominated diphenyl ethers, on human and animal gut microbiomes (Aguilera et al., 2020). As it expands, this evidence could potentially serve as an early cross-reference tier which would raise initial concerns, depending on the nature and chemical structure of a xenobiotic under RA.

Second tier – microbiome resilience

The second tier focuses on microbiome resilience. Other authors highlighted resilience, along with resistance to perturbation, as a key feature of healthy microbiomes, attributed to their rich and diverse metabolic pathways (Lloyd-Price et al., 2016; McBain et al., 2019; Cheng et al., 2022). Importantly, this tier assesses risk in relative terms. Thus, it circumvents the need to define the healthy microbiome, since it is only concerned about the return to its baseline state, independently of whether healthy or desirable. Of course, the length of exposure of the microbiome to the potential perturbator would be a critical consideration. Overall, however, this tier could be a reasonable approach to screen MDCs based on the resilience of the GM under various experimental approaches, extending from 'humanised' animals (NASEM, 2018) to *ex vivo* and *in vitro* models, such as simulator of the human intestinal microbial ecosystem (SHIME) (Van den Abbeele et al., 2012), minibioreactor arrays (Auchtung et al., 2015) and multi-compartment microfluidic-based gut-on-chip systems (De Gregorio et al., 2020; Signore et al., 2021).

Third tier – elucidating links between changes in the gut microbiome and health status

Finally, the third tier makes use of next-generation sequencing microbiome data in relation to host health status. This requires further development, is the most challenging tier and is, certainly, relevant to the RA of xenobiotics in the context of the GM. Métris et al. (2021) highlighted the requirement to focus not only on the microbiome's composition (including both relative and absolute abundances), but more importantly on its function. This is not surprising, given that microbiome variability between relatively homogeneous groups of people (e.g. healthy individuals of same sex and similar age) is less prominent at the functional level (Tian et al., 2020) and that compositional variation, more generally, might not necessarily impart key functional differences due to functional redundancy (NASEM, 2018). Regarding research methodology, metatranscriptomics, is an established approach to focus on the functional taxa in the microbiome. More recent methodologies, however, have combined flow cytometry with omics technologies to characterise active microbial fractions in the GM, revealing a number of taxa underrepresented by traditional 16S rRNA metagenomics (Peris-Bondia et al., 2011; Maurice et al., 2013). These approaches are likely to offer valuable insights in the extrapolation of this tier's approach to the GM, especially in the pursuit for key species or other types of biomarkers associated with host health or disease, which will be crucial for the incorporation of the GM in the RA of MDCs.

2.3.2. Identifying beneficial gut microbiome taxa/components for application under One Health

Taxa culturing strategies, in the context of the interactional triangle between EDCs (obesogens)-gut microbiota (dysbiosis vs eubiosis)-human health (obesity vs leanness), are key in obtaining and selecting strains (associated with pro-obesity and antiobesity phenotypes) with potential use as NGPs (López-Moreno et al., 2021a). The latter, unlike traditional probiotics, do not have a defined HoSU, and are thus subjected to more stringent regulatory requirements (O'Toole et al., 2017; Cerk and Aguilera-Gomez, 2022). Nevertheless, strains isolated from the human gut could more readily be used under OH, e.g. as probiotics for animals, plants and environmental protection and bioremediation.

Recent work has demonstrated that toxicomicrobiomics and culturomics are promising in exploring the potential of human GM taxa to metabolise obesogenic MDCs and selecting species able to tolerate or biodegrade BPA (López-Moreno et al., 2021b; López-Moreno et al., 2022). Moreover, whole-genome sequence (WGS) analysis of a relevant *Bacillus* species derived from the human gut microbiota shed light on the encoded metabolic pathways and key enzymes involved in BPA breakdown (Figure 2).

Thus, similar approaches, involving toxicomicrobiomics, culturomics and genomics, could be used going forward to explore the human GM as a source of beneficial microbes (NGPs), enzymes and bioactive compounds linked to MDC detoxification or biodegradation, with various potential applications under OH (Figures 1 and 3).

2.3.3. Gut microbiome and antimicrobial resistance under One Health

MDCs, such as triclosan and parabens, contribute to the AMR issue, primarily through resistance development against themselves (self-resistance), but also potentially through development of cross-resistance against antibiotics (Ribado et al., 2017). Although the evidence supporting cross-resistance development in situ is not conclusive (SCCS, 2010), the potential contribution of MDCs to AMR and their mechanisms merit further data compilation (Valkova et al., 2002; Hughes et al., 2020; Rozman et al., 2021). Moreover, given that antimicrobial MDCs would likely have higher potential to alter and perturb microbiomes (compared to non-antimicrobial xenobiotics), they have been proposed as candidate chemicals in investigations that would built our understanding around the xenobiotic-microbiome interactions in the context of xenobiotic RA (NASEM, 2018).

Nevertheless, even non-antimicrobial xenobiotics may pose resistance development issues in the context of the GM, as exposure to them may apply a selective pressure in favour of microbial taxa with specific enzymatic arsenals and metabolic pathways. For example, López-Moreno et al. (2022), associated BPA exposure and the obese phenotype in children to higher BPA biodegradation potential in their GM. Moreover, they reported that BPA-resistant strains isolated from human gut microbiota exhibited xenobiotic biodegradation and antimicrobial effects linked to polyketide biosynthesis (Torres-Sánchez et al., 2021). Therefore, in the presence of BPA, these strains may further modulate the composition and function of the human gut microbiota, potentially reducing GM diversity and inducing dysbiosis and adverse metabolic effects (Aguilera et al., 2020). The mechanisms, via which gut

microbiome taxa may be affected by non-antibacterial MDCs, potentially leading to dysbiosis, could include growth inhibition or promotion and metabolism modulation (Lindell et al., 2022). For example, several artificial sweeteners, spice extracts and food dyes have been shown to inhibit the growth of specific bacterial strains *in vivo*, while certain natural xenobiotics and food additives appear to promote the growth of other strains under similar conditions, likely acting as nutrient sources (Pan et al., 2012; de Bello González et al., 2016; Lu et al., 2017; Wang et al., 2018; Ruiz-Ojeda et al., 2019; Frame et al., 2020). Additionally, an alkaloid found naturally in coffee, trigonelline, has shown potential to alter the metabolism of a common human gut commensal in vivo (Anwar et al., 2018). Although limited, this evidence suggests that the potential for xenobiotic resistance development, in the context of the GM, may warrant further consideration and research, beyond antimicrobial resistance.

Overall, applying GM taxa and biocompounds able to metabolise antimicrobial MDCs to crosscutting areas under OH could potentially ameliorate AMR pressure (Figure 1).

3. Conclusions

The explored three areas of relevance to the GM in the context of OH open new avenues of research; the incorporation of the GM in RA of xenobiotics; the identification and application of beneficial GM components to various areas under OH, and specifically, in the context of AMR. This work took a first step with this combined approach and reached the following conclusions:

- Focusing on the GM's resilience circumvents some of the challenges. Moreover, looking at function, rather than composition, and exploring the active components of the GM can help establish biomarkers of health and disease, which is necessary for the incorporation of the GM in the RA of xenobiotics.
- The human GM may be a promising source of beneficial microbes (i.e. probiotics and NGPs), enzymes and bioactive compounds, with the potential to metabolise xenobiotics. These can be explored with toxicomicrobiomics and culturomics and may have potential applications in various areas under OH, e.g., as probiotics in animals or plants for xenobiotic detoxification or as xenobiotic biodegraders in environmental protection and bioremediation.
- This approach would be of particular interest for antimicrobials (such as triclosan or parabens), because applying relevant components isolated from the human GM to similar areas under OH could help ameliorate the risk of AMR development.
- Finally, the concept of resistance in the context of the GM could theoretically be extended from antimicrobials to xenobiotics, and the notion of xenobiotic resistance may warrant further consideration.

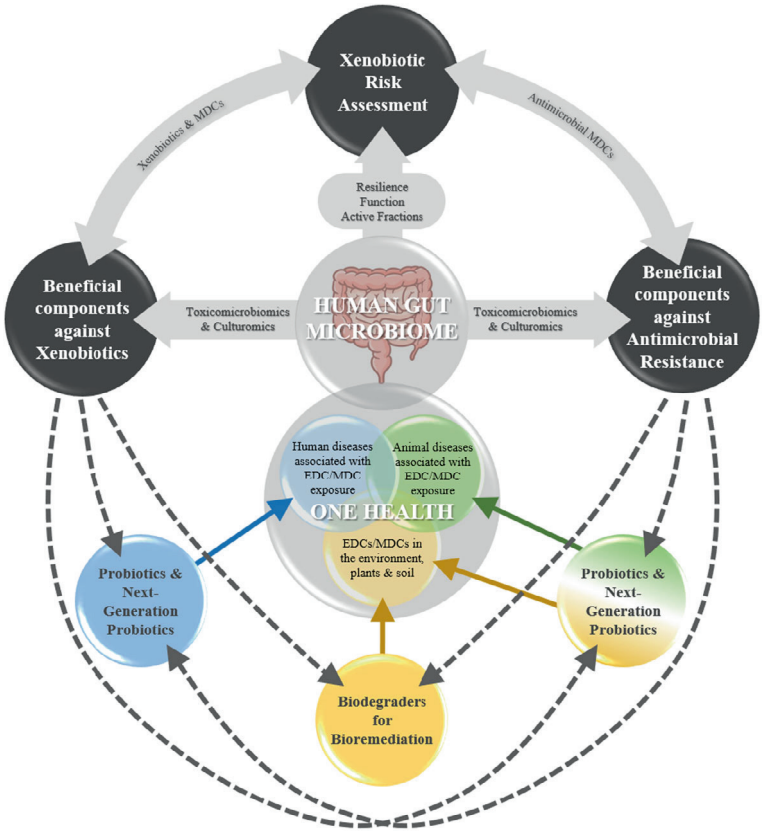

Figure 1: Three interlinked areas of relevance to the human gut microbiome (GM) in the context of One Health (OH); incorporation of the GM in food safety risk assessment of xenobiotics; identification and application of beneficial GM taxa and components (e.g., enzymes and bioactive compounds) to various areas under OH, and; specifically, in the context of antimicrobial resistance. EDC: endocrine disrupting chemicals, MDC(s): microbiota disrupting chemicals (Ampatzoglou et al., 2022)

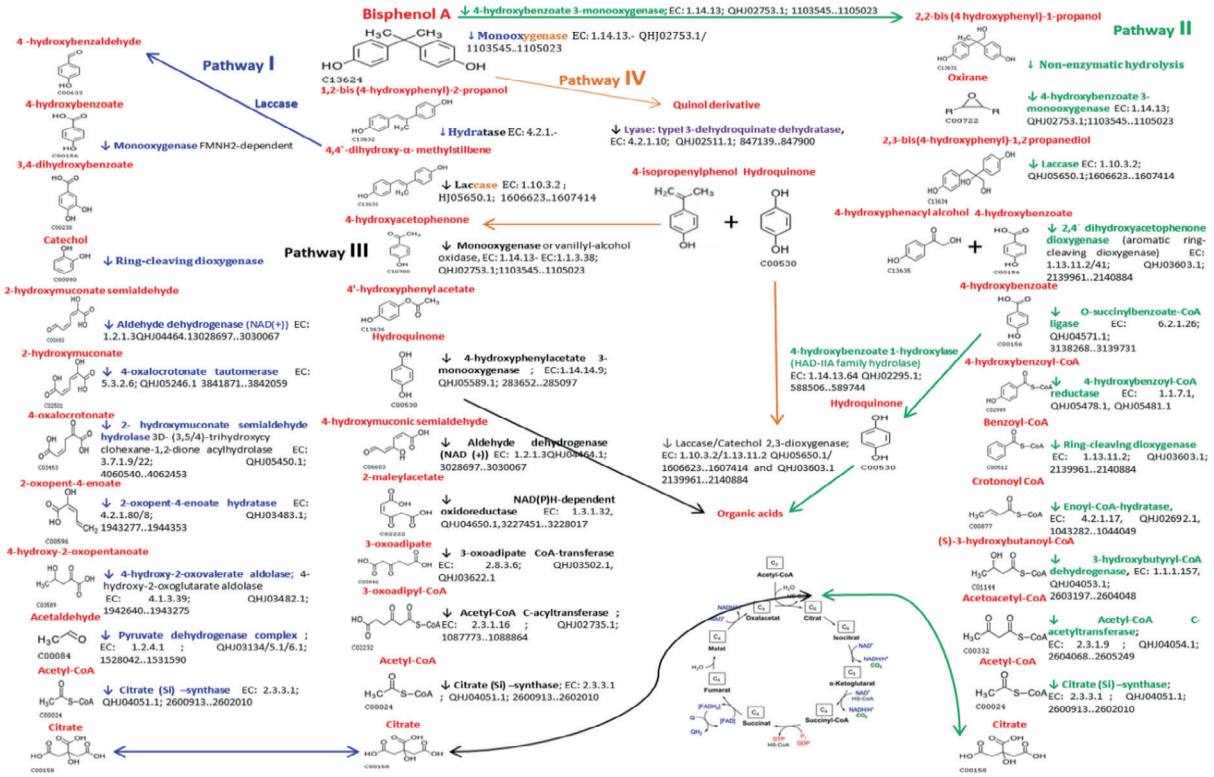

Figure 2: Proposed biodegradation pathways of bis-phenol A based on the whole-genome sequence analysis of *Bacillus* species AM1: reaction steps, enzymes, EC number, protein ID, and specific genes loci (with permission from López-Moreno et al., 2021b)

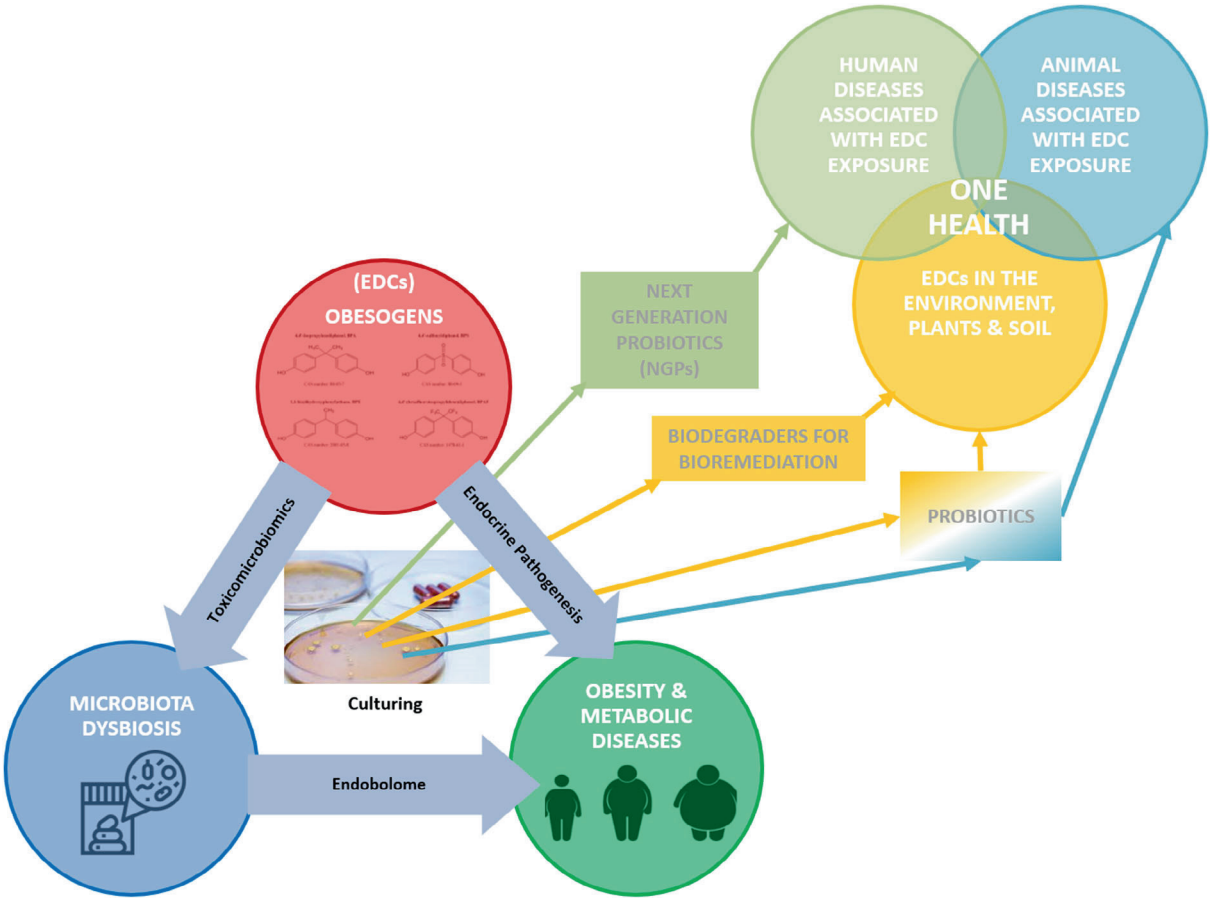

Figure 3: Next Generation Probiotics (NGPs), probiotics and biodegraders derived via culturing from the gut microbiota in the context of the triad micro-biome (dysbiosis vs eubiosis) – endocrine disrupting chemicals (EDCs)/xenobiotics/obesogens – human health (obesity vs normal weight) and their envisaged application in three areas under One Health (presented to the International e-Symposium on Probiotics, Prebiotics & Gut Microbiome: Key Regulators for Human & Animal Health, November 11, 2021, Ludhiana, India)

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Abbreviations

AMR	antimicrobial RESISTANCE
BPA	Bisphenol A
CAC	Codex Alimentarius Commission
CDC	Centers for Disease Control and Prevention
CIR	Cosmetic Ingredient Review Expert Panel
CNBBSV	Comitato Nazionale per la Biosicurezza, le Biotecnologie e le Scienze della Vita
EDCs	endocrine-disrupting chemicals
FCMs	food contact materials

GM	(human) gut microbiome
HoSU	History of Safe Use
INYTA	"José Mataix Verdú" Institute of Nutrition and Food Technology
MDCs	microbiota-disrupting chemicals
NASEM	National Academies of Sciences, Engineering, and Medicine
OH	One Health
RA	risk assessment
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
UGR	University of Granada
WGS	whole-genome sequence
WHO	World Health Organization

Appendix A – Additional activities undertaken by A. Ampatzoglou

The fellow joined the Biology and Biotechnology Group (BIO190, Halophilic Microorganisms and Environmental Bioremediation Research Group) at the hosting site (UGR), successfully applied to the UK Science Council for the Chartered Scientist (CSci) award, via his professional body, the Institute of Food Science and Technology (IFST) and attended the EU-FORA training modules and additional training opportunities. Salient examples are listed below.

- EU-FORA training modules by EFSA, the Austrian Agency for Health and Food Safety (AGES), the German Federal Institute for Risk Assessment (BfR) & the Hellenic Food Authority (EFET) on the following topics:
    - Data collection and reporting, 22–25 August 2022, online.
    - Emerging Risks, Nanomaterials, Omics in Risk Assessment & Risk Ranking, 6–10 Jun 2022, Athens, Greece.
    - Risk Perception, Risk Communication, Crisis Response & Media Training, 21–25 March 2022, Berlin, Germany and online.
    - Genetically Modified Organisms, Animal Health, Animal Welfare, Plant Health, Pesticides, Nutritional & Environmental Risk Assessment, 22–26 November 2021, Vienna, Austria and online.
    - EU Food Safety System & Legislation & a comprehensive overview of Microbiological & Chemical Risk Assessment (induction training), 30 August–17 September 2021, Parma, Italy and online.
- US Food and Drug Administration (FDA) and Alliance to Stop Foodborne Illness, Collaborating on Culture in the New Era of Smarter Food Safety, Food Safety Culture webinar series, including:
    - Building a coalition of food safety culture champions in your organisation, 18 May 2022
    - Making Leaders Risk Aware and Push to Reduce Risk, 16 February 2022.
    - Kick Off Meeting, 4 November 2021.
- BfR and Federal Office of Consumer Protection and Food Safety (BVL), Super(?)foods and Supplements – Risky or Healthy? 30 June–1 July 2022, online conference.
- EFSA, European Centre for Disease Prevention and Control (ECDC), European Chemicals Agency (ECHA), European Environment Agency (EEA), European Medicines Agency (EMA) & Joint Research Centre (JRC), ONE – Health, Environment, Society – Conference 2022, 21–24 June 2022, Brussels and online conference.
- Microbiome Virtual International Forum online webinar series, including:
    - Toward the development of defined microbial therapeutics, 16 June 2022.
    - Topic models for interpretable multidomain microbiome data, 9 February 2022.
- US FDA Grand Rounds online webinar, including:
    - One Health at FDA: From Concept to Application, 14 June 2022.
    - MinION Sequencing of Foodborne Pathogens, 14 April 2022.
- FoodSafety4EU EU Green Week Partner Event, How can we communicate food safety in the context of sustainable food systems? 1 June 2022, online event.
- The institute of Food Science and Technology (IFST), Spring Conference (SC22) – Minding the Gap; Communication, Skills and Technologies, 4–6 May 2022, online conference.
- The Frontiers Forum, The CRISPR health revolution, 31 March 2022, online webinar.
- EU-FORA training visit to the Spanish Agency for Food Safety & Nutrition (AESAN), covering Risk Assessment & the AESAN Scientific Committee, Risk Communication & Risk Management of Biological & Chemical Hazards, Nutritional Safety, Food Official Control & Alerts, 23–24 February 2022, Madrid.
- EU-FORA training visit to the Spanish National Centre for Food (CNA), covering Food Contact Materials, Food Processing Contaminants, Veterinary Drug Residues, Biotechnology, Microbiology & Antimicrobial Resistance, 25 February 2022, Majadahonda, Spain.
- University of Granada, Machine Learning and Big Data for Bioinformatics, 7 February–1 April 2022, massive open online course (MOOC).

- European Institute of Innovation and Technology (EIT) Food, The Human Microbiome, 24 January–11 February 2022, MOOC.
- EIT Food, The Future of Food Conference 2021, 30 November–1 December 2021, virtual event.
- Introduction to BioCyc for New Life Sciences Graduate Students and Post Docs, covering; Introduction to BioCyc; Smart tables and Comparative Analysis, and; Transcriptomics and Metabolomics Data Analysis, 3, 10 and 17 November 2021, online webinar series.
- Workshop OBEMIRISK-Knowledge platform for assessing the risk of Bisphenols on gut microbiota and its role in obesogenic phenotype: looking for biomarkers, 14–15 October 2021, Granada.

Annex A – Scientific output dissemination of EU-FORA fellowship project

The following outputs of this EU-FORA fellowship project have been published in scientific journals or communicated in scientific conferences.

A.1. Scientific papers

Torres-Sánchez A, López-Moreno A, Moreno A, Ortiz P, **Ampatzoglou A**, **Gruszecka-Kosowska A**, Ruiz-Rodríguez A, Monteoliva-Sánchez M, **Aguilera M**, 2022. Microbiome taxa and metabolite profiles altered in endocrine disorders or by xenobiotics and the counteraction with Next Generation Probiotics, International Journal of Molecular Sciences, review submitted for publication.

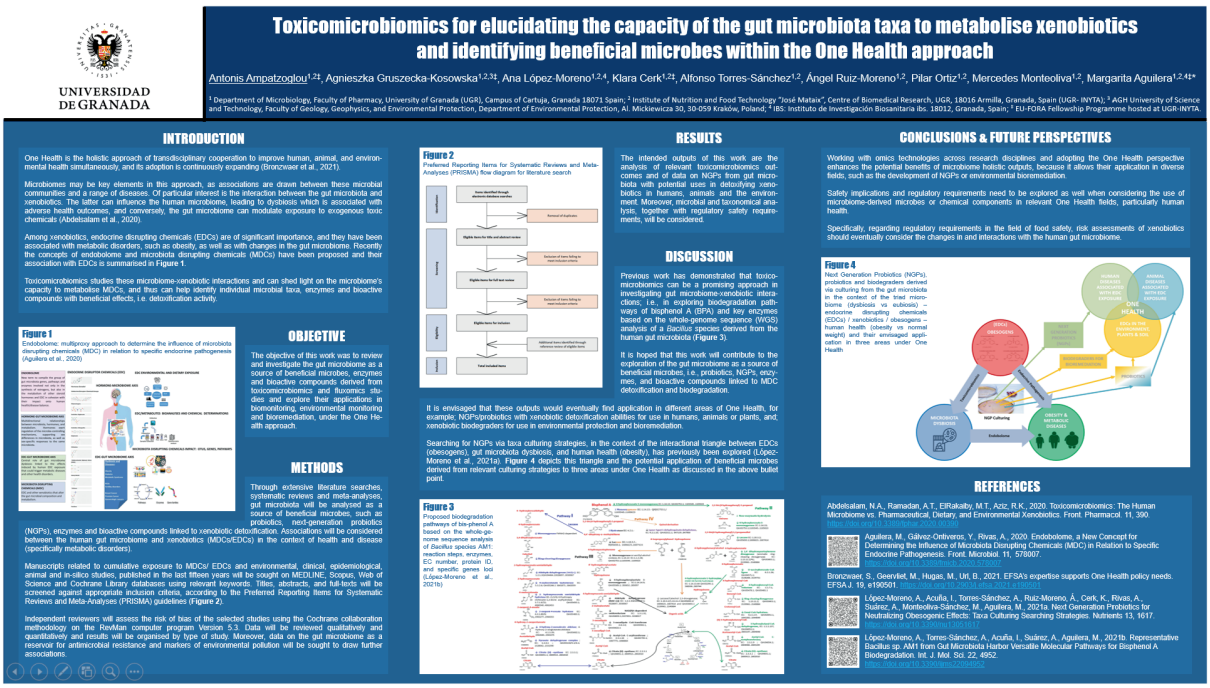
Gruszecka-Kosowska A, **Ampatzoglou A**, **Aguilera M**, 2022. Integration of Omics approaches enhances the impact of scientific research in environmental applications. International Journal of Environmental Research and Public Health, 19(14), 8,758. doi: 10.3390/ijerph19148758.

Ampatzoglou A, **Gruszecka-Kosowska A**, Torres-Sánchez A, López-Moreno A, Cerk K, Ortiz P, Monteoliva-Sánchez M, **Aguilera M**, 2022. Incorporating the gut microbiome in the risk assessment of xenobiotics & identifying beneficial components for One Health. Frontiers in Microbiology, 13, 872,583. doi: 10.3389/fmicb.2022.872583.

