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Foreword

I am very pleased to introduce the fourth collection of reports of EFSA's EU-FORA Fellowship Programme. EU-FORA started in 2016 as part of EFSA's efforts to support the development of next generations of Europe's food risk assessors. Five years later, it continues to empower food safety professionals, ensuring an interconnected community of experts, while stimulating the involvement of Member States in risk assessment work and building a common EU risk assessment culture.

The wide array of scientific work and experiences described in this document portrays the wide scope of the EU-FORA and the rich diversity of scientists and hosting sites that comprise the Programme.

The fruitful and timely execution of the programme would not have been possible without the support of EFSA colleagues across different departments, the resilience of the fellows and hosting sites, and the demonstrated excellence in the provision of virtual trainings by the training consortium, in the continuously uncertain epidemiological situation.

As EFSA, we put a great emphasis on Partnership and Cooperation, and we are both humbled and proud by the advancements made by EU-FORA towards the establishment of the European risk assessment community through the capacity building efforts of EFSA's and its partners'.

A big thank you to the participating organisations for the fruitful cycle and congratulations to our newly graduated fellows. We wish them success in all their future endeavours, and we hope to meet them again in a prominent role in the risk-assessment ecosystem.

Barbara Gallani

Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

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Introduction

We are very happy to present this special issue of the EFSA journal dedicated to EFSA's Fellowship Programme (EU-FORA). It brings together the risk assessment pursuits of the fellows and the work programmes they were engaged in during the 4th cycle of the Programme. The realisation of these programmes is a result of the efforts invested by those institutions and organisations of the EU-FORA network tasked with the hosting and training of the fellows.

We congratulate the fellowship graduates and would like to acknowledge their professionalism, engagement and dedication, demonstrated during the duration of the fellowship. In the past 12 months, they have managed to significantly increase their knowledge of food risk assessment and have contributed towards the creation of a risk-assessment community and knowledge development in their respective areas, despite the uncertainties arising from the COVID-19 pandemic.

This year, we also have the pleasure to announce our improved Programme – EU-FORA 2.0 Programme – which we hope will help broaden the scope and welcome new organisations, thus setting EU-FORA on a new trajectory towards reinforcing its nature as a successful cooperation tool, cultivating a strong risk-assessment expertise and knowledge exchange.

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, who ensured the continuity of the EU-FORA programme in a state of a pandemic, thus, empowering our fellows to become the pioneers in the first cycle entirely executed remotely.

On behalf of the EU-FORA Programme,

Victoria Villamar

Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

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Monitoring of pesticide amount in water and drinkable food by a fluorescence-based biosensor

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Abstract

The identification of pollutants is crucial to protect water resources and ensure food safety. The available analytical methodologies allow reliable detection of organic pollutants such as pesticides; however, there is the need for faster, direct and continuous methodologies for real-time monitoring of pesticides. Fluorescent-based biosensors have been recently proposed as a valid alternative due to their advantage of being easy, cheap and specific. In this context, the aim of the present EU-FORA fellowship programme was to develop and apply a fluorescence-based biosensing device for the detection of organophosphate (OP) pesticides in water samples and drinkable food. The study was addressed using a mutant of the thermostable esterase-2 from *Alicyclobacillus acidocaldarius* (EST2-S35C) as a bioreceptor for OP pesticides. The use of EST2 involves some significant advantages including specificity and affinity towards OPs, and high stability over time in a different range of temperatures and pH. The protein was labelled to the fluorescent probe IAEDANS and fluorescence measurements of quenching in solution and in immobilised form were performed. The results showed good stability and sensitivity, reaching low limits of detection and quantification and a constant signal intensity over time. The addition of paraoxon quenched the fluorescence of the complex, reaching a plateau at 100 pmol paraoxon. The decrease of enzymatic activity of EST2-S35C-IAEDANS in the presence of paraoxon correlated the inhibition of the labelled enzyme with the decrease in fluorescence. The results from the application of the biosensor with real samples showed a decrease in fluorescence in surface water samples, contaminated by OPs. The use of the developed fluorescence-based biosensor demonstrated its applicability for real samples monitoring and could ensure the production of large amounts of data in a short period of time which can be used to address environmental and food safety risk assessment.

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Keywords: organophosphate pesticides, biosensor, fluorescence, thermostable enzyme, esterase-2, environmental monitoring

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1. Introduction

Pesticides have been extensively used since the mid-20th century in public health to prevent agricultural pests and harmful organisms. Despite their undoubtable usefulness, their high use has contributed to a widespread contamination of the ecosystem, leading to high pesticides occurrence in water resources, soil, air and food. Exposure to pesticide residues has been proved to induce toxic effects to unwanted species, non-target organisms, and human health (Li and Fantke, 2022), raising concern about food safety-related issues. Pesticide monitoring has been achieved due to the use of modern state-of-the-art techniques such as gas chromatography and liquid chromatography coupled to mass spectrometry (GC/LC–MS) (De O. Silva et al., 2019; Song et al., 2019; Barbieri et al., 2020). However, these methodologies are not adequate for real-time pesticides monitoring, since they are time-consuming and require highly skilled personnel. To overcome this problem, fluorescent biosensors have been implemented as an easy, cheap and fast technique for direct pesticides monitoring (Bhattu et al., 2021). Several protein-based biosensors have been recently used for organophosphate (OP) pesticides detection, and most of them are based on acetylcholinesterase (AChE) inhibition mechanisms (Cao et al., 2020). Nevertheless, some limitations include low stability and specificity of AChE enzymatic activity, thus the need for new, efficient enzymes to be used as bioreceptors.

Esterase-2 (EST2) from *Alicyclobacillus acidocaldarius* have been recently proposed as a bioreceptor for a biosensor due to its stability over time at different temperatures, pH and organic solvents and specificity to OP compounds (Manco et al., 1998; Mandrich et al., 2005). Recently, our group designed and synthesised a mutant of EST2 to be used as bioreceptor for paraoxon detection (Carullo et al., 2018). In this context, we applied a biosensing device based on the use of EST2 labelled with a fluorescent probe as a sensitive method for the detection and quantification of OPs in real water samples.

The main goal of the present work, as part of the European Food Risk Assessment (EU-FORA) fellowship programme, granted by the European Food Safety Authority (EFSA), was the development of a fluorescence-based biosensor which allows fast, direct and continuous monitoring of OPs compounds in real environmental and food samples to address risk assessment.

The methodology provided reliable analytical performance, with fast time of analysis and high selectivity and sensitivity toward paraoxon, achieving limits of detection (LODs) and quantification (LOQs) comparable to other previous levels obtained for the analysis of OP pesticides in aqueous solution. The results obtained were confirmed by the detection of OPs in surface water samples collected in areas impacted by agricultural and/or industrial activities, supporting the use of the developed biosensing device for the monitoring of OPs in real samples. The direct, fast and cheap developed methodology is effective to support the use of fluorescence-based biosensors as a solid tool to obtain high amounts of data which can be used for pesticides risk assessment.

2. Description of work programme

2.1. Aims

The aims of the work programme can be categorised into four main parts. The first goal involved the preparation of the enzyme in free and immobilised form, to be used in the following activity as a bioreceptor for a fluorescent-based biosensor. The third activity aimed at validating the biosensor in the operative condition for the detection of OP pesticides in water and drinkable food, which have been collected as part of the last activity to acquire data on different real samples (including drinking water, beverages and surface water) to be used for a preliminary study of the risk assessment of pesticides in environmental and food samples.

2.2. Activities/Methods

2.2.1. Expression and purification of EST2-S35C bioreceptor

In the first part of the work programme, the fellow carried out the overexpression in mesophilic host *Escherichia coli* strain BL21 (DE3) of a mutant of the thermostable esterase-2 (EST2) from *Alicyclobacillus acidocaldarius* (Febbraio et al., 2011), to which the serine 35 was replaced by a cysteine residue (EST2-S35C) near the catalytic site. EST2-S35C was extracted and purified following a protocol already described in Carullo et al. (2018), with slight modifications (Rodrigues et al., 2021). The microorganism was grown in an appropriate medium, the biomass was recovered by

centrifugation and the protein was extracted by a sonication step. Subsequently, the protein was purified by thermoprecipitation steps, followed by a gel filtration step to obtain enzyme purity > 95%. The protein concentration was estimated following the Bradford method (Bradford 1976), with bovine γ-globulin as the standard.

2.2.2. Labelling of EST2-S35C bioreceptor

The fellow proceeded to the labelling of cysteine in the active site of the purified enzyme, incubating the protein in the presence of the fluorescent probe IAEDANS, selected for the specificity of the binding to cysteine residues. The enzyme was incubated with IAEDANS in a ratio 1:100 overnight at 4°C. When binding occurred, the excess of the probe was removed by dialysis, and the protein–probe concentration was determined using the Bio-Rad dye reagent, as previously described (Bradford, 1976). The fluorescence of the labelled enzyme was measured by fluorescence spectroscopy in the emission wavelength range from 400 to 550 nm exciting at 340 nm, and acquired in a Jasco FP-8200 fluorimeter using a quartz cuvette of 1.0 cm optical path. The enzyme was immobilised on a membrane for further analysis of the free and immobilised form for the measurement of OPs in aqueous solution, measuring the fluorescence quenching after OP binding to the catalytic site of the enzyme. Part of this activity was carried out in collaboration with a university group at the Department of Chemistry of the University of Naples Federico II (Italy).

2.2.3. Biosensor validation

In the third part of the work programme, the fellow accomplished the validation of the biosensor in the operative condition for OPs detection in water and drinkable food. The methodology was validated in terms of precision, accuracy, linearity, stability of bioreceptor, specificity and sensitivity. Measurements of the fluorescence quenching of inhibited EST2-S35C were performed by adding increasing concentrations of OPs, using paraoxon as organophosphate model. The linearity of data was assessed, as well as the LOD and LOQ. Tests on known and unknown concentrations of OP have been carried out to define the precision and accuracy of the method, as well as measurements at different storage times for assessing the stability of the bioreceptor.

2.2.4. Biosensor application

The last part of the work programme was dedicated to the organophosphates detection in real samples. Several drinking water samples and beverages (tea, milk, energy drinks) were purchased from different markets in Naples (Italy). Moreover, two sampling campaigns were carried out to collect surface water samples from Sarno and Tiber Rivers (Italy). Both of these rivers flow in the proximity of the metropolitan areas of Naples and Rome, respectively; thus they are representative of areas impacted by different anthropogenic pressures (e.g. agriculture, industries). The samples were collected in plastic bottles, transported under cool conditions to the laboratory, where they were stored upon arrival at −20°C in the dark. Prior to analysis, the samples were centrifuged to remove suspended particles, and aliquots of 50 mL were transferred to polypropylene centrifuge tubes.

3. Conclusions

3.1. Bioreceptor preparation

The overexpression of the EST2 mutant (Figure 1) and biochemical characterisation was well described in Carullo et al. (2018), demonstrating that the catalytic site is not affected by the replacement of the serine by a cysteine and that the structure-function relationship does not change. Moreover, EST2-S35C shows the same sensitivity of the wild type EST2 to be irreversibly inhibited by paraoxon. After conjugation of EST2-S35C with the fluorescent probe IAEDANS, the catalytic efficiency was measured, and the results demonstrated that the activity does not change compared to the unlabelled enzyme, continuing to be fully inhibited by paraoxon in a ratio 1:1. The labelling efficiency was improved by incubating the enzyme with increasing concentrations of IAEDANS, observing the best binding to occur at 1:100 protein:probe ratio.

The fluorescence intensity was measured for increasing amounts of protein and protein-probe complex, and good replicability and linear relationship were observed. At lower protein concentration, a better sensitivity was observed with the EST2-S35C-IAEDANS complex, confirming the advantage of

using IAEDANS as a fluorescent probe instead of only measuring the intrinsic fluorescence of the tryptophan of the EST2 enzyme.

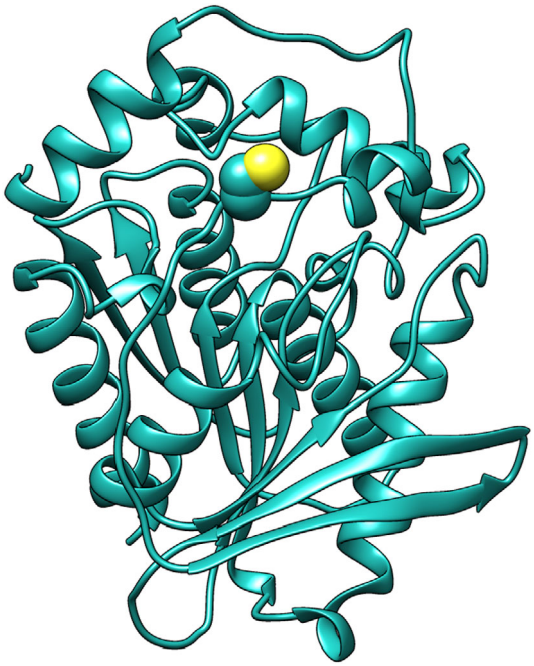

Figure 1: Representation of the EST2-S35C 3D structure with a detail of the mutated group, indicated using the van der Waals (VDW) structure

3.2. Biosensor application in real samples

A decrease in fluorescence intensity of EST2-S35C-IAEDANS was observed after paraoxon addition, demonstrating that the inhibition of the labelled enzyme by paraoxon quenches its fluorescence. The results showed that the addition of paraoxon aliquots (in the range from 40 to 140 pmol) to 300 pmol of EST2-S35C-IAEDANS, quenched the fluorescence of the complex, reaching a plateau at 100 pmol paraoxon. The fluorescence quenching observed in the covalently inhibited EST2 can be related to a structural rearrangement around the cysteine 35 residues at the entrance of EST2 catalytic site (Carullo et al., 2018), because the presence of paraoxon molecules inside the acyl pocket affects the accessibility to the surface of the IAEDANS probe bond to the cysteine 35. 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. Measurements carried out after 7 and 14 days of the labelled protein at 4°C gave the same results with no significant signal decay, accounting for a high stability of the bioreceptor. The low LODs and LOQs obtained stand for a high sensitivity of the fluorescent-based biosensor, reaching limits comparable to previous biosensor methodologies developed for the analysis of OP pesticides in aqueous solution.

The applicability of the developed biosensor was tested with real environmental and drinkable food samples for OPs monitoring. The samples analysed included five surface water samples from Sarno River and two surface waters from Tiber River (Italy), two tap water samples from Naples city, four commercially available drinking water samples, three tea samples, one milk sample and one energy drink sample. The results showed a decrease in fluorescence with increasing volumes of both surface water samples from the Tiber River, located in the centre of the city of Rome, therefore probably contaminated by the high domestic and industrial activities of the area, as well as the water samples 1 and 2 from Sarno River, thus contaminated by the presence of OP pesticides. These sampling points are located close to the sea, in urban areas with some industries, railway and other infrastructures, or in the proximity of agricultural fields (Figure 2). On the other hand, no decrease in fluorescence is observed in waters belonging to sampling sites at the source of the Sarno River, which are mostly characterised by small private crops, rural areas and small urban centres. As expected, no OP was detected in the commercial samples. However, an increase in intensity was observed at increasing volumes of tea samples, probably due to the presence of pigments which manifest an intrinsic

fluorescence that may interfere with the fluorescence measurements. To overcome this problem, the enzyme was immobilised on a membrane which was placed onto a support in a 3D adapter designed as part of the extracurricular activities developed during the 1-year fellowship programme. The 3D support allows the washing of the membrane to remove unwanted substances such as pigments, improving the fluorescence measurements for food samples and avoiding fluorescence interferences in the aqueous solution (Rodrigues et al., 2021).

These results support the use of the EST2-S35C-IAEDANS complex as a bioreceptor in biosensors for the monitoring and detection of organophosphate pesticides in food and environmental samples using fluorescence approaches.

The sensitivity of this bioreceptor could allow the analysis of a wide range of organophosphate pesticides which have the same molecular target (e.g. acetylcholinesterase), and support a more precise and complete risk assessment in terms of single compound, as well as total amount of specific pollutants.

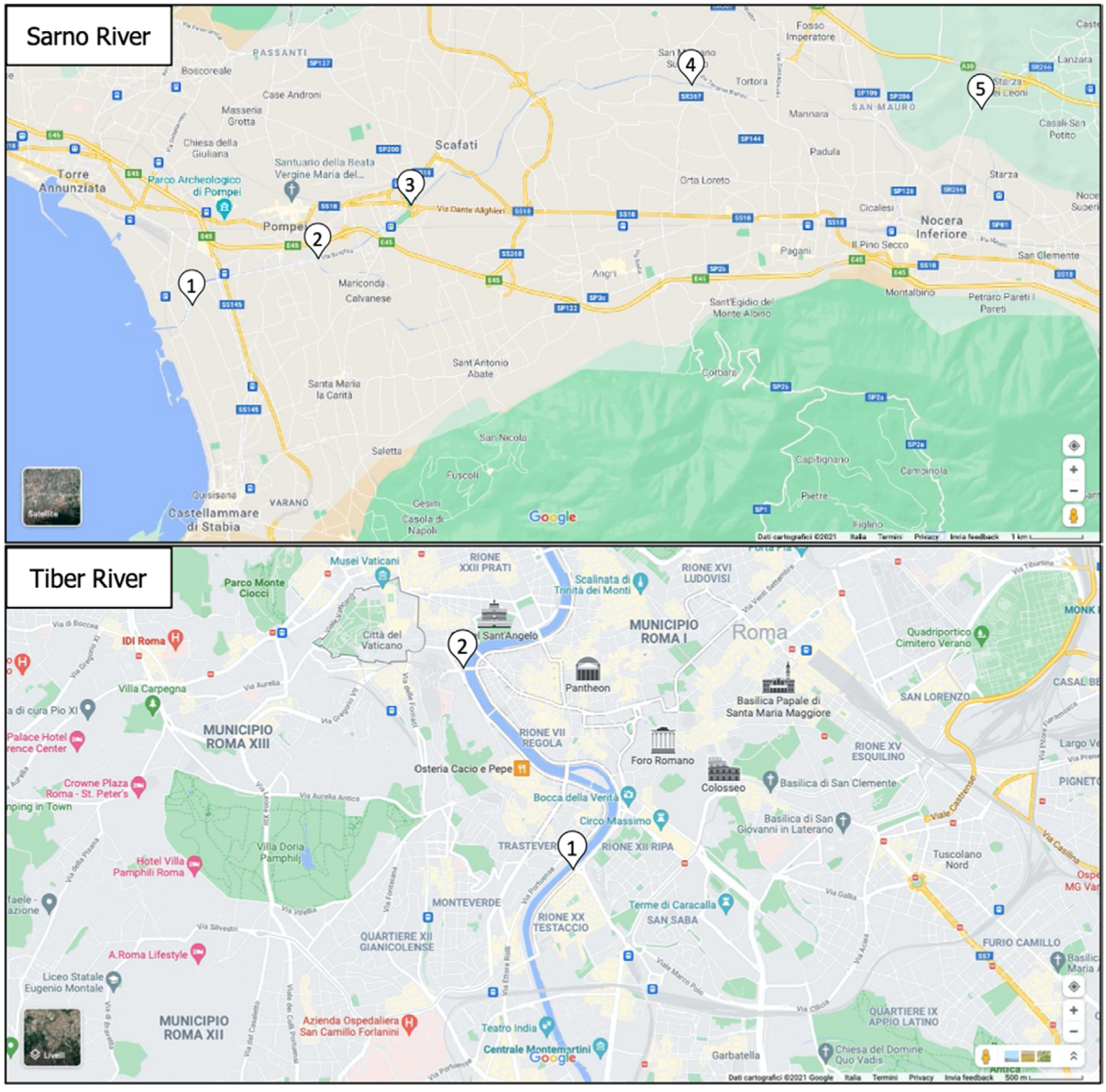

Figure 2: Map of the Sarno River and Tiber River (Italy) sampling campaigns, with a detail of the sampling locations

3.3. Additional scientific activities

Besides the working programme-specific goals, the fellow was involved in extracurricular activities, including the participation in the scientific international conference EUROTOX 2021, held online from 27 September to 1 October 2021 with a poster presentation 'Direct detection of organophosphate pesticides in water by a fluorescence-based biosensor' as the presenting author and as the co-author of the poster presentation 'A FRET approach to detect organophosphate pesticides using a fluorescent biosensor' (https://www.eurotox2021.com/abstracts/). Moreover, the fellow is the presenting author of the abstract 'Application of a fluorescence-based biosensing device for the detection of organophosphate pesticides in water samples' and the co-author of the abstract 'Detection of neurotoxic compounds at environmentally relevant concentrations by using a fluorescence-based biosensing device' accepted as poster presentations at ONE – Health, Environment, Society – Conference, 21–24 June 2022. Also, she took part in weekly internal institutional data clubs as a participant and twice as a speaker. In addition, the fellow developed part of her activities in collaboration with a university group at the Department of Chemistry of the University of Naples Federico II (Italy). This collaboration has been demonstrated by the publication of the scientific article 'A 3D printable adapter for solid-state fluorescence measurements: the case of an immobilised enzymatic bioreceptor for organophosphate pesticides detection' (Rodrigues et al., 2021), in which the fellow contributed as the first co-author. A further article is currently submitted to a peer-reviewed journal and another publication is in preparation. She also participated in the preparation of enzymes in the framework of a bilateral project between the IBBC institute and an Egyptian partner from Zewail City of Science and Technology, confirmed by her period as a visiting scientist at Zewail City of Science and Technology in 6th of October City (Giza, Egypt) from 5 to 15 November 2021.

3.4. 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 will be subsequently published in other scientific journals.

References

Barbieri MV, Monllor-Alcaraz LS, Postigo C and López de Alda M, 2020. Improved fully automated method for the determination of medium to highly polar pesticides in surface and groundwater and application in two distinct agriculture-impacted areas. Science of the Total Environment, 745, 140650. https://doi.org/10.1016/j.scitotenv.2020.140650

Bhattu M, Verma M and Kathuria D, 2021. Recent advancements in the detection of organophosphate pesticides: a review. Analytical Methods, 13, 4390–4428. https://doi.org/10.1039/D1AY01186C

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Cao J, Wang M, Yu H, She Y, Cao Z, Ye J, Abd El-Aty AM, Hacımüftüoğlu A, Wang J and Lao S, 2020. An overview on the mechanisms and applications of enzyme inhibition-based methods for determination of organophosphate and carbamate pesticides. Journal of Agricultural and Food Chemistry, 68, 7298–7315. https://doi.org/10.1021/acs.jafc.0c01962

Carullo P, Chino M, Cetrangolo GP, Terreri S, Lombardi A, Manco G, Cimmino A and Febbraio F, 2018. Direct detection of organophosphate compounds in water by a fluorescence-based biosensing device. Sensors and Actuators B: Chemical, 255, 3257–3266. https://doi.org/10.1016/j.snb.2017.09.152

De O. Silbva O, De Menezes MGG, De Castro RC, Crisiana DN, Milhome MAL and Do Nascimento RF, 2019. Efficiency of ESI and APCI ionization sources in LC-MS/MS systems for analysis of 22 pesticide residues in food matrix. Food Chemistry, 297, 124934. https://doi.org/10.1016/j.foodchem.2019.06.001

Febbraio F, Merone L, Cetrangolo GP, Rossi M, Nucci R and Manco G, 2011. Thermostable Esterase 2 from Alicyclobacillus acidocaldarius as biosensor for the detection of organophosphate pesticides. Analytical Chemistry, 83, 1530–1536. https://doi.org/10.1021/ac102025z

Li Z and Fantke P, 2022. Toward harmonizing global pesticide regulations for surface freshwaters in support of protecting human health. Journal of Environmental Management, 301, 113909. https://doi.org/10.1016/j.jenvman.2021.113909

Manco G, Adinolfi E, Pisani FM, Ottolina G, Carrea G and Rossi M, 1998. Overexpression and properties of a new thermophilic and thermostable esterase from Bacillus acidocaldarius with sequence similarity to hormone-sensitive lipase subfamily. Biochemical Journal, 332(Pt. 1), 203–212. https://doi.org/10.1042/bj3320203

Mandrich L, Merone L, Pezzullo M, Cipolla L, Nicotra F, Rossi M and Manco G, 2005. Role of the N terminus in enzyme activity, stability and specificity in thermophilic esterases belonging to the HSL family. Journal of Molecular Biology, 345, 501–512. https://doi.org/10.1016/j.jmb.2004.10.035

Rodrigues ACM, Barbieri MV, Chino M, Manco G and Febbraio F, 2021. A 3D printable adapter for solid-state fluorescence measurements: the case of an immobilized enzymatic bioreceptor for organophosphate pesticides detection. Analytical and Bioanalytical Chemistry, https://doi.org/10.1007/s00216-021-03835-1

Song N-E, Lee JY, Mansur AR, Jang HW, Lim M-C, Lee Y, Yoo M and Nam TG, 2019. Determination of 60 pesticides in hen eggs using the QuEChERS procedure followed by LC-MS/MS and GC-MS/MS. Food Chemistry, 298, 125050. https://doi.org/10.1016/j.foodchem.2019.125050

Abbreviations

AChE	acetylcholinesterase
EST2	esterase-2
EU-FORA	European Food Risk Assessment Fellowship Programme
GC	gas chromatography
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
OP	Organophosphate

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Microbiota analysis for risk assessment: evaluation of hazardous dietary substances and its potential role on the gut microbiome variability and dysbiosis

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Abstract

The expansion of fields related to probiotics, microbiome-targeted interventions and an evolving landscape for implementation across policy, industry and end users, signifies an era of important clinical translational changes. Characteristics and perception of traditional probiotics stemmed from the historical long-term use of fermented products. Although the distinction between probiotic microorganisms and fermentation-associated microbes is important, it is often confused as not all fermented foods are probiotic supplements. Current innovation in area of biotechnology and bioinformatics is emerging outside of the classical definitions and new probiotics will emerge from novel sources, challenging scientific as well as regulatory instructions. At the same time, the search for individual and group microbiome signatures – biomarkers in order to predict disease incidence, progression and response to treatment is a key area of microbiological and multidisciplinary research, enabled by efficient and powerful processing of large data sets. However, the regulation of marketed beneficial microbes and probiotics differs among countries and the basic level of classification, which depend on probiotic classification is not globally harmonised. At the same time, the regulation is very demanding to evaluate the safety of products on the market, so that only those products with scientific evidence benefits can obtain positive recognition in ways of health claims. Collaborative experimental and theoretical approaches and case studies have assisted the progress in this crosscutting area of research. There is a requirement to clearly specify criteria and provide details about ways and approaches of achieving those criteria with the intention that manufacturers can benefit from a transparent way of communicating product quality to end users.

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Keywords: knowledge, microbiota, obesity, next-generation probiotics, bisphenols, biomarkers, risk assessment

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1. Introduction

The human microbiota is a microbial community that lives on and in the human body. It varies according to several factors (e.g. age, diet and lifestyle) and play a very important role in maintaining the health homeostasis or eubiosis (López-Moreno et al., 2021). It has been demonstrated that gastrointestinal tract (GIT) disorders are linked to microbiota alterations patterns, also called GIT dysbiosis. Many of those disorders have been proved to be reversed by administration of probiotics (Bear et al., 2020).

Current and most adequate definition of probiotics is 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill et al., 2014). This definition was established and issued by a consensus panel convened by the International Scientific Association of Probiotics and Prebiotics (ISAPP), which met to discuss the modern relevance of the 2001 Food and Agriculture Organization (FAO)/World Health Organization (WHO) definition of probiotics (Sanders et al., 2018). Definition of probiotics will most likely still evolve over the time, and their characteristics include: (1) Qualified Presumption of Safety (QPS) at the species level by the European Food Safety Authority (EFSA); (2) targeting general sub-health population people; (3) isolated from gut, breast milk and fermented foods; (4) long history of use; (5) belong to limited genera (Chang et al., 2019; Lin et al., 2019; EFSA, 2021a).

Traditionally, lactobacilli, bifidobacteria and other lactic acid-producing bacteria (LAB) have been used as probiotics, primarily isolated from fermented dairy products and the faecal microbiome, mainly *Lactobacillus* spp. (Firmicutes) and *Bifidobacterium* spp. (Actinobacteria), and involve *Streptococcus* spp. (Firmicutes), *Bacillus* spp. (Firmicutes) and *Saccharomyces cerevisiae* (yeast), etc. (Marco et al., 2017; Pasolli et al., 2020; Taylor et al., 2020; Veiga et al., 2020). In 2002, the FAO and the WHO published the 'Guidelines for the Evaluation of Probiotics in Food'. This guideline established safety and efficacy standards for probiotics, systematising their discovery and selection (Araya et al., 2002). The identification of probiotic strains that efficiently produce reproducible effects on human health is still largely made through an empirical top-down approach, that is, studying microorganisms that are typically enriched in healthy individuals (Veiga et al., 2020; EFSA, 2021b).

Unique taxonomic profiles and specific genera and species have been associated with health and disease status as well as host biomarkers, dietary and lifestyle characteristics in large cross-sectional studies (López-Moreno et al., 2021). Probiotics presented as promising candidate interventions with the potential to 'transmit' disorder signatures towards health utilise multiple potential modes of action (Cunningham et al., 2021). The advent of molecular approaches, such as complete whole genome sequencing (WGS) increased our capacity to isolate and characterise new probiotic candidates for which cultivation was previously limited by their rigorous growth requirements, with potential health benefits and the opportunity to be developed as next-generation probiotics (NGP) and providing further potential for precision medicine intervention (O'Toole et al., 2017; Lin et al., 2019).

This current state of interest for discovery of new species includes the GIT, female urogenital tract, oral cavity, nasopharyngeal tract and skin. Species or genera associated with health in these regions are being investigated as potential interventions to restore microbial populations and therefore physiological homeostasis in disease states (Reid, 2012; George et al., 2016; Maguire and Maguire, 2017; Nakatsuji et al., 2017; Bourdichon et al., 2021).

The area of discussion that has great potential for probiotics is in detoxification of environmental pollutants and the need to prevent adsorption of these compounds into the body of humans, such as several endocrine disruptors (Reid, 2015; López-Moreno et al., 2021). The hormone system has an essential role in the regulation of many physiological functions such as body development, growth, reproduction, metabolism, immunity, inflammation and behaviour (Chrousos, 2007). Endocrine disruptor chemicals (EDCs) are exogenous compounds that interfere with any aspect of endogenous hormone system, including hormones production, release, transport, metabolism, binding, action or elimination, negatively affecting human health (Lee, 2018; Pouzaud et al., 2018). They represent a special and challenging form of toxicity as their effects depend on both the level and timing of exposure, being especially critical in developmental stages (WHO/UNEP, 2012). EDCs are highly heterogeneous chemicals – including pesticides, fungicides, plastics, plasticisers and heavy metals – with diverse applications at industrial, agricultural, pharmaceutical and cosmetic level, which result in contaminant residues in food and other consumer products leading to human exposure to EDC mixtures, which is continuously increasing (Schug et al., 2016; Gálvez-Ontiveros et al., 2020).

This Technical Report represents a description of the EUropean FOod Risk Assessment (EU-FORA) Fellowship work programme and its objectives: 'To promote and coordinate the development of

uniform risk assessment methodologies in the fields falling within its mission', founded by EFSA. The proposed project of 'Microbiota analysis for risk assessment improval: Evaluation of hazardous dietary substances and its potential role on the gut microbiome variability and dysbiosis' was developed within Faculty of Pharmacy, University of Granada (UGR), and 'José Mataix Verdú' Institute of Nutrition and Food Technology (INYTA - UGR) team projects that carry out microbiota analysis with different health purposes since 2003. Interaction among distinct scientific disciplines as microbiology, nutrition, toxicology, analytical chemistry, food safety and personalised medicine are needed to analyse factors and substances that affect human microbiota eubiosis/dysbiosis. Furthermore, omics technologies have a relevant role to achieve the elucidation of mechanisms leading many diseases, disorders and dysbiosis caused by dietary exposure to toxic compounds. They could be one of the strategies to understand the relation between microbiome and gut physiology status together with its axis interaction. Moreover, current interest of gut microbiota determinations for complementing risk assessment of metabolite traces of toxicants and xenobiotic substances in food is being of high relevance. Within this initiative, multidisciplinary consortium submitted the following EFSA Partnering Grant proposal (2019–2021) that has been successfully evaluated and awarded: 'KNOWLEDGE PLATFORM FOR ASSESSING THE RISK OF BISPHENOLS ON GUT MICROBIOTA AND ITS ROLE IN OBESOGENIC PHENOTYPE: LOOKING FOR BIOMARKERS' Acronym: OBEMIRISK. The programme was supervised by Dr. Margarita Aguilera-Gómez, Associate Professor at UGR. The programme consisted of three different modules based on on-going research project work and previous research interests.

2. Description of work programme

The EU-FORA work programme 'Microbiota analysis for risk assessment improval: Evaluation of hazardous dietary substances and its potential role on the gut microbiome variability and dysbiosis' was structured in three different modules that covered a wide range of aspects related to microbiota analysis for risk assessment improval. Taken together, all modules ensured a broad overview on the various methodologies, tools and applications of programme. Each module was organised into various related activities that were addressed step by step. Over the course of the year, Dr. Aguilera-Gómez monitored the progress of the programme and managed the evolution of the project's activities. Weekly meetings analysed in greater detail the progress of each module's deliverables and outcomes according to the programme timeline. Furthermore, specialists were chosen to co-supervise each module based on their experience and relevance.

2.1. Aims

Each module of the 'Microbiota analysis for risk assessment improval: Evaluation of hazardous dietary substances and its potential role on the gut microbiome variability and dysbiosis' work programme represented an independent project (Figure 1) and had specific deliverables and outcomes, as follows:

- Objective/Module 1. To focus on obtaining the upmost information about human microbiota variability and dysbiosis associated and/or putatively caused by diet hazardous substances exposure and consumption, and future perspectives of probiotics and next-generation probiotics.
- Objective/Module 2. To learn main available methods and omics technologies for gut microbiota analysis (composition/activity patterns) while exposed to different level of diet hazardous substances (e.g. bisphenol A and analogues).
- Objective/Module 3. To learn main methods for chemical determination of bisphenols and analogues in food samples and human sample specimens (saliva, urine, faeces) and elaboration of common questionnaires and surveys for food exposure estimation of the presence of BPA and analogues (design, improvement, validation and its implementation).

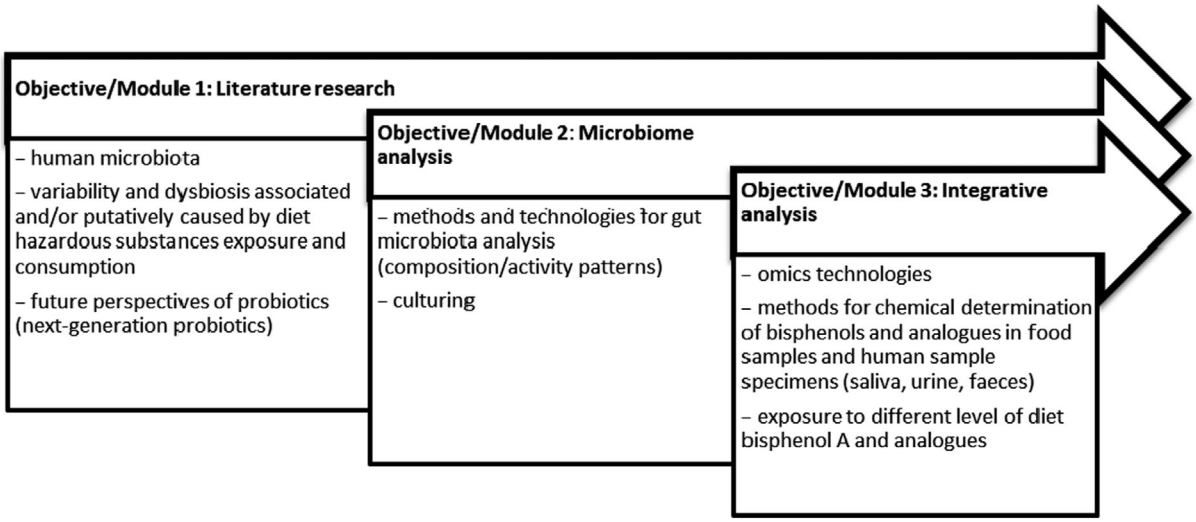


Figure 1: Diagram of proposed work programme as timeline

2.2. Activities/Methods

2.2.1. Objective/Module 1: Comprehensive revision of literature data

A systematic review, a meta-analysis and an extensive literature search methodology was taught and applied in different searching engine databases (Pubmed, Scifinder, Web of Science, Scopus, Embase) according to (EFSA, 2015). It has been designed with specific key words (microbiota, endocrine disruptors, bisphenols, obesity, insulin resistance, dyslipidaemia, hypertension, symptoms of metabolic syndrome, infertility, interventions, probiotics and omics technologies), together with specific question, specific exclusion and inclusion criteria, and categorisation of studies in order to obtain relevant documents for the holistic analysis of human microbiota and its role in risk assessment and to build Guidelines documents compiling Regulatory data and scientific evidence affecting microbiota and probiotics risk assessment and food safety aspects.

Safety assessment and regulatory framework

The regulation of marketed probiotics differs among countries and the basic level of classification is not globally harmonised. Therefore, probiotics can be sold as nutraceuticals, dietary supplements, or food. In the US, probiotics are categorised as nutraceuticals, life biotherapeutic agent, medical food, biological product or dietary supplement, which are regulated by Food and Drug administration (FDA), under Dietary Supplement Health and Education Act (DSHEA) or Biologic Licence Application (BLA). In Japan, probiotics are classified as functional foods and nutraceuticals and regulated by Ministry of Health and Welfare (MHLW), as Food for Specified Heath Use (FOSHU). In Canada, probiotics are classified as Natural Health Products and are regulated by The Canadian Food Inspection Agency (CFIA). In the EU, most bacteria that will be used in foods for human consumption need to comply with two different regulations (EC 258/1997 and EC 1924/2006), or if used as life biotherapeutic products (described in the European Pharmacopoeia - Ph. Eur.) (Cordaillat-Simmons, et al., 2020). In order to assess the safety of microorganisms, EFSA introduced the concept of the QPS to harmonise the safety evaluation of microorganisms used as food or feed additives, food enzymes, novel foods or pesticides, which has to follow certain criteria (EFSA, 2017, 2018, 2020) (Table 1).

Table 1: Transfer of general criteria for safety assessment of microorganisms isolated from human microbiota

	Criteria	Description
Identification	General information	Source, culture collection deposition, intended use, genetically modified microorganisms
	Sequencing	Whole genome sequencing (WGS), methodology of sequencing, assembly, annotation, quality control

	Criteria	Description
	In silico identification	Identification, phylogenetic relatedness (Alignment-free genome distance estimation (isDDH), Alignment-based calculation of average nucleotide identity (ANI))
Characterisation	Antimicrobial susceptibility	Determination of minimum inhibitory concentration (MIC) and antimicrobial resistance (AMR) genes from WGS
	Toxigenicity and pathogenicity	Determination of virulence factors from WGS, cytotoxicity test
	In vivo microbial studies	Impact on gut microbiota, compatibility with other additives showing antimicrobial activity
Production process	Industrial scaling	Production process (processes, culture media, impurities), stability, specifications (formulation and other ingredients)
Final product	Information	Compositional data, proposed uses and level of uses, route of administration, labelling, post-market surveillance

By the general guideline for qualifications of the QPS, unless the strain qualifies for the QPS approach or belongs to a taxonomic unit, known not to produce antimicrobials relevant to use in humans and animals, assessment should be made to determine the inhibitory activity of culture supernatants against reference strains, known to be susceptible to a range of antibiotics and the inhibitory substance (FAO, 2007; EUCAST, 2015). Slight difference has been made for the production strains, which have to demonstrate the absence of carry over into the final product together with the exact phase of the industrial scale manufacturing process and whether any critically important antimicrobials (CIAs) or highly important antimicrobials (HIAs) are used during the manufacturing of the product, to determine compatibility with other additives showing antimicrobial activity and furthermore possible co-/ cross-resistance (EFSA, 2018) and which might eventually be transferred via horizontal gene transfer to pathogenic bacteria during food manufacture or after consumption (EFSA, 2020). In addition to general guideline for qualifications of the QPS, EFSA made supplementary requirement for Bacillus species other than the *Bacillus cereus* group, where a cytotoxicity test should be made to determine whether the strain produces high levels of non-ribosomal synthesised peptides (EFSA, 2018).