Ortiz P, Torres-Sánchez A, López-Moreno A, Cerk K, Ruiz-Moreno Á, Monteoliva-Sánchez M, **Ampatzoglou A**, **Aguilera M**, **Gruszecka-Kosowska A**, 2022. Impact of cumulative environmental & dietary xenobiotics on human microbiota: risk assessment for one health. Journal of Xenobiotics, 12(1), 56–63. doi: 10.3390/jox12010006.

A.2. Poster communications at scientific conferences

Moreno A, Ortiz P, López-Moreno A, Torres-Sánchez A, **Ampatzoglou A**, **Gruszecka-Kosowska A**, Ruiz-Rodríguez A, Monteoliva-Sánchez M, **Aguilera M**, 2022. Representación de taxones microbianos cultivables inducidos por exposición a xenobióticos en microbiota de niños. XIX Taxon, Reunión del Grupo de Taxonomía, Filogenia y Biodiversidad, October 13–15, 2022, Mallorca, Spain (abstract accepted).**Ampatzoglou A**, **Gruszecka-Kosowska A**, López-Moreno A, Cerk K, Torres-Sánchez A, Ruiz-Moreno A, Ortiz P, Monteoliva M, **Aguilera M**, 2021. Toxicomicrobiomics for elucidating the capacity of the gut microbiota taxa to metabolise xenobiotics and identifying beneficial microbes within the One Health approach. International e-Symposium on Probiotics, Prebiotics & Gut Microbiome: Key Regulators for Human & Animal Health, November 11, 2021, Ludhiana, India (**Best poster award received-presented by A. Ampatzoglou**).



A.3. Oral communication at scientific conference

Ampatzoglou A, **Gruszecka-Kosowska A**, Torres-Sánchez A, López-Moreno A, Cerk K, Ortiz P, Monteoliva-Sánchez M, **Aguilera M**, 2022. Exploring the incorporation of gut microbiome omics data in next-generation risk assessment of xenobiotics in foods. Next Generation Challenges in Food Microbiology, FoodMicro 2022, August 28–31, 2022, Athens, Greece (abstract accepted).

EU-FORA SERIES 5

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Microbiota analysis for risk assessment of xenobiotics: cumulative xenobiotic exposure and impact on human gut microbiota under One Health approach

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Abstract

Human gut microbiota is the microbial community that, through the constant bidirectional communication with its host, plays the critical role of maintaining the state of eubiosis and health balance, contributing to food digestion, detoxification, and proper endocrine, neurological, immunological and potentially reproductive health. To this extent, gut microbiota is called the 'second brain' as well as the 'second liver'. Xenobiotics, including environmental pollutants, are widely spread in the environment and easily accessible in food, cosmetics, personal care products, drugs and medicinal products. Thus, the gut microbiota can be exposed to these xenobiotics, which in turn might alter its composition and metabolism that can trigger dysbiosis, and they seem associated with disorders and diseases in the host. A specific group of xenobiotics, called endocrine-disrupting chemicals, is particularly important due to relevant adverse health effects. A considerable challenge in risk assessment is the combined exposure to xenobiotics, for which the integrated approaches, including the One Health concept, are still under development. Nevertheless, recent research advancements focus on molecular data in the search for elucidating crucial microbiome biomarkers, associated with physiopathology and specific dysfunctions triggered by xenobiotic exposure. In this context, the application of meta-omics and integration of genomics, metagenomics, metabolomics, metatranscriptomics, proteomics and multidisciplinary approaches are particularly important.

Keywords: One Health, xenobiotics, microbiota-disrupting chemicals, unintentional mixtures, combined exposure, environmental omics, farm to fork

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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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1. Introduction

The microbiota, human-associated microbes, including coexisting bacteria, archaea, bacteriophages, eukaryotic viruses and fungi (Fan and Pedersen, 2021) are estimated to be present in the number of trillions in the human body (Looi, 2020). The human gut microbiota, meaning microorganisms inhibiting human intestines, is estimated to contain up to 10^{14} microbial cells/g (Valdes et al., 2018), mostly commensal or mutualistic microorganisms (Fan and Pedersen, 2021), representing over 1,000 different species, many of them showing relevant functionalities (Aguilera et al., 2012). In addition, the human gut microbiome refers to the collective genomes of the microorganisms and their metabolites in the gastrointestinal tract (Valdes et al., 2018; Acharya et al., 2022).

1.1. Eubiosis and dysbiosis

Between the gut microbiome and the host, together creating the holobiont, there is a continuous and bidirectional communication, in which a variety of bioactive compounds and metabolites, synthesised by the gut microbiota, exert pleiotropic effects on the human organism (Ortega et al., 2022). The gut microbiome is responsible for training the host immune system, digesting food, regulating gut endocrine function and neurological signalling, modifying xenobiotic action through their metabolism, eliminating toxins and producing various compounds that affect the host (Fan and Pedersen, 2021). The healthy state of balance between the gut microbial ecosystem and the host is often dubbed intestinal homeostasis or eubiosis (Iebba et al., 2016; Perrotta, 2021; Al-Rashidi, 2022).

In contrast, imbalances in the composition and function of these intestinal microbes are called dysbiosis and are associated with diseases ranging from localised gastroenterological disorders to neurologic, respiratory, metabolic, hepatic and cardiovascular illnesses (Lynch and Pedersen, 2016), like depression, anxiety, asthma, obesity, type 2 diabetes, cardiovascular diseases, inflammatory bowel conditions and autoimmune disorders, type 1 diabetes, rheumatoid arthritis (Acharya et al., 2022), and reproductive health and fertility disorders (López-Moreno and Aguilera, 2020; López-Moreno and Aguilera, 2021). The eubiosis of the human microbiome is individualised and depends on many endogenous and exogenous factors like prenatal dietary factors, mode of delivery during birth, infant breast-feeding or formula-feeding, diet, lifestyle, host genetic features, host immune response, xenobiotics, drugs, antibiotics and environmental microbial exposures (Lynch and Pedersen, 2016; Al-Rashidi, 2022). What is more, gut microbial communities differ between males and females across species, including mice, rats and humans (Acharya et al., 2022), as well as between various life stages (Al-Rashidi, 2022). Furthermore, states of dysbiosis and the gut microbiota's reduced diversity may impact and modify the endobolome, which in the long term may lead to the development of certain pathophysiological conditions (Aguilera et al., 2020). This is because the endobolome encompasses 'the group of gut microbiota, genes, pathways and enzymes involved not only in the synthesis of estrogens, but also in the metabolism of other steroid hormones and endocrine disruptor chemicals in cohesion with their impact onto human health/disease balance' (Aguilera et al., 2020).

1.2. Endocrine and metabolic pathogenesis

The endocrine system regulates different biological processes, including development, growth and reproduction, as well as systems, e.g. gastrointestinal, cardiovascular and renal, and the body's response to acute and chronic stress (Walling and Rosol, 2019). After observing that stress-induced neuroendocrine hormones can influence bacterial growth by Lyte and Ernst in 1992, research in microbial endocrinology began (Neuman et al., 2015). Today, it is well-demonstrated that the microbiome regulates endocrine systems and influences many aspects of hormone signalling (Williams et al., 2020). The gut microbiome is suggested to serve as a virtual endocrine organ since it can produce and regulate multiple compounds that reach and influence distal organs and systems, such as the nervous, endocrine and immune system (Garcia-Reyero, 2018). Moreover, it is considered a major 'virtual' organ or even a system in the organism, with many connections, functions and influences at different levels (Garcia-Reyero, 2018). Recent studies linking hormones and the gut microbiota, indicated that hormones regulated by the microbiota span all functional classes and exert broad influences on host behaviour, metabolism, appetite, growth, reproduction and immunity (Neuman et al., 2015).

Probiotics are reported to have a significant interventional role in maintaining the state of eubiosis in the host gut microbiome (López-Moreno et al., 2020; Perrotta, 2021; López-Moreno et al., 2021a). They are defined as 'live and vital microorganisms that confer health benefits on the host when

consumed, in adequate quantities, as part of a food or supplement' (Perrotta, 2021). Traditional probiotics have been demonstrated to have a positive impact on many disorders or diseases, however, the health effect is related to specific strains or species, mainly with *Lactobacillus* and *Bifidobacterium* genera (López-Moreno et al., 2021a). It has also been demonstrated that gastrointestinal tract (GIT) disorders linked with microbiota alterations can be treated with probiotics (López-Moreno et al., 2021a). In addition, new scientific approaches in high-throughput and -omics technologies allowed the identification and characterisation of new microorganisms called next-generation probiotics (NGPs), which combine the basic definition of a probiotic with better elucidation of the relation to target specific diseases and clinical outcomes (López-Moreno et al., 2021a).

1.3. Xenobiotics

Recently, the interactions between xenobiotics and the gut microbiome have gained significant scientific attention, including related adverse health effects on the host (Sutherland et al., 2020). Xenobiotics are defined as chemical substances not occurring naturally in the environment of living organisms. They are generally considered synthetic substances, but the term may also be used for naturally occurring chemicals when present in higher-than-usual concentrations, or produced by certain organisms as a defence mechanism, for example fungi, bacteria and herbs (Abdelsalam et al., 2020). In terms of metabolism, xenobiotics can be defined as chemicals that are extrinsic to the natural metabolism of a living organism (Abdelsalam et al., 2020). What is more, a variety of chemical compounds and environmental pollutants present in foods and medications are currently considered as xenobiotics for the human body (Koppel et al., 2017).

Among xenobiotics, a specific group of endocrine-disrupting chemicals (EDCs) stand out, because of their adverse impact on specific aspects of human health related with the endogenous hormone system, including hormone production, release, transport, metabolism, binding, action or elimination (Cerk and Aguilera-Gómez, 2022). The definition provided by the Endocrine Society describes an EDC as an 'exogenous (non-natural) chemical or a mixture of chemicals, that interferes with any aspect of hormone action' (Gálvez-Ontiveros et al., 2020). EDCs comprise various classes of chemicals, including heavy metals (e.g. lead, cadmium, arsenic), pesticides (e.g. atrazine and dichlorodiphenyldichloroethylene [DDT]), plasticisers (e.g. bisphenol A [BPA], parabens, polyvinyl chloride [PVC], phthalates), synthetic hormones (e.g. ethinylestradiol [EE] and diethylstilbestrol [DES]), and per- and polyfluoroalkyl substances (PFAS). Due to their wide usage in food packaging, building materials, cleaning products, personal care products, pharmaceuticals, and generally in the industry and agriculture (Acharya et al., 2022; Cerk and Aguilera-Gómez, 2022; Ortiz et al., 2022), exposure occurs via many parallel pathways. In addition, the term microbiota-disrupting chemicals (MDCs) was proposed by Aguilera et al. (2020) to refer to these EDCs and other xenobiotics that alter the gut microbial composition and its metabolic capacities, in order to distinguish the role of contaminants from natural microbiota modifiers such as those contained or released from diet, environment, physical activity and stress.

In addition, research on human exposure to xenobiotics also involves the impact of the surrounding environment, which is consistent with the concept of the exposome. According to various definitions, the term exposome refers to 'the totality of exposures from a variety of external and internal sources including chemical agents, biological agents, radiation, and more general exposures and determinants from conception onward, over a complete lifetime' (Vineis et al., 2020). Thus, based on this definition, the exposome includes biologically active chemicals induced by both the external and internal chemical environments (Vineis et al., 2020).

2. Description of work programme

2.1. Aims

The fellowship programme was performed in consonance with the legal framework for the EUropean FOod Risk Assessment (EU-FORA) Fellowship Programme and its objectives of 'promoting and coordinating the development of uniform risk assessment methodologies in the fields falling within the European Food Safety Authority (EFSA) mission'.

This technical report describes the EU-FORA Fellowship work programme entitled 'Microbiota analysis for risk assessment of xenobiotics and its potential impact on dysbiosis and endocrine pathogenesis: microbiota learning by doing' that was performed during Series 5, cycle 2021–2022.

The fellowship was held in the Faculty of Pharmacy, University of Granada (UGR) and "José Mataix Verdú" Institute of Nutrition and Food Technology (INYTA – UGR). Prof. Margarita Aguilera-Gómez was the dedicated supervisor for managing and monitoring the progress of the programme's deliverables and outcomes. During weekly meetings, both with the whole research team and the supervisor with the fellows, information and knowledge were shared, and discussions were performed to achieve planned outcomes on the programme's timeline. Moreover, specialists from the research team members were engaged to facilitate gaining solid knowledge and implementing specific parts of the programme.

2.2. Activities

The work programme was focused on harmonising and exchanging methodologies that could enlarge and enrich European food risk assessment practice, especially concerning the One Health (OH) approach. During the fellowship, the hosting site provided broad and specialised theoretical background in the scientific topics of the work programme to the fellows. Moreover, the fellows during the programme were involved in the three ongoing projects of the team. The common topic of the research projects was dietary exposure to xenobiotics, especially EDCs, and related xenobiotic-gut microbiota interactions and host health effects:

1) EFSA Partnering Grant (2019–2021): 'OBEMIRISK - Knowledge platform for assessing the risk of bisphenols on gut microbiota and its role in obesogenic phenotype: looking for biomarkers', 2019–2021.
2) INFRAESTRUCTURE Project: Human microbiota reference laboratory: estación de manipulación de microorganismos en anaerobiosis y accesorios para la optimización y armonización del análisis fenotípico y genotípico de la microbiota y su impacto en nutrición y salud, 2020–2022.
3) Fondo de Investigación en Salud (FIS) Institute Carlos III ENDOMICROMICS Project: 'Influence of endocrine disrupters chemicals on gut microbiota: a missed link in childhood obesity', 2021–2023.

As described in the research works of Aguilera et al. (2020, 2022), the necessary future of analysing substances and factors that affect human microbiota eubiosis/dysbiosis is facilitated by interactions among distinct scientific disciplines. Such interdisciplinary cooperation has a pivotal role in explaining mechanisms leading to various diseases, disorders and dysbiosis caused by dietary exposure to toxic compounds. These interdisciplinary approaches are critical for understanding the relationship between xenobiotics, gut physiological status and the microbiome, together with the interactions between the latter and various axes. In nutrition, these concepts are expected to define the impact of individual risks of exposure (the exposome) in the biology of individuals (genomics, epigenomics). Metagenomic approaches in food risk assessment are expected to help address the challenges in dysbiosis causality, e.g. causally linking obesity modulators with gut microbiota imbalance and the obese phenotype through exposure to chemical obesogens.

Furthermore, the application of the genomic approach is expected to greatly contribute to the search of non-invasive biomarkers in the form of microbiome data, which is the ultimate objective of this biomedical research. MDCs might promote specific changes in the microbiota that can ultimately cause intestinal and chronic or long-term systemic diseases in the host.

Thus, for establishing links between the triad of MDCs, microbiota dysbiosis and host disease, both the effects of the MDCs on the gut microbiota and the impact of microbiota metabolism of MDCs on host susceptibility should be investigated.

2.3. Outcomes

The following sections are based on the research outcomes achieved by the team during the fellowship programme. These have been published in scientific journals, as well as presented at scientific conferences. The list of the scientific contributions is presented in **Appendix A**.

2.3.1. Impact of diet exposure to EDCs on human gut microbiota

Microbial and molecular dysbiosis in the human gut microbiota has been related to the cumulative exposure to a wide variety of xenobiotic substances that act as EDCs through microbiota interaction and inducing obesogenic phenotypes (López-Moreno et al., 2022). In the last decade, EDCs were

revealed to also behave as obesogens, altering the endocrine system and leading to the onset of obesity (Stecca et al., 2022). EDCs are common in foods after their processing, packaging, transportation, and storage and prolonged exposure to EDCs might affect human health by triggering obesity, insulin resistance, metabolic syndrome and even infertility (Aguilera et al., 2021; Robles-Aguilera et al., 2021). Human exposure to EDCs with obesogenic effects during early life may disrupt neuroendocrine-mediated processes that are critical for growth, energy metabolism, appetite control, adipogenesis and glucose–insulin regulation, thereby increasing the risk of childhood obesity (Aguilera et al., 2022). In their research on the role of EDCs in children's neurodevelopment, Ramírez et al. (2022a) stated that postnatal EDC exposure is related to adverse neurobehavioral outcomes in children, however, the underlying mechanisms of action remain unclear.

EDCs encompass a variety of chemical groups. BPA is demonstrated to have obesogenic properties, just as and its analogues: bisphenol B (BPB), bisphenol S (BPS), bisphenol F (BPF) and bisphenol E (BPE), due to the same basic chemical structure (Robles-Aguilera et al., 2021). BPA is one of the most common EDCs found in the environment (Ramírez et al., 2021). Parabens, methyl- (MetPB), ethyl- (EthPB), propyl- (PropPB), buthyl- (ButPB) parabens, that are especially used in cosmetics, pharmaceuticals, foodstuffs and beverages as antimicrobials and in food products for preservation purposes, have been associated with metabolic, endocrine, neurological and hormonal diseases (Monteagudo et al., 2021; López-Moreno et al., 2021b). Even at low concentrations, chronic exposure to EDCs represents a toxicological concern, with the risk significantly increasing when it comes to additives in or leaching of plasticisers into children's food (García-Córcoles et al., 2018).

Among other EDCs, phthalates have been investigated to induce obesogenic and adverse reproduction system effects and modify key glycaemic parameters. Perchlorates, organophosphates and other pesticides stimulate symptoms of diabetes and obesity (López-Moreno et al., 2021b).

Investigations of Gálvez-Ontiveros et al. (2021) on the occurrence of parabens and bisphenols in food commonly consumed in Spain revealed that these xenobiotics were frequently present in analysed foods. Among parabens and bisphenols, MetPB and BPA were the most frequently detected chemicals, respectively. The estimated dietary exposure to individual bisphenols and parabens did not exceed the tolerably daily intakes (TDIs) established by EFSA; however, the cumulative effect was not assessed.

Results of Robles-Aguilera et al. (2021) investigations on adolescents from Spain revealed the positive association between total bisphenols and BPA dietary exposure and body mass index (BMI) suggesting that exposure to their even low concentrations could be related to health effects. The same study revealed gender-related differences in the association between BPA exposure and obesity. In addition, investigations of Monteagudo et al. (2021) on estimated dietary exposure to parabens and BMI in Spanish adolescents according to gender showed positive associations between dietary exposure to parabens and overweight/obesity in girls.

Research of Ramírez et al. (2022b) on the effects of genetic polymorphisms on BMI in relation to dietary exposure to bisphenols and parabens indicated that individuals highly exposed to these two groups of xenobiotics and carriers of risk G allele at LEPR rs9436303 were significantly more likely to have elevated BMI.

Investigations by López-Moreno et al. (2022) revealed that specific and differential gut enriched microbial isolates or consortia that resist, tolerate or biodegrade BPA were present in human-associated microbial communities, and they harboured specific genes encoding enzymes involved in biodegrading BPA and other obesogens.

Research by Torres-Sánchez et al. (2022) suggested that the human gut microbiota might be able to modulate the host metabolome and affect its homeostasis. In the same studies, the authors indicated the importance of considering the role of the cumulative exposure to xenobiotics on the host organism in the research on human gut microbiota, since they appear to be important disturber and determinant of altered homeostasis in humans and animals.

2.3.2. Cumulative exposure to xenobiotics under One Health

For establishing interactions between xenobiotics and the gut microbiota large-scale human studies are necessary. However, these are challenging because of various confounding factors, which are difficult to control, like lifestyle, diet or medication intake (Lindell et al., 2022). Translation of findings from animal models to humans may be difficult, as animals differ in their physiological responses to xenobiotics, microbiota composition and specifics of host–microbiota interactions, as well as it is challenging to establish causal links between xenobiotics and microbiota and pinpoint underlying mechanisms (Lindell et al., 2022).

Although there are three main exposure pathways, oral intake is the primary route regarding various xenobiotics entering the human body (Nogacka et al., 2019). However, it is not only about food consumption. Environmental pollutants might also be ingested with water or even air pollutants inhaled (i.e. particulate matter (PM)), and as a consequence alter gut microbial composition and abundance (Acharya et al., 2022). Moreover, over the decades, substantial modifications have been introduced in food production and processing, lifestyle, family structure and the environment that could directly affect the gut microbiome (Acharya et al., 2022).

Since it all starts with soil in the food chain, its health has become one of the most important topics globally. For this reason, the European Union (EU) have emphasised numerous actions for protecting soil health, including the: European Green Deal; Soil Deal Mission; Farm to Fork Strategy; Climate Adaptation Strategy; EU Soil Strategy; EU Biodiversity Strategy for 2030; Forest Strategy; Organic Action Plan; Zero Pollution Action Plan for air, water and soil; long term vision for EU's rural areas; communication on food security and resilience of food systems.

What is often overlooked is that human activity in industrial and urbanisation processes causes significant environmental pollution. As all environmental compartments are linked with each other, soils might be the sinks for all types of chemicals. Agricultural soils are currently under particular concern due to two reasons. First, their area is decreasing due to factors like erosion, desertification or progressive urbanisation. Second, the quality of existing agricultural soils is decreasing due to, for instance, intensive farming practices that introduce a variety of chemicals to them. And, thus, the risk arises that these xenobiotics might finally end up in our plates (Ortiz et al., 2022).

For example, EFSA considers the presence of residues of veterinary medicinal products and certain substances in live animals and animal products (meat, egg, milk, honey) in the EU, Iceland and Norway. In 2020 it was reported that among 331,789 targeted samples, 0.27% were non-compliant with EU regulations and exceeded the permissible level of xenobiotics, such as antithyroid agents, steroids, beta-agonists, antibacterial products, other veterinary drugs and environmental contaminants (EFSA, 2022a). The highest percentage (3.71%) of non-compliant samples in the group 'other substances and environmental contaminants' was found for the subgroup 'chemical elements' (B3c) with copper, cadmium, total mercury and lead being the most frequent elements causing non-compliance (EFSA, 2022a). Importantly, non-compliance might result from the presence of one or more substances in the food sample at the same time.

Defining the human risk related to the cocktail effect of unintentional mixtures of xenobiotics in the farm-to-fork chain is still a challenge for scientists. Adoption and integration of new approach methodologies (NAMs) into the next generation risk assessment (NGRA) is still under development as animal studies become less relevant with time and integrated approaches to testing and assessment (IATAs) are required (Escher et al., 2022). However, the vision for 2030 is to develop and implement a harmonised approach for the assessment of human health risks resulting from both dietary and non-dietary exposure to multiple chemicals (EFSA, 2022b).

Since the term OH defines the holistic approach for simultaneously improving human, animal and environmental health through transdisciplinary cooperation (Bronzwaer et al., 2021; Buschhardt et al., 2021; Ampatzoglou et al., 2022), the human gut microbiome needs to also be included as an essential element of the NGRA approach as suggested in Figure 1 (Ortiz et al., 2022).

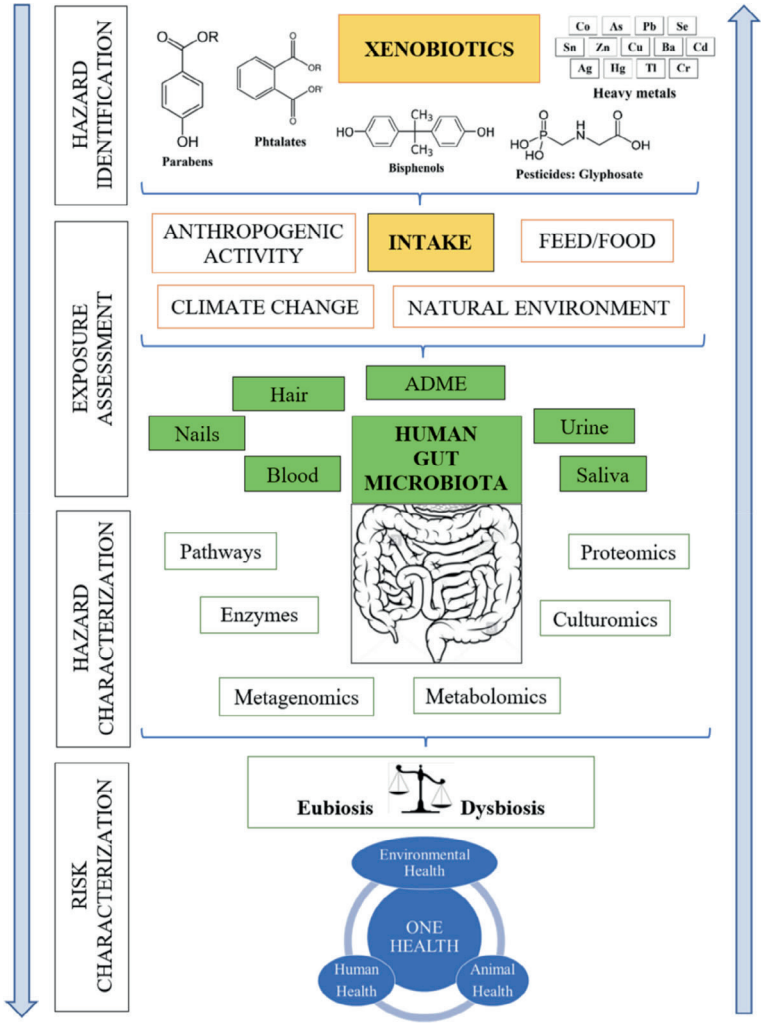

Figure 1: Graphical interactions between xenobiotics and human gut microbiome under One Health approach (Ortiz et al., 2022)

Omics	**•Techniques to study biological molecules for characterizing structure, function, and dynamics of organism(s)**
Genomics	•Study of DNA sequences to predict properties of (micro)organisms
Transcriptomics	•Study of RNA transcripts in organism(s)
Proteomics	•Study of set of proteins produced in organism(s)
Metabolomics	•Study of metabolites in cells, biofluids, tissues, and organism(s)
Lipidomics	•Study of cell lipids in biological system(s)
Phenomics	•Study of environment and individual lifestyle on gene expression
Epigenomics	•Study of epigenetic modifications across cell(s) and organism(s)
Fluxomics	•Study of the rates of metabolic reactions in organism(s)
Ionomics	•Study of elemental composition of organism(s) on biological response
Meta-omics	•Study of metagenomes from several organisms
Multi-omics	•Usage of multiple omics approaches
Integromics	•Study of data-integration

Figure 2: Omics techniques to use in cumulative xenobiotics exposure for environmental research (Gruszecka-Kosowska et al., 2022)

As xenobiotics may alter the microbiota composition, leading to a state of dysbiosis, adverse health outcomes like increased toxicity of some xenobiotics or multiple diseases, it is important to realise the different nature of these bidirectional interactions (Ampatzoglou et al., 2022; Ortiz et al., 2022); individual gut microbiome components might be negatively affected by several contaminants or xenobiotics with pathophysiological impact through triggering microbial composition disequilibrium; gut microbiota could protect against xenobiotics by degrading or biotransforming them to less harmful compounds or facilitating their excretion; gut microbiota may detoxify xenobiotics or may reverse the detoxification caused by host metabolism; gut microbiota is capable of transforming xenobiotics towards lower toxic and mutagenic substances, decreasing the chances of dysbiosis effects. As various classes of xenobiotics might alter the composition of the gut microbiota, it stresses the need to include microbiota-mediated impacts on health status in the risk assessment (Lindell et al., 2022).