Therefore, different legal procedure has to be conducted in order for the product to reach the market, which depend on previously explained classification. At the same time the regulation is very demanding to evaluate the safety of products on the market, so that only those products with scientific evidence that claim health benefits can obtain positive recognition (Degnan, 2012; Morovic et al., 2017; Li et al., 2018; Jagadeesan et al., 2018; de Simone, 2019). EFSA, responsible for authorisation of health claims, has rejected all submitted health claims regarding probiotics. The dossiers submitted in support of the claims have been deemed to not establish a cause-and-effect relationship between a probiotic product and the claimed health effect. The applied standard in the EU is the 'highest possible standard' of evidence and all studies must be conducted on healthy subjects to be considered (Sanders et al., 2018). EFSA also does not permit the use of the word probiotic on the label of products containing the GRAS strains (de Simone, 2019). However, to the extent a probiotic is added to a fermented food, or included in the production of a fermented food, then that fermented food would also be a probiotic food. Indeed, EFSA has approved a health claim for yogurt, having determined that the yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) at the species level can help improve digestion of lactose among individuals with lactose maldigestion (Sanders et al., 2018).

While there is a general consumer view that probiotics and fermented foods are beneficial, there is still a gap in understanding on definitions of the terms 'probiotics', their benefits to health, how they function, and where to find the best sources in food and healthcare products (Cunningham et al., 2021). Probiotic supplements are often conceived by the public and recommended by clinicians to their patients as homogenous beneficial microorganisms (Veiga et al., 2020). And regardless of all preventive effects, the consumption of probiotics may not be thoroughly safe in certain cases or physiological states (O'Toole et al., 2017). In this context, several bacterial species from genera other than *Lactobacillus* and *Bifidobacterium* with proven efficacy, which are considered as potential NGP may be strain-by-strain assessed in order to obtain sufficient research data, and to grant probiotic status on the species level (Hill et al., 2014).

Information of beneficial results provided by the NGP will comprise comprehensive understanding of their targeted diseases. On top of these, the underlying molecular mechanisms on how NGP work and interacts with the host have to be clarified (Lin et al., 2019). It is important to characterise in vitro bacterial physiology, genomic analysis of potential virulence and antimicrobial resistance genes, investigations on the presence or absence of potential genes involved in transferring antibiotic resistance gene, and in vivo acute toxicity study in both healthy and immunosuppressed mice (Saarela, 2019).

2.2.2. Objective/Modules 2 and 3: Practical work

Next-Generation Sequencing (NGS), Bioinformatics Analyses and Omics Data Integration

The search for individual and group microbiome signatures together with the rapid evolution of cultivation-independent, next-generation sequencing and meta-omics technologies, has allowed for the integration and analyses of large datasets for the study of the diversity, complexity and functional role of human gut microbiome in health and disease (Miyoshi et al., 2020). A large part of the detected bacteria has never been cultivated (Amrane et al., 2019), therefore, an integrative approach using both metagenome and metabolome-based characterisations of the gut microbiome together with bioinformatic and statistical filters and algorithms can provide strain-level taxonomic resolution of the taxa present in microbiomes, assess the potential functions encoded by the microbial community and quantify the metabolic activities within a complex microbiome (Dhakan et al., 2019). Based on these data, there is significant interest in targeted strategies to modulate microbial composition within hosts on a personalised or population subgroup level (Cunningham et al., 2021).

The various platforms and reference databases developed for the marker gene (16S rRNA), metagenomics, or meta-transcriptomics analysis (Table 2) often use similar stepwise approaches with different bioinformatic tools (Knight et al., 2018; Swann et al., 2020; Graw et al., 2021).

Table 2: Different genomic analyses for evaluation of microbial communities

Method	Cons (+) and pros (–)
High-level community profiling: Marker gene analysis (16S rRNA, ITS or 18S rRNA)	+Simple and inexpensive method for sample preparation and analysis +Large already existing public data available for comparisons of different datasets +Higher-level analysis –No live, death or active discrimination –Several biases introduced through amplification, choice of primers and variable regions –Negative controls are required –Functional information is limited
Functional profiling: Whole metagenome analysis	+Can directly infer the relative abundance of microbial functional genes; microbial taxonomic and phylogenetic identity to species and strains level is attainable for known organisms +No sequencing-related biases as with marker gene analysis +Higher-level analysis –Relative expensive, complex and laborious method for sample preparation and analysis –No live, death or active discrimination –Default pipelines don't have well annotated viruses and plasmids and together with host-derived DNA and organelles it may introduce ambiguous microbial signatures and assembly artefacts
Real-time functional profiling: Metatranscriptome analysis	+Can estimate which microorganisms and their activity in a community are actively transcribing when paired with marker gene analysis, including the responses to interventions (intra-individual variation) +Can discriminate between active vs. dormant or dead microorganisms and extracellular DNA +Higher-level analysis –Relative expensive, complex and laborious method for sample preparation and analysis, together with collection and storage –Host micro RNA contamination and rRNA must be removed –Several biases introduced due to organisms with high transcription rates

The microbiomics give us a great insight into the regulation of gut microbiota. However, in order to understand the complex biological pathways behind diseases, the identification of novel -omics biomarkers, such as identification of genes (genomics), gene expressions and phenotype (epigenomics), messenger RNA and micro RNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics, lipidomics, glycomics) could bring forward knowledge on probiotics and their effects on obesity and its modulation of pathophysiological mechanisms that have links with chronic diseases (Graw, et al., 2021).

Integrating multi-omics datasets is an innovative assignment (Figure 2), due to the increased complexity and diversity of the collected data (Knight et al., 2018). This integration is increasingly reliant on efficient bioinformatics tools and advanced statistical methods (Valles-Colomer et al., 2016; Mallick et al., 2017; Knight et al., 2018). Identifying microbial taxa that explain differences between communities is particularly challenging because microbiome data sets are high-dimensional (that is, they include thousands of taxa), sparse and compositional. Furthermore, understanding and modelling the confounding effects of the microbiome environment, such as host ethnicity and lifestyle, body site etc., on the microbiome remains challenging. NGS data require intensive analysis and would benefit from some standardisation of approach in the scientific community especially when different methods applied in the same population yield inconsistent results (Knight et al., 2018; Poussin et al., 2018).

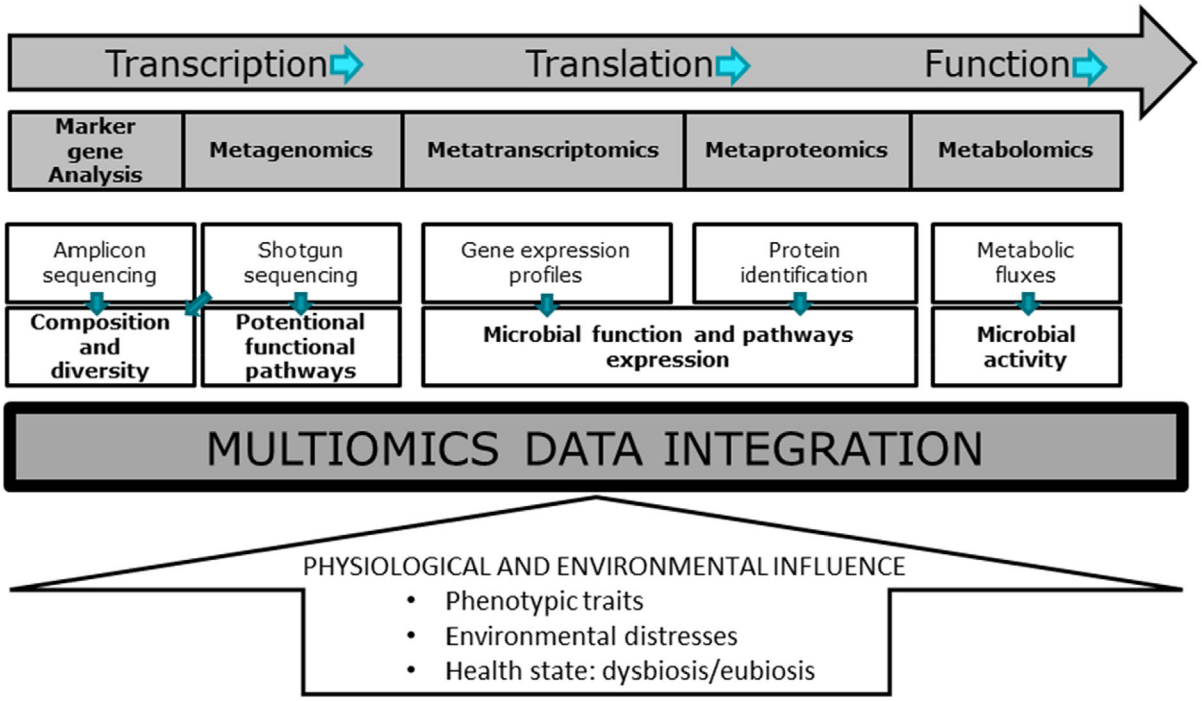


Figure 2: Multiomics data integration approach for elucidating the role of microbiota

Therefore, multi-omics data integration still poses challenges, but integration of multiple meta-omics datasets lays out a promising approach to comprehensively characterising the composition, functional, and metabolic activity of microbiomes. This is of particular importance for microbiome research to be translated into clinical applications, together with an increased demand for larger prospective cohort studies to validate findings and determine biomarker reproducibility before they can find applications for further improvement of human health management (Zhang et al., 2019).

2.2.3. EU-FORA Fellowship additional activities and trainings

EU-FORA Fellowship additional activities: In addition to the work at the Unit of INTYA - UGR, the Fellow attended the four EU-FORA modules organised by EFSA (Italy), AGES (Austria), BfR (Germany) and EFET (Greece) where a wide training in risk assessment provided her extremely useful knowledge and practice. Moreover, additional activities positively contributed the work development and results dissemination, as well as her training and learning:

- The BIOSAFE webinar series: 'Update your knowledge on Transparency Regulation and In vitro efficacy studies', 17th February, 2021.
- The Microbiome Informatics webinars of the Ohio State University, 2nd March - 4th May 2021.
- The GEN webinar series: 'Solving Metadata Management from Sample Provenance through Omics' 6th of April 2021.
- The BIOSAFE webinar series: 'Update your knowledge on antimicrobial resistance', 15th April 2021.
- The LAS-ICMSF Webinar: 'Update on Food Safety', 13–15 April 2021.
- The Greater Copenhagen Microbiome Summit 2021, 22nd April 2021.
- Canadian Bioinformatics Workshop Series: 'PNA: Pathway and Network Analysis', 10–12 May 2021.
- The STOA (The European Structural and Investment Funds - ESIFs and Horizon Europe) online workshop: 'Health and economic benefits of microbiomes', 11th May 2021.
- The BIOSAFE webinar series: 'Update your knowledge on Microbiome research and WGS analysis of secondary metabolites', 11th May 2021.
- Canadian Bioinformatics Workshop Series: 'MLE: Machine LEarning', 25–26 May 2021.
- Canadian Bioinformatics Workshop Series: 'MET: METabolomics analysis', 7–11 June 2021.
- Canadian Bioinformatics Workshop Series: 'AUR: Analysis Using R', 28–29 June 2021.
- Canadian Bioinformatics Workshop Series: 'MIC: MICrobiome analysis', 26th August and 1–3 September 2021.
- Canadian Bioinformatics Workshop Series: 'RNA: RNA-seq Analysis', 8–10 September 2021.
- Canadian Bioinformatics Workshop Series: 'HTG: High Throughput Genomics analysis', 27–29 September 2021.
- RAFA 2021 - Virtual event highlighting current Trends and Views: 'Recent advances in food analysis', 3–4 November 2021.
- The series of 'Introduction to BioCyc for New Life Sciences Graduate Students and Post-Docs in the life sciences', 3–17 November 2021.

and participation at congresses (Annex A):

- Abstract acceptation and iPoster presentation at the World Microbe Forum 2021, 20–24 June 2021 (online worldwide); in collaboration with the team (Ana López-Moreno, Alfonso Torres-Sánchez, Ángel Ruiz-Moreno, Pilar Ortiz, Marina Úbeda, Jesús Pardo, Margarita Aguilera; Faculty of Pharmacy, University of Granada, Granada and 'José Mataix Verdú' Institute of Nutrition and Food Technology (INYTA), Granada).
  Title: *Safety Assessment Criteria Implemented for the Gut Microbiota Taxa with Potential Use in Metabolization of Dietary Endocrine Disruptors*.
- Oral communication at THE EFSA-OBEMIRISK WORKSHOP Granada meeting Action: OBEMIRISK-Knowledge platform for assessing the risk of Bisphenols on gut microbiota and its role in obesogenic phenotype: looking for biomarkers (Granada, Spain; 14–15 October 2021) in collaboration with the team (Ana López-Moreno, Alfonso Torres-Sánchez, Ángel Ruiz-Moreno, Pilar Ortiz, Antonis Ampatzoglou, Agnieszka Gruszecka-Kosowska, Margarita Aguilera; Faculty of Pharmacy, University of Granada, Granada and 'José Mataix Verdú' Institute of Nutrition and Food Technology (INYTA), Granada):
  Title: Metagenomic analysis of children gut microbiota: challenges and standardization
- Abstract acceptation and Poster presentation at the EFFoST conference 2021, from 1 to 4 November 2021, Lausanne; Switzerland in collaboration with the team (Ana López-Moreno, Alfonso Torres-Sánchez, Ángel Ruiz-Moreno, Pilar Ortiz, Antonis Ampatzoglou, Agnieszka Gruszecka-Kosowska, Margarita Aguilera; Faculty of Pharmacy, University of Granada, Granada and 'José Mataix Verdú' Institute of Nutrition and Food Technology (INYTA), Granada):
  Title: 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

2.3. Results and discussion

Faecal sampling previously clustered from children (n = 109) population, aged between 3 and 13 years old, according to different level of bisphenols and analogues measurement and estimation will be analysed through 16S rRNA gene sequencing, metagenomics and data processing. Genomic DNA was extracted and microbiota in faecal samples population was analysed by sequencing the V4 region of microbial 16S rDNA, using an Illumina MiSeq platform as described by Ruiz et al. (2017) and Cerdó

et al. (2016). Sequences were further demultiplexed and filtered. The amplicon sequence variants (ASVs) were defined at 99% and taxonomic classifications and were assigned using the naive Bayesian algorithm CLASSIFIER of SILVA database. Furthermore, faecal supernatants were prepared from the most relevant samples to assess microbiota anaerobic culturing.

To identify microbiota composition, dysbiosis phenotypes patterns, reduced rank regression (RRR) models were used to derive combinations of phenotypes maximising the explained variability of gut microbiota within-sample diversity (each α-diversity index; Chao1, Shannon, and Faith PD). Later we will examine partial Spearman's correlation coefficients between the α-diversity dietary pattern and relative abundance (% ASV) of major phyla including F/B ratio and genera within the major phyla of the human gut microbiota with different variables within population, and sample batch as covariates and with multiple comparison corrections using false discovery rate (FDR). Enterotypes of gut microbiota were explored by a combination of principal coordinate analysis (PCoA) based on between-sample (β-) diversity indices (unweighted and weighted UniFrac and Bray-Curtis), and then k-means cluster analysis based on the PCoA scores of the first two principal coordinates. All analyses were performed using the R statistical software.

Microbiome data collection, collation, comparison and integration of data contribute to strength the dysbiosis phenotype-xenobiotic/toxic compound. As the proposed outcome, Microbiota knowledge integration platform database together with Standard operational procedures could serve as further improvements and guidelines for future legislation. This proposal contributes to the EFSA's scientific assessments through the expected findings that enlarge crosscutting knowledge about bisphenols exposure, its impact on gut microbiota, dysbiosis and obesity.

The final deliverables and complete work will be published in open access publications.

3. Conclusions

In short, microbiome analysis could contribute to improve area of risk assessment and food safety. Moreover, probiotic strains must be sufficiently characterised by next generation sequencing, safe for the intended use, assessed through pathogenicity, immunotoxicity, colonisation, antimicrobial susceptibility and genetic stability, supported by human clinical trials, conducted according to generally accepted scientific standards and alive in sufficient numbers in the product at an efficacious dose throughout shelf life. However, also legal and ethical matters must be addressed in the development of next generation probiotics, taking into account the proposed use and in the case of isolating microbes from humans ensuring appropriate informed consent.

Food safety risk assessment of EDCs should eventually consider the changes in and interactions with human microbiome. However, research in this field, including the variability of the human microbiome and its association with health outcomes, is still at its very first phase of development, requiring integrative expertise and holistic analyses. Furthermore, global harmonization and consensus of all stakeholders involved in the probiotic market is important since boundaries between differently regulated markets became minimal. Therefore, product approval procedures should be globally enforced, together with clear, reliable and truthful labelling for consumers stating general safety and true nature of the product they are using together with the following parameters: the genus, species and strain used, the CFU / g or ml of product (colony forming units), the recommended use and the daily dose, as well as quality parameters of the product: trademark, magisterial formula, ingredients, expiration date, storage conditions.

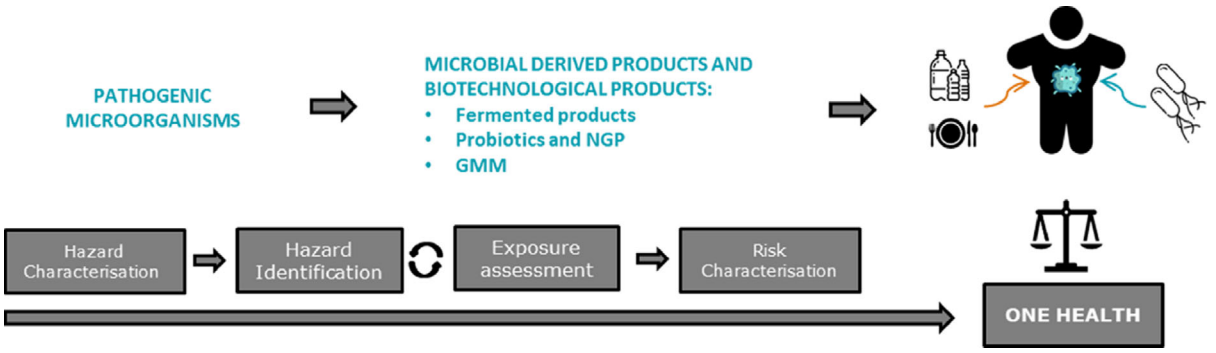


Figure 3: Evolution of microbial risk assessment under One Health approach

Microbiological risk assessment was initially designed to evaluate and prevent the impact of pathogenic microorganisms on human health. However, the era of biotechnology and extensive use of new microorganisms and their products need a more holistic risk analysis. In line, risk assessors and managers request actions and implementation of practical approaches under the One health concept. Therefore, this work contributes to establish know-how for the integration of microbiota biomarkers and next generation of probiotics impacting and modulating global health (Figure 3).