For example, specific transitory gut taxa identified with high potential for BPA biodegradation might be used for environmental bioremediation purposes or as plant probiotics; BPA could be removed from soil using bacterial strains of *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella* sp. and *Pantoea* sp.; degradation of BPA by *Pseudomonas putida* YC-AE1 and consortium of *Terrimonas pekingensis* and *Pseudomonas* sp. isolated from river sediment; gut bacteria harbouring laccases used for detoxification of several hazardous dietary contaminants and EDCs through a bioreactor with novel biocatalytic system based on active membranes and immobilised laccase technology (López-Moreno et al., 2021c).

Because cumulative exposure to xenobiotics and overall outcome might enormously impact health research, interactions among distinct scientific disciplines as microbiology, nutrition, toxicology, environmental epidemiology and personalised medicine, are required (Ortiz et al., 2022). Moreover, multiple technologies, including omics technologies (Figure 2), bioinformatics and machine learning, when applied in this multidimensional research, might lead to a paradigm shift in understanding mechanisms of diseases and associated exposure factors (Gruszecka-Kosowska et al., 2022). Genomics, metagenomics, metabolomics, metatranscriptomics, proteomics and multidisciplinary approaches are considered crucial in scientific research for characterising the function, metabolism and composition of microbiomes in relation to environmental sciences (Gruszecka-Kosowska et al., 2022). Moreover, as environmental and dietary exposure and the related interaction with host genetic factors may have an essential role in common chronic diseases, the latest achievements in research technology have already allowed exposome to become a novel research paradigm in biomedical sciences (Gruszecka-Kosowska et al., 2022).

Based on the current scientific research, xenobiotic absorption, distribution, metabolism and excretion (ADME) processes involve genes, enzymes and pathways both of human and human microbiota origin. Moreover, innovative microbiota biocomponents and functional analyses could contribute to increase the metabolites, analytes and enzymatic repertoire beyond the microbial taxonomic principal components analyses that were widely used in studies of the microbiome and toxicant exposures. Thus, the future of this interdisciplinary research will focus on molecular data in the search for microbiome biomarkers associated with diseases and dysfunctions triggered by xenobiotic exposures (Ortiz et al., 2022).

2.3.3. Additional trainings and activities

During the one-year fellowship, Agnieszka Gruszecka-Kosowska attended the five EU-FORA risk assessment training modules. The modules were organised by the European Food Safety Agency EFSA (Italy), the Austrian Agency for Health and Food Safety AGES (Austria), the German Federal Institute for Risk Assessment BfR (Germany) and the Hellenic Food Authority EFET (Greece). The EU-FORA training modules provided the latest knowledge and practical training on current topics related to food safety.

- *Data Collection and Reporting*, 22–25 August 2022, online.
- *Emerging Risks, Nanomaterials, Omics in Risk Assessment and Risk Ranking*, 6–10 June 2022, Athens, Greece.
- *Risk Perception, Risk Communication, Crisis Response and Media Training*, 21–25 March 2022, online.
- *Genetically Modified Organisms, Animal Health, Animal Welfare, Plant Health, Pesticides, Nutritional and Environmental Risk Assessment*, 22–26 November 2021, online.
- *EU Food Safety System, Legislation, Microbiological and Chemical Risk Assessment* (induction training), 30 August – 17 September 2021, online.

Additional trainings during the EU-FORA fellowship programme were based on the fellow's background and professional interests. These are listed in the **Appendix B**.

During the fellowship the fellow joined the *BIO-190: Biology and Biotechnology. Halophilic Microorganisms and Environmental Bioremediation* research group in the University of Granada and Junta de Andalucía.

The fellow initiated the inclusion procedure of the fellow's parent organisation the AGH University of Science and Technology in Krakow (AGH-UST) on the EFSA Art. 36 Competent Organisation list.

3. Conclusions

Based on the research activities performed during the fellowship programme the following conclusions were formulated:

- As all environmental compartments, abiotic and biotic, are interrelated through various pathways, the need is underlined to incorporate the OH approach in the risk analysis.
- As unintended mixtures constitute the reality of exposure, the cumulative risk assessment approach, emphasises the necessity of focusing on xenobiotics cocktails in investigating the mechanisms of adverse health effects.
- As various xenobiotics impact and alter the composition and metabolism of the gut microbiota, the need to include microbiota-mediated impacts on health in the risk assessment, becomes increasingly more imminent.
- As the food chain starts with soil, the requirement to include environmental sciences, and environmental epidemiology in the food safety analysis is increasingly gaining more traction.
- Since the exposome represents the totality of external exposures, it induces the need to incorporate omics techniques like genomics, metagenomics, metabolomics, metatranscriptomics, proteomics and multi-omics integration and the requirement to overcome discipline-specific silos, in the pursuit of ground-breaking knowledge.

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Abbreviations

ADME	absorption, distribution, metabolism and excretion
AGES	Austrian Agency for Health and Food Safety
AGH-UST	AGH University of Science and Technology in Krakow
BfR	German Federal Institute for Risk Assessment
BIO-190	Biology and Biotechnology. Halophilic Microorganisms and Environmental Bioremediation Research Group
BMI	body mass index
BPA	bisphenol A
BPB	bisphenol B
BPE	bisphenol E

BPF	Bisphenol F
BPS	bisphenol S
ButPB	buthylparaben
DDT	dichlorodiphenyldichloroethylene
DES	diethylstilbestrol
EDCs	Endocrine-disrupting chemicals
EE	ethinylestradiol
EFET	Hellenic Food Authority Hellenic Food Authority
EthPB	ethylparaben
EU-FORA	EUropean FOod Risk Assessment
GIT	gastrointestinal tract
IATAs	Integrated Approaches to Testing and Assessment
INYTA	"José Mataix Verdú" Institute of Nutrition and Food Technology
MDCs	microbiota-disrupting chemicals
MetPB	methylparaben
NAMs	new approach methodologies
NGPs	next generation probiotics
NGRA	next generation risk assessment
OH	One Health
PFAS	per- and polyfluoroalkyl Substances
PM	particulate matter
PropPB	propylparaben
PVC	polyvinyl chloride
TDI	tolerably daily intake
UGR	University of Granada

Appendix A – Scientific outcomes of EU-FORA fellowship programme

A.1 Scientific papers

Torres-Sánchez A., Lopez-Moreno A., Moreno A., Ortiz P., **Ampatzoglou A.**, **Gruszecka-Kosowska A.**, Ruiz-Rodríguez A., Monteoliva-Sánchez M., **Aguilera M**. 2022. **Microbiome taxa and metabolite profiles altered in endocrine disorders or by xenobiotics and the counteraction with Next Generation of Probiotics, *International Journal of Molecular Sciences*, review** submitted for publication.

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A.2 Poster presentations at scientific conferences

Moreno M.A., Ortiz P., López-Moreno A., Torres-Sánchez A., **Ampatzoglou A.**, **Gruszecka-Kosowska A.**, Ruiz-Rodríguez A., Monteoliva-Sánchez, **Aguilera M**. 2022. Representación de taxones microbianos cultivables inducidos por exposición a xenobióticos en microbiota de niños. XIX Reunión del Grupo de Taxonomía, Filogenia y Biodiversidad, 13–15 October 2022, Mallorca, Spain.

Ampatzoglou A., **Gruszecka-Kosowska A.**, Torres-Sánchez A., López-Moreno A., Cerk K., Ortiz P., Monteoliva-Sánchez M., **Aguilera M**. 2022. Exploring the incorporation of gut microbiome omics data in next-generation risk assessment of xenobiotics in foods. Next Generation Challenges in Food Microbiology, FoodMicro 2022, 28–31 August 2022, Athens, Greece.

Ampatzoglou A., **Gruszecka-Kosowska A.**, López-Moreno A., Cerk K., Torres-Sánchez A., Ruiz-Moreno A., Ortiz P., Monteoliva M., **Aguilera M**. 2021. Toxicomicrobiomics for elucidating the capacity of the gut microbiota taxa to metabolise xenobiotics and identifying beneficial microbes within the One Health approach. International e-Symposium on Probiotics, Prebiotics & Gut Microbiome: Key Regulators for Human & Animal Health, 11 November 2021, Ludhiana, India (**Best poster award received**).

Cerk K., López-Moreno A., Torres-Sánchez A., Ruiz-Moreno Á., Ortiz P., **Ampatzoglou A.**, **Gruszecka-Kosowska A.**, **Aguilera M**. 2021. *Safety assessment of Bacillus sp. AM1 isolated from human gut microbiota, with the ability to metabolize dietary endocrine disruptors, as potential product used in food production chain*. Poster presentation on the 35th European Federation of Food Science and Technology (EFFoST) International Conference, 1–4 November 2021, Lausanne, Switzerland.

López-Moreno A., Ruiz-Moreno Á., Pardo J., Cerk K., Torres-Sánchez A., Ortiz P., **Ampatzoglou A.**, **Gruszecka-Kosowska A.**, **Aguilera M**. 2021. Bisphenol A directed-culturing for human gut microbiota taxa metabolizing dietary obesogens. Poster communication in the Workshop OBEMIRISK – Knowledge platform for assessing the risk of Bisphenols on gut microbiota and the role in obesogenic phenotype: looking for biomarkers, 14–15 October 2021, Granada, Spain.

Appendix B – Additional training during the EU-FORA fellowship programme undertaken by Agnieszka Gruszecka-Kosowska

Additional training during the EU-FORA fellowship programme was based on the fellow's background and professional interests:

US FDA Grand Round: *One Health at FDA: From Concept to Application*. U.S. Food and Drug Administration, 14 July 2022, online webinar.

Super(?)foods and Supplements - Risky or Healthy?, 30 June – 1 July 2022, German Federal Institute for Risk Assessment (BfR) and Federal Office of Consumer Protection and Food Safety (BVL) Conference, Berlin, online participation.

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), European Chemicals Agency (ECHA), European Environment Agency (EEA), European Medicines Agency (EMA) and Joint Research Centre (JRC), *ONE – Health, Environment, Society – Conference 2022*, 21–24 June 2022, Brussels, online participation.

FoodSafety4EU EU Green Week Partner Event. *How can we communicate food safety in the context of sustainable food systems?*, 1 June 2022, online.

The diverse roles of human and animal gut microbiota in health and disease, Microbiome Webinar 2022, 18 May 2022, online.

Dish cluster. Towards healthy and safe diet, FoodSafety4EU, 6 April 2022, online event.

EIT Food: *Farm to Fork: Sustainable Food Production in a Changing Environment*, online course.

EFSA/RIVM training: *Risk of combined exposure to multiple chemicals (mixture risk assessment)*, 21–24 February 2022, online course.

EU-FORA training visit to the Spanish Agency for Food Safety and Nutrition (AESAN), covering Risk Assessment and the AESAN Scientific Committee, Risk Communication and Risk Management of Biological and Chemical Hazards, Nutritional Safety, Food Official Control and Alerts, 23–24 Feb 2022, Madrid.

EU-FORA training visit to the Spanish National Centre for Food (CNA), covering Food Contact Materials, Food Processing Contaminants, Veterinary Drug Residues, Biotechnology, Microbiology and Antimicrobial Resistance, 25 Feb 2022, Majadahonda, Spain.

EFSA Scientific Colloquium N°26 on *Risk Benefit Assessment of combined exposure to Nutrients and Contaminants through food*, On-line meeting, 15–17 February 2022, online (recording).

EFSA/Fraunhofer ITEM training: *Risk assessment: Nanoscience and Nanotechnologies on nanotoxicity*, 14–18 February 2022, online course.

EFSA Workshop *From NOAEL to BMD approach*, Day 1 and 2, 2 February 2022, online (recording).

EFSA/Fraunhofer ITEM: *E-learning on In-Silico Toxicology*, online training, access 10 January – 28 February 2022.

FDA and Alliance to Stop Foodborne Illness, Collaborating on Culture in the New Era of Smarter Food Safety, *Making Leaders Risk Aware and Push to Reduce Risk*, 16 February 2022, online.

Microbiome Virtual International Forum #6, *Topic models for interpretable multidomain microbiome data*, 8–9 February 2022, online.

University of Granada, *Machine Learning and Big Data for Bioinformatics*, 7 February – 1 April 2022, massive open online course (MOOC).

European Institute of Innovation and Technology (EIT) Food, *The Human Microbiome*, 24 January – 11 February 2022, massive open online course (MOOC).

European Institute of Innovation and Technology (EIT) Food, *Food for Thought: The Relationship Between Food, Gut and Brain* EIT Food, from 24 January – 11 February 2022, online course.

Adverse outcome pathway (AOP) co-operative activities between scientific journals and the OECD, 25 January 2022, online webinar.

FoodSafety4EU *EU FOOD SAFETY FORUM Sustainable food: how to keep it safe?*, 15 December 2021, online.

European Institute of Innovation and Technology (EIT) *The Future of Food* Conference 2021, 30 November – 1 December 2021, virtual event.

Introduction to BioCyc for New Life Sciences Graduate Students and Post Docs. Introduction to BioCyc, 3 November 2021; *Smart tables and Comparative Analysis*, 10 November 2021, T*ranscriptomics and Metabolomics Data Analysis*, 17 November 2021, online webinar series.

International e-Symposium on Probiotics, Prebiotics & Gut Microbiome: Key Regulators for Human and Animal Health, 11 November 2021, online.

EFSA *International Workshop on Risk Assessment of Combined Exposure to Multiple Chemicals*, 18–20 October 2021, online.

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Developing a framework for open and FAIR data management practices for next generation risk- and benefit assessment of fish and seafood

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Abstract

Risk and risk–benefit assessments of food are complex exercises, in which access to and use of several disconnected individual stand-alone databases is required to obtain hazard and exposure information. Data obtained from such databases ideally should be in line with the FAIR principles, i.e. the data must be Findable, Accessible, Interoperable and Reusable. However, often cases are encountered when one or more of these principles are not followed. In this project, we set out to assess if existing commonly used databases in risk assessment are in line with the FAIR principles. We also investigated how access, interoperability and reusability of data could be improved. We used the OpenFoodTox and the Seafood database as examples and showed how commonly used freely available open-source tools and repositories can be implemented in the data extraction process of risk assessments to increase data reusability and crosstalk across different databases.

Keywords: FAIR, OpenFoodTox, Seafood database, R, Shiny, Zenodo

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2. Description of work programme

2.1. Aims

This project assessed how to apply FAIR data principles (Wilkinson et al., 2016) in risk and risk–benefit assessments of food. Focusing on key databases recently used in a risk–benefit assessment of fish and seafood in the Norwegian diet (VKM et al., 2022a), the OpenFoodTox and the Seafood database, we aimed to demonstrate how open-source software tools can be used to make data stored in publicly available repositories more Accessible, Interoperable and Reusable.

2.2. Methods

Using the programming language R (R Core Team, 2021) and data obtained from the ESFA OpenFoodTox Tool (Dorne et al., 2020; Kovarich et al., 2022) and from the Institute of Marine Research (IMR) Seafood database (Institute of Marine Research, 2022), we assessed if programmatical optimisation of access and the creation of a web-tool for selection and merging of subsets of the stored data improved accessibility, interoperability, and reusability of the data. In this section, a brief description of both data and tools used is provided.

2.2.1. R

The programming language and environment R has been designed for the statistical analysis of data and the creation of graphics (R Core Team, 2021). Over the past years, R has increasingly gained interest in the scientific research community (Hackenberger, 2020) as it is effective for data handling and includes many tools for basic and advanced data analysis (R Core Team, 2021). R is a well-developed non-static language, which means that its base features can easily be extended via packages that can provide new functions and functionalities for different data science challenges, including bioinformatics and data mining (Giorgi et al., 2022). In addition to this, R is supported by a big open-source community actively using this language and continuously adding new functionalities. R is licensed under the terms of the Free Software Foundation's GNU General Public License in source code form (R Core Team, 2021). To facilitate the programming with R, we used RStudio, an integrated development environment for R (RStudio Team, 2022).

2.2.2. Shiny

As commented above, R can be expanded through packages, being one commonly used 'Shiny' (Chang et al., 2021). This package was designed with the idea of creating interactive web applications which use R in the backend. While the creator of a web-based Shiny-tool does need to know R, the end user of the web application created with Shiny does not need to have any knowledge of R. In addition to local installations of R and Shiny, Shiny web app also can be stored on a server, which users can access through their web browser. In both cases, the appearance and functionalities of the applications are the same, and the underlying R code can be shared freely.

2.2.3. Git and GitHub

Git is a version control system designed to allow different users to work on the same programming project ensuring the traceability of progress and changes in the project. One of the most widely used providers of internet hosting for software development and version control using Git is GitHub (Microsoft, 2022). GitHub implements Git and offers a free version, in which users can host different smaller projects and scripts providing an easy way to share codes created in R and other programming languages on the web. The scripts generated during this project will be hosted and accessible on GitHub in this repository: https://github.com/J-Pineda-Pampliega/EU_FORA_Project (Pineda-Pampliega, 2022).

2.2.4. Zenodo

Under the European OpenAIRE program, and with the idea of championing the sharing of scientific data, the Zenodo (European Organization for Nuclear Research,, and OpenAIRE, 2013) open repository

was developed and operated by CERN (European Council for Nuclear Research). This open-source repository was developed for scientific data in a broad way, allowing to deposit not only research papers, but also data sets, software, reports, supplementary data and any other research-related digital artefacts. Submissions to Zenodo obtain a persistent digital object identifier (DOI), which facilitates the citation of the stored items and allows the sharing of data prior to their publication in peer-reviewed journal.

For a speedy exchange of evidence and supporting materials which could be used in food and feed safety risk assessments, EFSA has created a curated open repository called the 'Knowledge Junction' within Zenodo. In addition to EFSA, several other institutions use Knowledge Junctions to share different data related to food security. For example, The Norwegian Scientific Committee for Food and Environment (VKM), which is part of this project, uses this Zenodo repository to upload finished reports (i.e. risk assessment and risk–benefit assessment) and supplementary materials of interest (i.e. literature searches, datasets, codes, etc.). To date, for VKM, the most recent example of the use of Zenodo is the opinion on the 'Risk-Benefit Assessment of Sunscreen' (VKM et al., 2022b). For this opinion, the fellow Javier Pineda-Pampliega contributed to the preparation of the public sharing of the report's supplementary material including datasets and R codes currently hosted on the VKM Knowledge Junction (Norwegian Scientific Committee for Food and Environment (VKM), 2022).

Zenodo recently implemented the possibility to import GitHub workspaces; it now is possible to host completed GitHub projects also on Zenodo. This offers the advantage of obtaining a DOI for one's code, which simplifies the traceability and proper citation of code used to create the results.

2.2.5. OpenFoodTox

The EFSA's Chemical Hazards Database (OpenFoodTox) (Dorne et al., 2020; Kovarich et al., 2022) is a structured database summarising the outcomes of hazard identification and characterisation for human and animal health and for the environment. It includes all regulated products and contaminants and provides open-source data for the (1) substance characterisation, (2) links to EFSA outputs, and the values of (3) reference points, (4) reference values and (5) genotoxicity. This database has become an essential tool for risk assessors and has provided the basis for the development and implementation of new approach methodologies (NAMs) in food and feed safety research. OpenFoodTox is hosted both on the EFSA webpage (as an interactive web tool) and on Zenodo in the EFSA Knowledge Junction.

2.2.6. Seafood database

The Institute of Marine Research in Norway routinely collects samples of key marine species for national and international monitoring programs. Their ISO 17025 accredited laboratories perform analyses of contaminants and nutrients using state-of-the-art methods. All the data generated, comprising multiple data points for over 25,000 individuals collected over a period of up to 15 years are aggregated in a large in-house database. This database can be accessed freely through the online Seafood database portal (Institute of Marine Research, 2022), where the user can select between fish, shellfish and seaweed divided by wild or farmed, and even prepared products, which can be found in Norwegian supermarkets. The database holds data of both Nutrients (separated into five categories: Amino acids, Fatty acids, Macro nutrients, Minerals and Trace elements and Vitamins) and Contaminants (separated into four categories: 'Drug residues', 'Heavy metals', 'Organic pollutants' and 'Other undesirable substances').

2.3. Activities

With the aim to investigate the application of FAIR data principles in risk–benefit assessment of seafood, it was essential to evaluate opportunities and limitations in the OpenFoodTox and the Seafood database. Once evaluated, we developed publicly available R and Shiny code, which attempts to address potential limitations found and to add new functionalities for sub-setting and improved crosstalk between hazard and occurrence data repositories.

2.3.1. Evaluation and actions on the OpenFoodTox

The OpenFoodTox database can be used in two different ways. The first (1) option is through the EFSA-hosted web application. The EFSA-hosted web application of the OpenFoodTox tool presents a classical interface, where different compounds can be searched by name. When searching, selected

substances appear in five different categories of results: Substance characterisation, EFSA outputs, Reference points, Reference values and Genotoxicity. The resulting output represents the main limitation, as each category only can be downloaded individually (either in pdf, csv or xlsx format). In other words, after a search, the users need to download five different files and manually merge the data.

The second (2) option to access data is to download the entire OpenFoodTox database in xlsx format (Microsoft Excel Open XML Spreadsheet) from Zenodo. The data comprises five individual spreadsheets providing data on (1) Substance characterisation, (2) EFSA outputs, (3) Reference points, (4) Reference values, and (5) Genotoxicity results. There is another 'complete' spreadsheet which is a combination of the five spreadsheets commented above (each one in a different tab) in addition to a Dictionary spreadsheet (Dorne et al., 2020). This makes data Interoperable. However, as was described in the example above, to work with subsets of data spreading across the different spreadsheets, data aggregation and merging again must be performed manually using additional software for tabular data files. The most common among these tools is Excel, which is part of the commercial Microsoft Office Suite, but other free alternatives such as OpenOffice, LibreOffice or online tools such as Google Drive Sheets also can be used. In any case, for merging the large individual datasets, the user needs to be proficient in the terminology of terms and use of spreadsheet tools for efficient filtering, merging and sub-setting of the data in the desired format.

To evaluate potential complementary solutions to access, subset, and merge data stored in the EFSA OpenFoodTox database on Zenodo, in the present project using R (vers. 4.1.2) running in RStudio (vers. 2022.2.3.492), functions (i.e. pieces of code which work together for a common purpose) were written using R markdown, being characterised by the following features:

- Data is downloaded directly from the OpenFoodTox URL to eliminate the need for the user to search for and/or download the data in Excel.
- The possibility to search for up to 15 elements at the same time, with an implemented control of any repeated entry values. In the case of repetition, the repeated value is indicated, but not considered in the search.
- If a search is entered for a general term and several compounds appear in the database, an indication for the number of the different compounds is provided. For example, the search "lead" returns "4" results, because the components identified in the database are: "Lead", "Lead (II)", "Lead sulphate" and "Tetraethyl lead".
- To increase the (computational) reusability of the data in automated analysis pipelines, the information is downloaded in a plain text file (txt). This is a standard format of plain text that can be open in many different software tools. However, also the possibility to download data in csv (comma-separated values) is provided.
- To increase traceability information on the OpenFoodTox database version and the date and time when the file was created are automatically appended to the name of the downloaded file.

After the creation of the R script, to increase the number of potential users of this tool, we assessed if an additional approach that does not require knowledge and use of R could be developed. For this, the creation of a web-based application using Shiny was attempted. The use of Shiny opens the possibility to access and subset OpenFoodTox data using an internet browser only and also allows for the implementation of additional functions into our R code. That is, in addition to the characteristics of the function described above, the Shiny application developed in this project (Figure 1) has the following extra functions:

- Increased traceability: an indication of which version of the OpenFoodTox database used has been included. At the time of writing this report, the 5th iteration of the OpenFoodTox was released (and published on Zenodo on 16 June 2022).
- Implementation of interactive tables, allowing to filter results in real-time.
- Initially, tables will show all columns in the dataset but tools for sub-setting and selection of individual columns to be retained are provided. This functionality makes it easier to take snapshots only of the columns of interest for further uses.
- With one of the objectives of this project being to facilitate the interaction and crosstalk between databases of interest to risk assessors, the option to add links to PubChem for each selected compound was implemented. PubChem is a database of chemical molecules and their activities, maintained by the National Centre for Biotechnology Information (NCBI) of the United States.

2.3.2. Evaluation and actions on the Seafood database

The Seafood database contains information collected over a period of up to 15 years, with different data points for over 25,000 individual samples. This represents a comprehensive data repository of nutrients and contaminants in fish and seafood comprising more than 700,000 records. Due to the experience gained in the previous work with the OpenFoodTox tool, we directly designed a web application using Shiny to work with the Seafood database. As with OpenFoodTox, the first step was to evaluate the potential limitations and challenges of the existing system to access the database, which for the general public currently occurs via a web interface (https://sjomatdata.hi.no/). Having gotten access to the data underlying the web-based tool hosted at the IMR, in the present project, we assessed alternative solutions by addressing issues of the current web application using R and Shiny (Figure 2). We also set out to include additional functions potentially of use to risk assessors. The Seafood database Shiny web application is characterised by the following features:

- The publicly available web interface of the Seafood database is not version controlled. Furthermore, it is not updated with a defined periodicity, as it depends on data from different projects which are made available at different times throughout the year. This could be a challenge for the traceability of results and repeatability of analysis. As an attempted solution, we suggested for the database to be version controlled and to be updated at defined intervals only, e.g. annually. In addition, we implemented code to show a message highlighting the date when the database was last updated (Figure 3A). In a new version of our code, we also will include a button in the Shiny app to select which version of data the user wants to retrieve (i.e. to select the data regarding the day of the update).
- One common situation users of the Seafood database often encounter is the interest in the comparison of the presence of different compounds in different species or products. In the current web interface of the Seafood database, to check all the substances evaluated, it is only possible to select species or products one by one. In addition, to compare the concentration of different substances between species or products, the maximum number of substances is 10 by search. This makes it difficult to prepare a subset of desired data for further comparisons downstream. As a solution, in the prepared Shiny-based application, the user can select up to 15 species or products simultaneously, with information on all nutrients or contaminants. In addition, if the user is interested in only a particular set of compounds, up to 15 nutrients and another 15 contaminants can be selected.
- The R of FAIR means reusability of the data. This implies that for performing additional data analyses not yet envisaged by the data providers, users of a database should be able to access data presented in a non-aggregated way. Currently, the Seafood database does not provide this option; the results of searches are presented as numerical summaries (with sample size, mean, minimum and maximum values for each parameter). This makes it difficult to reuse this data in new evaluations. In the present project, at the IMR access to all data contained in the Seafood database was provided and two tables are presented in the Shiny application developed: one with a summary of the data (as in the IMR web interface), and another table with the non-aggregated data (Figure 3B).
- Continuing with reusability; in addition to access to non-aggregated data, also the format in which data can be downloaded by the user is important to consider. The Seafood database allows downloading in 'Portable Document Format' (pdf) format only. This format is widely used to present documents which include text and images and has the advantage of being immutable, i.e. independent of application software, hardware and operating systems documents are displayed always in the same way. However, this characteristic is a weakness for sharing data intended to be used in downstream analyses. For this, the data needs to be Reusable and Interoperable. The newly developed Shiny application allows for the download of selected data in txt or csv, being the most typical format to share data which could be used for further analysis. Both data from the summary table and the non-aggregated data can be downloaded in the desired formats. In addition, to ensure traceability when files are downloaded, the name consists of the date and the time of the creation and also incorporated the version of the database (the date of the latest update of the data; Figure 3C).

In addition to addressing specific limitations of the Seafood database listed above, we added extra functionalities in the Shiny code that we considered could be useful for the users:

- An increase in the number of inputs could entail an increase in the number of mistakes due to a repetition of terms in a search. To avoid this, our application indicates if a value is repeated but, even more important, the repeated value is not considered for the search, showing the same results as if the value was introduced only once (Figure 3A).
- Despite quality control measures in place, databases may contain erroneous entries (e.g. the inclusion of text in numeric rows and vice versa, or empty values). The Shiny application developed here includes a filter to flag and eliminate any rows which potentially contain mistakes. In addition, an indication of the number of eliminated entries is provided (Figure 3A).
- During the quantification of substances, it is possible that values are below the limit of quantification (LOQ) of a specific analysis. The LOQ is the lowest concentration of an analyte that can be quantified with a given certainty. In the Seafood database, values for contaminants below the LOQ are routinely reported using 'Upper bound' summation where the LOQ is used as if it were the actual concentration measured. This may result in many data points of the same value and such data sets are referred to as 'Left censored'. The web interface of the Seafood database indicates which values are below the LOQ, and also lists the numerical value of the respective LOQ for each method and compound in question. The Shiny application developed here, in addition, allows for further modification of the data and the possibility to calculate 'Lower bound' (substitute values < LOQ by 0) or 'Upper bound' (substitute values < LOQ with the actual LOQ) (Figure 3D).
- To increase data interoperability and crosstalk between different databases, common unique identifiers must be found. In our opinion, codes of the chemical substances in question provide a good option. Different unique identifiers do exist including InChI (International Chemical Identifier) or SMILES (Simplified Molecular Input Line Entry System), which both are included in the Seafood database. In addition to these, the 'paramCode' was added in the Shiny App, which is suggested by EFSA to be used when reporting on different substances in food and feed (EFSA, 2019).

One general challenge we found when working with the Seafood database is that its web interface is designed to share aggregated occurrence data with the public; access to non-aggregated data is limited to in-house use and can be made available on request to risk assessors. Hazard data from OpenFoodTox on the other hand can be accessed both via a web interface for quick screening of information and through a dedicated Zenodo repository for bulk download and direct reuse (e.g. in exposure calculations for risk assessments). In addition, data is version controlled and linked to persistent citable DOIs. This, in our view, strongly facilitates the timely dissemination of information and the reproducibility of the data analysis performed. The benefits of publishing data on an open repository such as Zenodo sparked a discussion at the IMR on how seafood data could be made available to a wider audience in the future, which is an important first step towards further implementation of the FAIR data principles.

2.3.3. Spin off activities in implementing FAIR data management practices

In addition to the work described above, during the project period supporting activities were carried out to improve communication in project work relying on coding and data sharing across different work groups and institutes. Within the Marine Toxicology group at the IMR several software tools are used to advance work on several cross-disciplinary projects. Microsoft Teams is used to communicate between members of the group through video calls or chat. Microsoft SharePoint is used as a document repository and for interactive document creation and editing. Linking SharePoint to OneDrive, within the group R code could be developed locally using RStudio. This allowed for efficient local collaboration between members of the team. To share different elements for a project externally, in addition to Teams, GitHub accounts were set up, and using RStudio scripts created earlier, were directly uploaded. This workflow was shown to VKM, which implemented this workflow, for the 'Risk-benefit assessment of sunscreen' (VKM et al., 2022b), that for the first time shares supplementary codes and datasheets interactively on Zenodo (Norwegian Scientific Committee for Food and Environment (VKM), 2022). Lastly, the fellow also engaged in discussions with IMR IT staff about modern software development and recent developments in micro-services architectures with standardised and structured data representation formats for sharing information between systems and services such as JSON and XML.

2.3.4. Other spin off activities during the project

The performed work is not only represented in this report but was also presented on a poster at the 'ONE Health, Environment, Society' conference in Brussels in June 2022. This conference also invites to participate in a video contest, where a short summary of the project was also presented. In addition to the project work related to FAIR data management, the EU-FORA programme also has offered further opportunities. Being integrated into the working group, the fellow had the opportunity to familiarise himself with a new field, participating in a paper regarding proteomics. Finally, to continue training in food security, the fellow also carried out the training 'Risk assessment in biotechnology' offered by the European Commission as part of the training initiative 'Better Training for Safer Food' (BTSF).

3. Conclusions

Large amounts of data that could be used in food safety risk assessments are available in different database. However, this steady increase of data has not been always followed by an improvement in the ways to easily access these data, data traceability or easy data reuse. To tackle these challenges, it is recommended that data for risk assessments must follow the FAIR principles, i.e. data must be Findable, Accessible, Interoperable and Reusable. Based on publicly available databases and open-source software tools, this project has been attempting to provide a proof of concept to show how using custom-made codes and alternative approaches could improve some characteristics of well-known databases including the OpenFoodTox Tool and the Seafood database. The use of platforms such as GitHub or Zenodo could make the data more Findable and Interoperable. The creation of web applications with Shiny could increase the accessibility to the data and make easy interaction between databases. The reusability was obtained through the selection of the appropriate formats for the data downloaded and the application of adequate systems to ensure traceability. Following these FAIR principles in the different databases is an essential step to ensuring the success of the future risk–benefit assessment timely and with adequate spending of human and economic resources.

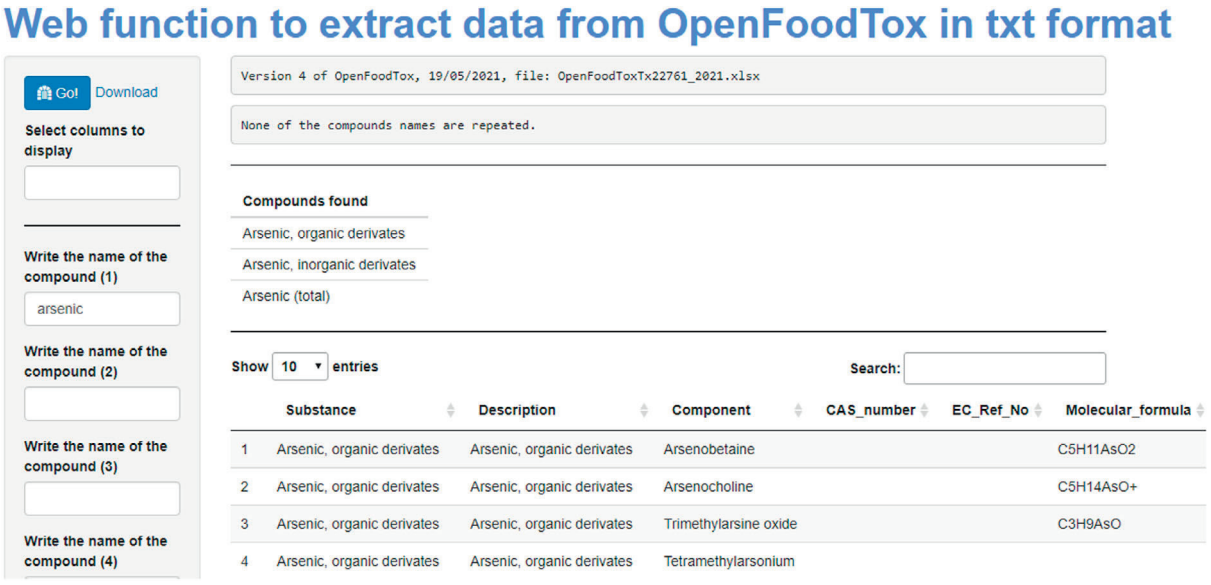

Figure 1: User interface of the application designed with Shiny to access and work with the OpenFoodTox database

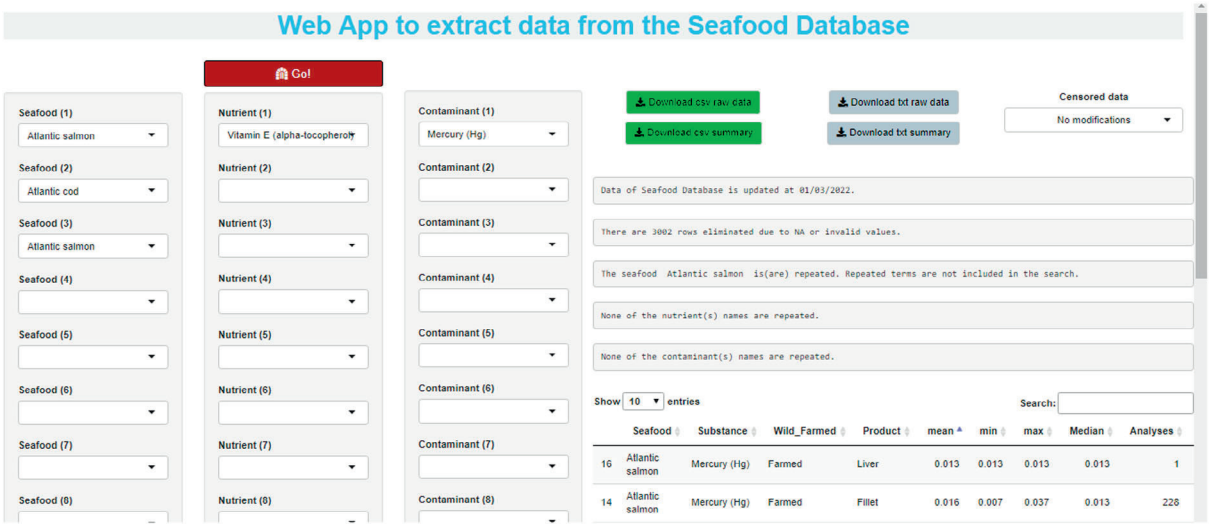

Figure 2: User interface of the application designed with Shiny to access and work with the Seafood database

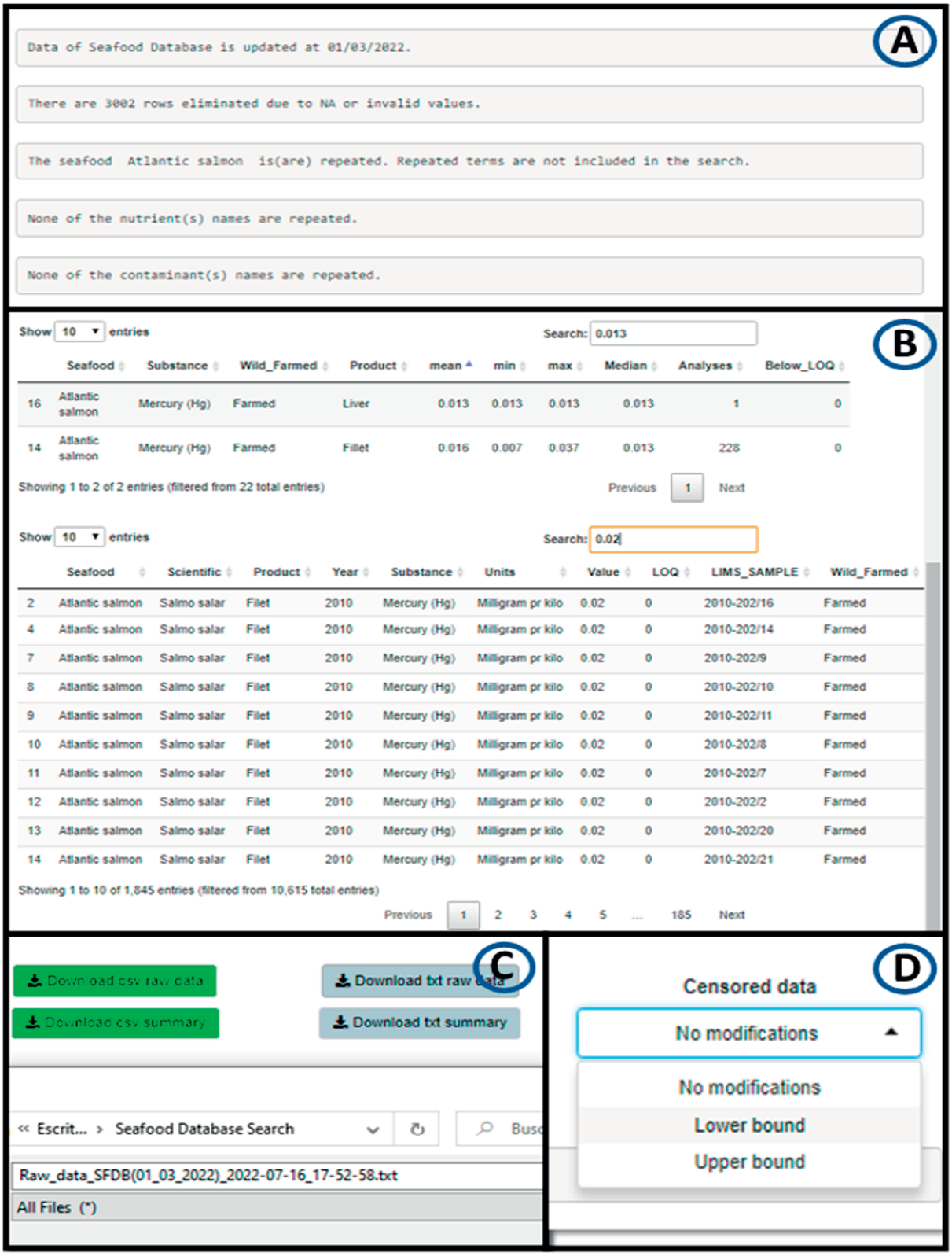

Figure 3: Results of the search in the Shiny application designed to work with the Seafood database. (A) Version of the database used, number of registers eliminated for errors and control of repeated inputs. (B) Examples of the summary and non-aggregated tables. (C) Options to download the results and the name of the file. (D) Options to control left-censored data

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Abbreviations

DOI	digital object identifier
IMR	Institute of Marine Research
NAM	new approach methodology
VKM	Norwegian Scientific Committee for Food and Environment

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Risk assessment of enteric viruses along the food chain and in the population

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Abstract

Food-borne microbial illness contributes up to one third of global disease burden. The largest category of food-borne illness is gastroenteritis, the majority of which is caused by enteric viruses. Viruses like these are transmitted to food either by waste-contaminated waters, or by handling and transfer during processing. An important tool for reducing or controlling food-borne microbial risk is risk analysis. This framework has been adopted globally to manage risks associated with microbial contamination in food. Several hundred microbial risk assessments (MRAs) have been published by different national and international organisations, for different food-hazard combinations. The use of MRAs in controlling and understanding virus risk has, to date, been limited, compared with the efforts made on bacterial pathogens. Given the large disease burden that viruses are responsible for, this disparity should be addressed. The main reasons for the relative lack of risk assessments are the difficulty in detecting and monitoring viruses compared with bacteria. This means less data on prevalence, concentration and inactivation, and allows viruses to remain silent contributors to global disease. There are also key conceptual differences between virus risk assessment and bacterial risk assessment. This project aimed to assess the current state of the art for food-borne virus risk assessment, then to progress the field further by using the data available to produce risk rankings and risk assessments. This was done by a combination of literature reviewing and various risk assessment tools. The result was an assessment of the overall evidence base in the literature, a semi-quantitative ranking comparison between the viruses and foods of most concern, and a survey of inactivation methods, leading to a quantitative ranking of the effectiveness of each in reducing and managing food-borne virus risk.

Keywords: microbial risk assessment, viruses, virus risk assessment, virus inactivation, norovirus, hepatitis

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1. Introduction

1.1. Background

Viruses are a major food-borne hazard, with enteric viruses causing over half of the food-borne disease burden (FAO and WHO, 2008; Hall et al., 2014; Havelaar et al., 2015). Unlike bacteria, viruses will not grow on food once contaminated and pose little risk of spoilage. Because the most common viruses can be infectious at low doses, it is important to ensure as much as possible that they are removed or inactivated before consumption (Bradshaw and Jaykus, 2016).

Food safety risk management should be based on solid scientific evidence and on transparent and reproducible processes and procedures that use available knowledge to arrive at public policy decisions. The importance of risk assessment lies not only in its ability to estimate public health risk, but also in its use as a framework for organising data and allocating responsibility for analysis. Risk assessment is a process that can include a variety of methods and models to reach its conclusions. The FAO and WHO distinguish risk assessment from risk management and communication but recognise that risk assessment and risk management have a number of significant interfaces. For example, establishing priorities and policies for risk assessment often includes input from risk management consideration (FAO and WHO, 1995).

1.2. Microbial risk assessment

Microbial risk assessment (MRA) focuses on biological hazards, mainly pathogenic bacteria. However, adverse health effects of other biological agents such as food-borne viruses and parasites are increasingly considered for assessment. Among MRA methods, quantitative microbial risk assessment (QMRA) is a tool used in analysing and managing the risk of food-borne infectious disease quantitatively. The same principles of QMRA apply for all pathogens, but there are important reasons to treat viruses as a separate category of hazard (FAO and WHO, 2008; CAC, 2012; EPA/USDA-FSIS, 2012). Microbial risk assessment emerged as a separate discipline to chemical risk assessment by the mid-1990s (Foegeding et al., 1994; Bradshaw and Jaykus, 2016; Nauta, 2021). Since 2008, the FAO and WHO have formally acknowledged viruses in food as a hazard category of importance (FAO and WHO, 2008). Food safety measures for bacteria are insufficient in dealing with virus risk, due to some key difference in persistence and infectivity (FAO and WHO, 2008; Bradshaw and Jaykus, 2016). This can lead to diverging standards for virus food safety criteria. Some of the earliest examples of published MRAs focused on viruses as a hazard (Haas, 1983; Gerba and Haas, 1988; Haas et al., 1993; Gerba et al., 1996). Since then, however, most published risk assessments have been for bacterial hazards. Virus hazards do not require a new paradigm of risk, just some differences in detail, similar to the separation between chemical and microbial hazards (Havelaar and Rutjes, 2008; Bradshaw and Jaykus, 2016). Rather, the problem is a lack of data on certain key question, and the difficulty in obtaining this data by present detection.

The hazard identification step for virus risk assessment is a qualitative description of a food product, a virus and the effect of both on a population of concern. Viruses are estimated to cause more than 50% of the world's food-borne disease burden (Koopmans and Duizer, 2004; Havelaar et al., 2015). The most significant viruses, as identified by the FAO/WHO, are hepatitis A, norovirus and rotavirus, with emerging viruses like hepatitis E also of particular interest (FAO and WHO, 2008). The foods most associated with virus outbreaks are shellfish, fresh produce and ready-to-eat or preprepared foods (Duizer and Koopmans, 2008; Le Guyader and Atmar, 2008).

The exposure assessment stage for virus risk assessment has a specific set of concerns and challenges that are important to consider. Since most viruses of concern do not grow outside of a human or animal host, the main factors to consider will be transmission, detection, inactivation, survival or persistence, and consumption. Much data for exposure modelling falls into these categories (FAO and WHO, 2008; Bradshaw and Jaykus, 2016).

Transmission for food-borne viruses occurs in stages. First, food comes into contact with a virus reservoir, then the virus attaches to the food matrix, and finally, the virus persists on the food matrix until consumption (Le Guyader and Atmar, 2008). The principal transmission routes identified by the original FAO/WHO committee are wastewater contamination, unhygienic handling by infected handlers, and animal or zoonotic sources (FAO and WHO, 2008). The main foods of concern for virus transmission are fresh produce, ready-to-eat food, shellfish, with pigmeat an emerging concern for zoonotic viruses like hepatitis E virus. For fresh produce, virus is adsorped onto the surface of fruits

and vegetables and has also been observed getting taken directly into the plant tissue (Katzenelson and Mills, 1984; Chancellor et al., 2006).

The problem of virus risk assessment has been known for decades. However, only recently has the opportunity to meet this challenge begun. Better detection methods will lead to more information on prevalence, concentration and control. Given the contribution of viruses to the food-borne disease burden, greater attention should be put on them in the years ahead. The output rate of virus risk assessment will continue to increase as greater data becomes available. The control of virus hazards will be improved by greater awareness of virus attachment and virus inactivation especially the variability possible. Microbiological control criteria are always needed, and with the next generation of tools and data, the future of QMRA can correctly address the role of enteric viruses as significant food-borne hazards.

2. Description of work programme

2.1. Aims

The work programme covered the topic of risk assessment of food-borne viruses, divided into three general categories.

First, the fellows were to gain experience and knowledge of two topics: risk assessment of food-borne viruses and risk management of the same. The training was based on the EU-FORA training modules, and on additional workshop activities, detailed in Table 1. It also included comprehensive literature reviews on the two topics, and networking activities with other fellows and risk assessment professionals.

The tools and experience gained during training were to increase the capacity for the second aim: the research activities. Having established the current state of the art of food-borne virus risk assessment, the fellows would use this knowledge to carry out original research, of benefit to the broader research community, relating to both risk assessment and risk control.

The third and final aim for the project was communication, relating to the earlier activities. The fellows were to organise presentations of their work at conferences and other venues, and wrote manuscripts intended for publication.

2.2. Activities/Methods

2.2.1. Training and capacity building

2.2.1.1. Training modules

Training in general risk assessment concepts and tools was done as part of the EU-FORA training programme. In addition, a 2-week workshop was arranged in collaboration with the University of Córdoba in Spain, which covered practical aspects of predictive microbiology and quantitative risk assessment in more depth. This included tutorials in the MicroHibro software, whose consortium is based in Córdoba. These activities were tailored towards the questions of viruses in food where possible.

2.2.1.2. Literature reviewing

Comprehensive literature reviews were prepared by both fellows in collaboration on the two main topics of the research program: (1) risk assessment of viruses in food, and (2) control and inactivation of food-borne viruses.

2.2.1.3. Networking activities

As well as the EU-FORA training activities, and the research visits to Córdoba, the fellows based in Spain were hosted for a 1-week visit to AESAN, the Spanish Agency for Food and Nutrition in Madrid, Spain. The activities of AESAN as a regulatory agency were explained, and the relationship to the overall EU food safety framework. All training and networking activities are listed in Table 1.

2.2.2. Research activities

The research activities built on the earlier training and reviewing, extending current knowledge based on systematic reviewing of the literature. They were guided by the risk-ranking workflow descriptions in EFSA opinions and guidance documents.

2.2.2.1. Risk assessment and risk ranking

The risk assessment contribution of the research was a risk ranking exercise for food-borne viruses in the EU. Following guidance and examples from institutions like the FAO, WHO and EFSA, the fellows identified (1) the most significant food-borne viruses present in the EU, (2) potential emerging viruses, as defined by EFSA guidance, and (3) the quantitative criteria most useful in ranking the impact of each virus. Systematic reviewing of the literature provided a map of available evidence for estimating each criteria, and the most significant data gaps. This exercise provides a survey for the state of the art for food-borne virus risk ranking, and profiles of each virus that will be useful in guiding future laboratory work or expert knowledge elicitation efforts.

Where data was available, the most significant virus-food combinations were ranked semi-quantitatively. This was done using the risk ranking tools covered in earlier training, whose different approaches could be compared and contrasted. Sources of uncertainty were considered throughout the process. This 'top-down' approach to risk ranking gives an overview of the most significant food-virus combinations and the data available for risk assessment.

2.2.2.2. Risk management and inactivation modelling

After the significant food–virus combinations were identified, and the amount of information in the literature mapped, the next research contribution was a review of quantitative predictive models for inactivation of viruses during processing. The literature was systematically reviewed for data on inactivation and reduction by thermal and non-thermal technologies. These models were presented in tabular format, with key parameters extracted for each inactivation method.

With this information, a 'bottom-up' fully quantitative risk ranking exercise was carried out, comparing the impact of each intervention method on consumer health, and discussing the costs and benefits of each method. This collection of inactivation models could be incorporated in online software tools and databases like MicroHibro, Combase and RAKIP.

2.2.3. Communication and outreach

2.2.3.1. International conferences

The fellows organised a full symposium at the International Association for Food Protection in Munich, Germany, comprising three 30 min presentations on food-borne viruses, food-borne virus risk assessment and food-borne virus control. This was done in collaboration with two other fellows from the same cohort. The fellows will also present results of their research activities at FoodMicro2022, in Athens, Greece, as poster and oral presentation (Table 1).

2.2.3.2. Other formal meetings

The fellows presented themselves and their work in other venues during the course of the fellowship, both internally at the host organisation, and during the visit to the Spanish Agency for Food and Nutrition (AESAN) (Table 1).

2.2.3.3. Publication outputs

The results of the initial literature reviewing during the training formed two book chapters in a forthcoming book on Food and Environmental Virology (Hunt and Trząskowska, 2022; Trząskowska and Hunt, 2022). Other manuscripts relating to the research activities are in preparation. For this reason, results of the research activities are not being presented in this summary.