Disclaimer

The individual results of the analysis are not included in this report to avoid copyright claims as this research is part of an ongoing research project (being the EU-FORA Fellowship Programme) and the results are intended to be subsequently published in other scientific journals.

References

Amrane S, Hocquart M, Afouda P, Kuete E, Pham TP, Dione N, Ngom II, Valles C, Bachar D, Raoult D and Lagier JC, 2019. Metagenomic and culturomic analysis of gut microbiota dysbiosis during Clostridium difficile infection. Scientific Reports, 9, 12807.

Araya M, Morelli L, Reid G, Sanders ME, Stanton C, Pineiro M and Ben Embarek P, 2002. Guidelines for the evaluation of probiotics in food. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food, London (ON, Canada). pp. 1–11. 30 April and 1 May.

Bear TLK, Dalziel JE, Coad J, Roy NC, Butts CA and Gopal PK, 2020. The role of the gut microbiota in dietary interventions for depression and anxiety. Advances in Nutrition, 11, 890–907.

Bourdichon F, Patrone V, Fontana A, Milani G and Morelli L, 2021. Safety demonstration of a microbial species for use in the food chain: Weissella confusa. International Journal of Food Microbiology, 339, 109028.

Cerdó T, García-Valdés L, Altmäe S, Ruiz A, Suárez A and Campoy C, 2016. Role of microbiota function during early life on child's neurodevelopment. Trends in Food Science and Technology, 57, 273–288.

Chang CS, Ruan JW and Kao CY, 2019. An overview of microbiome based strategies on anti-obesity. Kaohsiung Journal of Medical Sciences, 35, 7–16.

Chrousos GP, 2007. Organization and integration of the endocrine system: the arousal and sleep perspective. Sleep Medicine Clinics, 2, 125–145.

Cordaillat-Simmons M, Rouanet A and Pot B, 2020. Live biotherapeutic products: the importance of a defined regulatory framework. Experimental and Molecular Medicine, 52, 1397–1406.

Cunningham M, Azcarate-Peril MA, Barnard A, Benoit V, Grimaldi R, Guyonnet D, Holscher HD, Hunter K, Manurung S, Obis D, Petrova MI, Steinert RE, Swanson KS, van Sinderen D, Vulevic J and Gibson GR, 2021. Shaping the future of probiotics and prebiotics. Trends in Microbiology, 29, 667–685.

Degnan FH, 2012. Clinical studies involving probiotics: when FDA's investigational new drug rubric applies-and when it may not. Gut Microbes, 3, 485–489.

Dhakan DB, Maji A, Sharma AK, Saxena R, Pulikkan J, Grace T, Gomez A, Scaria J, Amato KR and Sharma VK, 2019. The unique composition of Indian gut microbiome, gene catalogue, and associated fecal metabolome deciphered using multi-omics approaches. Gigascience, 8, giz004.

EFSA (European Food Safety Authority), 2015. Tools for critically appraising different study designs, systematic review and literature searches. EFSA Supporting Publication 2015;EN-836, 65 pp. https://doi.org/10.2903/j.efsa.2015.836

EFSA (European and Food Safety and Authority), 2017. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. EFSA Journal 2017;15(3):4664, 178 pp. https://doi.org/10.2903/j.efsa.2017.4664

EFSA (European and Food Safety and Authority), 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

EFSA (European and Food Safety and Authority), 2020. Scientific Opinion on the update of the list of QPS recommended biological agents intentionally added to food or feed as notified to EFSA (2017-2019). EFSA Journal 2020;18(2):5966, 56 pp. https://doi.org/10.2903/j.efsa.2020.5996

EFSA (European and Food Safety and Authority), 2021a. Scientific Opinion on the safety of pasteurised *Akkermansia muciniphila* as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2021;19(9):6780, 18 pp. https://doi.org/10.2903/j.efsa.2021.6780

EFSA (European and Food Safety and Authority), 2021b. General scientific guidance for stakeholders on health claim applications (Revision 1). EFSA Journal 2021;19(3):6553, 44 pp. https://doi.org/10.2903/j.efsa.2021.6553

EUCAST (European Committee on Antimicrobial Susceptibility Testing), 2015. The European Committee on Antimicrobial Susceptibility Testing. Routine and extended internal quality control as recommended by EUCAST. Version 5.0, 2015. Available online: https://www.eucast.org [Accessed: 23 June 2021].

FAO (Food and Agriculture Organization of the United Nations), 2007. Analytical methods, test procedures and laboratory solution used by and referenced in the food additive specifications. Combined compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives; FAO JECFA Monographs: Rome, Italy, 2007, 4, 1–331.

Gálvez-Ontiveros Y, Páez S, Monteagudo C and Rivas A, 2020. Endocrine disruptors in food: impact on gut microbiota and metabolic diseases. Nutrients, 12, 1158.

George VT, Varghese MM, Vaseem M, Thomas A and Sreejith C, 2016. The promising future of probiotics: a new era in periodontal therapy. Journal of International Oral Health, 8, 404–408.

Graw S, Chappell K, Washam CL, Gies A, Bird J, Robeson II MS and Byrum SD, 2021. Multi-omics data integration considerations and study design for biological systems and disease. Molecular Omics, 17, 170–185.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC and Sanders ME, 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology, 11, 506–514.

Jagadeesan B, Gerner-Smidt P, Allard MW, Leuillet S, Winkler A, Xiao Y, Chaffron S, Van Der Vossen J, Tang S, Katase M, McClure P, Kimura B, Ching Chai L, Chapman J and Grant K, 2018. The use of next generation sequencing for improving food safety: translation into practice. Food Microbiology, 79, 96–115.

Knight R, Vrbanac A, Taylor BC, Aksenov A, Callewaert C, Debelius J, Gonzalez A, Kosciolek T, McCall LI, McDonald D, Melnik AV, Morton JT, Navas J, Quinn RA, Sanders JG, Swafford AD, Thompson LR, Tripathi A, Xu ZZ, Zaneveld JR, Zhu Q, Caporaso JG and Dorrestein PC, 2018. Best practices for analysing microbiomes. Nature Reviews in Microbiolology, 16, 410–422.

Lee DH, 2018. Evidence of the possible harm of endocrine-disrupting chemicals in humans: ongoing debates and key issues. Endocrinology and Metabolism, 33, 44–52.

Li B, Zhan M, Evivie SE, Jin D, Zhao L, Chowdhury S, Sarker SK, Huo G and Liu F, 2018. Evaluating the safety of potential probiotic *Enterococcus durans* KLDS6.0930 using whole genome sequencing and oral toxicity study. Frontiers in Microbiology, 9, 1–15.

Lin TL, Shu CC, Lai WF, Tzeng CM, Lai HC and Lu CC, 2019. Investiture of next generation probiotics on amelioration of diseases – strains do matter. Medicine in Microecology, 1–2, 100002.

López-Moreno A, Acuña I, Torres-Sánchez A, Moreno Á, Cerk K, Rivas A, Suarez A, Monteoliva-Sánchez M and Aguilera M, 2021. Next generation probiotics for neutralizing obesogenic effects: taxa culturing searching strategies. Nutrients, 13, 1617.

Maguire M and Maguire G, 2017. The role of microbiota, and probiotics and prebiotics in skin health. Archives of Dermatological Research, 309, 411–421.

Mallick H, Ma S, Franzosa EA, Vatanen T, Morgan XC and Huttenhower C, 2017. Experimental design and quantitative analysis of microbial community multiomics. Genome Biology, 18, 1–16.

Marco ML, Heeney D, Binda S, Cifelli CJ, Cotter PD, Foligné B, Gänzle M, Kort R, Pasin G, Pihlanto A, Smid EJ and Hutkins R, 2017. Health benefits of fermented foods: microbiota and beyond. Current Opinion in Biotechnology, 44, 94–102.

Miyoshi J, Rao MC and Chang EB, 2020. Navigating the human gut microbiome: pathway to success from lessons learned. Gastroenterology, 159, 2019–2024.

Morovic W, Roper JM, Smith AB, Mukerji P, Stahl B, Rae JC and Ouwehand AC, 2017. Safety evaluation of HOWARU® Restore (Lactobacillus acidophilus NCFM, Lactobacillus paracasei Lpc-37, Bifidobacterium animalis subsp. lactis Bl-04 and B. lactis Bi-07) for antibiotic resistance, genomic risk factors, and acute toxicity. Food and Chemical Toxicology, 110, 316–324.

Nakatsuji T, Chen TH, Narala S, Chun KA, Two AM, Yun T, Shafiq F, Kotol PF, Bouslimani A, Melnik AV, Latif H, Kim JN, Lockhart A, Artis K, David G, Taylor P, Streib J, Dorrestein PC, Grier A, Gill SR, Zengler K, Hata TR, Leung DY and Gallo RL, 2017. Antimicrobials from human skin commensal bacteria protect against *Staphylococcus aureus* and are deficient in atopic dermatitis. Science Translational Medicine, 9, 1–12.

O'Toole PW, Marchesi JR and Hill C, 2017. Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. Nature Microbiology, 2, 17057.

Pasolli E, De Filippis F, Mauriello IE, Cumbo F, Walsh AM, Leech J, Cotter PD, Segata N and Ercolini D, 2020. Large-scale genome-wide analysis links lactic acid bacteria from food with the gut microbiome. Nature Communications, 1, 2610.

Poussin C, Sierro N, Boué S, Battey J, Scotti E, Belcastro V, Peitsch MC, Ivanov NV and Hoeng J, 2018. Interrogating the microbiome: experimental and computational considerations in support of study reproducibility. Drug Discovery Today, 23, 1644–1657.

Pouzaud F, Thierry-Mieg M, Burga K, Verines-Jouin L, Fiore K, Beausoleil C, Michel C, Rousselle C and Pasquier E, 2018. Concerns related to ED-mediated effects of Bisphenol A and their regulatory consideration. Molecular and Cellular Endocrinology, 475, 92–106.

Reid G, 2012. Probiotic and prebiotic applications for vaginal health. Journal of AOAC International, 95, 31–34.

Reid G, 2015. The growth potential for dairy probiotics. International Dairy Journal, 49, 16–22.

Ruiz A, Cerdó T, Jáuregui R, Pieper DH, Marcos A, Clemente A, García F, Margolles A, Ferrer M, Campoy C and Suárez A, 2017. One-year calorie restriction impacts gut microbial composition but not its metabolic performance in obese adolescents. Environmental Microbiology, 19, 1536–1551.
Saarela M, 2019. Safety aspects of next generation probiotics. Current Opinion in Food Science, 30, 8–13.
Sanders ME, Merenstein D, Merrifield CA and Hutkins R, 2018. Probiotics for human use. Nutrition Bulletin, 43, 212–225.
Schug TT, Johnson AF, Birnbaum LS, Colborn T, Guillette Jr LJ, Crews DP, Collins T, Soto AM, Vom Saal FS, McLachlan JA, Sonnenschein C and Heindel JJ, 2016. Minireview: endocrine disruptors: past lessons and future directions. Molecular Endocrinology, 30, 833–847.
de Simone C, 2019. The unregulated probiotic market. Clinical Gastroenterology and Hepatology, 17, 809–817.
Swann JR, Rajilic-Stojanovic M, Salonen A, Sakwinska O, Gill C, Meynier A, Fança-Berthon P, Schelkle B, Segata N, Shortt C, Tuohy K and Hasselwander O, 2020. Considerations for the design and conduct of human gut microbiota intervention studies relating to foods. The European Journal of Clinical Nutrition, 59, 3347–3368.
Taylor BC, Lejzerowicz F, Poirel M, Shaffer JP, Jiang L, Aksenov A, Litwin N, Humphrey G, Martino C, Miller-Montgomery S, Dorrestein PC, Veiga P, Song SJ, McDonald D, Derrien M and Knight R, 2020. Consumption of fermented foods is associated with systematic differences in the gut microbiome and metabolome. mSystems, 5, e00901-19.
Valles-Colomer M, Darzi Y, Vieira-Silva S, Falony G, Raes J and Joossens M, 2016. Meta-omics in inflammatory bowel disease research: applications, challenges, and guidelines. Journal of Crohn's and Colitis, 10, 735–746.
Veiga P, Suez J, Derrien M and Elinav E, 2020. Moving from probiotics to precision probiotics. Nature Microbiology, 5, 878–880.
WHO/UNEP, 2012. State of the science of endocrine disrupting chemicals – 2012. An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme and World Health Organization.
The European Parliament and the Council of the European Union. Regulation (EC) No 258/1997 of the European Parliament and of the Council of 27 January 1997. Concerning novel foods and novel food ingredients. Official Journal of the European Union, 1997, 43, 0001–0006.
The European Parliament and the Council of the European Union. Regulation (EC) No 1924/2006 of the European Parliament and the of the Council of 20 December 2006. on nutrition and health claims made on foods. Official Journal of the European Union, 2006404 pp. 9–25.
Zhang X, Li L, Butcher J, Stintzi A and Figeys D, 2019. Advancing functional and translational microbiome research using meta-omics approaches. Microbiome, 7, 154.

Abbreviations

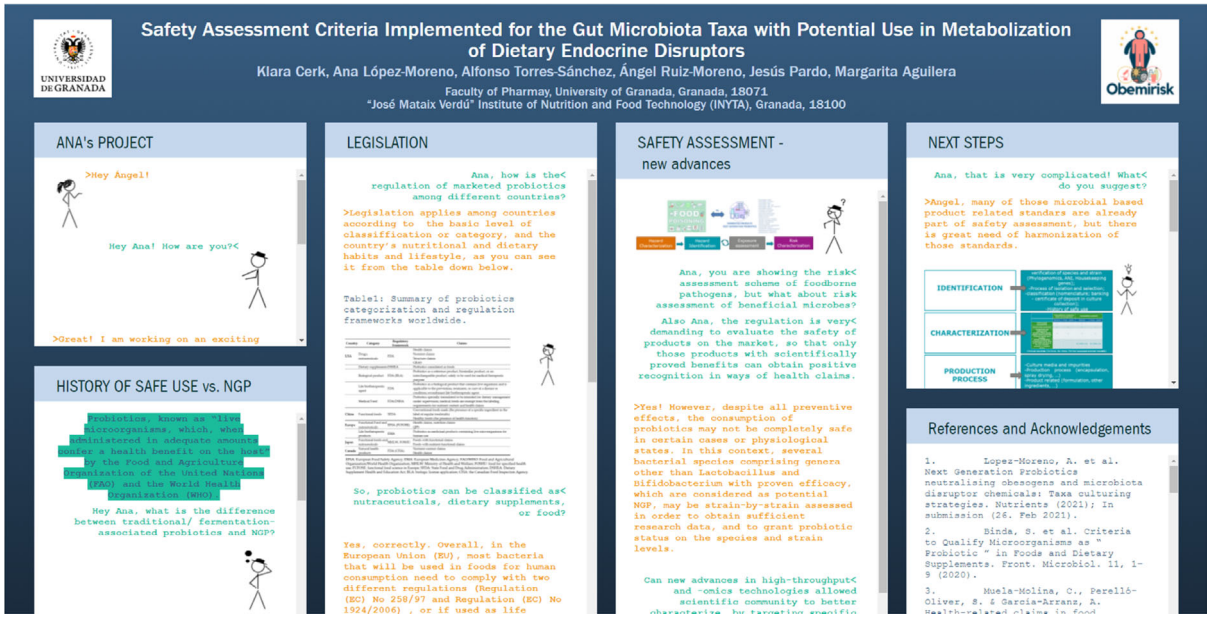
AGES	Agency for Health and Food Safety
AMR	antimicrobial resistance
ANI	alignment-based calculation of average nucleotide identity
ASV	amplicon sequence variant
BPA	bisphenol A
BfR	Federal Institute for Risk Assessment
BLA	biologic license application
CFIA	Canadian Food Inspection Agency
CFU	colony forming units
CIA	critically important antimicrobial
DSHEA	Dietary Supplement Health and Education Act
EFET	Hellenic Food Authority
EFFOST	European Federation of Food Science and Technology
EMA	European Medicines Agency
EU-FORA	European Food Risk Assessment
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FDR	false discovery rate
FOSHU	food for specified health use
F/B	Firmicutes/Bacteroidetes
GMM	genetically modified microorganism
GIT	gastrointestinal tract
GRAS	generally recognized as safe
HIA	highly important antimicrobials
INTYA	Institute of Nutrition and Food Technology
ISAPP	International Scientific Association for Probiotics and Prebiotics

isDDH	alignment-free genome distance estimation
MDC	microbiota-disrupting chemicals
MHLW	Ministry of Health and Welfare
MIC	minimum inhibitory concentration
NGP	next-generation probiotics
NGS	next-generation sequencing
Ph. Eur.	European Pharmacopoeia
PCoA	principal coordinate analysis
QPS	qualified presumption of safety
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RRR	reduced rank regression
SFDA	State Food and Drug Administration
UGR	University of Granada
WGS	whole genome sequencing
WHO	World Health Organization

Annex A – Posters presented at conferences:

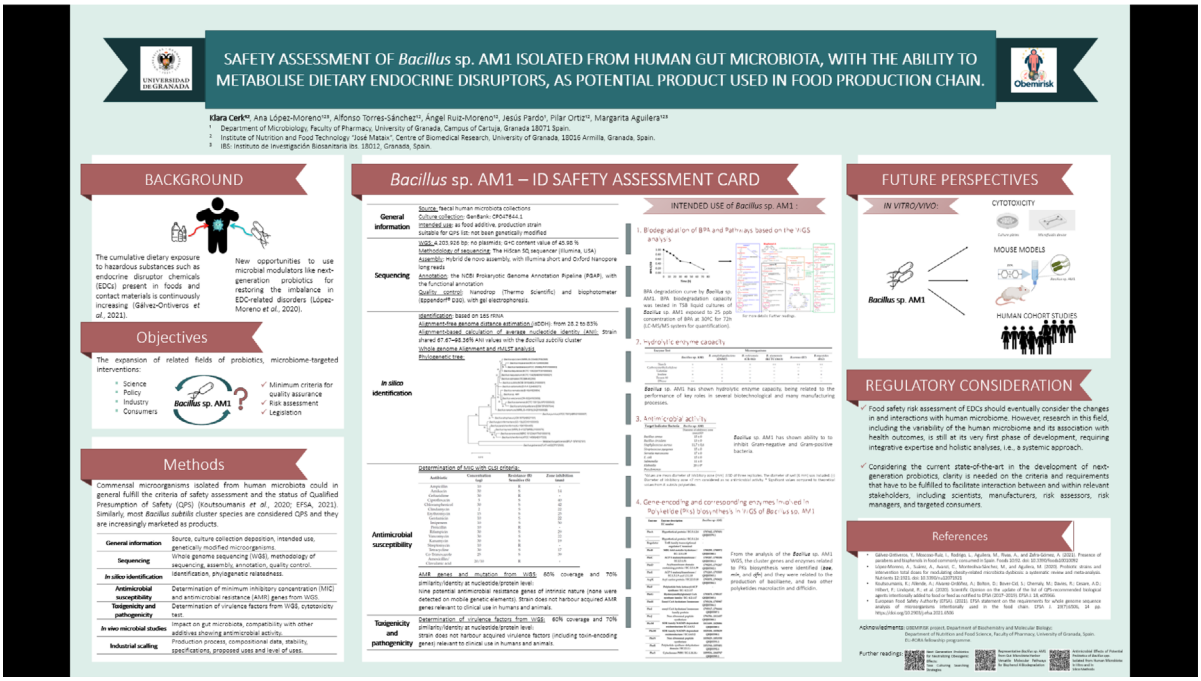
- The World Microbe Forum 2021, 20-24 June 2021

Title: Safety Assessment Criteria Implemented for the Gut Microbiota Taxa with Potential Use in Metabolization of Dietary Endocrine Disruptors.



- The EFFoST conference 2021, from 1-4 November 2021

Title: 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.