Table 1: Summary of courses, conferences and seminars attended by the fellows

Course/seminar	Title	Date
Training sessions provided by EFSA	Introduction training of the European Food Risk Assessment Fellowship Programme	30.8–17.9.2021
	Module 1 training of the European Food Risk Assessment Fellowship Programme – nutrition, animal welfare and health, animal health, regulated products, GMO	22–26.11.2021
	Module 2 training of the European Food Risk Assessment Fellowship Programme – risk communication and crisis response	21–25.3.2022
	Module 3 training of the European Food Risk Assessment Fellowship Programme – emerging risks, AOP, MoA, applications of OMICs in RA, risk ranking	6–10.6.2022
	Module 4 training of the European Food Risk Assessment Fellowship Programme – data collection and reporting	22–25.8.2022
Other training sessions	Training sessions at University of Cordoba – working with Prof. Antonio Valero, international expert on predictive microbiology	11–22.7.2022
Conferences and workshops	Workshop on predictive microbiology, held at University of Burgos	15–16.11.2021
	IAFP'S European Symposium on Food Safety, Munich, Germany Organisation of Symposium titled 'New Hazards and Old Threats; Foodborne Viruses and Risk Assessment in Food Safety' Presentation: The next frontier in risk assessment in food: quantitative viral risk assessment (KH) Presentation: Control of Foodborne Virus Risk in the Context of Risk Assessment (MT)	4–6.5.2022
	ONE – Health, Environment, Society –Conference 2022 Attended virtually	21–24.6.2022
	FoodMicro2022 Next Generation Challenges in Food Microbiology, Athens, Greece Oral presentation: Quantitative microbial risk assessment for foodborne viruses: past, present, and future (KH) Poster presentation: The control and management of foodborne virus hazards, from 2022 and beyond (MT)	28–31.8.2022
Other activities	National workshop for EU-FORA fellows based in Spain, satellite meeting	23–25.2.2022

3. Conclusions

3.1. Summary of work

Food-borne virus risk assessment is an important topic in food safety, and is becoming more relevant as new detection methods are developed. Although most microbial risk assessments have focused on bacterial hazards, and on modelling growth, there are compelling reasons to consider viruses as a separate subcategory for risk assessment purposes. The prevention and control of viruses during production in particular should be carefully considered in the future, and new or emerging technologies assessed for their inactivation potential.

3.2. Future directions

The work done by the fellows can form the basis of a new and comprehensive risk-ranking for food-borne viruses in the EU. This has been carried out for parasitic hazards and bacterial pathogens in the past, and could be repeated for viruses using data from the literature and expert elicitation.

Software and databases designed for predictive microbiology can and should incorporate more data on virus inactivation, and consider the impact of the food matrix on this inactivation.

The map of available evidence assembled during the systematic review process indicates that more laboratory experiments are needed for modelling virus inactivation throughout the production process of uncooked foods.

Given the persistence of viruses in the environment, more data and models for transfer between surfaces and equipment would also be of use.

Finally, better detection methods for infectious viruses at low levels of concentration or prevalence should continue in development. Virus risk assessment is as critical and urgent as bacterial, and has been neglected to date due to the difficulties in detecting and quantifying. With these barriers eliminated, more and more virus risk assessments will continue to be published in the future.

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Abbreviation

AOP	adverse outcome pathway
FAO	Food and Agriculture Organization
KH	Kevin Hunt
MoA	mode of action
MRA	microbial risk assessment
MT	Monika Trząskowska
QMRA	quantitative microbial risk assessment
WHO	World Health Organization

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Risk–benefit assessment of shifting from traditional meat-based diets to alternative dietary patterns

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Abstract

The consumption of plant-based meat alternatives has recently transitioned from the niche market to the mainstream. However, changing the dietary patterns may lead to new health challenges referred to possible higher exposure to natural toxins found in plant-based food. The present project aimed to train the fellow in the field of chemical risk assessment and provide a comprehensive overview on how a change towards plant-based meat alternatives may represent a driver for emerging risks. Thus, within the EU-FORA programme the fellow engaged in the following activities: (i) perform a systematic review to analyse the distribution of natural toxins in the most common plant-based meat alternatives in Europe, (ii) risk–benefit assessment of shifting from meat-based diets to soy-based meat alternatives by modelling the substitution of meat with soy, and (iii) determine the occurrence of mycotoxins in plant-based meat alternatives from local markets in Parma, Italy. The fellow learnt the chemical risk assessment procedures applied by the researchers from the Department of Food and Drug of Parma University, thus gaining an in-depth expertise in all the steps. A risk–benefit assessment was performed modelling the intake of aflatoxin B1-contaminated soy-based meat analogues. The health impact due to intake of soy and exposure to aflatoxin B1 was estimated. Within the research group, the fellow also worked on developing a multi-mycotoxin determination method for plant-based meat alternatives matrices. The results of the project provide a picture reflecting the occurrence of natural toxins in plant-based meat alternatives and the need of upgraded regulation frameworks that take into account new products and dietary patterns. The EU-FORA fellowship was a great opportunity for the fellow to expand his professional network and increase his expertise in food safety by gaining new skills in chemical risk assessment, risk–benefit assessment and analytical chemistry.

Keywords: risk–benefit assessment, mycotoxin, contamination, DALY, soy, plant-based meat alternative

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1. Introduction

Worldwide meat consumption increased in the last five decades from 23.1 kg/capita per year in 1961 to 42.2 kg/capita per year (Sans and Combris, 2015). By 2050, the world's population is expected to increase to 10 billion, which means that the current sources of animal protein (livestock and poultry) will not be sustainable for the whole population (Willett et al., 2019).

In recent years, reducing the consumption of animal-based foods has been seen as an essential part of a sustainable and nutritious diet. In Europe, the market for plant-based meat alternatives was valued at €4.4 billion in 2019; it is anticipated to grow by 70% over the next six years, reaching €8.3 billion by 2025. Often referred to as 'plant-based meat substitutes', plant-based meat analogues are typically made from wheat gluten or legumes like chickpeas, peas, soybeans or lupins (Kyriakopoulou et al., 2018).

1.1. Plant-based meat alternatives

Although meat alternatives based on proteins from fungi (Sha and Xiong, 2020), insects (Megido et al., 2016) and microalgae (Percival, 2019) have recently entered the market, and cultured-meat is becoming of growing interest for the research community, the research and innovation efforts of the agri-food industry is nowadays mainly focused on plant-based meat analogues (He et al., 2020; Santo et al., 2020).

The consumption of processed plant-based protein products can trace back since ancient times in countries such as China and India, and have become over time well-accepted protein sources in developed countries vegetarian diets, especially with products such as tofu, tempeh and seitan. In particular, tofu and tempeh are obtained from soymilk and fermented soybeans, respectively, while wheat gluten is used for seitan production.

However, these is a health halo effect associated with plant-based meat alternatives that influences consumers' perception (Edge and Garrett, 2020). The launch of more than 4,400 plant-based meat substitutes over the past six years (Mintel Group Ltd, 2019) along with a higher consumption of plant-based foods in alternative dietary patterns, may have changed the dietary exposure taken into account for risk assessments that served as the foundation for the current regulatory framework. Additionally, taking into account climate change, this is be especially important for naturally toxins (such as mycotoxins and plant alkaloids) present in plant products used as meat substitutes (Mihalache et al., 2022a).

It is known that mycotoxins may accumulate in a wide range of crops at both pre- and post-harvest, among them grains, seeds and beans. More than 400 mycotoxins have been identified so far but, as a consequence of gaps in toxicological and occurrence data, only a few are regulated in crops, among them aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), deoxynivalenol (DON) and zearalenone (ZEN). Other mycotoxins often referred as 'emerging' have been found in legumes and grains, such as enniatins (ENs), beauvericin (BEA) and moniliformin (MON) as well those produced by *Alternaria* spp. (Schollenberger et al., 2007; Uhlig et al., 2013; Tolosa et al., 2017; EFSA, 2018; Rodríguez-Carrasco et al., 2019).

Besides mycotoxins, plant alkaloids are a wide group of natural toxins synthesised in plants as secondary metabolites (Jing et al., 2014). They have demonstrated over centuries a wide range of biological activities of pharmacological importance (Debnath et al., 2018). However, the uncontrolled exposure of animals or humans to plant alkaloids through the diet can be of toxicological relevance, especially for pyrrolizidine, tropane and β-carboline alkaloids (Diaz, 2015; Herraiz, 2016). While some alkaloids may inherently occur in legumes, such as quinolizidine alkaloids in lupins (EFSA, 2019a), usually plant toxins are found in seeds and pulses following a cross contamination at harvesting or along the production chain.

1.2. Risk–benefit assessment

The risks and benefits of food consumption have recently become an important health topic (Nauta et al., 2018). In recent years, new tools such as the risk–benefit assessment (RBA) tool have been developed to provide guidance on dietary habits and establish new policies (EFSA, 2010).

RBA, a decision-support tool, is used to develop and/or improve dietary guidelines for population groups and offer public health guidance (EFSA, 2010). This type of assessment can also help policymakers take improved risk – benefit decisions (Verhagen et al., 2012). When the RBA question is related to a food product, the possible adverse and positive health effects are associated with different

food compounds or contaminants (Nauta et al., 2018). RBA is an emerging multidisciplinary tool and until now has been used either for fish (Hoekstra et al., 2012; Cardoso et al., 2018), meat (Mota et al., 2021), cereal-based food (Assunção et al., 2021) or rice consumption (Fang et al., 2021), the substitution of red and processed meat with fish (Thomsen et al., 2018), and the substitution of unprocessed red meat with pulses (Fabricius et al., 2021).

The disability-adjusted life year (DALY) is the most often used health indicator for quantitative RBA, with one DALY equalling one year of healthy life lost (Membré et al., 2021). The World Health Organization (WHO) also employs this metric to assess the global burden of illnesses (Hay et al., 2017) and foodborne diseases (Devleesschauwer et al., 2015).

2. Description of work programme

2.1. Aims

The aims of the work programme for the fellow were the following:

- Assess the distribution of natural toxins in plant-based meat alternatives in Europe.
- Perform an RBA of shifting from meat-based diets to alternative dietary patterns.
- Determine the occurrence of mycotoxins in plant-based meat alternatives from the local markets (Parma, Italy).

The activities described in Section 2.2 are in line with the aims of the programme.

2.2. Activities/Methods

2.2.1. Systematic review

One of the first main activities of the fellow was to perform a systematic review regarding the monitoring of natural toxins in plant-based meat alternatives. Hence, focusing on the European scenario, the objectives were to emphasise the occurrence and/or co-occurrence of mycotoxins and plant alkaloids in plant-based meat imitates and to provide a summary of the current state of the art related to natural toxins regulations and the chemical risks associated with the increased consumption these foods.

The Preferred Reporting Item for Systematic Reviews and Meta-analysis (PRISMA) statement process was followed for the screening of titles, abstracts and full texts in order to ensure the scientific quality of this review and to reduce the possibility of bias (Page et al., 2021). The extensive literature search was performed using three databases (Scopus, WebofScience and PubMed) within the timeframe of January 2000–December 2021.

The systematic review was recently published (Mihalache et al., 2022a) and it provides a picture reflecting the current data regarding the occurrence and concentration of natural toxins in plant-based meat alternatives. Of outmost importance, it explains the need of upgraded exposure assessments correlated with current consumption trends.

2.2.2. RBA of shifting from traditional diets to alternative dietary patterns

Plant-based meat substitutes, also known as meat analogues, are typically made from wheat gluten or legumes like chickpeas, peas, or soybeans (Kumar et al., 2017; Boukid, 2021). Soy proteins, in particular, are of considerable interest for meat analogues due to their high protein content (Kumar et al., 2017),

Initially, the fellow performed research regarding the framework of RBA, the steps of the assessment and previous RBA case studies to get a more comprehensive understanding of the whole process. The steps of the RBA are presented in Figure 1.

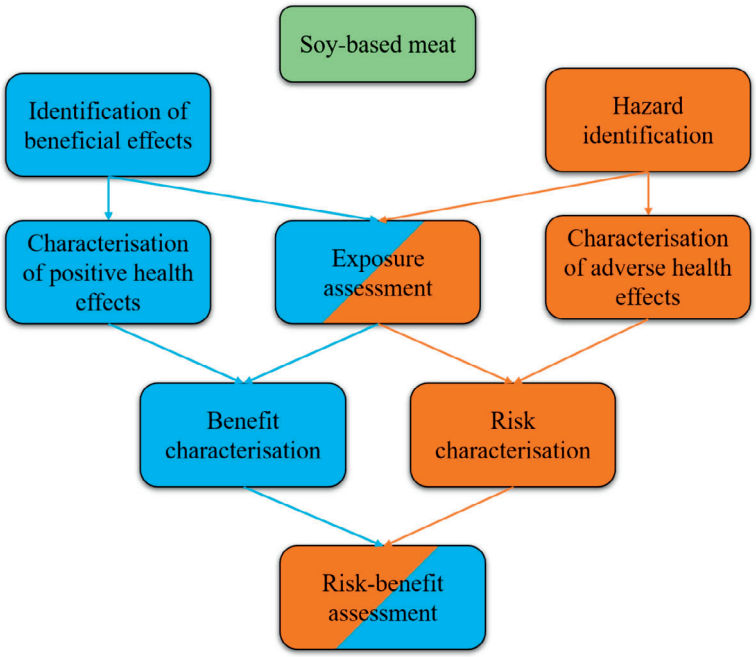

Figure 1: Risk–benefit assessment framework adapted from EFSA (2010)

Considering the increased consumption of meat analogues and mycotoxin occurrence of aflatoxin B1 (AFB1) and ochratoxin A (OTA) (based on the systematic review of Mihalache et al., 2022a and EFSA, 2020a,b), the aim of the RBA was to assess the potential health concern due to an increased mycotoxin exposure in soy-based diets. Soy consumption has been linked to a decrease in the risk of cancer (Fan et al., 2022) while AFB1 and OTA are classified as carcinogenic and possibly carcinogenic agents by International Agency for Research on Cancer (IARC).

For each step of the RBA, research was performed in international expert reports from organisational bodies such as the European Food Safety Authority (EFSA), Food and Agriculture Organization (FAO), World Health Organization (WHO), and WHO & Joint FAO/WHO, and meta-analyses with dose–response relationships. The literature search was performed in Scopus, Web of Science and PubMed with a publication date up to June 2022.

For the collection of food consumption, the fellow used the hierarchical system for classifying and describing food (FoodEx2) (EFSA, 2011). Three consumption scenarios were created using consumption data from the Italian National Food Consumption Survey INRAN-SCAI 2005-06. A baseline scenario (BS) with consumption data from the survey and two alternative scenarios (AS): AS1 with an increased consumption of soy-based food of 50% and AS2 with an increased consumption of soy-based food of 100%. Since soy-based food were not present in the survey, a simulation model was created by substituting each meat product with soy-based analogues, thus reflecting exposure based on a complete substitution of meat with soy, such as is the case for vegans and/or vegetarians.

Three types of exposure scenarios were modelled based on data from EFSA (EFSA, 2020a,b): (a) the optimistic scenario (OS) – mean lower bound (LB) contamination level, (b) the pessimistic scenario (PS) (conservative) mean upper bound (UB) contamination level and (c) the worst-case scenario (WCS) 95th percentile (P95) UB contamination level.

Exposure assessment and risk characterisation were performed as indicated by EFSA using the estimated daily intake (EDI) and the margin of exposure (MOE) approach for the mean and P95 values of AFB1 and OTA (EFSA, 2019b).

The health effects were expressed as increased/decreased risk of liver cancer based on AFB1 exposure/soy ingestion following the procedures recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (WHO and FAO/WHO, 2017).

For the estimation of the relative risk (RR) of hepatocellular carcinoma (HCC)/reduced risk of cancer the fellow used the RRs from the literature and modelled the simulation based on the exposure to AFB1 and intake of soy. Then to show the impact of shifting towards alternative diets, the potential impact fraction (PIF) was calculated. PIF is a measure used to express the proportional change in disease, burden or mortality (Barendregt and Veerman, 2010).

The health effects were finally integrated in the health metric of DALY. The averted/gained DALYs were calculated using data from the Global Burden Disease (GBD, 2019). The final step was to compare each scenario through the calculation of the difference between the alternative scenarios and baseline scenario (ΔDALYs). ΔDALY > 0 indicates loss of healthy years of life due to exposure to AFB1, while ΔDALY < 0 was considered as a health gain due to intake of soy. All of the calculations were realised using Microsoft Excel 19 (Microsoft, Redmond, Washington), SPSS Statistics 26 (IBM Software Group, Chicago, IL), Dietary Exposure (DietEx) and the Rapid Assessment of Contaminant Exposure (RACE) tools (https://www.efsa.europa.eu/en/science/tools-and-resources).

This is a proof of concept and a first attempt at evaluating the risks and benefits of a full replacement of meat with soy-based analogues related to mycotoxin exposure (Mihalache et al., 2022b).

The results have been integrated into a manuscript currently under review (preprint available: https://doi.org/10.21203/rs.3.rs-1821191/v1) which underlines the importance of proper regulatory frameworks correlated with current consumption trends.

Using the above methodology, in this report a third alternative scenario (AS3) was created in which a simulation of 200% increased consumption of soy-based food was modelled for Italian consumers. In order to show a glimpse of the benefits and risk of shifting towards soy-based foods, the reader can check Figures 2 and 3. Based on the type of consumption and contamination scenario, the extra number of cancer cases varied between 0.02 and 0.66/100,000 individuals, while the number of prevented cases of cancer was between 0.0006 and 0.18/100,000 individuals. Converting the number of cancer cases into DALYs would translate in to the gain of 0.2–0.6 DALYS/100,000 individuals due to AFB1 exposure and averting between 0.006 and 1.8 DALYs/100,000 due to soy intake.

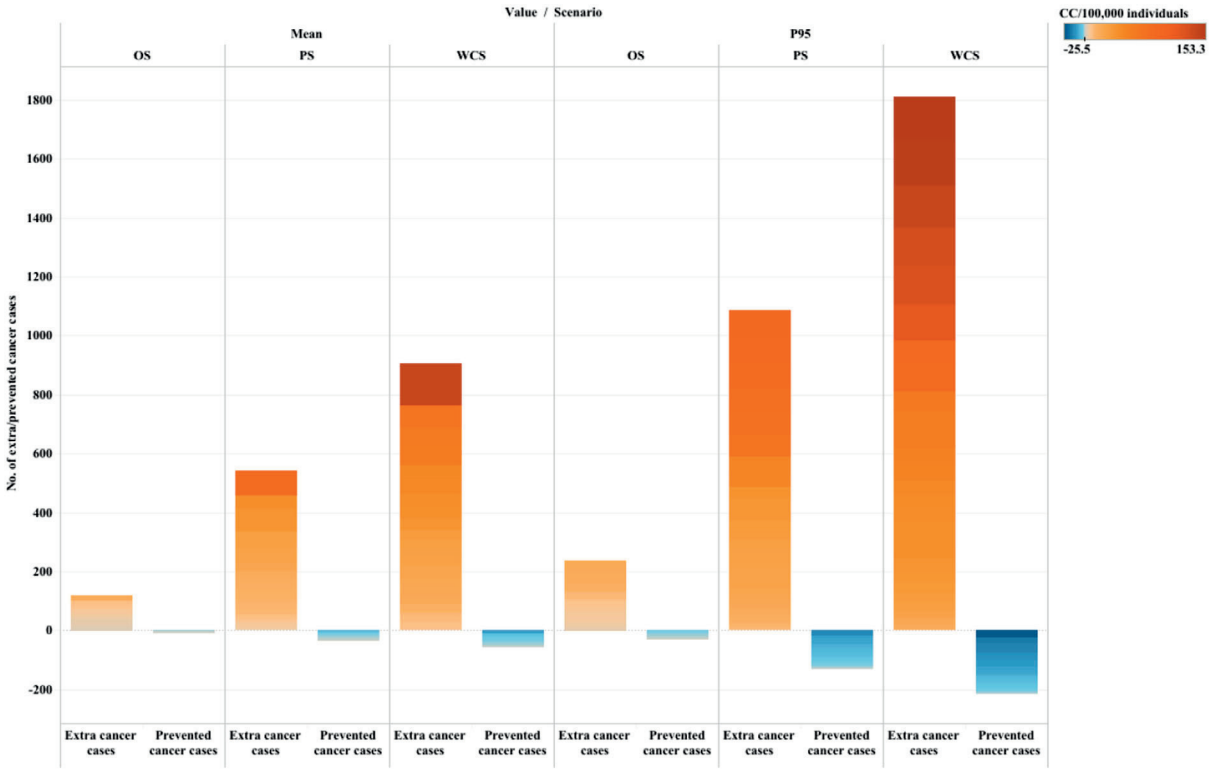

Figure 2: Mean and P95 extra cancer cases (coloured orange) due to AFB1 exposure and prevented cancer cases (coloured blue) due to soy intake in the alternative scenario based on three contamination scenarios optimistic scenario (OS), pessimistic scenario (PS) and worst-case scenarios (WCS); CC = cancer cases

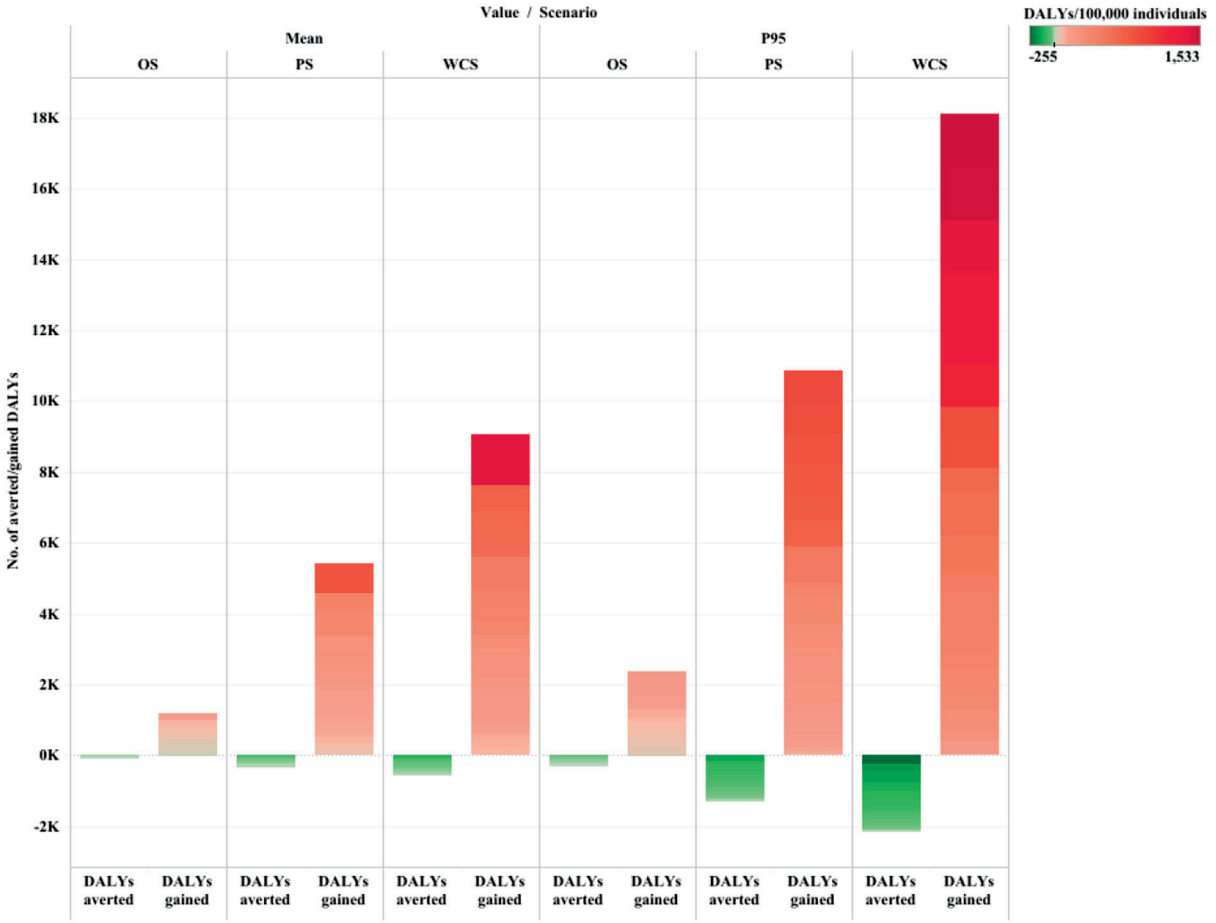

Figure 3: The number of DALYs gained (coloured red) due to AFB1 burden for the mean and P95 values and the number of DALYs averted (coloured green) due to mean and P95 soy intake in the alternative scenario based on three contamination scenarios: optimistic scenario (OS), pessimistic scenario (PS) and worst-case scenarios (WCS)

2.2.3. Multi-mycotoxin determination in plant-based meat alternatives

As already mentioned in this report, mycotoxins have been previously found in legumes and plant-based meat alternatives (Kunz et al., 2020; Mihalache et al., 2022a; Rodríguez-Carrasco et al., 2019). However, data regarding the contamination of the most used plant-based meat alternatives is limited. Consumers' exposure to natural toxins needs to be reassessed as the consumption of meat imitates is in a continuous uptrend.

Hence the fellow's aim for this activity was to optimise an HPLC–MS/MS method for the determination of mycotoxins in plant-based meat alternatives and perform a preliminary exposure assessment based on the assumption of a full substitution of meat with plant-based meat alternatives.

Meat consumption data for Italian consumers were retrieved from FAO (2018) and then substituted with plant-based meat alternatives.

Exposure assessment and risk characterisation were performed as recommended by EFSA (2019b).

The fellow worked together with the group from the Department of Food and Drug from Parma University where he learnt chemical analytical procedures for the determination of mycotoxins in food. Since there is a lack of analytical methods for multi-mycotoxin determination in plant-based meat alternatives, the fellow together with the working group optimised a salting-out-assisted liquid–liquid extraction (SALLE) method followed by HPLC–MS/MS. The method was optimised, characterised and validated using real samples.

A manuscript, currently under review (Mihalache et al. submitted, 2022), presents the results of this activity which consist of the occurrence and concentration of mycotoxins found in the meat alternatives and a preliminary exposure based on a replacement model of meat with plant-based foods.

To be able to carry out these activities, the fellow had regular meetings and discussions with the supervisor.