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A risk assessment model for *Salmonella* spp. in swine carcasses

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Abstract

Salmonellosis is one of the most important food-borne outbreaks that occurs in the EU/EEA. From the first production stages at slaughter, meat is susceptible to spoilage and can be a substrate for the pathogenic microorganisms growth. Among the pathogens, the presence of *Salmonella* is mainly due to mishandling during the evisceration stage. For the year 2019, according to the collected data from MSs, on the 17.9% of all food-borne outbursts, the presence *Salmonella* was confirmed. Pork meat is considered as one of the four most commonly reported foods in cases of salmonellosis. For the training purposes of this project, *Salmonella* isolation and identification along with RA for carcass contamination, was performed. Pig carcasses were sampled using the non-destructive technique. The sampling took place post dressing and before the stage of chilling. For the *Salmonella* detection, a three phases process was performed (pre-enrichment, enrichment, isolation). A total of 757 samples were collected, 19 were found to be positive for *Salmonella*. The most common was found to be *Salmonella* Derby, which was identified eight times. The main objective of the project was to determine the prevalence of *Salmonella* spp. in swine carcasses. Moreover, certain parameters were evaluated in terms of their influence on the prevalence of *Salmonella*. A stochastic simulation model was developed in Microsoft Office Excel 2019 by using the add-in @Risk v.8.1. The prevalence was estimated to be 2.6%. For the pigs sampled, the average value of the distance from farm to slaughterhouse was 200.92 km. Additionally, the average weight of the carcasses was 127.97 kg. The prevalence of *Salmonella* between the samples that came from farms with a distance above the average, was higher by 1.7 units, while the prevalence for the samples with weight above the average was higher by 0.2 units. According to the stochastic model, it is specified that the prevalence is higher with greater distance, and there is an 8.1% probability the prevalence will exceed the legislation's – hygiene criteria. In addition, the prevalence of *Salmonella* was shown to increase, as well in the case of samples from weightier animals, but to a lesser extent.

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Keywords: *Salmonella* spp., Risk assessment, @Risk, Swine, Carcass, Slaughterhouse

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1. Introduction

1.1. Food safety and hygiene

During the slaughter of animals and meat processing, the possibility of contamination by mishandling is high. From the first stages of the slaughter, meat is susceptible to spoilage and can be an ideal substrate for the growth and multiplication of pathogenic microorganisms. The application of inappropriate hygiene practices during handling, processing, storage, and distribution, catalyses the type and extent of contamination, causing unwanted degradation of quality and potentially serious consequences for the safety and health of consumers. During the last years, continuous improvement actions are carried out in the production processes with the main aim of implementing suitable food safety protocols. Despite these, incidents and their graveness remain high, causing increased concern and a considerable rise in uncertainty. In cases involving the growth of pathogenic microorganisms in meat, the type or intensity of the infection may be influenced by factors such as the origin or other specific characteristics of the animal (Koutsoumanis and Sofos, 2004). Among the main causes of contamination in meat are, direct contact with faeces residues, hides during the skinning process, lymph nodes and gastrointestinal tract during the process of evisceration (Mann et al., 2016; Mrdovic et al., 2017; Peruzy et al., 2021).

According to EU Regulation 853/2004, 7°C is defined as the maximum storage temperature for carcasses, which needs to be applied once the dressing stage is been completed. At this temperature, most of the pathogenic microorganisms do not grow, but the activity of some of them is not completely inhibited. Among the pathogens is the presence of *Salmonella*, which is mainly due to mishandling during the evisceration stage (Choi et al., 2013; Sánchez-Rodríguez et al., 2018, Grispoldi et al., 2020).

1.2. Reported food-borne data

Salmonellosis is one of the most significant food-borne outbreaks that occurred in the EU/EEA on an annual basis. It is the second most frequent gastrointestinal infection in the human population. First in the ranking is campylobacteriosis. Between the years 2018 and 2019, the reported cases of salmonellosis in the European Union (EU) remained at the same level, interpreted as 20 recorded cases for every 100,000 people. During the last 5 years, there have been stabilising trends regarding the cases of *Salmonella* Enteritidis, as well. In absolute numbers, the confirmed cases, for 2019, reached the level of 88,000. From the collective data of the 23 Member States (MS), for 2019, a total of 926 salmonellosis cases were recorded. More than 9,000 people fell ill, leading to seven declared deaths. For the 17.9% of all food-borne outbursts, *Salmonella* was confirmed as the main cause, while 72.4% of them were due to *S*. Enteritidis (EFSA, 2021).

Pork meat and its products are one of the four most commonly reported foods in cases of salmonellosis. For the years 2017 to 2019, the officially declared data, of the results of the *Salmonella* control by the food companies, show continuously low percentages of positive samples in pork carcasses. Nevertheless, the presented values, are often lower than the results of the inspections carried out by the competent authorities. From the MS' reports on the serotyped isolates, derived both from food and animal sources, 12% concerned the case of pork origin. The serotyped isolates with the highest incidence responsible for infections in the human body were *S. Infantis* at 29.7%. Followed by *S*. Enteritidis, the single-phase variant of *S*. Typhimurium*, S*. Typhimurium and finally *S*. Derby, with percentages of 6.9%, 4.5%, 3.9% and 3.7%, respectively (EFSA, 2021).

1.3. Legislation's requirements

According to Annex I of the EU Regulation 2073/2005, it is stated that after three successive positive samples, but no longer after five positives, the food business operators must adopt immediately suitable preventive measures. For instance, the general upgrading of hygienic conditions during slaughter or revaluation of the process controls. Therefore, it must be certified that the percentage of carcasses that do not fulfil the process hygiene criteria, is less than 6%.

The EU Regulation 218/2014, inserts further information regarding the official inspection controls concerning porcine species, with the aspect of respecting the application of the microbiological criteria. At the slaughterhouse, the business operator should carry out a fixed annual sampling plan with a minimum of 49 samples. Each sample from a different animal. The competent authority must plan the method most suitable for each slaughterhouse.

In the case of having one or more positive samples, out of the total 49 animals tested, a ≥ 6% *Salmonella* prevalence is assumed, with a 95% confidence margin. As a result, the competent Authority shall investigate further the possible non-conformities to determine if the critical percentage of 6% is exceeded or not. This percentage can also be interpreted as the minimum percentage of samples that need to meet the legislation – hygiene criteria in the official sampling plan before corrective actions are needed.

Furthermore, the sampling plan can be modified accordingly and comprise smaller number of samples. This can be achieved only with relevant approvement by the official veterinarian authority, after taking into account the risk assessment of the slaughterhouse, and aspects such as the size of the slaughterhouse, hygienic conditions, the facilities of the establishment, number of suppliers, distance between suppliers' farms and premises. The implementation of all samplings should always be in respect of EU Regulation 2073/2005.

All results and data from the business operator's self-control and from authorities sampling, must be reported in an annual base to European Commission in accordance with 2003/99.

2. Description of work programme

2.1. Importance

Active enrolment in the *Salmonella* isolation and identification along with RA for carcass contamination, will cover all aspects of the risk assessment process, including knowledge transfer to state veterinarians and food business operators. The topic of choice is of extreme actuality and arises from the background of a progressive streamlining of food controls on abattoir on behalf of visual inspection and limitation of palpation and cuts. For a comprehensive risk assessment, this approach has to consider the presence of bacteria on the carcass as per regulation 2073/2005.

2.2. Activities/Methods

This study was carried out at a slaughterhouse in central Italy between October 2018 and October 2021. During this period, 757 pig carcasses were sampled using the non-destructive technique. The four sampling sites tested for *Salmonella* presence on swine carcasses were: rump, belly, thorax, and neck. The sampling areas of each of the four sites were approximately 100 cm^2, like a square with 10 cm length on each side. The sampling took place post-dressing and before the stage of chilling.

The technique used is the following: for the sampling, a sponge in a sterile bag (Hydrated Speci-Sponge® Bags) is moistened in 10 mL of peptone water. Make sure that the sponge is adequately soaked. After identifying the sampling sites, delimit the four areas each of 100 cm^2. Apply on both halves of the carcass, by exerting sufficient pressure to the skin surface area. Sterile disposable gloves should be used, to avoid any contamination of the samples. The sponge should be swiped across the surface to be sampled horizontally, vertically, and diagonally, approximately 10 times in each direction, inside the limits of the designated areas. Then return the sponge into the sterile bag, seal it and label it with lot number of the sampled carcass.

Salmonella detection involves a process of three successive phases. The first is pre-enrichment, the second is enrichment, and the last one the isolation. For the pre-enrichment phase, test tubes containing 25 mL of peptone water were used (ISO 17604/2015). These tubes were poured inside the sterile sponge bags and after sampling they were incubated at 37°C for 24 h. For the enrichment phase, 0.1 mL was taken from the liquid derived from the sponge, after pre-enrichment, and sown in tubes containing a selective broth for the isolation of salmonellae, Rapport-Vassiliadis (ISO 6579-1 2017/AMD 1:2020). These tubes were incubated at 42°C for 24 h. This broth was developed specifically to exploit the four characteristics that differentiate *Salmonella* from other Enterobacteriaceae, namely: endurance to high osmotic pressures, growth at lower pH values, malachite green resistance, minor nutritional requirements. For the isolation, the samples were sown, by using sterile loops, on Petri dishes containing xylose lysine deoxycholate (XLD), a selective medium for the isolation of salmonellae, suitable for clinical and food samples. These plates were then incubated at 37°C for an additional 24 h. Salmonellae use xylose and decarboxylate lysine, thereby changing the pH to higher alkalinity levels. Change of the soil's colour is observed. The initial light red turns to bright red/violet. Furthermore, salmonellae presence can be spotted from the black-coloured colonies. The hydrogen sulfide, which is produced as a catabolite, forms a bind with the ammonium iron citrate, found in the soil, precipitating in the form of a black compound (ISO 6579-1 2017/AMD 1:2020).

Colonies showing phenotypic characteristics attributable to the genus *Salmonella* were isolated, cultivated in purity, and stored in a freezer at −80°C for subsequent analysis. The stems were then thawed and grown in brain–heart infusion broth at 37°C for 24 h. Subsequently, a suspension in physiological solution of the second degree of the McFarland scale was prepared for each bacterial stem. This suspension was inoculated in an API 20E diagnostic gallery and incubated at 37°C for 24 h. API 20E is a standardised system used, not only for the identification of Enterobacteriaceae but for other non-demanding Gram-negative bacilli as well. It includes 21 miniaturised biochemical tests, in addition to a specific database. The API 20E gallery consists of 20 microtubes, containing dehydrated substrates. The tubes are inoculated with a bacterial suspension that reconstitutes the media. The reactions produced during the incubation period result in spontaneous colour changes or may be revealed by the addition of reagents. The reading of these reactions is carried out using the reading table while the identification is obtained with the analytical index or with the identification software. The stems identified as *Salmonella* spp. were sent to the 'Togo Rosati' Experimental Zooprophylactic Institute of Umbria and Marche for serotyping (ISO 6579-1 2017/AMD 1:2020).

Serological typing is carried out through serum agglutination, with a rapid method on a slide. This method is applied to the identification of somatic O antigens and H flagellar antigens and is based on the identification scheme developed by Kauffmann-White, Le Minor (Grimont and Weill, 2007).

For the 19 positive samples, the serotype was specified, as described above. From more to less frequently identified: *Salmonella derby*, eight times; *Salmonella typhimurium* and *Salmonella London*, three times; *Salmonella give* and *Salmonella Brandenburg*, two times; *Salmonella gold coast* one time respectively (Figure 1, Table 1).

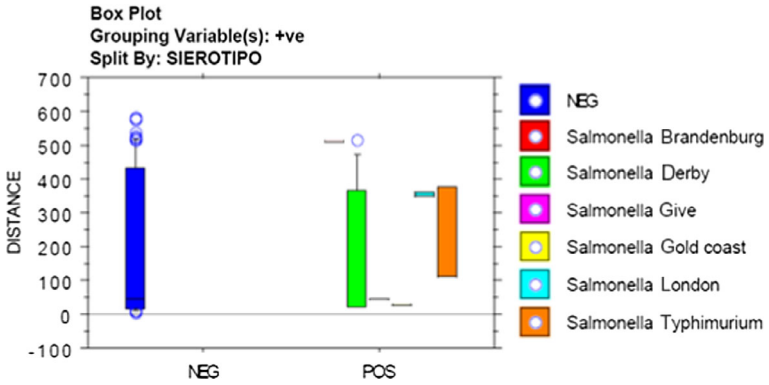

Figure 1: Box Plot – Serotype grouping with distance

2.3. Application of simulation model

To analyse the risk and calculate the prevalence, a stochastic simulation model was developed in Microsoft Office Excel 2019 with the use of the add-in programme @Risk v.8.1 for Excel (Palisade, Ithaca, USA). @Risk is based on a Monte Carlo simulation that can provide beneficial outcomes and allow to overcome uncertainty in quantitative analysis. Monte Carlo simulation can perform risk analysis through the substitution of individual points of uncertain inputs with the distribution of possibilities. These are randomly tested over and over, for many interactions, and the model calculation to create large sets of possible data which can then be further analysed.

A total of 757 samples were collected and the data were processed with the application of @Risk. Of that 19 samples were found to be positive for *Salmonella*. The prevalence of *Salmonella* was estimated to be 2.6%. The pigs came from farms in various parts of Italy (Figure 2). The distance from farm to slaughterhouse ranged from 7.6 to 584 km and the average value was 200.92 km (Figure A.1a). The weight of the carcasses ranged from 51 up to 207.8 kg with an average of 127.97 kg (Figure A.1b). The prevalence between the samples that came from farms with a distance greater than 200 km (distance above the average), concerning the prevalence of *Salmonella* for all the samples, was higher by 1.7 units, with an estimated prevalence value of 4.3%. The stochastic model gives a direct correlation between the distance and the expected prevalence of *Salmonella*. A possible cause, can be the increased length of stay, of the animals, in the transport vehicles with inadequate hygiene conditions and close contact among animals (Simons et al., 2016). The considerable higher prevalence value (4.3%) designates the need to increase monitoring and sampling frequency in these

cases (Figure A.2). On the other hand, the prevalence of *Salmonella* for the samples that weighed more than 130 kg (weight above the average), concerning the prevalence of *Salmonella* for all the samples, was higher by 0.2 units, with an estimated prevalence value of 2.8% (Figure A.3) (Graphs **Appendix A**).

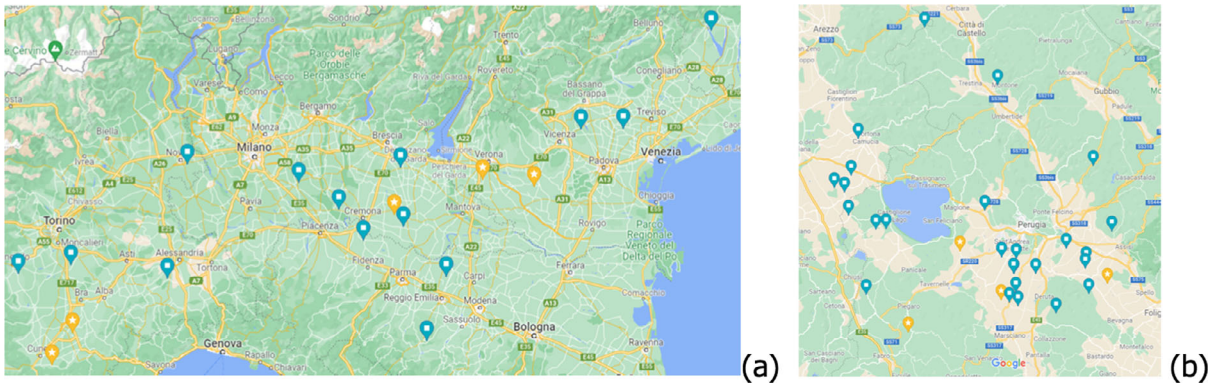

Figure 2: Locations of the farms (a) Northern Italy and (b) Central Italy – Region of Umbria (Google Maps). Yellow pins: Farms with samples tested positive for *Salmonella*

2.4. Additional relevant activities and learning opportunities

Besides the specific activities on the risk assessment model for *Salmonella* spp. in swine carcasses, the fellow participated in a full range of activities of the research unit, which is indeed a group young and committed, so the fellow took part in the exciting and numerous activities of the unit: participations to the master degree workshops and the continuing education training for the public health and risk assessment. Moreover, the fellow has fully involved in the organisation and participation in the monthly scientific events of the master degree in Veterinary public health and food hygiene (**Appendix B**).

3. Conclusions

The main objective of the project was to determine the prevalence of *Salmonella* in swine carcasses from regular and emergency slaughter. This research will clarify the role of slaughterhouses and procedures as a source of pathogens' contaminations and focus the attention on the importance of RA along with the accurate and detailed inspection of the carcasses despite modern trends and revisions of procedures. For all carcasses sampled, additional accompanying data were collected for processing and analysis. All information came from the competent audit authority responsible for the operation of the sampling slaughterhouse. Furthermore, certain parameters were evaluated in terms of their influence on the prevalence of *Salmonella*. According to the stochastic model, it is specified that the prevalence is higher with greater distance, and there is an 8.1% probability the prevalence will exceed the critical percentage of 6%. In addition, the prevalence of *Salmonella* shown to increase, as well in the case of samples from weightier animals, but to a lesser extent. The probability the prevalence will exceed the critical percentage of 6%, is 0.1%.

Risk assessments are beneficial studies that in fact can help decision-making. Based on the RA results, FBOs can achieve more targeted resource management. The qualitative model's outcome can be utilised for the reassessment of existing priorities in the inspection technics. Moreover, interventions with great efficacy are made easier to plan and apply throughout the production chain (Pouillot et al., 2012, Costa et al., 2020).

Table 1: Data of samples tested positive in *Salmonella*, including the identified serotype, the lot number – slaughter number, the category, the farm code, Distance farm to slaughterhouse, weight of carcasses, breeder name, the freezer number of the sample when archived

	Slaughter number	Category	Farm code	Distance in km	Weight in kg	Breeder name	Internal reference number
Serotype							
Salmonella Give	043177	S	020TR***	46.6	132.6	B*****	977
	043176	S	020TR***	46.6	121.4	B*****	976
Salmonella Gold Coast	113275	S	039PG***	25.9	144.4	L*****	979
Salmonella Derby	120278	S	163CN***	518	86.2	D*****	980
	120304	S	027PG***	21.5	128	B*****	982
	120313	S	027PG***	21.5	137	B*****	983
	120302	S	006PG***	22.3	131.8	U*****	981
	190236	S	094VR***	365	121	S*****	995
	190237	S	094VR***	365	117.8	S*****	996
	190238	S	094VR***	365	129	S*****	997
	190239	S	094VR***	365	139	S*****	998
Salmonella Brandenburg	127259	S	019CN***	511	138.4	V*****	984
	134234	S	019CN***	511	118.4	V*****	985
Salmonella Typhimurium	148272	S	107CR***	377	156.2	F*****	988
	148273	S	107CR***	377	163.6	F*****	989
	176199	S	006PG***	22.3	121.4	U*****	994
Salmonella London	351184	S	027VR***	351	133.8	A*****	1000
	351188	S	027VR***	351	132.8	A*****	1001
	351283	S	094VR***	365	111.8	A*****	1002

References

Choi YM, Park HJ, Jang HI, Kim SA, Imm JY, Hwang IG and Rhee MS, 2013. Changes in microbial contamination levels of porcine carcasses and fresh pork in slaughterhouses, processing lines, retail outlets, and local markets by commercial distribution. Research in Veterinary Science, 94, 413–418. https://doi.org/10.1016/j.rvsc.2012.11.015

EFSA (European Food Safety Authority), 2021. The European Union One Health 2019 Zoonoses Report. EFSA Journal 2021;19(2):6406, 45 pp. https://doi.org/10.2903/j.efsa.2021.6406

European Commission, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union, 50, 1–26.

Freitas Costa E, Cardoso M, Kich JD and Corbellini LG, 2020. A qualitative risk assessment approach to microbial foodborne hazards in Brazilian intensive pork production: A step towards risk prioritization. Microbial Risk Analysis, 15, 2352–3522. https://doi.org/10.1016/j.mran.2020.100105

Grimont PAD and Weill FX, 2007. Antigenic Formulae of the *Salmonella* Serovars, 9th Edition. World Health Organization Collaborating Center for Reference and Research on Salmonella, Institute Pasteur, Paris.

Grispoldi L, Karama M, Hadjicharalambous C, de Stefani F, Ventura G, Ceccarelli M, Revoltella M, Sechi P, Crotti C, D'Innocenzo A, Couto-Contreras G and Cenci-Goga B, 2020. Bovine lymph nodes as a source of *Escherichia coli* contamination of the meat. International Journal of Food Microbiology, 16. https://doi.org/10.1016/j.ijfoodmicro.2020.108715

ISO (International Organization for Standardization), ISO. 6579-1 2017/ AMD 1:2020. Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella.

Koutsoumanis K and Sofos JN, 2004. Microbial contamination of carcasses and cuts. Encyclopedia of Meat Sciences, Elsevier Academic Press, 727–737. https://doi.org/10.1016/B0-12-464970-X/00070-2

Mann E, Wetzels SU, Pinior B, Metzler-Zebeli BU, Wagner M and Schmitz-Esser S, 2016. Psychrophile spoilers dominate the bacterial microbiome in musculature samples of slaughter pigs. Meat Science, 117, 36–40. https://doi.org/10.1016/j.meatsci.2016.02.034

Mrdovic B, Raseta M, Brankovic-Lazic I, Milijasevic M, Baltic B and Ivan N, 2017. Pigs and cattle slaughter process hygiene in a large - scale and a small - scale abattoir: a report from one county in Serbia. Scientific Journal Meat Technology, 58, 65–72.

Peruzy MF, Houf K, Joossens M, Yu Z, Proroga YTR and Murru N, 2021. Evaluation of microbial contamination of different pork carcass areas through culture-dependent and independent methods in small-scale slaughterhouses. International Journal of Food Microbiology, 336, 108902. https://doi.org/10.1016/j.ijfoodmicro.2020.108902

Pouillot R, Garin B, Ravaonindrina N, Diop K, Ratsitorahina M, Ramanantsoa D and Rocourt J, 2012. A risk assessment of campylobacteriosis and salmonellosis linked to chicken meals prepared in households in Dakar, Senegal. Risk Analysis, 32, 1798–1819. https://doi.org/10.1111/j.1539-6924.2012.01796.x

Sánchez-Rodríguez JA, Navas L, Vinuesa FM, Castells C, Martínez MA, López A, Lindez B and Cabrera-Vique C, 2018. New insights on the risk factors associated with the presence of *Salmonella* on pig carcasses. Lessons from Small Slaughterhouses. Food Control, 87, 46–52. https://doi.org/10.1016/j.foodcont.2017.12.016

Simons RR, Hill AA, Swart A, Kelly L and Snary EL, 2016. A transport and Lairage model for *Salmonella* transmission between pigs applicable to EU member states. Risk Analysis, 36, 482–497. https://doi.org/10.1111/risa.12390

Abbreviations

AGES	Austrian Agency for Health and Food Safety
BfR	Bundesinstitut für Risikobewertung
EEA	European Economic Area
EFET	Hellenic Food Authority
EU-FORA	European Food Risk Assessment Programme
FBO	food business operator
ISO	International Organization for Standardization
MS	Member State
RA	risk assessment
UniPG	University of Perugia
XLD	xylose lysine deoxycholate

Appendix A – Distribution Graphs @Risk

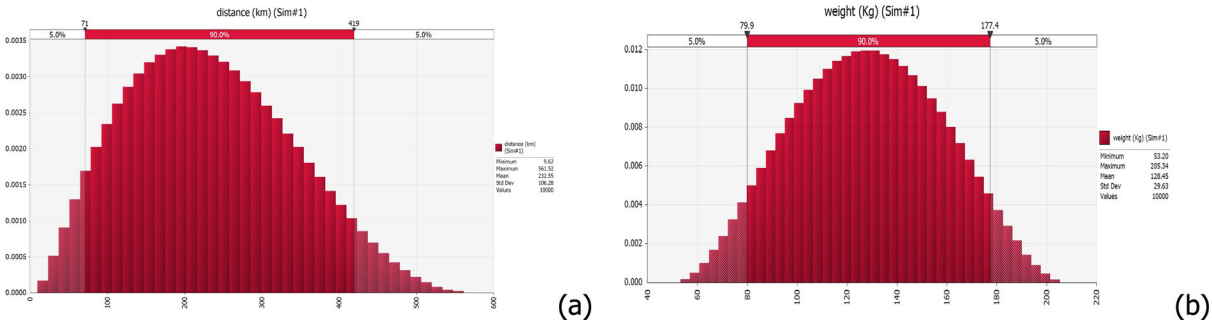

Figure A.1: (a) Distribution of distance values (farm to slaughterhouse), (b) Distribution of weight values

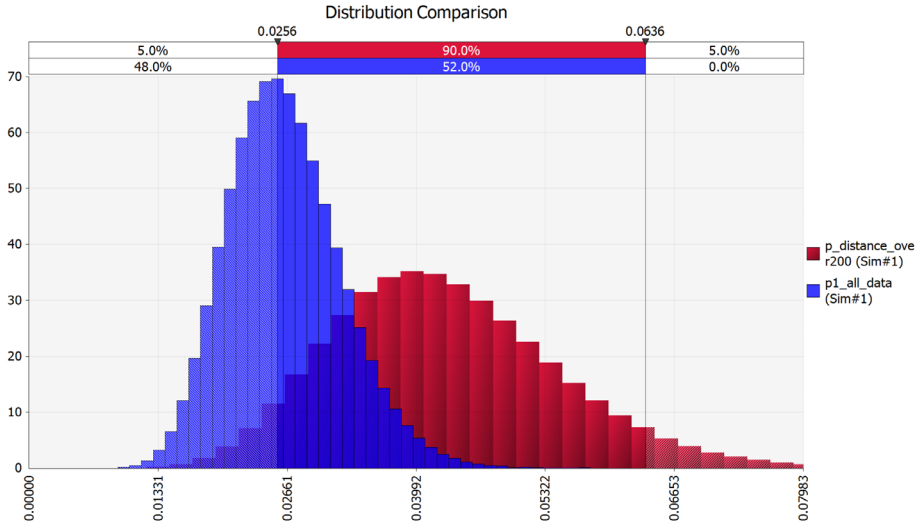

Figure A.2: Prevalence of *Salmonella* in all samples (blue), in comparison with prevalence of *Salmonella* for the samples from farms with distance 200 km and greater (red)

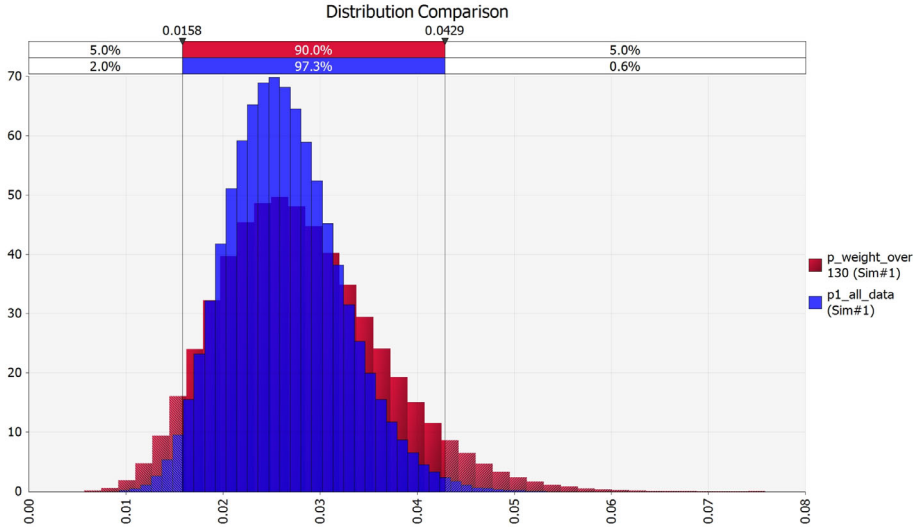

Figure A.3: Prevalence of *Salmonella* in all samples (blue), in comparison with prevalence of *Salmonella* for the samples with weight of 130 kg and up (red)

Appendix B – Side projects and activities throughout the fellowship

Alongside of the main risk assessment project, the fellow had participated actively in all the ongoing projects of the department:

1) eLearning courses on Quantitative risk analysis models in Excel, with @RISK, Palisade.
2) Participation in the preparation and writing of research papers and ongoing projects of the department: A review manuscript on *E.coli*, currently untitled project/A quantitative risk assessment of *Listeria monocytogenes*/A study on the application of natural extracts as alternatives to sodium nitrite in canned meat/Hygienic Characteristics and Detection of Antibiotic Resistance Genes in Crickets (*acheta domesticus*) Breed for Flour Production/ Evolution and Antimicrobial Resistance of *Enterococci* Isolated from Pecorino and Goat Cheese.
3) Regular visits to a slaughterhouse in Umbria Central Italy, for the observation of animal's behavior prior to and after certain animal-welfare interventions.
4) Participation in the experimental part of undergraduate student's thesis project: Effect of packaging and storage conditions on some qualitative characteristics of beef meat.
5) Webinars on Programming with Python: Getting started with Python/Python Data Structure
6) Presentation of the Eu-Fora fellowship programme to Erasmus and undergraduate students of UniPG.
7) Day trip to a meat processing company in Umbria Central Italy, for the supervision of the production line, processing and packaging of Chianina beef meat.
8) Day trip to an automatic – robotic milk farm in Umbria Central Italy, where cattle moving and handling techniques were examined on Holstein Friesian cattle, in accordance with animal welfare principals.
9) Cibo Sovrano. Le guerre alimentari globali al tempo del virus – "Sovereign Food. Global food wars at the time of the virus" by Maurizio Martina, Vice Director of the Food and Agriculture Organization (FAO), Round table discussion.
10) EU-FORA Training Courses:
    - Induction training of the European Food Risk Assessment Fellowship Programme (EFSA) (11–29 January 2021).
    - Module 1 training of the European Food Risk Assessment Fellowship Programme – Risk Communication (BfR–EFSA) (22–26 March 2021).
    - Module 2 training of the European Food Risk Assessment Fellowship Programme – Emerging Risks (EFET–EFSA) (7–14 June 2021).
    - Module 3 training of the European Food Risk Assessment Fellowship Programme – Data Collection and Reporting (EFSA) (4–7 October 2021).
    - Module 4 training of the European Food Risk Assessment Fellowship Programme – Novel Foods (AGES–EFSA) (22–26 November 2021).

EU-FORA SERIES 4

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Monitoring AMR in *Campylobacter jejuni* from Italy in the last 10 years (2011–2021): Microbiological and WGS data risk assessment

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Abstract

Campylobacter jejuni is considered as the main pathogen in human food-borne outbreaks worldwide. Over the past years, several studies have reported antimicrobial resistance (AMR) in *C. jejuni* strains. In Europe, the official monitoring of AMR comprises the testing of *Campylobacter* spp. from food-producing animals because this microorganism is responsible for human infections and usually predominant in poultry. Food-producing animals are considered to be a major source of campylobacteriosis through contamination of food products. Concerns are growing due to the current classification of *C. jejuni* by the WHO as a 'high priority pathogen' due to the emergence of resistance to multiple drugs such as those belonging to the fluoroquinolones, macrolides and other classes, which limits the treatment alternatives. Knowledge about the contributions of different food sources to gastrointestinal disease is fundamental to prioritise food safety interventions and to establish proper control strategies. Assessing the genetic diversity among *Campylobacter* species is essential to the understanding of their epidemiology and population structure. Using a population genetic approach and grouping the isolates into sequence types within different clonal complexes, it is possible to investigate the source of the human cases. The work programme was aimed for the fellow to assess the AMR of *C. jejuni* isolated from humans, poultry and birds from wild and urban Italian habitats. Given the public health concern represented by resistant pathogens in food-producing animals and the paucity of data about this topic in Italy, the aim was to identify correlations between phenotypic and genotypic AMR and comparing the origin of the isolates. The work programme allowed the fellow to acquire knowledge, skills and competencies on the web-based tools used by IZSAM to process the NGS data and perform bioinformatics analyses for the identification of epidemiological clusters, the study of AMR patterns in *C. jejuni* isolates, and the assessment of the human exposure to such AMR pathogens. Furthermore, the fellow became able to transfer the acquired knowledge through innovative web-based didactical tools applied to WGS and clustering of specific food-borne pathogens, with particular reference to *C. jejuni*. To achieve this objective, 2,734 *C. jejuni* strains isolated from domestic and wild animals and humans, during the period 2011–2021 were analysed. The resistance phenotypes of the isolates were determined using the microdilution method with EUCAST breakpoints, for the following antibiotics: nalidixic acid, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, streptomycin, tetracycline. The data were complemented by WGS data for each strain, uploaded in the Italian information system for the collection and analysis of complete genome sequence of pathogens isolated from animal, food and environment (GENPAT) developed and maintained at IZSAM; information like clonal complex and sequence type to understand the phylogenetical distance between strains according to their origins were also considered. This work underlines that a better knowledge of the resistance levels of *C. jejuni* is necessary, and mandatory monitoring of *Campylobacter* species in the different animal productions is strongly suggested.

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Keywords: Campylobacter, antimicrobial resistance (AMR), multidrug resistance (MDR), multilocus sequence typing, resistance genes, MLST

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1. Introduction

Antibiotic-resistant bacteria can spread through many routes. When antimicrobial resistance (AMR) occurs in zoonotic bacteria present in animals and food it can also compromise the effective treatment of infectious diseases in humans.

In the field of food safety, policymakers need to protect consumers from risks related to the food chain and to establish the best control options to reduce such risks. Scientists and risk assessors are examining the factors which may lead to the presence of antimicrobial-resistant bacteria in food and animals to provide appropriate scientific advice to decision-makers (Allard et al., 2012).

1.1. Description of the pathogen

Campylobacter is a Gram-negative, aerobic, microaerophilic, mobile bacterium. It presents pleomorphic forms, being observed as curved, spiral, comma and coccoid bacilli, the last one is observed especially in old cultures (Baker, 2009). They can grow at two temperatures: 37°C and 42°C (Best et al., 2005). The taxonomy of this genus has changed dramatically since its discovery, in 1963, by Sebald and Véron (Buettner et al., 2010); (Cecilia et al., 2013). Currently, it comprises 25 species, 2 provisional species and 8 subspecies, many of which are of clinical and economic importance (Cevenini et al., 1985). *Campylobacter jejuni* is one of the most important species, which comprises two subspecies: *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. The *jejuni* subspecies, referred to as *C. jejuni*, have been recognised as the most isolated bacterium from humans with gastroenteritis since 1970. In addition, it is involved in other diseases (Diaz-Sanchez et al., 2013), such as proctitis, septicaemia, meningitis, abortion and autoimmune diseases (Reiter's syndrome and Guillain-Barré syndrome) (Baker, 2009). In chickens and other species of birds, as well as in dogs, pigs, sheep and livestock, *C. jejuni* is considered a commensal organism, making it one of the most important pathogens present and transmitted by foods of animal origin (Dingle et al., 2001). Most cases of *Campylobacter enteritis* do not require treatment, as they are generally short-lived, self-limited events. However, when symptoms are prolonged or very severe, antimicrobial therapy is necessary. For these occasions, erythromycin is the antibiotic of choice. Some *Campylobacter* species are resistant to penicillin, ampicillin and cephalosporins. The increase in resistance to fluoroquinolones coincides with their administration in poultry and veterinary medicine in general. Most strains of *C. jejuni* are still susceptible to erythromycin, azithromycin, gentamicin, tetracycline and chloramphenicol. Erythromycin and azithromycin shorten the duration of illness when given early in gastrointestinal infection (EFSA and ECDC, 2014; EFSA Antimicrobial Resistance, 2021).

1.2. Situation

Campylobacter is the most common cause of bacterial gastroenteritis in Europe. The incidence of human campylobacteriosis is increasing worldwide, as well as the number of isolates resistant to fluoroquinolones which are one of the primary classes of antimicrobials used to treat *Campylobacter* infection in human therapy and thus considered of high public concern (Kittl et al., 2013). In 2020, in the European Union, *Campylobacter* is still the most commonly reported cause of bacterial food-borne illness, as it has been since 2005. It represented more than 60% of all the reported cases in 2020 (EFSA and ECDC, 2021). Poultry is a natural reservoir of *Campylobacter* species, constituting the most important source of human infection. The consumption of undercooked poultry meat or the mishandling of raw poultry products is considered to be the main risk factor associated with human campylobacteriosis (Lucarelli et al., 2016; Manfreda et al., 2016). In Italy, the notification rate was 1.410 reported human cases per 100.000 population (Di Giannatale et al., 2016; EFSA and ECDC, 2021). Human clinical cases are not regularly reported; Therefore, the real incidence of the disease in man has not been established. However, a pilot surveillance programme for *Campylobacter* infection in humans has been recently implemented. The preliminary results confirmed previous observations (Parkhill et al., 2000; Mughini Gras et al., 2012), namely that the pathogen is widespread, and that human cases frequently occur, particularly in children (Pendleton et al., 2013). With regard to animals and animal products, studies carried out in Italy have demonstrated the presence of *Campylobacter* in poultry, poultry products and pork meat (Piantieri et al., 1985; Pezzotti et al., 2003a,b; Piccirillo et al., 2014; EFSA and ECDC, 2021). However, the presence of the pathogen at a national level needs to be better investigated.

1.3. EFSA's Role in AMR

EFSA provides independent scientific support and advice to risk managers on the risks to human and animal health related to the possible emergence, spread, and transfer of AMR in the food chain and animals. EFSA takes an integrated approach to its work on antimicrobial resistance involving a number of its Scientific Panels and Units as it is a concern for the entire food chain. On this topic, EFSA cooperates closely with other relevant EU agencies such as the European Centre for Disease Prevention and Control (ECDC) and the European Medicines Agency (EMA) (Allard et al., 2012).

1.4. NGS in Campylobacteriosis

The investigation of the food-borne disease outbreaks related to the origin of the pathogen strains could be time-consuming in most cases. The available molecular tools, including the 'gold standard' pulsed-field gel electrophoresis (PFGE) method, do not always distinguish the outbreak-related strains from other genetically similar strains unassociated with the same outbreak (Sánchez de la Barquera and Herrera, 2002; Lévesque et al., 2008; Man, 2011). Thus, next-generation sequencing (NGS) tools and whole genome sequencing (WGS) in particular, provide a powerful approach for epidemiological trace-back efforts (On, 2005). The tracking and tracing of living animals and animal-derived food within the food chain is part of the monitoring process (Harlizius et al., 2004; Sarhangi et al., 2021). In addition to epidemiological tracing, NGS provides additional data to investigators that can include identification of specific markers for detection efforts and assessment of unique virulence factors that may be strain specific.