2.2.4. Additional activities

During the EU-FORA fellowship, the fellow took part into the following activities:

- Getting acquainted with the chemical risk assessment procedures that take place at hosting site.
- Preparation of three manuscripts out of which one is already published and two are under review in peer-reviewed journals.
- Weekly meeting with the supervisor and working group from the Food and Drug Department.
- Participation to the World Mycotoxin Forum 2022 with a poster presentation within the EU-FORA activities.
- Learning chemical analytical procedures within the working group.
- Participation to FoodMicro2022 (to take place on 28–31 August 2022).

3. Conclusions

The work programme carried out at the Food and Drug Department, Parma University enriched the fellow with expertise in chemical risk assessment, RBA, and food and analytical chemistry.

At the hosting site, the fellow was able to perform for the first time an RBA showing the importance of proper regulatory frameworks in regards to the regulation of natural toxins in plant-based meat alternatives using soy as a meat replacer. The fellow got familiarised with food classification systems and dietary guidelines as well as tools used by EFSA for exposure assessment and risk characterisation, thus advancing his skills as a future risk assessor.

Through collaboration with the research group, the fellow was challenged to learn analytical chemistry techniques and optimise a method for the detection and quantification of mycotoxins using meat imitates matrices. Therefore, the fellow has advanced his scientific skills and has gained multidisciplinary expertise in terms of risk assessment and food chemistry.

In addition to these activities, the fellow was exposed to the supervisor's and hosting site scientific network providing opportunities for knowledge exchange and future collaborations.

Thereby, the EU-FORA programme and the research group from Parma University were a rewarding experience for the fellow both in terms of professional and personal development.

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Abbreviations

AFB1	aflatoxin B1
Afs	aflatoxins
AS	alternative scenario
BEA	beauvericin
DALY	disability-adjusted life year
DietEx	Dietary Exposure
DON	deoxynivalenol
EDI	estimated daily intake
EU-FORA	European Food Risk Assessment Fellowship Programme
FAO	Food and Agriculture Organization
FBs	fumonisins
GBD	Global Burden of Disease
HCC	hepatocellular carcinoma
HPLC-MS/MS	high-performance liquid chromatography–mass spectrometry/mass spectrometry
IARC	International Agency for Research on Cancer
INRAN-SCAI	Italian National Food Consumption Survey
MOE	margin of exposure
OS	optimist scenario
OTA	ochratoxin A
P95	95th percentile
PIF	potential impact fraction
PRISMA	Preferred Reporting Item for Systematic Reviews and Meta-analysis
PS	pessimistic scenario
RACE	Rapid Assessment of Contaminant Exposure
RBA	risk–benefit assessment
RR	relative risk
SALLE	salting-out-assisted liquid–liquid extraction
WCS	worst-case scenario
WHO	World Health Organization
ZEN	zearalenone

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Risk assessment of food contact materials

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Abstract

In the EU, any material or article intended to come into contact with food, which is placed on the market, has to comply with the requirements of the Regulation (EC) No 1935/2004 – the so called 'framework regulation' for food contact materials (FCM). FCM covers a wide range of materials, including plastics, paper, metal and glass, which contain chemicals that might migrate into food. These chemicals must not migrate into the foodstuff in quantities that could endanger human health, bring about an unacceptable change in the composition of the food, or bring about a deterioration in the organoleptic characteristics thereof. Despite of this general regulation, the safety of new and specific materials that are not covered must be assessed case-by-case. In addition, national authorities can set their own regulations, and in this context, the BfR sets recommendations, which are not legal norms, but represent a standard for the production of materials not subjected to any specific legislation and are well accepted by other European Commission member states according to the mutual recognition principle. The BfR Unit 74 is responsible not only to deal with chemical risk assessment of FCM but also to evaluate application dossiers to include new substances in the positive list of FCM chemicals. In the proposed EU-FORA programme, the fellow had the opportunity to gain experience in the evaluation of toxicological data from applicant dossiers and in the methodologies of migration tests performed in the laboratories. Moreover, the fellow also made a bibliographic review on scientific literature on the migration studies from starch-based materials.

Keywords: chemical risk assessment, food contact materials, FCM, migration, biopolymers, starch-based materials

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1. Introduction

Food contact materials (FCM) are all materials intended to come into contact with food during its production, processing, storage, preparation and serving. FCM include a wide variety of materials such as plastics, paper, ceramics, metals, rubbers and inks used in food packaging and containers, industrial equipment kitchenware and tableware. Once the material is in direct or indirect contact with food, it can release its compounds, which might migrate into foodstuff. Migration is the process of mass transfer of chemical compounds from packaging to food and is affected by different factors: nature of food and material, type and temperature of contact, properties and concentration of migrating substances present in the material (Arvanitoyannis and Kotsanopoulos, 2013).

During the manufacture of FCM, many substances, which can contain impurities, are incorporated to achieve the required properties. The interaction between the different compounds present in the material can originate non-intentionally added substances (NIAS), which can also migrate into food. The Commission Regulation (EU) No 10/2011 (European Commission, 2011) on plastic materials and articles intended to come into contact with food defines NIAS as 'an impurity in the substances used or a reaction intermediate formed during the production process or a decomposition or reaction product'.

In the European Union (EU), in order to be considered safe, materials and articles intended to be in contact with food must comply with the Regulation (EC) No 1935/2004 (European Commission, 2004), which is considered the framework regulation for FCM. It sets general requirements for FCM, that shall be manufactured in compliance with good manufacturing practices, according to Commission Regulation (EC) No 2023/2006 (European Commission, 2006a), and must not transfer their constituents to food in quantities that could '(a) endanger human health, (b) bring about unacceptable change in the composition of the food or (c) bring about a deterioration in the organoleptic characteristics thereof'.

The EU rules on FCM can be of general scope, i.e. apply to all FCM, or apply to specific materials only. EU law regulation may be complemented with member states national legislation, in accordance with Article 6 of Regulation (EC) No 1935/2004 (European Commission, 2004), if specific EU rules do not exist.

In Germany, although the BfR Recommendations on Food Contact Materials (https://bfr.ble.de/kse/faces/DBEmpfehlung_en.jsp) are not legal norms, they 'represent the current state of the scientific and technical knowledge for the conditions under which consumer goods made of high polymer substances meet the requirements of § 31 paragraph 1 of the German Food and Feed Code (LFGB) and of the Regulation (EC) No 1935/2004 (European Commission, 2004) in respect to their health safety'. BfR Recommendations are also well accepted by other European Commission member states due to the mutual recognition principle.

Manufacturers that want to include new substances in the BfR Recommendations on Food Contact Materials must send an application following the EFSA Note for Guidance for the Preparation of an Application for the Safety assessment of a Substance to be used in Plastic Food Contact Materials (EFSA CEF Panel, 2008) (https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2008.21r). The evaluation of dossiers include the verification of the compliance with the requirements set in the EFSA Note of Guidance and the assessment of the scientific information provided by the applicant.

1.1. Enamels

Enamel is a glassy, vitreous and usually opaque substance. It is used as a protective or decorative coating on metal, glass or ceramic ware. Vitreous enamel, or porcelain enamel, is produced by fusion of powdered glass applying fire, between 750°C and 850°C. After melting, it can be applied on surfaces that withstand this high temperature and then hardens resulting in a smooth vitreous coating.

As a FCM, enamel has been tested in the same way as ceramic materials, i.e. at room temperature for 24 h using acetic acid 4% as a food simulant (FS). However, these conditions do not mimic properly the possible uses of enamelled fcm. Thus, the International Organization for Standardization (ISO) (2022) published a document (DIN EN ISO 4531:2022–04 'Vitreous and porcelain enamels - Release from enamelled articles in contact with food - Methods of test and limits') setting migration conditions and release limits for metals from vitreous and porcelain enamelled articles in contact with food.

1.2. Biomaterials

Bioplastics are being developed as an alternative to fuel-based plastics as a result of current environmental concerns about the production and disposal of conventional plastic materials. Bioplastics can be either bio-based, biodegradable or both. Polybutylene adipate terephthalate (PBAT), polycaprolactone (PCL), polyhydroxyalkanoate (PHA), polyhydroxybutyrate (PHB), polylactic acid (PLA)

and thermoplastic starch (TPS) are some examples of bioplastic materials (European Bioplastics, 2018). As starch is naturally present in the human diet, it is generally considered safe when used as FCM. However, when it undergoes chemical modification and/or additives are incorporated, migration tests to identify and quantify possible compounds and/or NIAS that could migrate into food should be carried out.

2. Description of work programme

2.1. Aims

The objective of the programme was to acquire knowledge on how risk assessment of FCM is performed. In this way, first, the fellow participated in the evaluation of toxicological data from dossiers for adding new substances to the BfR Recommendations on Food Contact Materials. It included analysis of toxicological data of *in vivo* and *in vitro* tests to verify the agreement with EFSA's Note for Guidance. Later on, the fellow was involved in practical work conducted in the German National Reference Laboratory for Food Contact Materials, which is located at the BfR. In this occasion, the fellow learned how to conduct a migration test in the laboratory and how to handle the available equipment. In parallel, the fellow made a bibliographic research on risk assessment of biomaterials, focused on starch-based materials intended to be used as FCM, which will be further detailed in this document.

2.2. Activities/methods

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

a) Scientific evaluation of one dossier, whose data will not be included due to confidential agreement. It involved the review of toxicological data of a substance to be included in the BfR Recommendations on Food Contact Materials. The dossier was submitted by the applicant in accordance to the EFSA Note for Guidance. Due to data confidentiality, the data related to this item will not be detailed in the next sub sessions.
b) Determination of release of the elements aluminium, antimony, arsenic, barium, cadmium, cobalt, chromium, copper, iron, lead, lithium, manganese, molybdenum, nickel, titanium, vanadium and zinc from enamelled plates into food and food simulants (FS). Apparatuses according to DIN EN ISO 4531:2022–04 (International Organisation for Standardisation, 2022) were used for the release test and inductively coupled plasma-mass spectrometry (ICP-MS) was used to quantify the elements. The release tests were conducted at 70°C and 95°C for 2 h, in accordance to ISO 4531. The FS proposed were artificial tap water acidified with citric acid to pH 3.5, citric acid 0.5%, acetic acid 3% and 4%, respectively.
c) Bibliographic research on accurate definition of biomaterials, and on migration results, focusing especially on starch-based materials. For more details see Section 2.2.2.

2.2.1. Migration study

FCM cover a wide range of materials, including ceramic and vitreous materials; enamel and enamelled materials are one of them. It can be applied on the surface of metal items with protection or decorative purposes. Metals such as lead and cadmium are present in those materials and can migrate into food when used as FCM. However, not only those metals are present but also aluminium, arsenic, chromium, nickel and others can be detected in those materials, which can bring risks to human health if they migrate into foodstuff in quantities resulting in exceedance of health-based guidance values.

As enamels are very resistant to high temperatures, they can be used as kitchenware coatings and be heated during food preparation. As elements migration is dependent on the temperature of contact and the nature of food, it is important that the migration tests are conducted in a way that the conditions simulate the worst-case scenario, e.g. with respect to food simulant, temperature and time.

The preliminary tests were conducted with the objective to make the fellow familiar with the release test apparatus, according to ISO 4531:2022 (International Organisation for Standardisation, 2022), and with an ICP-MS equipment. Water MilliQ was used and the chosen conditions were 70°C and 95°C for 2 h in order to mimic the use of enamelled kitchenware used for cooking, such as pans and cake pans. The tests were performed in the German National Reference Laboratory for FCM, located at the BfR.

The migration vessels are made of borosilicate glass, cylindrical-shaped with an internal diameter (d) of 80 mm and a height of 36 mm. Between the cylinders and plates, silicon rubber rings with the same internal diameter of the vessels are used to seal the chambers properly and avoid leakage. Each

silicon rubber has a thickness of 2 mm, so then, when the chambers are assembled, the total height (h) is 40 mm as silicon rings are placed in both ends in contact with the sample. Given that, it is possible to calculate the volume (V) of FS, multiplying the contact surface area (S) by the height of the chamber.

$$S = \pi \times d^2/4 = 5,025 \text{ mm}^2.$$

$$V = S \times h = 201,000 \text{ mm}^3.$$

The apparatus was assembled in order to have three chambers (Figure 1). Thus, there were two surfaces of releasing enamel material in each chamber. If it is the case, the surface area (S) might be multiplied by 2. It is important that the chambers are filled completely in order to have correct surface and volume for migration calculation. Element concentration in the FS was determined via ICP-MS. Three consecutive tests were performed on the same day and only the FS solution of the third test was analysed in order to account for repeated use of enamel articles. Blank tests were also conducted together with the samples.

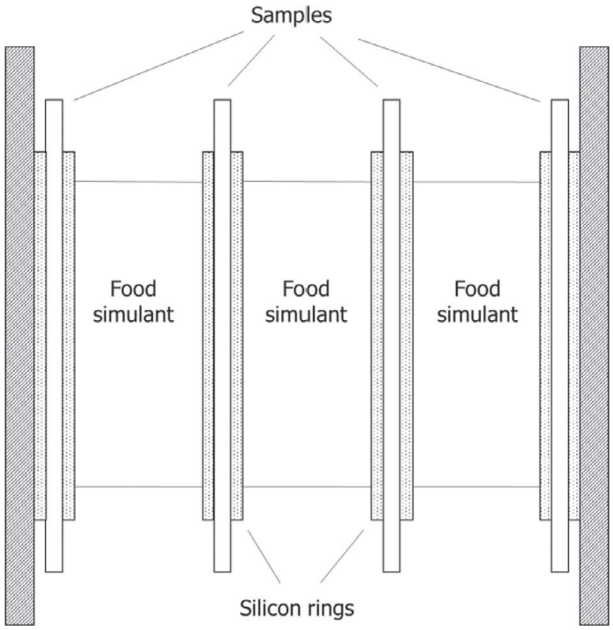

Figure 1: Schematic representation of a release test apparatus, according to ISO 4531:2022, with three testing chambers

In order to get reproducible results, it is very important to closely follow the conditions set by the ISO – especially keeping the temperature constantly in a very narrow range. To ensure this, tests were conducted prior to analysis to establish a method representing hot fill conditions (i.e. pouring boiling liquid food/drink into a fillable article and let it cool down). The method was optimised to keep the temperature of the FS at 70 ± 2°C or 95 ± 2°C, respectively. Therefore, the temperature was monitored every 60 s.

2.2.2. Risk assessment review for starch-based materials used as FCM

Biopolymers have been widely explored as a more eco-friendly alternative to plastics derived from petroleum. There are already a large number of companies producing biomaterials, usually blended with conventional plastics, to be applied as FCM. Nevertheless, biopolymers present some drawbacks that must be overcome, such as low stability, poor mechanical and barrier properties. Therefore, additives are usually incorporated in their production to achieve the desired properties (Scarfato et al., 2015). Those additives and possible NIAS, derived from their degradation or interaction with polymers or other additives, might migrate when in contact with food. It is necessary and importance to perform a risk assessment for these new materials in accordance to EFSA's Note for Guidance.

Starch is one of the most abundant macronutrients in nature and is composed by amylose and amylopectin (Figure 2). It has good potential to become a raw material in the manufacture of

bioplastics, thus, there is currently a huge amount of researches on biomaterials using both native and modified starch (García-Guzmán et al. 2022). However, most of the researches do not focus on the safety aspects of the materials, and only few of them report results of migration studies.

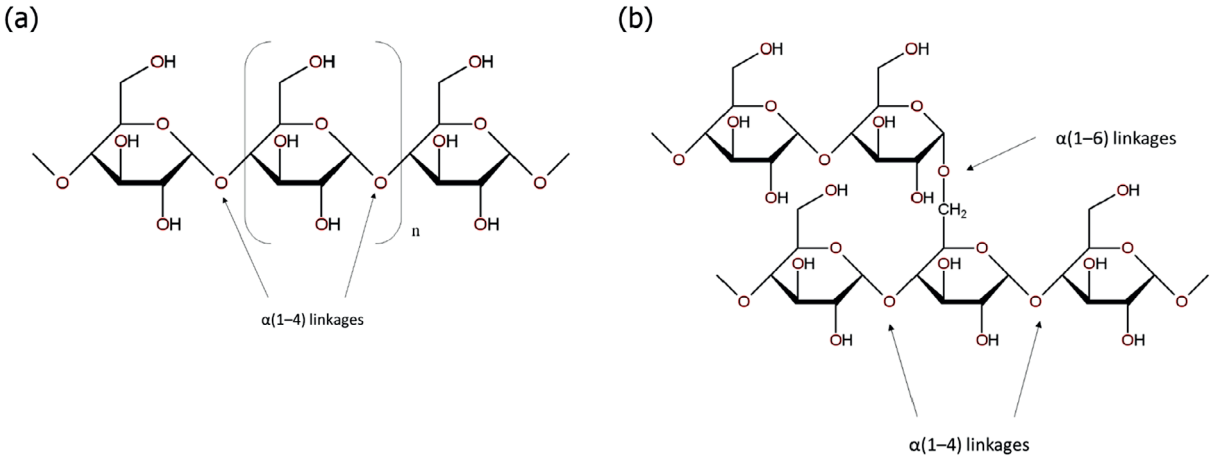

Figure 2: Chemical structure of (a) amylose and (b) amylopectin

Regulation (EU) No 10/2011 (European Commission, 2011) includes biomaterials in its scope if they are produced by a conventional polymerisation process, by microbial fermentation or by chemical modification of natural polymers; so, materials based on native starch are not included in its scope. In the BfR Recommendations XXXVI on paper and board for food contact and XXXVI/2 on paper and paperboard for baking purposes, native and modified starches are listed as production aids and sizing agents.

According to Regulation (EC) No 1907/2006 (European Commission, 2006b), concerning Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH), non-chemically modified substances obtained from natural sources are exempted from registration, excepted those which are dangerous, flammable, skin or eye irritants, or persistent, bio accumulative and toxic, or very persistent and very bio accumulative.

Following those regulations, and taking into account Article 3 of Regulation (EC) No 1935/2004 (European Commission, 2004), the use of pure native starch as a FCM is not submitted to any previous registration or risk assessment. However, starch-based materials are brittle and present poor water permeability barrier, which makes the use of additives or blends very common to manufacture bioplastics with suitable properties.

The fellow carried out a bibliographic study on Scopus scientific database of the last five years using the combinations of keywords presented in Table 1. However, the large number of publications resulting from the search for 'bioplastics', 'biopolymers' or 'biomaterials', they include a wide range of applications other than FCM, such as medical, automotive, agricultural and others. Also, the studies on starch covered a wide range of different subjects not related to FCM. It could be noticed that even when the researchers published data on migration studies, most of them focused on the migration process modelling rather than the safety aspects. The fellow evaluated the available publications, extracted data on use, possible additives, migration and risk assessment considerations. From reviewing these, a prioritisation for necessary information and evaluation was concluded in order to improve risk assessment for starch based bioplastics in contact with food.

Table 1: Keywords and combinations in Scopus on 15 July 2022

Scopus – keyword	Total	2022	2021	2020	2019	2018
Bioplastic(s)	2,253	424	694	473	373	289
Biopolymer(s)	17,688	2,704	4,730	3,928	3,299	3,027
Biomaterial(s)	42,431	5,198	10,249	9,354	9,194	8,436
(biobased OR bio-based) AND plastic(s)	1922	352	539	409	357	265
(biobased OR bio-based) AND material(s)	6,518	1,167	1830	1,435	1,182	904
Biodegradable AND plastic(s)	5,164	967	1,432	1,068	980	717
Biodegradable AND polymer(s)	13,301	1978	3,379	2,882	2,638	2,424

Scopus – keyword	Total	2022	2021	2020	2019	2018
Biodegradable AND material(s)	14,762	2,248	3,799	3,251	2,942	2,522
Bioplastic(s) AND (food AND contact AND material(s))	23	3	10	6	2	2
Biopolymer(s) AND (food AND contact AND material(s))	86	11	21	23	17	14
Migration AND bioplastic(s)	18	2	6	2	7	1
Migration AND biopolymer(s)	220	24	57	45	64	30
Migration AND biomaterial(s)	1701	180	429	399	374	319
(risk AND assessment) AND bioplastic(s)	24	8	9	2	4	1
(risk AND assessment) AND biopolymer(s)	46	10	15	6	11	4
(risk AND assessment) AND biomaterial(s)	282	35	66	53	61	67
Starch	34,823	5,165	8,724	7,659	6,913	6,362
Starch AND (food AND contact AND material(s))	85	17	26	13	17	12
Migration AND starch	239	46	66	49	42	36
(risk AND assessment) AND starch	1,074	146	284	262	187	195

3. Conclusions

The EU-FORA programme was an opportunity for the fellow to acquire theoretical and practical knowledge on chemical risk assessment of FCM. First, the fellow was placed together with the experts on Safety on Food Contact Materials and could participate on the evaluation of a dossier of a substance to be included in the BfR Recommendations list. Then, the fellow was invited to carry on a literature review on the safety of bioplastics intended to be used as FCM, with special attention to starch-based materials. In parallel, the fellow followed a laboratory work on preliminary tests carried out in the German National Reference Laboratory for food contact materials, located at the BfR. The aim of this project was to establish proper methodologies to use migration cells for testing of enamel articles under hot fill conditions.

All in all, the programme not only allowed the fellow to learn different tools and gain expertise to assess risks related to food contact materials, but also has been a unique opportunity for the fellow to build connections with experts and outstanding professionals in food risk assessment.

4. Disclaimer

The results of the risk assessment review for starch-based materials used as FCM as well as the results of the migration studies are intended to be published in a peer-reviewed journal. In order to avoid copyright claims, they were not included in the technical report.

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Abbreviations

BfR	Bundesinstitut für Risikobewertung
d	internal diameter of the releasing material in the testing chamber
FCM	food contact material
FS	food simulant
h	total height of the glass chamber
ICP-MS	inductively coupled plasma-mass spectrometry
LFGB	German abbreviation for German Food and Feed Code
NIAS	non-intentionally added SUBSTANCES
PBAT	polybutylene adipate terephthalate
PCL	polycaprolactone
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
PLA	polylactic acid
REACH	Registration, Evaluation, Authorisation and Restrictions of Chemicals
S	surface area of the sample in the testing chamber
TPS	thermoplastic Starch
V	volume of food simulant inside the testing chamber

Appendix A – Training Activities

Type of event	Title	Contribution	Location	Date
Meeting	27th Toxicology Subcommittee of the BfR Commission for Consumer Goods	Oral Presentation	BfR	9.11.2021
Training	Occupational safety	Attendance	BfR	14.1.2022
Training	Introduction to the Quality Management System	Attendance	BfR	15.2.2022
Meeting	28th Toxicology Subcommittee of the BfR Commission for Consumer Goods	Attendance	Online	26.4.2022
Meeting	Meeting of the BfR Commission for Consumer Goods	Attendance	Online	27.4.2022
Seminar	Product analytics	Attendance	Online	5.5.2022
Seminar	Control of procedures and evaluation strategies	Attendance	Online	17.5.2022
Training	Biological Substance and Genetic Engineering Safety Ordinance	Attendance	Online	1.6.2022
Seminar	Fiber- and nano-toxicology	Attendance	Online	14.6.2022
Conference	ONE Conference	Attendance	Brussels	21–24.6.2022

EU-FORA SERIES 5

PROF. WACLAW DABROWSKI INSTITUTE OF AGRICULTURAL AND FOOD BIOTECHNOLOGY STATE RESEARCH INSTITUTE

INSTYTUT BIOTECHNOLOGII PRZEMYSŁU ROLNO-SPOŻYWCZEGO im. prof. Wacława Dąbrowskiego PAŃSTWOWY INSTYTUT BADAWCZY

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Quantitative microbiological risk assessment of traditional food of animal origin produced in short supply chains in Poland

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Abstract

Polish raw-milk cheeses produced in short supply chains may pose a threat to consumer safety due to pathogen presence. *Listeria monocytogenes* is a bacterium of great importance for the food safety of refrigerated RTE foods due to its ability to grow at refrigeration temperatures. During the EU-FORA fellowship, a stochastic risk assessment was designed and executed to estimate the risk for consumers from *L. monocytogenes* in these products. The aim was to develop a probabilistic QMRA model that would incorporate the variability and uncertainty of the model's inputs such as prevalence, initial concentration levels, product intrinsic factors, domestic storage temperature and consumer behaviour. The project involved data collection and analysis, growth model selection, mathematical modelling and Monte Carlo analysis in R programming language. Microbiological and physicochemical testing were carried out throughout the year on two types of cheeses in combination with a domestic refrigerator temperature survey and accompanying consumption questionnaire. Collected data were fitted to probability distributions using R. The appropriate growth model for the pathogen was selected based on an inoculation study performed on one of the raw-milk cheeses and the chosen mathematical model was written into the R script developed for the QMRA. The dose–response model used the ingested dose calculated from the modelled concentration of *L. monocytogenes* at the time of consumption and the single serving size from the questionnaire to estimate the probability of illness. The final risk was expressed as probability of listeriosis for Polish consumers per serving of raw-milk cheese.

Keywords: *Listeria monocytogenes*, QMRA, raw-milk cheese, risk assessment

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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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1. Introduction

Polish cheese consumption is dominated by Polish white-fresh cheeses, which account for 54% of total sales volume in the country **(WORLD AND POLAND PER CAPITA CHEESE CONSUMPTION,** n.d.**)**. The most popular among these are 'twarog', an acid-set cheese, and 'ser podpuszczkowy', a rennet cheese, made from naturally acidified milk. These types of cheeses from unpasteurized cow's milk are commonly produced by small scale farms and sold at a local level by producers directly to customers (Lucey, n.d.). Consumer Interest in small-scale locally produced cheeses, such as the aforementioned, has for the past years been on the rise in Poland **(Soroka and Wojciechowska-Solis,** 2019**)**.The biological risk associated with the consumption of such products has not been thoroughly assessed in Poland. The small scale of production by farmers and the use of unpasteurised milk raises concerns about the safety of these products. Products produced locally, often using traditional methods,14 are rarely controlled in terms of safety, and the farmers/producers themselves often lack extensive knowledge related to good hygiene and production practices.

Food of animal origin can be a very good substrate for the growth of microorganisms. Specifically, for fresh cheeses, the high nutrient content, high moisture, low-temperature heat treatment and lack of additives, conduce to the growth of various microorganisms, including possibly dangerous pathogens, such as *Listeria monocytogenes*, that could pose a threat to consumers of these products. The small dose required for illness and the severity of listeriosis in humans warrant high vigilance for all RTE foods including cheeses (The European Union One Health 2019 Zoonoses Report, 2021; The European Union One Health 2020 Zoonoses Report, 2021).