The lack of a method for distinguishing *Campylobacter* strains makes it more difficult to trace back sources in outbreaks. Multiple typing methods have been developed including antibiotic resistance, phage typing, serotyping and several emerging genetically based methods. Advanced research in the field of genome-based methods (multilocus sequence typing and microarrays) has allowed the identification of different clonal groupings of *C. jejuni* (Troso et al., 1997; Manning et al., 2003; Kittl et al., 2013a). Also, the development of real-time PCR Taqman allelic discrimination assays permitted the rapid detection of *C. jejuni* isolates and preliminary strain identification (Véron and Chatelain, 1973). However, the high plasticity of *Campylobacter* relegates the use of some of the genotyping technology in advance of more advantageous methods like WGS (Sebald and Veron, 1963; Troso et al., 1997; Young et al., 2007 Stahl and Stintzi, 2011). The development of WGS of *C. jejuni* offers the opportunity to detect genes and proteins involved in the pathogenesis of *Campylobacter* and also genes of unknown function (Zanetti et al., 1996; Zhang et al., 2009). The understanding of the function of many of these proteins and genes could lead to the increasing of knowledge of the role of *Campylobacter* in the colonisation of chicken gut, the immune response and finally could cause the improvement of current practices and potential intervention strategies for the control of campylobacteriosis (Zhang et al., 2009).

1.5. Data collection

The development of integrated information systems is a guarantee for farmers and consumers because these systems allow to turn data into actions. The Italian Ministry of Health appointed the IZSAM as National Reference Centre for WGS of microbial pathogens: database and bioinformatics analysis. For this reason, the IZSAM has realised a platform (GENPAT) (Genpat Platform, 2017) for the collection and storage of genomic sequences of pathogenic microorganisms, to perform bioinformatic analyses, to archive and to share the results. IZSAM provides the technical infrastructure, developers team, and service desk needed for the management of data, ensuring the interoperability with other national information systems in the veterinary field through an online platform. The GENPAT system provides IT tools and data that are quickly available, usable and helpful in outbreak situations allowing to link molecular typing and bioinformatics analyses result with time and geographical position of sampling as well as the others epidemiological information available. In this way, it is possible to compare the classification results obtained by WGS for two or more distinct isolates and to measure their relatedness in order to support an epidemiological investigation as experienced in 2015 during a severe listeriosis outbreak in the Marche region (Duranti et al., 2018).

Access to information allows knowing the health risk to which the livestock and food processing chain is exposed and how the risks can affect public health, performing real-time assessments to adjust technical and organisational responses, plan actions and check their effectiveness afterward. The national platform for the collection and analysis of complete genome sequence together with the veterinary information systems adopted in Italy during the years fully meets these needs (Cito et al., 2018).

2. Description of the work programme

2.1. Aims

The work programme allowed the fellow to acquire knowledge, skills and competencies on the application of NGS methods and bioinformatics analyses for the identification of epidemiological clusters, the study of AMR patterns in *C. jejuni* isolates and the assessment of the human exposure to such AMR pathogens. Furthermore, the fellow became able to transfer the acquired knowledge through innovative web-based didactical tools applied to WGS and clustering of specific food-borne pathogens, with particular reference to *C. jejuni*.

2.2. Activities/Methods

WP1. Acquisition of knowledge and skills to use didactic web-based tools (February–April 2021)

The fellow had the opportunity to acquire new knowledge and skills through theoretical and practical training on didactic web-based tools. The training was carried out at distance in cooperation with subject matter experts and trainers working at the ISO 9001:2015 certified International Centre for Veterinary Training and Information (CIFIV). This learning-by-doing approach allowed the fellow to apply his knowledge by contributing to the development of technical and scientific content for an e-learning course devoted to professionals engaged with genomics applied to food safety risk assessment (e.g. microbiologists, epidemiologists, bioinformaticians, statisticians and veterinarians). This WP allowed maximising the impact of the fellowship by enhancing cascade training and scalability of the acquired competencies to other scientific domains.

WP2. NGS technology and production of WGS data (March–May)

NGS has been used for WGS of viral and bacterial pathogens, and it is also applied for metagenomics and transcriptomic studies of microorganisms. At the genomic laboratory, the fellow acquired knowledge and practical skills on different NGS technologies, starting from the sample (the bacterial isolate or different samples for metagenomics analysis).

Different aspects of the WGS workflow were taken into consideration:

- General requirements for a genomic laboratory, use of the laboratory management system, and specific worksheets.
- DNA extraction for short and long-read sequencing, evaluation of quantity and quality of DNA.
- Sequencing on Illumina and Nanopore NGS platforms, quality metrics of the NGS run.
- Best practices for quality management of the WGS workflow, use of positive and negative control, genomic library Quality Control.
- Principles of validation of WGS workflow.
- Participation in proficiency tests for microbial WGS.

WP3. Collection, normalisation and organisation of data (June–August)

IZSAM collects and registers a well-defined set of data for each sample tested in its laboratories. In addition, several samples were collected in the framework of national control plans (all related data are registered into the National Veterinary Information System) and European and national projects managed by IZSAM. All these factors allowed IZSAM to retrieve relevant epidemiological data for all tested samples.

The WP3 activity was focused on data related to a selection of *Campylobacter* isolates obtained from different sources, at various stages of the chicken meat production chain (farm, slaughterhouse and retail). A detailed data analysis plan was prepared, including the description of the dataset retrieved, the type of data quality checks to be performed and the format of the resulting validated databases, using data from 2,734 *C. jejuni* strains isolated from domestic and wild animals and humans, during the period 2011–2021.

WP4. Bioinformatics analysis (July–October)

The fellow was involved in the development of a detailed data analysis plan, including the description of the bioinformatics pipelines used to analyse WGS data and metadata obtained from the *Campylobacter* isolates arriving at IZSAM. The data were analysed to identify 'epi-clusters', considering the outcomes of the molecular typing methods using WGS data, such as *in silico* MLST, and gene-by-gene or single nucleotide polymorphisms (SNPs)-based analysis. Moreover, different bioinformatic tools such as Resfinder, ARIBA, Plasmidfinder and databases like CARD were applied to WGS data aiming to identify the AMR genes of interest and associated mobile genetic elements within the genome of *Campylobacter* isolates.

WP5 Exposure assessment (November–December)

The results of the analysis performed in WP4 allowed to development of AMR risk assessment models, considering different exposure pathways. For instance, data concerning the dissemination and persistence of *C. jejuni* in the farm's environment and the production chain were taken into account together with the presence of AMR determinants and genetic mobile elements in the strain's genome.

2.3. Secondary activities

The fellow has assisted and completed complementary activities to reinforce the experience (description in Appendix A).

3. Results and conclusions

3.1. AMR

The proportion of completely susceptible strains was very similar in isolates from humans and domestic animals (67,63% and 61,55%, respectively), while strains from the wild animal population found a significantly higher prevalence (95,49%). Most of these strains were collected from domestic animals (95,01%), largely poultry samples (81,21%), that showed a high level of resistance to nalidixic acid, ciprofloxacin and tetracycline (67,39%, 67,27% and 55,63%, respectively). The human isolates reproduced the same patterns reinforcing the direct association between the increase in the resistance profiles over time with veterinary practices in the control of pathogens in poultry (Moore et al., 2006; Ruiz-Palacios, 2007). Fluoroquinolones had very high rates of resistant profiles since 2011 with a stable trend over the last decade. Conversely, erythromycin showed a slight increase in resistance levels mostly for poultry and swine, while wild animals and human strains had stable trends. In conclusion, antibiotics released in animal production environments can interfere with the development of resistance profiles.

3.2. Genetic approach

Within the samples we have identified 11 clonal complexes (CCs) (443-49-179-446-42-206-354-45-353-828-21), the most prevalent being CC-828, CC-21 and CC-353, with 30,5%, 19,7% and 16,9% respectively. This CCs were often described in the literature for its global distribution. Despite the high genetic diversity usually detected within *Campylobacter* populations, a remarkable similarity among isolate collections on a national and international scale, even across different continents, can be documented (Di Giannatale et al., 2016; Cito et al., 2018). CC-21 is a multihost lineage shared among different sources (De Haan et al., 2010), one of the largest CCs found to date, comprising 26% of all the isolates submitted to PubMLST, with a total of 152 different STs (https://pubmlst.org/campylobacter). This CC is frequently associated with cases of human disease and is one of the most frequently reported CCs in poultry (Dingle et al., 2001; Kärenlampi et al., 2007; Kittl et al., 2013b; Manfreda et al., 2016; EFSA and ECDC, 2021); however, CC-21 has also been found in cattle (Pezzotti et al., 2003; EFSA and ECDC, 2021), sheep (Lucarelli et al., 2016), turkey (Lo Schiavo et al., 1991) and the environment (Dingle et al., 2001). Several studies were performed in Italy and show how common this CC is not only in chickens but also in strains isolated from turkeys, cattle and bulk tank milk (Lo Schiavo et al., 1991; Bianchini et al., 2014a,b). The most common CC among chicken *C. jejuni* isolates was the CC-21 according to that documented worldwide (Pezzotti et al., 2003b; EFSA and ECDA, 2014; Lucarelli et al., 2016; Cito et al., 2018; Sarhangi et al., 2021). CC-828 was also described as predominant and with similar results to CC-21 and shared between chicken and human campylobacteriosis (Pezzotti et al., 2003a). The predominance of the CC-828 and CC-353 was expected since several studies demonstrated that these CCs are globally spread in both human and chicken *Campylobacter* populations (Sánchez de la Barquera and Herrera, 2002; Pezzotti et al., 2003b; Colles and Maiden, 2012; Lucarelli et al., 2016; Cito et al., 2018).

The most diverse CCs are related to more prevalent sequence types. This proposes that probably their diversity is a mirror of their replication frequency and circulation which affects their gene content and efficiency (On, 2005; Ragimbeau et al., 2008). This data demonstrates the importance of rapid reporting of gastroenteritis cases to local public health authorities to perform a timely epidemiological and microbiological investigation. Mandatory notification for *Campylobacter* would be useful for better estimation of the disease, as well as identification of the source of infection and proper control measures to protect public health (Sheppard et al., 2009a,b). The more prevalent CCs we have found

are concordant to those found worldwide, keeping track of molecular epidemiology provides a universal picture of the movement of dominant *Campylobacter* strains.

3.3. Conclusions from the participation in the EU-FORA programme

It was a valuable opportunity for the fellow to obtain experience in AMR risk assessment of a food-borne pathogen like *C. jejuni*. This was also an excellent opportunity to consolidate his specialised knowledge and skills in food safety, particularly in bacterial microbiology, by working according to European and international guidelines and standards for AMR. The general risk assessment methodology applied for this specific project is expected to be further extended and applied by the fellow in future positions, to expand knowledge. Moreover, the EU-FORA programme provided a great environment to build a strong professional and personal network that will be an open door for future collaborations and references.

References

Allard MW, Luo Y, Strain E, Li C, Keys CE, Son I, Stones R, Musser SM and Brown EW, 2012. High-resolution clustering of *Salmonella enterica* serovar Montevideo strains using a next-generation sequencing approach. BMC Genomics, 13, 1–19.

Baker CD, 2009. Red Book Atlas de enfermedades infecciosas en pediatria/Red Book Atlas of Pediatric Infectious Diseases. Ed. Médica Panamericana.

Best EL, Fox AJ, Frost JA and Bolton FJ, 2005. Real-time single-nucleotide polymorphism profiling using Taqman technology for rapid recognition of *Campylobacter jejuni* clonal complexes. Journal of Medical Microbiology, 54, 919–925.

Bianchini V, Borella L, Benedetti V, Parisi A, Miccolupo A, Santoro E, Luini M, 2014a. Prevalence in bulk tank milk and epidemiology of *Campylobacter jejuni* in dairy herds in Northern Italy. Applied and Environmental Microbiology, 80, 1832–1837.

Bianchini V, Luini M, Borella L, Parisi A, Jonas R, Kittl S and Kuhnert P, 2014b. Genotypes and antibiotic resistances of *Campylobacter jejuni* isolated from cattle and pigeons in dairy farms. International Journal of Environmental Research and Public Health, 11, 7154–7162.

Buettner S, Wieland B, Staerk KDC and Regula G, 2010. Risk attribution of *Campylobacter* infection by age group using exposure modeling. Epidemiology & Infection, 138, 1748–1761.

Cecilia HC, Arreola MGA and Graciela CE, 2013. *Campylobacter jejuni*: ¿ A forgotten bacteria? Its situation in Mexico. Enfermedades Infecciosas Y Microbiología, 33, 77–84.

Cevenini R, Varoli O, Rumpianesi F, Mazzaracchio R, Nanetti A and La Placa M, 1985. A two-year longitudinal study on the etiology of acute diarrhea in young children in Northern Italy. Microbiologica, 8, 51–58.

Cito F, Di Pasquale A, Cammà C and Cito P, 2018. The Italian information system for the collection and analysis of complete genome sequence of pathogens isolated from animal, food, and environment. International Journal of Infectious Diseases, 73, 296–297.

Colles FM and Maiden MCJ, 2012. *Campylobacter* sequence typing databases: applications and prospects. Microbiology, 158, 2695–2709.

De Haan CPA, Kivistö R, Hakkinen M, Rautelin H and Hänninen ML, 2010. Decreasing trend of overlapping multilocus sequence types between human and chicken *Campylobacter jejuni* isolates over a decade in Finland. Applied and Environmental Microbiology, 76, 5228–5236.

Di Giannatale E, Garofolo G, Alessiani A, Di Donato G, Candeloro L, Vencia W, Decastelli L and Marotta F, 2016. Tracing back clinical Campylobacter jejuni in the Northwest of Italy and assessing their potential source. Frontiers in Microbiology, 7, 887.

Diaz-Sanchez S, Hanning I, Pendleton S and D'Souza D, 2013. Next-generation sequencing: the future of molecular genetics in poultry production and food safety. Poultry Science, 92, 562–572.

Dingle KE, Colles FM, Wareing DRA, Ure R, Fox AJ, Bolton FE, Maiden MCJ, 2001. Multilocus sequence typing system for *Campylobacter* jejuni. Journal of Clinical Microbiology, 39, 14–23.

Duranti A, Sabbatucci M, Blasi G, Acciari VA, Ancora M, Bella A, Busani L, Centorame P, Cammà C, Conti F, De Medici D, Di Domenico M, Di Marzio V, Filippini G, Fiore A, Fisichella S, Gattuso A, Gianfranceschi M, Graziani C, Guidi F, Marcacci M, Marfoglia C, Neri D, Orsini M, Ottaviani D, Petruzzelli A, Pezzotti P, Rizzo C, Ruolo A, Scavia G, Scuota S, Tagliavento G, Tibaldi A, Tonucci F, Torresi M, Migliorati G and Pomilio F, 2018. A severe outbreak of listeriosis in central Italy with a rare pulsotype associated with processed pork products. Journal of Medical Microbiology, 67, 1351–1360.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2014. The European Union Summary REPORT on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA Journal 2014;12(2):3547, 55 pp. https://doi.org/10.2903/j.efsa.2014.3547

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2021. The European Union one health 2020 zoonoses report. EFSA Journal, 2021;19(12):6971, 324 pp. https://doi.org/10.2903/j.efsa.2021.6971

EFSA Antimicrobial Resistance, 2021. Available online: https://www.efsa.europa.eu/en/topics/topic/antimicrobial-resistance

Genpat Platform, 2017. Ministero della Salute, con Decreto del 30 maggio 2017 (G.U.R.I. n. 196 del 23 agosto 2017), ha attivato il Centro di Referenza Nazionale per Sequenze Genomiche di microrganismi patogeni: banca dati e analisi di bioinformatica presso la sede centrale dell'IZSAM Available online: https://genpat.izs.it/cmdbuild/ui/#login

Harlizius B, van Wijk R and Merks JW, 2004. Genomics for food safety and sustainable animal production. Journal of Biotechnology, 113, 33–42.

Kärenlampi R, Rautelin H, Schönberg-Norio D, Paulin L and Hänninen ML, 2007. Longitudinal study of Finnish *Campylobacter jejuni* and *C. coli* isolates from humans, using multilocus sequence typing, including comparison with epidemiological data and isolates from poultry and cattle. Applied and Environmental Microbiology, 73, 148–155.

Kittl S, Heckel G, Korczak BM and Kuhnert P, 2013a. Source attribution of human *Campylobacter* isolates by MLST and fla-typing and association of genotypes with quinolone resistance. PLoS One, 8, e81796.

Kittl S, Korczak BM, Niederer L, Baumgartner A, Buettner S, Overesch G and Kuhnert P, 2013b. Comparison of genotypes and antibiotic resistances of *Campylobacter jejuni* and *Campylobacter coli* on chicken retail meat and at slaughter. Applied and Environmental Microbiology, 79, 3875–3878.

Lévesque S, Frost E, Arbeit RD and Michaud S, 2008. Multilocus sequence typing of *Campylobacter jejuni* isolates from humans, chickens, raw milk, and environmental water in Quebec. Canada. Journal of Clinical Microbiology, 46, 3404–3411.

Lo Schiavo A, Minniti A, Panebianco A and Iannuzzi L, 1991. Isolamento di Campylobacter jejuni da polli totalmente eviscerati. Archivio Veterinario Italiano, 42, 18–21.

Lucarelli C, Dionisi AM, Trezzi L, Farina C, Passera M, Kärki T, Luzzi I, 2016. Molecular and epidemiological analysis of a *Campylobacter jejuni* outbreak in Northern Italy in November 2013. Foodborne Pathogens and Disease, 13, 490–494.

Man SM, 2011. The clinical importance of emerging *Campylobacter* species. Nature Reviews Gastroenterology & Hepatology, 8, 669–685.

Manfreda G, Parisi A, De Cesare A, Mion D, Piva S and Zanoni RG, 2016. Typing of *Campylobacter jejuni* isolated from turkey by genotypic methods, antimicrobial susceptibility, and virulence gene patterns: a retrospective study. Foodborne Pathogens and Disease, 13, 93–100.

Manning G, Dowson CG, Bagnall MC, Ahmed IH, West M and Newell DG, 2003. Multilocus sequence typing for comparison of veterinary and human isolates of *Campylobacter jejuni*. Applied and Environmental Microbiology, 69, 6370–6379.

Moore JE, Barton MD, Blair IS, Corcoran D, Dooley JSG, Fanning S, Kempf I, Lastovica AJ, Lowery CJ, Matsuda M, McDowell DA, McMahon A, Millar BC, Rao JR, Rooney PJ, Seal BS, Snelling WJ and Tolba O, 2006. The epidemiology of antibiotic resistance in *Campylobacter*. Microbes and Infection, 8, 1955–1966.

Mughini Gras L, Smid JH, Wagenaar JA, de Boer AG, Havelaar AH, Friesema IHM and van Pelt W, 2012. Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis.

On SL, 2005. Taxonomy, phylogeny, and methods for the identification of *Campylobacter* species. Campylobacter: molecular and cellular biology, 13–42.8-.

Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Barrell BG, 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature, 403, 665–668.

Pendleton S, Hanning I, Biswas D and Ricke SC, 2013. Evaluation of whole-genome sequencing as a genotyping tool for *Campylobacter jejuni* in comparison with pulsed-field gel electrophoresis and flaA typing. Poultry Science, 92, 573–580.

Pezzotti G, Serafin A, Luzzi I, Mioni R, Milan M and Perin R, 2003. Aparición y resistencia a antibióticos de Campylobacter jejuni y Campylobacter coli en animales y carne en el noreste de Italia. Revista Internacional De Microbiología Alimentaria, 82, 281–287.

Pezzotti G, Serafin A, Luzzi I, Mioni R, Milan M and Perin R, 2003. Occurrence and resistance to antibiotics of *Campylobacter jejuni* and *Campylobacter coli* in animals and meat in northeastern Italy. International Journal of Food Microbiology, 82, 281–287.

Piantieri G, Mamolo G, Caffarelli A, Bossi G, Bignamini ML and Bo G, 1985. Presenza di *Campylobacter jejuni* nel pollo di allevamento. Ig. Mod, 83, 510–517.

Piccirillo A, Giacomelli M, Salata C, Bettanello S, De Canale E and Palù G, 2014. Multilocus sequence typing of *Campylobacter jejuni* and *Campylobacter coli* from humans and chickens in North-Eastern Italy. New Microbiologica, 37, 557–562.