Commission Regulation (EC) No 2073/2005 on microbiological criteria, distinguishes between RTE products able to support *L. monocytogenes* growth and those that inhibit growth. The physicochemical characteristics of these two products, pH and water activity (a_w), in this case place them in category 1.2, for which two criteria apply. These are absence in 25 g at the end of production and $<$ 100 CFU/g during its shelf life. These safety criteria are required to achieve the appropriate level of protection (ALOP) for EU consumers.

The Rapid Alert System for Food and Feed (RASFF) is a European system for reporting food safety issues within the Union, and is a key feature of EU food safety. The RASFF report for 2020 included 3 alerts regarding raw-milk cheeses out of a total of 25. The Polish chief sanitary inspectorate (Główny Inspektorat Sanitarny), has enforced 6 recalls of cheeses due to detection of *Listeria* from 2019 to 2021 **(Główny Inspektorat Sanitarny,** 2021**)**. These included cheeses made from raw-milk, and traditional products. Pyz-Łukasik et al studied the occurrence of *L. monocytogenes* in artisanal Polish cheeses and found the prevalence to be much higher than the European average reported by EFSA. Their findings suggest a prevalence of 6.2% for artisanal cheeses, thus warranting the assessment of listeriosis risk (Pyz-Łukasik et al., 2021).

To ascertain the listeriosis risk associated with the consumption of such products, a quantitative microbiological risk assessment (QMRA) was conducted. QMRA is a mathematical modelling method used to estimate the risk from a hazard-food combination for a specific population. The methodology involves the steps of hazard identification, hazard characterisation, exposure assessment and risk characterisation. The process aims to quantitatively describe the fate of the studied pathogen in the food from production to consumption and associate the consumers' subsequent exposure with the risk of an adverse health outcome. Considering the variability and uncertainty associated with all the model inputs (food intrinsic factors, contamination levels, consumption patterns, etc.), the stochastic approach was selected to better portray real-life scenarios. In the stochastic approach, the point estimate values of QMRA inputs are substituted by probability distributions describing variability. Subsequently the final outcome of the risk assessment (RA) is a probability distribution of risk.

2. Description of work programme

2.1. Aims

The working programme aimed to familiarise the fellow with all aspects of an QMRA by providing hand-on experience in performing a RA of a traditional RTE food of animal origin. The fellow carried out activities spanning from relevant data collection to mathematical modelling in R. By applying a stochastic approach, emphasis was placed on describing the variability and uncertainty of most of the model inputs. Throughout the fellowship's duration the fellow undertook learning to code in R, an open-source programming language, popular in the RA community. Thus, a custom R script was

written to fit probability distributions to data, estimate kinetic parameters, model bacterial growth for *L. monocytogenes* and lactic acid bacteria (LAB), perform Monte Carlo analysis and produce a final risk estimate for listeriosis arising from the consumption of raw-milk cheeses produced in short supply chains in Poland.

2.2. Activities/Methods

2.2.1. Prevalence estimation – Cyclical microbiological testing

Microbiological analyses were performed on 46 raw-milk cheese samples (26 twarog and 20 ser podpuszczkowy) from a farmer/producer based in the Silesian voivodeship. Testing for *L, monocytogenes* was carried out according to EN ISO 11290-1:2017 and EN ISO 11290-2:2017 aiming to detect the presence, as concentrations were expected to be low. In cases of detection, *L. monocytogenes* was biochemically verified (LISTERIA-ID Microgen Bioproducts, Camberley Surrey, UK). *L. monocytogenes* was detected in 3.85% of acid-set cheeses and 10% of rennet cheeses, for a total product prevalence of 6.52%. In their study of Polish artisanal cheeses, Pyz-Lukasik et al. estimated a mean prevalence of 6.2% for Listeria monocytogenes.

An important parameter affecting the fate of *Listeria* in dairy products is their indigenous lactic acid microflora which is characteristic of these cheeses, as they are produced without the addition of a starter culture. LAB act by acidifying the food matrix via lactic acid production, competing for resources and producing antimicrobial peptides such as bacteriocins. *Listeria* growth suppression due to the action of LAB is referred to in literature as the 'Jameson Effect' (Mellefont et al., 2008; Sip et al., 2012; de Niederhäusern et al., 2020). To include this important parameter in the final QMRA model, mesophilic LAB were enumerated following ISO 15214:2002.

Additionally, cyclical microbiological ISO standard-based testing was carried out on samples for presence of *Salmonella* spp., *Campylobacter* spp., *Clostridium perfringens*, coagulase-positive *Staphylococcus aureus*, *Escherichia coli* O157:H7, *E. coli*, total number of microorganisms, coliforms, Enterobacteriaceae, moulds and yeasts.

2.2.2. Product intrinsic factors

The ability of *L. monocytogenes* to grow in food and the rate at which it can proliferate is dependent on temperature, water activity, pH and organic acids such as lactic acid (Mellefont et al., 2008; Sip et al., 2012; de Niederhäusern et al., 2020). The product intrinsic factors influencing bacterial growth were measured for every sample and used to better describe the batch-to-batch variability in product characteristics. Water activity was measured with an Aqualab 4TE metre (METER Group, Pullman Washington, USA) with $\pm$ 0.003 accuracy, and pH was measured with an accuracy of $\pm$ 0.01 using a FiveEasy Benchtop pH-metre (Mettler Toledo, Columbus Ohio, USA). Additionally, cheese samples (n = 6) were sent to the institute's chemical department in Warsaw for lactic acid quantification by HPLC.

2.2.3. Domestic refrigerator temperature survey

Domestic refrigerated storage is a crucial part of the cold chain and proper storing temperatures contribute to food safety. Refrigerators can vary considerably and temperature fluctuates constantly during the cooling cycle. This variability in storage temperatures is of paramount importance in a stochastic quantitative microbial risk assessment. Since artisanal and traditional farmer products are mainly sold directly to consumers, the majority of storage time is spent in households. Due to this temperature variation, contaminated products may end up in an environment that could allow for additional growth of the pathogen, thus increasing the final dose consumed. The fact that *L. monocytogenes* is one of the few food-borne pathogens that can adapt and grow slowly under refrigeration temperatures emphasised the need for domestic storage temperature data (Taoukis et al., n.d.; Tasara and Stephan, 2006). As there was no data regarding storage temperatures in Poland in the literature, a refrigerator temperature survey was conducted in order to incorporate this source of variability into the exposure assessment. The survey included 78 inhabitants of Lodz, Poland, and measured the temperature of the middle shelf with a data logger (LogTag model TRIX-8) at 5-min intervals for 24 h. The results of this survey are under consideration for publication for use in future risk assessments.

2.2.4. Consumer questionnaire

Alongside the temperature survey, a consumption questionnaire was provided to participants. Questions were related to personal characteristics of the consumers, cheese storage habits, consumption patterns and refrigerator use. An English version of the questionnaire is provided in the Appendix A. The received questionnaires (n = 56) were used to estimate the variability in portion size (single serving consumed in one sitting), and time of consumption for consumers of these traditional products. The information collected was incorporated into the final model.

2.2.5. Inoculation study – model selection

To select an appropriate predictive microbiology model for *L. monocytogenes* in the fresh raw-milk cheeses, a 10-day inoculation study was performed on ser podpuszczkowy. A *L. monocytogenes* inoculum strain mix was prepared from two reference strains for food (ATCC 19111, WDCM 1/2a) and one strain isolated from the product. The working culture strains were prepared from reference stock following the procedures of EN ISO:11133:2014. Individual cheese samples in replicates were inoculated to achieve an initial concentration level of 4.04 logCFU/g. The aerobically packed, inoculated samples were stored at temperatures of 5°C and 15°C for 10 days. The sample's physicochemical properties were measured (pH, a_w, lactic acid) on the first and last day. Enumeration was performed for *L. monocytogenes* and LAB on each sampling day (days 0, 2, 4, 7, 8, 10) by plating out the appropriate dilution on OCLA and MRS media (Oxoid, Basingstoke Hampshire, UK) respectively (Figure 1).

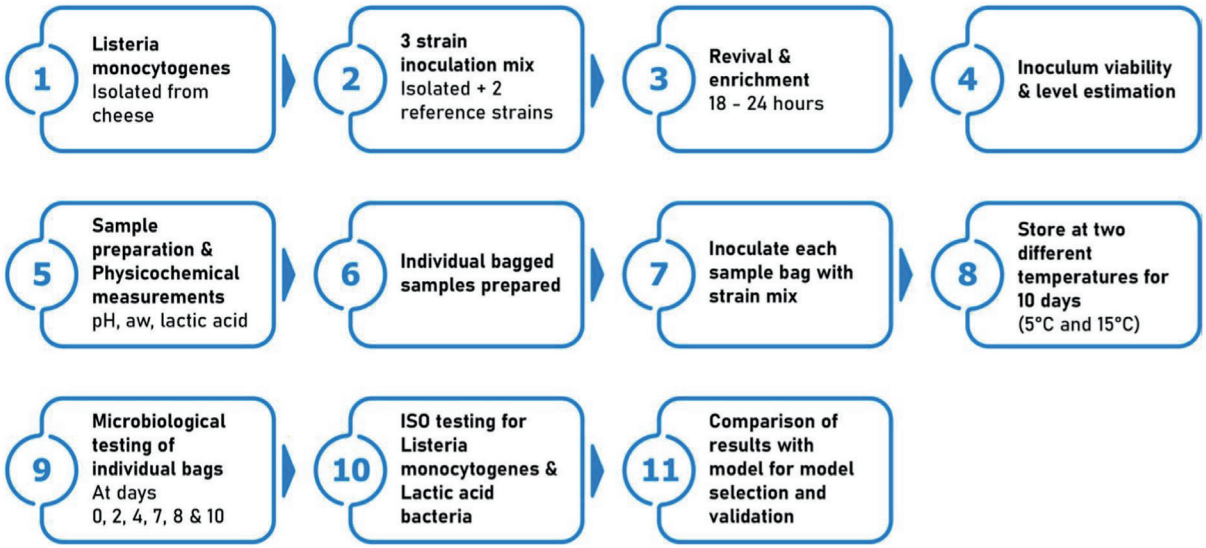

Figure 1: Inoculation study plan of *Listeria monocytogenes* on ser podpuszczkowy

Enumeration results were graphically compared for model selection, taking into account plate count uncertainty of 0.3–0.6 logCFU (Jarvis et al., 2007). The Food Spoilage and Safety Predictor (FSSP), developed by DTU, was selected as it included LAB and lactic acid concentration as an input in its *L. monocytogenes* cottage cheese model ('Growth of *Listeria monocytogenes* in cottage cheese in combination with LAB') (Østergaard et al., 2014) (Figure 2).

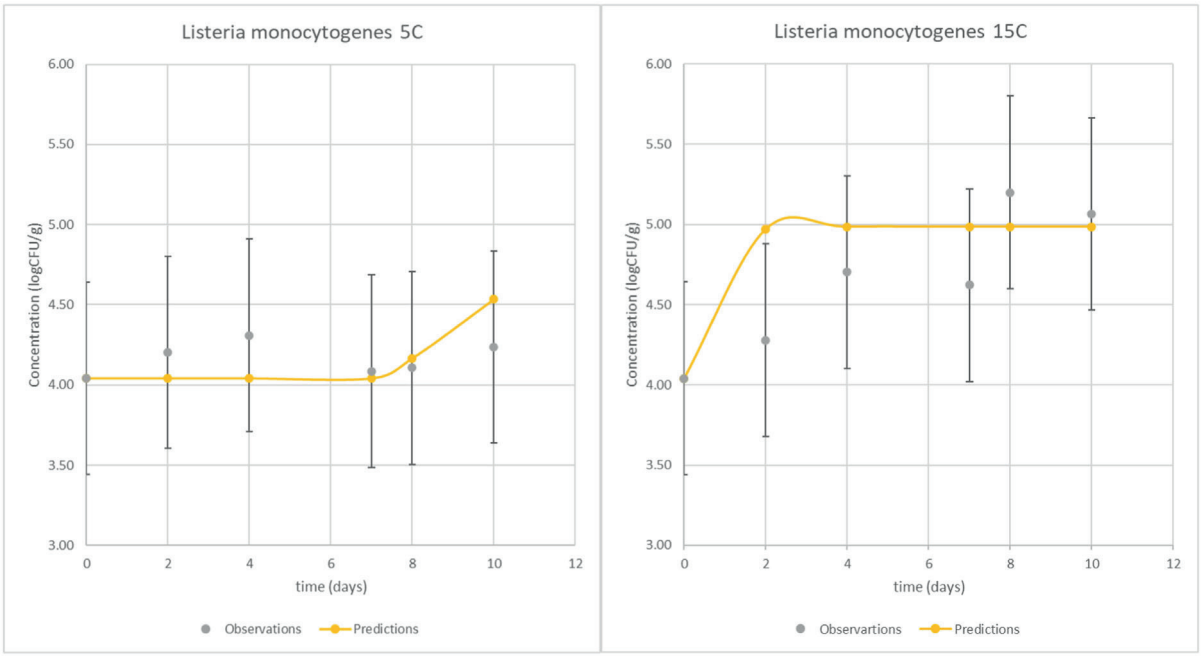

Figure 2: Graphical comparison of *L. monocytogenes* FSSP cottage cheese model prediction (line) and average observed counts (points) from the inoculation study at 5°C and 15°C

The relative error % (RE) for each set of predictions-observations was calculated according to equation (1). At 5°C, all predictions were within the ±10% RE zone while at 15°C 50% of the predictions were within the ±10% RE zone, with one outside of the ±20% RE zone.

$$Relative\ Error\%(RE) = \frac{N_{observed} - N_{predicted}}{N_{observed}} \times 100 \quad (1)$$

2.2.6. QMRA in R

R is an open-source programming language used for statistical analysis and graphics. The language has become a popular choice for risk assessors due to its flexibility and transparency, an important aspect of risk assessment. During the fellowship, the fellow learned to code in R and developed a custom R script for use in stochastic quantitative risk assessment of cheeses.

Probability distribution fitting scenarios of the collected data were statistically tested for goodness of fit. The distribution selection was made based on the chi-square test, the Kolmogorov–Smirnov test, the Cramer–Von Mises test, the Anderson–Darling Test, the Akaike information criterion and the Bayesian Information Criterion. Based on the summary of these statistics, appropriate distributions were selected to describe the model inputs where possible. In cases where the data was insufficient or fitting based on different goodness of fit tests was not conclusive, variables were assigned distributions according to scientific literature. In the case of *L. monocytogenes*, initial concentration N0 was assumed to follow a beta-general distribution as described in the EFSA opinion on *L. monocytogenes* contamination of RTE foods (Ricci et al., 2018). The variability in a_w and pH values was described by applying the beta-pert distribution based on the minimum, maximum and most likely values. The cumulative distribution function was used for the time of consumption and serving size to describe consumer behaviour. The mean lactic acid concentration was used to calculate the undissociated lactic acid (uLA) in mM, based on the product pH value according to equation (2).

$$uLA = \frac{\frac{LAC}{Mr_{lactic\ acid}}}{\left(1 + 10^{(pH - pka_{lactic\ acid})}\right)} \quad (2)$$

For the prevalence of *L. monocytogenes* in the tested products a beta distribution was chosen to incorporate the uncertainty resulting from the limited sample size (Figure 3).

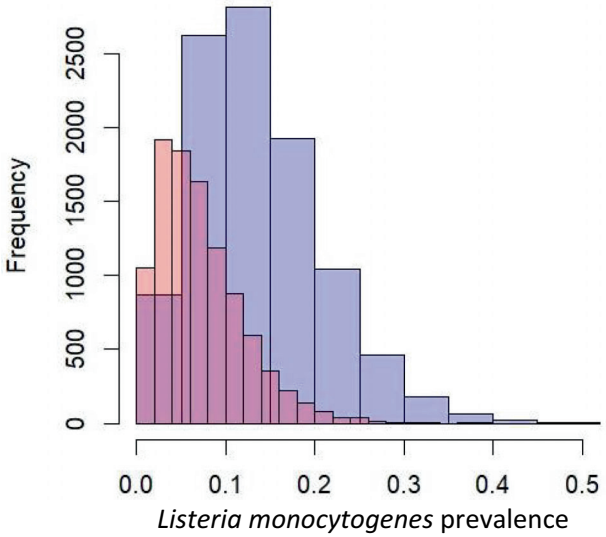

Figure 3: Estimated prevalence distribution of *Listeria monocytogenes* in raw-milk twarog (pink) and ser podpuszczkowy (purple)

To predict the fate of Listeria in the contaminated products, the growth model of Østergaard et al from FSSP was coded into the R script (Østergaard et al., 2014). The FSSP model follows the Jameson effect approach, illustrating the inhibiting influence of mesophilic LAB on the growth of *L. monocytogenes*. This model also includes the term ξ for quantifying the effects of interactions between temperature, pH and organic acids on the growth/no growth limit (le Marc et al., n.d.). The model estimated the LAG time, maximum growth rate $\{_{max}$, the growth rate of *Listeria* in the presence of LAB $\{_{lm}$ and the final concentration N for each simulated scenario. A total of 10,000 simulations were run for the complete model, sampling from the inputted distributions for the Monte Carlo analysis, calculating the final concentration of *L. monocytogenes* at the time of consumption. These results were multiplied with the distribution of the single serving size resulting from the cumulative distribution, to estimate the dose ingested by consumers (lamda) (Figure 4, Table 1).

Table 1: Overview of the R stochastic model parameters for both products

Model parameters	Description	Probability Distribution/Value		Units	Source
		Twarog	Ser podpuszczkowy		
N0	Initial concentration of *Listeria monocytogenes*	rbetagen(iterations, shape1 = 0.194, shape2 = 3.177, min = −1.69, max = 7)		logCFU/g	*EFSA Journal* 2018;16(1):5134, 173 pp. Gombas et al. (2003)
LAB	Initial concentration of lactic acid bacteria	rweibull(iterations, shape = 7.659300, scale = 7.859085)	rweibull(iterations, shape = 10.066707, scale = 7.959734)	logCFU/g	Microbiological analysis
pH	Product pH	rpert(iterations, min =3.98, max = 4.66, mode = 4.32)	rpert(iterations, min = 4.67, max = 5.95, mode = 5.84)	–	Physicochemical testing
aw	Product water activity	rpert(iterations, min = 0.9550, max = 0.9985, mode = 0.9980)	rpert(iterations, min = 0.9696, max = 0.9951, mode = 0.9832)	–	Physicochemical testing
LAC	Lactic acid concentration	Mean = 10,340	Mean = 8,395	ppm	Physicochemical testing
Temp	Domestic refrigerator Temperature	Truncated normal distribution(TBP)		°C	Temperature survey

Model parameters	Description	Probability Distribution/Value		Units	Source
		Twarog	Ser podpuszczkowy		
times	Time of consumption during product's shelf life	recdf(tofcon[(a)], iterations)		Days	Consumption questionnaire
SSS	Single Serving Size	recdf(SS[(b)], iterations)		g	Consumption questionnaire
lamda	Dose received	$=10^{N} \times$ SSS		CFU	WHO/FAO (2004)
iterations	Number of iterations for the Monte Carlo analysis	10,000		–	–
P_Contam	Prevalence of *Listeria monocytogenes* in tested products	rbeta(iterations, s + 1 = 2, n-s + 1 = 26)	rbeta(iterations, s + 1 = 3, n-s + 1 = 19)	–	Microbiological testing
P_ill	Probability of illness	$= 1 - e(-r^{(c)} \times$lamda)		–	WHO/FAO (2004)
Risk	Listeriosis risk for consumers of Polish raw-milk cheeses produced in short supply chains	$=$ P_Contam $\times$ P_ill		–	–

TBP: to be published.
(a): tofcon:Time of consumption Data from Consumption questionnaire.
(b): SS: Single Serving Data from Consumption questionnaire.
(c): $r = \frac{nb\ of\ cases\ in\ the\ subpopulation}{nb\ of\ L.monocytogenes\ ingested\ by\ the\ population}$.

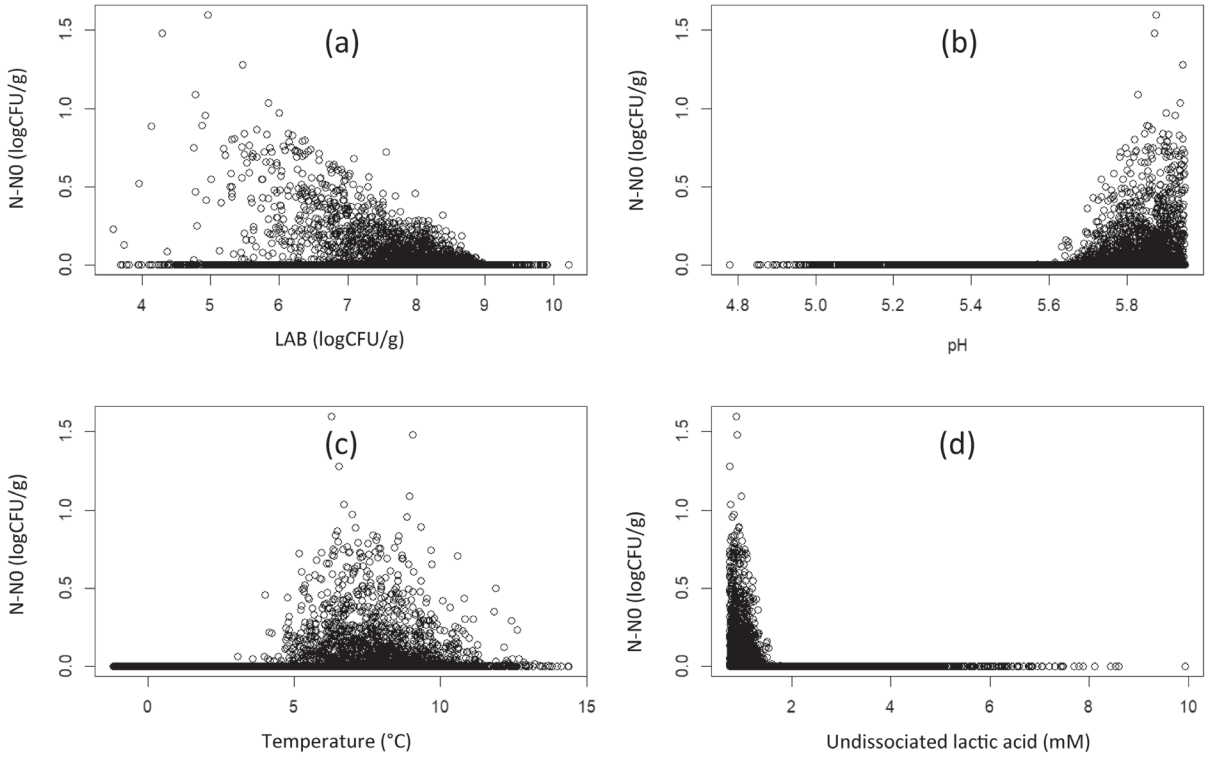

Figure 4: Ser podpuszczkowy *L. monocytogenes* growth in relation to LAB concentration (a), pH (b), Temperature (c) and undissociated lactic acid (d) for each of the 10,000 Monte Carlo simulations

The exposure assessment results were then inserted into the exponential dose response model of WHO/FAO (2004) for the hazard characterisation. Two scenarios were examined for listeriosis infection resulting from the consumption of fresh raw-milk cheeses, one for the healthy general population and one for susceptible consumers with the r parameter values being $2.37 \times 10^{(-14)}$ and $1.06 \times 10^{(-12)}$, respectively. The resulting probability of illness was a probability distribution which was used in the risk characterisation. The final risk to consumers of these products was calculated by multiplying the probability of illness by the prevalence of Listeria in each product.

The Monte Carlo analysis allowed for estimating the risk of foodborne illness per serving with the simulation model, drawing the values of input variables such as prevalence, initial *L. monocytogenes* concentration, initial LAB concentration, storage temperature, pH, a_w, time of consumption and individual serving size from the described probability distributions (Figure 5).

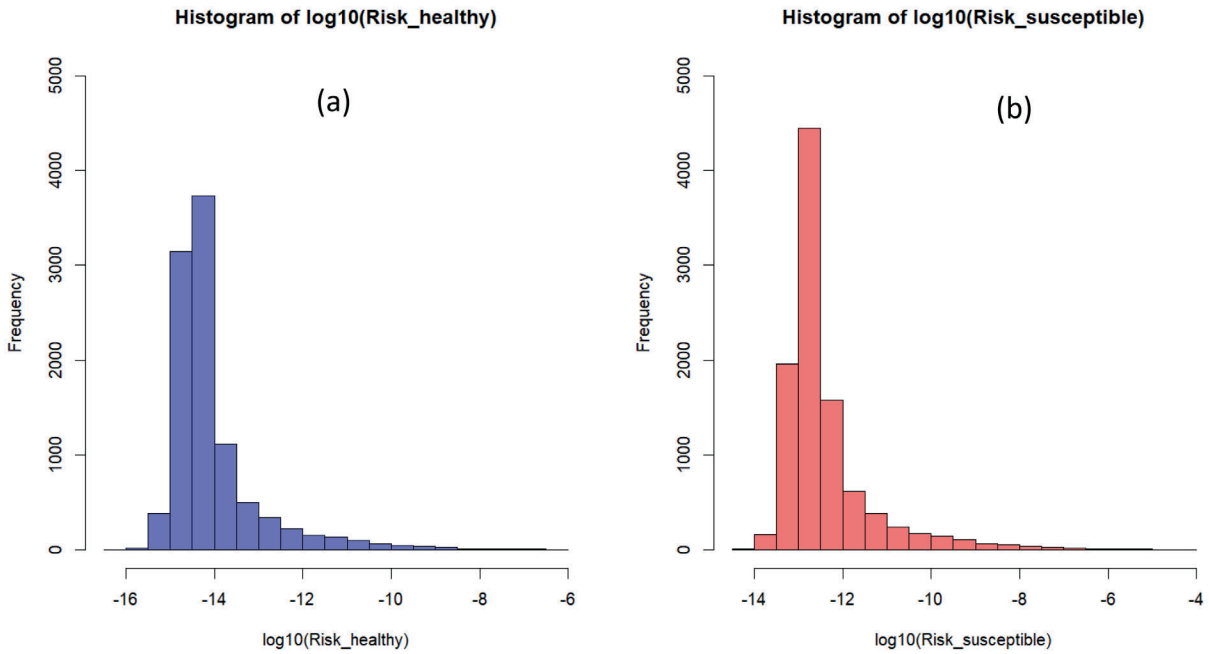

Figure 5: Probability distribution of Log10(Risk) of listeriosis associated with the consumption of one serving of raw-milk ser podpuszczkowy cheese by (a) typical healthy consumers and (b) susceptible high-risk consumers

3. Conclusions

The main aim of the project was the development and application of a stochastic quantitative microbial risk assessment model for estimating the health risk for listeriosis arising from the consumption of RTE animal origin products produced in small supply chains in Poland. The working programme enabled the fellow to gain expertise in the many components of a QMRA by applying the methodology on the specific food-hazard combination of raw-milk cheese and *L. monocytogenes*. In the course of the fellowship, the fellow became familiarised with appropriate data selection from scientific literature, collection of data to fill in identified case-specific data gaps, designing a risk assessment pathway, mathematical modelling of bacterial growth and developing a complete QMRA model for risk estimation, taking into account input and output variability and uncertainty. A particularly valuable facet of this process has been the introduction to R language programming, which has allowed for the model to be conceived and designed probabilistically with the use of Monte Carlo analysis.

Based on 10,000 simulations of the developed model, *Listeria* did now grow in twarog but displayed limited growth in 1.17% of the ser podpuszczkowy simulations. The mean log10(Risk) per serving for healthy consumers of local raw-milk twarog and ser podpuszczkowy was estimated −14.40 (min = −16.80, median = −14.66, max = −6.73, sd = 1.05) and − 14.05 (min = −16.08, median = −14.35, max-6.11, sd = 1.02), respectively. In the case of susceptible consumers, the mean log10(Risk) was increased, at −12.75 (min = −15.84, median = −13.01, max = −4.77, sd = 1.04) for twarog and − 12.40 (min = −14.49, median = −12.70, max = −4.22, sd = 1.03) for ser podpuszczkowy. These estimates are due to the short shelf life, low pH values and high lactic acid concentration due to LAB the products. Thus, the risk of Listeriosis arising from the consumption of these products can be considered as very low, although translating the risk into qualitative terms should be done with caution when communicating with risk managers as there is no consensus on translating probabilities (Scientific Opinion on Risk Assessment Terminology, 2012).

The fellowship programme has been an invaluable opportunity for the fellow to obtain new scientific knowledge and skills, and join the thriving and growing network of the EU-FORA fellows and alumni, promoting the dissemination of risk assessment expertise in Europe.

4. Additional activities during the EU-FORA fellowship

The hosting site arranged a visit to the on-farm facilities of the producer in the Silesian voivodeship participating in the project. During the visit, the facilities were inspected and samples from the production environment were taken and analysed for pathogen and spoilage microorganisms. Throughout the year the fellow was also given the opportunity to attend in person and virtually various scientific conferences and webinars to expand his knowledge in the fields of food safety and risk analysis.

- Attended virtually the Recent Advances in Food Analysis (RAFA) 2021 conference.
- Presented in person at the IAFP 2022 scientific conference in Munich on the topic of quantitative risk assessment of food-borne viruses (QVRA).
- Attended virtually the 4th BVL/MRI Course on Food Safety, Food Authenticity and Risk Management
- Followed a presentation by researchers from the Institute Of Technology Of Agricultural Products ITAP of Greece on photometric methods for microbial spoilage assessment.
- Attended virtually the EFSA ONE HEALTH conference 2022.
- Took part in a 10-h intensive QMRA in R workshop, PredMicro2022.
- Participated in the setting up of a QDA sensory profiling of Polish cheeses.
- Took Polish language classes at a Polish Language School for Foreign students.

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Abbreviations

ALOP	Appropriate Level of Protection
CFU	colony forming units
DTU	Danmarks Tekniske Universitet
FAO	Food and Agriculture Organization
FSSP	Food Safety and Spoilage Predictor
HPLC	high-performance liquid chromatography
LAB	lactic acid bacteria
MRS	de Man Rogosa and Sharpe agar
OCLA	Oxoid Chromogenic Listeria Agar
QMRA	quantitative microbiological risk assessment
RA	risk assessment
RASFF	Rapid Alert System for Food and Feed
RTE	ready to eat
uLA	undissociated lactic acid
WHO	World Health Organization

Appendix A – Consumption Questionnaire

INSTYTUT BIOTECHNOLOGII
PRZEMYSŁU ROLNO-SPOŻYWCZEGO
im. prof. Wacława Dąbrowskiego
PAŃSTWOWY INSTYTUT BADAWCZY

Questionnaire

The information provided will be used exclusively for research purposes by IBPRS.
Thank you for participating.

Date: ...../...../.....

General Information

- Age: ○ 18 – 30 ○ 31 – 40 ○ 41 – 50 ○ 51 – 60 ○ > 60
- Gender: ○ Male ○ Female
- Number of kids (< 18 years) living at home: .....
- Number of adults living at home: .....
- Employment: ○ Full-time ○ Part-time ○ Remote work ○ Retired ○ Domestic ○ Unemployed
- Education: ○ Elementary school ○ High school ○ Higher education ○ Other (please specify .....)
- Do you or anyone in your household take antacid digestion medication: ○ Yes ○ No
- Do you or anyone in your household have a long-term disease or allergy: ○ Yes ○ No
  If yes, please mention the condition: .....
- Do you or anyone in your household take immunosuppressant medication: ○ Yes ○ No

Q1 Do you work in the food industry or food science field?
- ○ Yes
- ○ No

Q2 Who in your household does the food shopping?
- ○ Always me
- ○ Mainly me, occasionally someone else
- ○ Usually someone else, occasionally me
- ○ Always someone else

Q3 How often do you buy farmer raw milk cheese?
- ○ Once a month
- ○ Once a week
- ○ I don't buy artisanal cheeses
- ○ Other (please specify .....)

Q4 How much cheese do you usually purchase?
- ○ < 200 g
- ○ 200 – 300 g
- ○ 300 – 400 g
- ○ 400 – 500 g
- ○ > 500 g (please specify .....)

INSTYTUT BIOTECHNOLOGII
PRZEMYSŁU ROLNO-SPOŻYWCZEGO
im. prof. Wacława Dąbrowskiego
PAŃSTWOWY INSTYTUT BADAWCZY

Q5 How long do you store cheese for?

- 1 – 3 days
- 3 – 5 days
- A week
- Other (please specify .....)

Q6 How much cheese do you usually consume in one sitting (portion size)?

Plate diameter: 27 cm

Q7 Do you check the expiration date on food packaging?

- Always
- Never
- Sometimes

Q8 Where in the fridge do you usually store cheese?

- Door shelf
- Top shelf
- Middle shelf
- Bottom shelf

Q9 How is your refrigerator's temperature regulated?

- Automatically
- Manually

Q10 Do you personally regulate your refrigerator's temperature settings?

- Yes
- No

Q11 How long does it take you to return home from the purchasing point?

- 5 minutes
- 5 – 10 minutes
- 15 – 30 minutes
- > 30 minutes (please specify .....)

Q12 Do you use a special insulated bag when transporting refrigerated products?

- Yes
- No

Q13 Time in: ..... Time out: .....

EU-FORA SERIES 5

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Use of biosensors for rapid and sensitive detection of pesticides in food samples for food safety chemical risk assessment

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Abstract

The utility of pesticides in the agricultural field is unquestionable, but at the same time pesticide use presents serious hazards to the environment and the human health. For that reason, detection of pesticides and their biotransformation products in food is of utmost importance. According to previous studies, esterase-based biosensors have been proposed as a viable and efficient solution for the detection of organophosphate pesticides. In this project, a double mutant of the thermostable esterase-2 (EST2) from *Alicyclobacillus acidocaldarius* was studied as a potential biosensor, for its ability to detect residual amounts of pesticides. Initial characterisation of the enzyme was performed, that included determination of optimal pH, thermophilicity, as well as kinetic analysis. Subsequently, the enzyme was studied by enzymatic activity assays with and without the presence of various organophosphate compounds. The effect of the organophosphates on the enzymatic activity was measured and complete inhibition of the enzyme was observed after incubation with paraoxon. These experiments were followed by an additional method involving labelling of the enzyme with a fluorescent probe. In this case, the effect of different pesticides on the EST2 enzyme was monitored by measuring the fluorescence quenching upon addition to the enzyme. Fourteen compounds were screened with this method and significant fluorescence quenching was observed in the presence of paraoxon and methyl-paraoxon when used in equimolar amounts with the enzyme in the range of nanomolar. This biosensor has been also used to test the presence of pesticides in real food samples, like fruits and juices. This research represents a starting point to develop effective fluorescence-based biosensors aiming at the screening of mutants with different pesticide selectivity profiles. The use of this enzyme-based biosensor can have applications in the field of food traceability as well as environmental monitoring, to control the presence of toxic chemicals, in particular organophosphate pesticides.

Keywords: EST2, biosensors, organophosphates, thermostable enzyme, fluorescence

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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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1. Introduction

The use of pesticides in agricultural activity has become inevitable since the last century, but with that, the hazards to the entire ecosystem, including humans have increased. On top of the strict regulations imposed by European law, monitoring pesticide fate is always necessary. Especially in food, detection of pesticides and their biotransformation products is of utmost importance.

Organophosphate compounds (OPs) belong to a family of chemicals with widespread use as pesticides but at the same time present a hazard to human health by their accumulation in the environment and in food (Georgiadis et al., 2018). OPs act by irreversibly inhibiting acetylcholinesterase (AChE), thus interrupting the function of the pest nervous system. However, this mode of action is so ubiquitous that contact with OPs can affect many non-target organisms including humans. Inhibition of AChE leads to accumulation of acetylcholine (Ach) at the synapses causing neurotoxicity followed by loss of metabolic balance which can ultimately cause death in absence of any effective prevention or treatment (Ranjan et al., 2018).

Traditional methods for the detection and quantification of OPs include gas chromatography (GC) and liquid chromatography coupled to mass spectrometry (LC–MS) (Park et al., 2021). Such methods can be highly sensitive and selective but on the other hand they are time consuming and require high levels of technical expertise to be applied. In recent years, attempts have been made for the development of fast, easy and cheap methodologies for the detection of pesticides, that are based on fluorescent biosensors (Carullo et al., 2015; Pundir et al., 2019). Towards that aim, cholinesterase (Pohanka et al., 2009) and carboxylesterase (Manco et al., 2009) biosensors have emerged during the last decades.

One such carboxylesterase, the thermostable esterase EST2 from the bacterium *Alicyclobacillus acidocaldarius* has recently been studied as a potential biosensor for the detection of OP pesticides (Febbraio et al., 2011). This enzyme is known to bind the OP paraoxon with high affinity (Febbraio et al., 2008) resulting in the irreversible inhibition of the enzymatic activity. This characteristic, combined with the tolerance of EST2 to high temperatures and the overall stability of the enzyme, make it a good candidate for biosensor studies. Previous studies have also investigated various mutants of EST2 for their properties (Pezzullo et al., 2013) and biosensor capabilities (Porzio et al., 2018).

In this project, we studied the double mutant of EST2, named here '2m-EST2'. This version of the enzyme has been modified to carry a cystein at position 35, where the fluorophore IAEDANS can be conjugated, and an additional mutation that was studied for potential diverse selectivity towards OPs. The enzyme was characterised and optimal conditions were determined. A set of available pesticides including paraoxon and other OPs were tested for fluorescence quenching of the double mutant. Indeed, the presence of paraoxon was detectable by fluorescence with a very low limit od detection (LOD) value, making this a good starting point for rapid detection of OPs.

2. Description of work programme

2.1. Aims

The aim of the project was to develop and study an enzyme-based biosensor in the field of food traceability and monitoring, to control the presence of toxic chemicals, particularly organophosphate pesticides, in foods. As part of this working programme, the fellow was planned to develop a fluorescence biosensor for fast and sensitive monitoring of pesticides in water and food samples.

To this aim, several activities were planned, including the bioreceptor preparation, the characterisation of the enzyme, followed by labelling of the enzyme with a fluorescent probe, the testing and evaluation of all the parameters for efficient detection of different pesticides, and finally, testing of the optimised biosensor in real samples, such as water and/or food. In addition, the work plan allowed the fellow to apply and improve expertise in the fields of biochemistry, molecular biology, microbiology and risk assessment.

2.2. Activities/Methods

2.2.1. Bioreceptor preparation: overexpression and purification

The enzyme studied in this project was the thermostable double mutant of the carboxylesterase EST2 from *A. acidocaldarius*, called '2m-EST2'. Preparations of the enzyme were made starting from an

existing plasmid vector pT7-7, containing the mutated esterase gene, that was used to chemically transform *E. coli* BL21 (DE3) cells. Upon induction of protein production, the enzyme was purified from the bacterial culture by using different steps: lysis by sonication, thermoprecipitation, anion exchange and gel filtration chromatography. The purity and activity of the enzyme were monitored through every step of the preparation process by enzymatic activity assay and analysis on SDS–PAGE, showing a > 95% final purity.

2.2.2. Enzymatic activity assays

Enzymatic activity assays were implemented to characterise the enzyme. The assays were performed by using as substrate the carboxylester *p*-nitrophenyl caprylate (*p*NP-C8). The enzyme hydrolyses this compound, and the activity was detected by following the release of the product (*p*-nitrophenol) at 405 nm at the spectrophotometer (Cary 100 spectrophotometer (Varian, Australia)).

Standard assays were performed at 25°C, and complete characterisation was performed to determine various important parameters of the enzyme, including: the optimal pH and the optimal temperature for the esterase activity; the specific activity value and the kinetic parameters in the optimal defined conditions.

The standard assay has been used to evaluate the inhibition effect of different pesticides on the esterase activity of the enzyme.

2.2.3. Labelling of the enzyme with a fluorescence probe and fluorescence spectroscopy analyses

Fluorescence is a very useful and powerful tool for the analysis of biological systems. In this project, it was used to detect changes of fluorescence signal caused by the presence of different pesticides in the sample. For this purpose, the 2m-EST2 enzyme was conjugated with the fluorescent probe 5-({2-[(iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid (IAEDANS) that specifically binds to the cysteine residues, Cys35 (Figure 1).

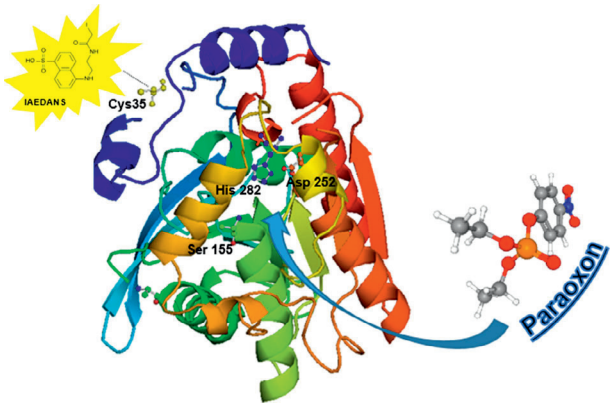

Figure 1: Crystal structure (pdb:1EVQ) of the thermophilic carboxylesterase EST2 from *Alicyclobacillus acidocaldarius*, with indication of the modified Cys35 residue where the fluorescence probe IAEDANS has been conjugated and the position where paraoxon interacts in the active site

After conjugation, the modified enzyme was purified by size exclusion chromatography to remove the excess of the unbound probe. The fluorescence of the labelled enzyme was measured by fluorescence spectroscopy using a spectrofluorimeter (Jasco-FP-8200). Measurements were recorded at emission wavelength of 472 nm, after excitation at 340 nm.

Subsequently, different concentrations of pesticides were added to the conjugated enzyme and new measurements were performed in order to determine the potential fluorescence quenching. An exhaustive list of all the available compounds that were used for these experiments can be found in Table A.1 (Appendix A).

The inhibition detection method was further improved by performing a quicker analysis of pesticides screening on 96-well plates, at a Microplate Reader (Victor Nivo, Pelkinelmer), at 25°C, by using two amounts of bioreceptor (80 and 160 pmoles). The specificity of the bioreceptor 2m-EST2

towards oxo- and thio-organophosphorus pesticides has been evaluated. Moreover, the enzyme, labelled and not, has been immobilised on PVDF membrane, and its stability has been evaluated over time.

2.2.4. Oxidation of thio-organophosphorous pesticides

Some phosphorothionate compounds (including tolclofos, chlorpyrifos and parathion) were oxidised in the presence of *N*-bromosuccinimide (NBS). The products of the oxidation reaction were subsequently tested both with fluorescence and with enzymatic activity assay, as oxidised compounds are expected to inhibit more efficiently the enzyme EST2.

3. Conclusions

The double mutant 2m-EST2 of the carboxylesterase from *A. acidocaldarius* has been easily expressed and purified with high yields, by recovering an amount of 50 mg of pure enzyme starting from 4 L of bacterial culture. This amount was sufficient for all the experiments of this project, as the enzyme was quite stable over time, being fully active after more than 5 months. However, a second purification was performed for reproducibility purposes and for further confirmation of the results.

3.1. Characterisation of the enzyme

The characterisation of the 2m-EST2 enzyme was performed to determine the optimal conditions for the carboxylesterase activity. As expected, since this is a thermophilic enzyme, the optimal temperature was determined to be 70°C. Additionally, the enzyme showed the best activity at optimal pH value of 7.0 in phosphate buffer.

Since the biosensor is intended for use at ambient temperature, the specific activity and the kinetic parameters were evaluated at both 70°C (optimal temperature) and 25°C (temperature of intended use). The observed k_{cat} value at 70°C was 3 times higher, but the affinity towards the substrate pNP-C8 was almost the same, around 15 μM, indicating that the active site maintains a good conformation at room temperature, compatible with its use as a biosensor.

3.2. Inhibition of enzymatic activity

The effect of various organophosphate compounds on the enzymatic activity of 2m-EST2 was measured via enzymatic activity assays in the presence of the organophosphate pesticides paraoxon, parathion, tolclofos, chlorpyrifos and glyphosate. Paraoxon demonstrated complete inhibition of the enzyme, while no inhibition was observed for the remaining four pesticides (Figure 2).

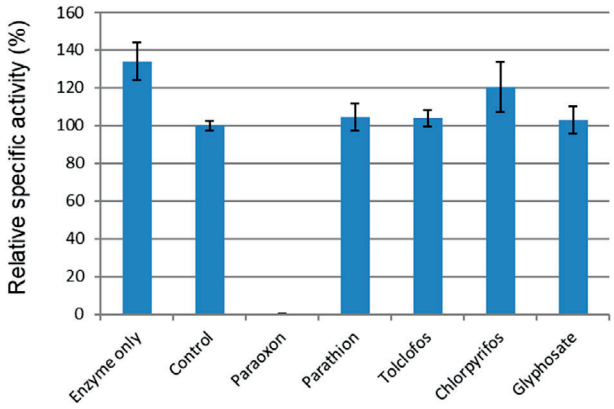

Figure 2: Comparative specific activity (%) relative to control assay containing 4% of corresponding solvent. Equimolar amounts of enzyme and organophosphate were incubated for 5 min at RT. After incubation, 3 pmol of enzyme were transferred into the assay mixture and activity was measured at 405 nm

3.3. Effect of the pesticides on fluorescence quenching

The enzyme was labelled with the fluorescent probe IAEDANS by means of a covalent bond with the residue Cys35. Upon this modification, the labelled enzyme was used to screen 16 different pesticides (presented in Appendix A) for their effect in fluorescence quenching, on a microplate reader. Paraoxon and methyl-paraoxon displayed fluorescence quenching of 18 ± 1% and 14 ± 2% respectively, when used in equimolar amounts with the enzyme in the range of nanomolar. However, oxidation of thio-OPs by NBS did not further improve the inhibition effect of these pesticides.

Therefore, a detailed fluorescence analysis was performed using paraoxon. A decrease in fluorescence intensity of the labelled enzyme was observed after paraoxon addition, demonstrating that the inhibition of 2m-EST2 by paraoxon quenches its fluorescence, with addition of pesticide aliquots in the range from 20 to 200 pmol. A linear response was observed considering the ratio (I_0/I) between the fluorescent intensity in the absence (I_0) and presence (I) of increasing amounts of paraoxon. The estimated limit of detection (LOD) was relatively low, corresponding to 15 pmol of paraoxon.

3.4. Detection of pesticides in real food samples

The applicability of the developed biosensor was tested with real food samples (fruits and juice) as a model of food containing pesticides (Figure 3).

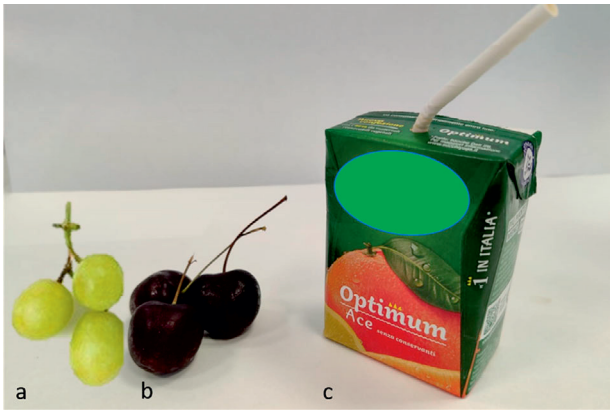

Figure 3: Real food samples used for testing model matrix by which pesticide can be extracted: (a) grapes; (b) cherries; (c) commercial juice. Paraoxon has been added and after extraction, the presence of pesticide has been detected by using the fluorescence-labelled bioreceptor 2 m-EST2

By using paraoxon as a pesticide, the compound was extracted in organic solvent from grapes, cherries and fruit juice, and the samples were analysed by using the thermostable bioreceptor 2m-EST2-IEADANS. The bioreceptor was able to detect the presence of pesticide in a range under 100 pmoles present in the foods. Detection was further confirmed by HPLC analysis.

3.5. Final conclusions

The labelled 2m-EST2 enzyme maintains activity that is inhibited at different extents by some of the organophosphate pesticides tested. More specifically, paraoxon results in complete inhibition of the enzymatic activity when used at equimolar ratio. Fluorescence quenching was also observed and increased linearly with the amounts of paraoxon added, with a LOD of 15 pmol. Compared to the enzymatic activity assays, a method utilising fluorescence, such as the one presented here, provides very rapid detection making this research a starting point to develop effective fluorescence enzyme-based biosensors. This kind of bioreceptor showed high sensitivity and can have applications in the field of food traceability as well as environmental monitoring, to control the presence of toxic chemicals, in particular organophosphate pesticides.

4. Other activities

For the duration of the EU-FORA programme, the fellow participated in weekly laboratory meetings in the presence of supervisors and other colleagues from the host lab, by presenting as speaker her own experimental results. The purpose of those meetings was to present weekly experimental progress, discuss the projects of the group and receive feedback or guidance.

Additionally, the fellow had the opportunity to attend the 23rd Bologna Winter School. 'Structural Bioinformatics in the era of AlphaFold2', an online event organised by the University of Bologna that included lectures spanning between 9 and 25 February 2022.

Thanks to the EU-FORA programme, the fellow was also able to present the results of her research at 'The Biochemistry Global Summit' in Lisbon, Portugal, between 9 and 14 July 2022. This was an international congress combining the 25th IUBMB Congress, the 46th FEBS Congress and the 15th PABMB Congress. The submitted abstract was presented as a poster with title 'A thermostable esterase as a biosensor for rapid and sensitive detection of pesticides'.

5. Disclaimer

Detailed results obtained from the method development, sample analysis and risk assessment 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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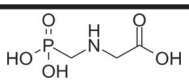
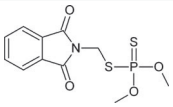
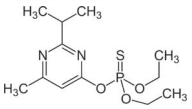
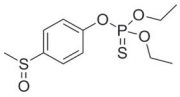
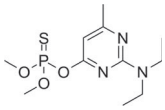
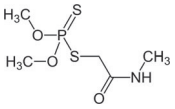
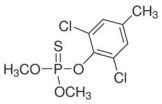
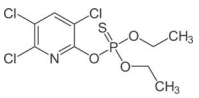
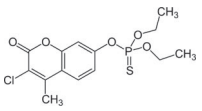
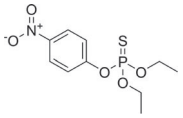
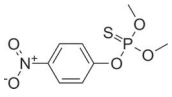
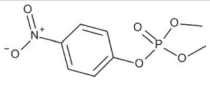
Abbreviations

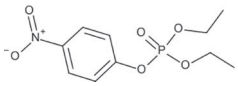
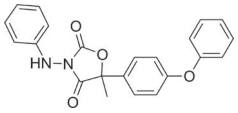
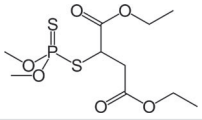
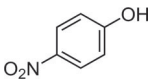
2m-EST2	double mutant of EST2
EST2	esterase-2
IAEDANS	5-({2-[(iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid
LC–MS	liquid chromatography coupled to mass spectrometry
LOD	limit of detection
NBS	N-Bromosuccinimide
OP	organophosphate
pNP-C8	p-nitrophenyl caprylate

Appendix A – List of pesticides used in this study

A detailed list of all the pesticides used in this study is presented below (Table A.1). All 16 compounds were used in fluorescence quenching experiments, while a subset of the compounds was also used for inhibition of enzymatic activity as described in Section 3.2.

Table A.1: Complete list of the chemical compounds used for the fluorescence experiments of this project

	Name	MW[(a)]	Chemical structure	Preferable solvent[(b)]
1	Glyphosate	169.07		Water
2	Phosmet	317.32		Organic solvents
3	Diazinon	304.35		Organic solvents
4	Fensulfothion	308.36		Water
5	Pirimiphos-methyl	305.33		Organic solvents
6	Dimethoate	229.26		Water
7	Tolclofos-methyl	301.13		Organic solvents
8	Chlorpyriphos	350.59		Organic solvents
9	Coumaphos	362.77		Organic solvents
10	Parathion	291.26		Organic solvents
11	Parathion-methyl	263.21		Organic solvents
12	Paraoxon-methyl	247.14		Organic solvents

	Name	MW[(a)]	Chemical structure	Preferable solvent[(b)]
13	Paraoxon-ethyl	275.2		Water
14	Famoxadone	374.39		Organic solvents
15	Malathion	330.36		Organic solvents
16	4-nitrophenol	139.11		Water

(a): Molecular weight (g/mol).
(b): For the compounds insoluble in water, acetonitrile was used as the solvent.

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