Ragimbeau C, Schneider F, Losch S, Even J and Mossong J, 2008. Multilocus sequence typing, pulsed-field gel electrophoresis, and fla short variable region typing of clonal complexes of *Campylobacter jejuni* strains of human, bovine, and poultry origins in Luxembourg. Applied and Environmental Microbiology, 74, 7715–7722.

Ruiz-Palacios GM, 2007. The health burden of Campylobacter infection and the impact of antimicrobial resistance: playing chicken.

Sánchez de la Barquera RM and Herrera BIF, 2002. Diarrea por Campylobacter. In: En Romero CR and Herrera BIF (eds.), Síndrome diarreico infeccioso. México-España. Ed. Médica Panamericana, pp. 204–205.
Sarhangi M, Bakhshi B and Peeraeyeh SN, 2021. High prevalence of *Campylobacter jejuni* CC21 and CC257 clonal complexes in children with gastroenteritis in Tehran. Iran. BMC Infectious Diseases, 21, 1–13.
Sebald M and Veron M, 1963. Base DNA content and classification of vibrios. In Annales de l'Institut Pasteur (Vol. 105, pp. 897–910).
Sheppard SK, Dallas JF, MacRae M, McCarthy ND, Sproston EL, Gormley FJ, Strachan NJC, Ogden ID, Maiden MCJ and Forbes KJ, 2009a. *Campylobacter* genotypes from food animals, environmental sources and clinical disease in Scotland 2005/6. International Journal of Food Microbiology, 134, 96–103.
Sheppard SK, Dallas JF, Strachan NJ, MacRae M, McCarthy ND, Wilson DJ, Forbes KJ, 2009b. *Campylobacter* genotyping to determine the source of human infection. Clinical Infectious Diseases, 48, 1072–1078.
Stahl M and Stintzi A, 2011. Identification of essential genes in C. jejuni genome highlights hyper-variable plasticity regions. Functional & Integrative Genomics, 11, 241–257.
Troso O, Gallo T, Gallo G, Crescente MD and Flora ME, 1997. A surveillance system for childhood gastroenteritis. Igiene Moderna, 107, 411–420.
Véron M and Chatelain R, 1973. Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter* fetus (Smith and Taylor) Sebald and Véron. International Journal of Systematic and Evolutionary Microbiology, 23, 122–134.
Young KT, Davis LM and DiRita VJ, 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. Nature Rev. Microbiol, 5, 665–679.
Zanetti F, Varoli O, Stampi S and De Luca G, 1996. Prevalence of thermophilic *Campylobacter* and *Arcobacter butzleri* in food of animal origin. International Journal of Food Microbiology, 33, 315–321.
Zhang MJ, Xiao D, Zhao F, Gu YX, Meng FL, He LH, Zhang JZ, 2009. Comparative proteomic analysis. of *Campylobacter jejuni* cultured at 37 C and 42 C. Japanese Journal of Infectious Diseases, 62, 356–361.

Abbreviations

AMR	antimicrobial resistance
CC	clonal complex
CIFIV	Centre for Veterinary Training and Information
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicines Agency
IZSAM	Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise
LEGO	Learning Genomics for Food Safety
MDR	multidrug resistance
MLST	multilocus sequence typing
NGS	next-generation sequencing
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
WGS	whole genome sequencing

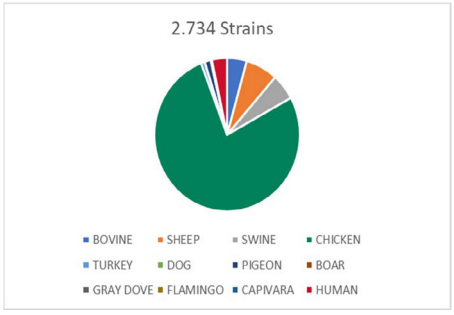
Appendix A – Secondary Activities

Additional relevant activities and learning opportunities completed by the fellow:

- 'Induction training of the European Food Risk Assessment Fellowship Programme' (EFSA) (11– 29 January 2021).
- Content Reviewing LEGO Project In the framework of the Erasmus+ Programme Unit 1. eLearning course for Food Microbial Bioinformatician (1 February–29 March 2021).
- 'Module 1 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (22– 26 March 2021).
- eLearning course for Food Microbial Bioinformatician LEGO Project In the framework of the Erasmus+ Programme (29 March–31 December 2021) https://www.learngenomics.eu/2021/02/04/call-for-applications-e-learning course.
- A course in the Italian language (April–July 2021). Provided by CPIA Municipality of Teramo.
- Illumina Practical Training (Provided by Illumina Technical Expert). (31 May–1 June 2021).
- 'Module 2 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (7–14 June 2021).
- 4th European Summer School on Nutrigenomics (virtual edition, 21–25 June 2021) https://www.unicam.it/nutrigenomics/ Food as Medicine: Food and our Genome https://www.futurelearn.com/courses/fam-genome. Scholarship.
- CampyUK. Liverpool International congress. Abstract Submitted 'Monitoring Antibiotic Resistance in Campylobacter jejuni from Italy in the last 10 years (2011–2021)- Conesa, A.; Garofolo, G.; Janowicz, A.; Di Marcantonio, L.; Di Pasquale, A.; Camma, C. LigthTalk Presentation. (8–10 September 2021).
- Parma Summer School 'Food Safety Aspects of Integrated Food Systems'-EFSA. (28–30 September 2021).
- 'Module 3 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (4–7 October 2021)
- 'Module 4 training of the European Food Risk Assessment Fellowship Programme' (EFSA) (22–26 November)
- Online Course- Introduction to programming for Bioinformatics with Python. October 2021. Udemy.
- Online Course- Practical Bioinformatics: Play with Genes On Your Screens. October 2021. Udemy.
- Antimicrobial Resistance- Theory and Methods. 5 weeks course. Authorized by the Technological University of Denmark (DTU) Trough Coursera. Grade: 90.61%. October–November 2021 https://coursera.org/share/5a1c35b77a615678e17657a88c786cca
- Cohesive Symposium One Health EJP- European Joint Programme Promoting One Health in Europe through joint actions on foodborne zoonoses, antimicrobial resistance, and emerging microbiological hazards. 8–10 November 2021. The Netherlands (On remote).
- Short Training Workshop EFSA Partnering Grants GP/EFSA/ENCO/2020/03 GA3- Basic Bioinformatics Skills Relating to Using Genotypic/Phenotypic techniques for Risk Assessment/ Predictive Microbiology – Online 'virtual' workshop from 9 to 11 November 2021. Dublin, Ireland (On remote).
- Giornata di studio del Centro di Referenza Nazionale per Sequenze Genomiche di microrganismi patogeni: banca dati e analisi di bioinformatica (GENPAT). by IZSAM G. Caporale, Teramo, IT. 30/11/2011. 4.5 Credits.

Appendix B – Graphics

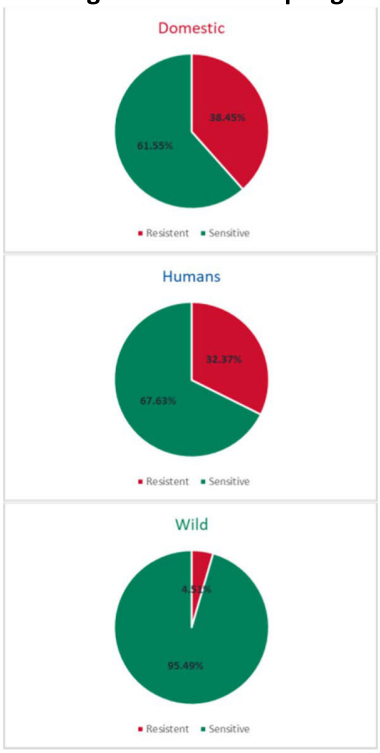
Total Number of Strains in the study and origin of the sample



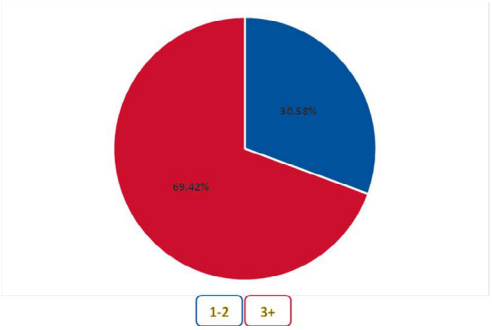
Percentage of total resistance during the decade for the principal antibiotics

HOST	PROFILE	CIP	NAL	TET	ERY
CHICKEN	R	89,49%	87,65%	73,98%	12,78%
	I+S	10,50%	12,34%	26,04%	87,2%
HUMAN	R	77,55%	62,5%	65,30%	6,1%
	I+S	22,44%	37,5%	34,69%	93,87%

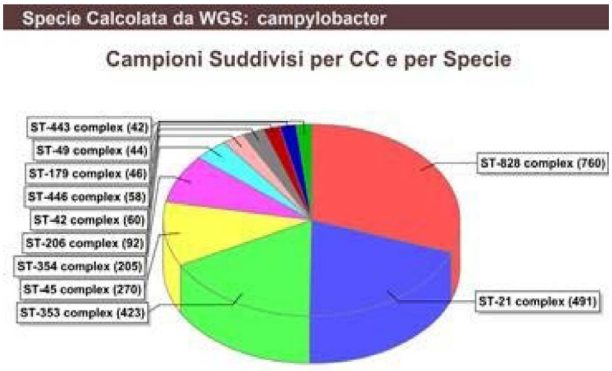
Percentage of Resistance per group



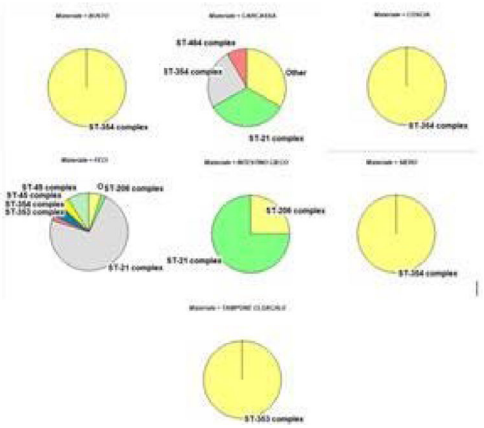
Percentage of total strains resistant to 1-2 vs 3+antibiotics



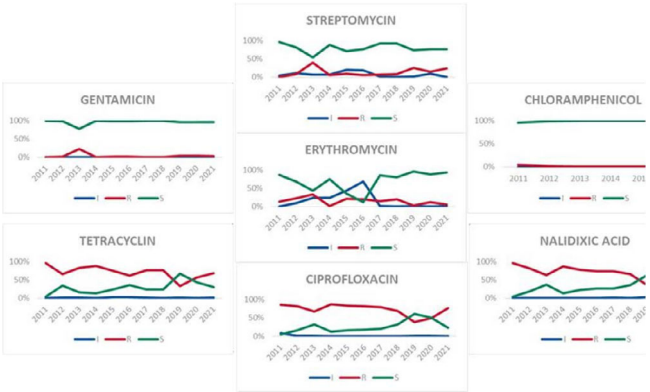
Total cases of each CC (GenPat platform Report)



Example of some CC per substrate of the sampling (GenPat platform output)



Resistance timeline per antibiotic during the decade



EU-FORA SERIES 4

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Risk Assessment of Food Contact Materials II

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Abstract

Food contact materials (FCMs) are materials and articles intended to be placed in direct or indirect contact with foodstuffs, or which can reasonably be expected to come into contact with food under normal or foreseeable conditions of use. Substances intentionally used to manufacture FCMs, as well as non-intentionally added substances resulting from impurities, by-products and/or degradation products, can migrate from FMCs into food and, consequently, are taken up by humans. To protect consumers' health, EU legislation requires that FCMs must be sufficiently inert to prevent substances from being transferred into the food in quantities that could endanger human health. At the German Federal Institute for Risk Assessment (BfR), Unit 74 'Safety of Food Contact Materials' deals with the risk assessment of FCMs and provides recommendations on the use of substances for the production of FCMs for which no specific European measures exist yet (e.g. silicone, rubber, paper and board). The BfR 'Recommendations on Food Contact Materials' are not legally binding; however, they represent the current state of the scientific and technical knowledge for the conditions under which these materials meet the requirements for consumer safety. As part of the EU-FORA programme, the fellow was involved in the risk assessment tasks and projects undertaken by Unit 74, which include: (i) the scientific evaluation of analytical and toxicological data from dossiers for adding new substances to the database 'BfR Recommendations on Food Contact Materials'; (ii) the hazard assessment of cyclic volatile methylsiloxanes (cVMS) migrating from silicone FCMs into foodstuff; and (iii) *in vitro* metabolic stability study of cyclic methylsiloxanes in the presence of S9 fraction, performed in the BfR laboratories. Moreover, the EU-FORA fellowship was a great opportunity for the fellow to build a strong network of food safety experts and to be part of an international community of risk assessment professionals.

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Keywords: chemical risk assessment, food contact materials, FCM, BfR recommendations, toxicology, migration

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Table of contents

1. Introduction

Risk analysis is a process consisting of three components: risk assessment, risk management and risk communication (CAC, 2015). The first component, risk assessment, is the scientific foundation of risk analysis, intended to estimate the risk to a given target organism, system or (sub)population, including the identification of attendant uncertainties, following exposure to a particular agent, taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system (IPCS, 2004). The risk assessment (RA) process begins with problem formulation and includes four additional steps: (i) hazard identification; (ii) hazard characterisation; (iii) exposure assessment; and (iv) risk characterisation (WHO, 2021). To develop the next generation of European food risk assessors and to build a common culture for RA, the European Food Safety Authority (EFSA) created the European Food Risk Assessment Fellowship (EU-FORA) Programme. Within the scope of the EU-FORA programme, the goal of this fellowship is to gain insight into the RA of Food Contact Materials (FCMs), in order to protect consumers from health risks associated with exposure to migrating chemicals from FCMs into food. An FCM is any material or article intended to be placed in direct or indirect contact with foodstuffs, or which can reasonably be expected to come into contact with food under normal or foreseeable conditions of use. To ensure food safety, FCMs must be sufficiently inert to prevent substances from being transferred into the food in quantities large enough to endanger human health or to bring about an unacceptable change in the composition of the food or a deterioration in its organoleptic properties, as laid down in Article 3 of the European Framework Regulation (EC) No 1935/2004 on materials and articles intended to come into contact with food (European Commission, 2004). Despite the enforcement of safety requirements, several food safety crises have been associated with FCMs. In 2005, Italian authorities withdrew 30 million litres of infant milk from the market due to high level of 2-isopropylthioxanthone (ITX), a photoinitiator used in UV-inks (Morlock and Schwack, 2006). In 2009, another photoinitiator, 4-methylbenzophenone, was notified by German and Belgian authorities due to its migration from food packaging into cereal products (EFSA, 2009). Over the last years, several chemicals used in FCM applications have been demonstrated to pose a health risk if consumers are exposed to those substances above safety levels. For instance, certain primary aromatic amines (PAAs) were shown to possess genotoxic and carcinogenic properties. Food contamination with PAAs can originate from printing azo-dyes, azo-pigments, isocyanate-based adhesives, monomers present in plastics and printed or recycled paper used for food packaging (Trier et al., 2010; Campanella et al., 2015; Yavuz et al., 2016). Some substances in the group of phthalates and perfluoroalkyl and polyfluoroalkyl substances (PFAS), as well as bisphenol A (BPA), have been classified as endocrine-disrupting chemicals (EDCs), exogenous substances or mixtures that alter functions of the endocrine system and consequently cause adverse effects in an intact organism, or its progeny, or (sub) populations (Zoeller et al., 2012). With more than 12,000 intentionally added substances (IAS) that could be possibly used to make FCMs worldwide (Groh et al., 2021) and the potential formation of non-intentionally added substances (NIAS), such as impurities, by-products, side reaction products and degradation products, FCMs can be a significant source of chemical food contamination (Grob et al., 2006). It does not necessarily imply that food contamination with substances migrating from FCMs leads to adverse health effects in humans. Nevertheless, the potential health risks resulting from the exposure to migrating non-evaluated chemicals, or due to improper conditions of use of an evaluated/authorised substance (e.g. level used, time, temperature, food types) or manufacturing process (e.g. curing), need to be assessed. At the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung – BfR), Unit 74 'Safety of Food Contact Materials' has the task to assess the nature and likelihood of harms resulting from human exposure to chemicals used in FCMs. The chemical/analytical and toxicological data provided by applicants in course of the inclusion of new substances into the BfR recommendations on food contact materials or data gathered from the literature and/or authoritative sources serve as the basis for the FCM risk assessment. Finally, the BfR publishes the results in the form of statements and publications.

2. Description of the work programme

2.1. Aims

The aim of the work programme was to gain insight into the RA of FCMs performed at the BfR, in accordance with the EFSA guidelines. In particular, the fellow acquired hands-on experience in the evaluation of analytical and toxicological (*in vitro* and *in vivo*) data for the inclusion of new substances

into the database 'BfR Recommendations on Food Contact Materials' (https://bfr.ble.de/kse/faces/DBEmpfehlung_en.jsp). He gained relevant knowledge of risk assessment tools, such as the PROAST software for Benchmark Dose Modelling (BMD), the FoodEx2 database and *in silico* toxicology tools (OECD Toolbox, Toxtree). In addition, part of the work programme included practical experience in the German national reference laboratory for materials in contact with food (NRL-FCM).

2.2. Activities/Methods

In order to achieve the training objectives, the fellow was involved in the ongoing risk assessment tasks undertaken by the BfR Unit 74, providing his contribution to the risk assessment issues. The following activities and projects were carried out:

i) Scientific evaluation of two dossiers for the inclusion of new substances in the 'BfR Recommendations on Food Contact Materials'. Due to the confidentiality concerning the dossiers, the data will not be disclosed.
ii) Hazard assessment of cyclic volatile methylsiloxanes (cVMS) that migrate from silicone FCMs into foodstuff.
iii) *In vitro* metabolic stability study of cyclic methylsiloxanes using S9 fraction.

2.2.1. Data evaluation in the context of an application of new substances into the 'BfR Recommendations on Food Contact Materials'

In the absence of specific European or national regulation for certain material groups, the BfR provides recommendations for the safe use of substances for the production of FCMs through the publicly available 'BfR Recommendations on Food Contact Materials'. The goal of these recommendations is to ensure that FCMs do not release substances into foods in quantities that could cause a health risk for consumers. Of the 17 material types of FCMs listed in Annex I of Regulation (EC) No 1935/2004, only four are currently covered by EU legislation on specific materials: (i) plastic materials and recycled plastic materials, (ii) active and intelligent materials, (iii) ceramics, (iv) regenerated cellulose film. Consequently, the BfR deals with those materials that are not subject to any harmonised EU regulation, such as silicones, natural and synthetic rubbers, papers, cartons and cardboards. It should be noted that BfR recommendations on plastic materials also exist, but are restricted only to components of catalytic systems and polymerisation auxiliaries, which are not yet accounted by the respective EU regulation. In addition, some BfR recommendations deal with a quite narrow scope of application (or intended use) of these materials (e.g. artificial sausage skins, temperature-resistant coatings for cooking, paper and paperboard for baking purposes). Even though BfR recommendations are not legally binding, they represent the current state of the scientific and technical knowledge for the conditions under which not specifically regulated materials meet the requirements for consumer safety as laid down in the framework Regulation (EC) No 1935/2004. Consequently, materials and articles that come into contact with food are often required to be manufactured in accordance with these provisions. Nevertheless, FCMs shall always be manufactured in compliance with good manufacturing practices stated in Regulation (EC) No 2023/2006 (European Commission, 2006). In order to include a new substance into the BfR 'Recommendations on Food Contact Materials', an application must be submitted to BfR. The dossier must follow the guidelines laid down in 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). The manufacturer has to supply information on the identity of the substance, data on chemistry and technology, conditions of use, migration into food (including the analytical methods used), data on substance's residual content in the FCM, antimicrobial properties (if antimicrobial substances are incorporated into FCMs) and toxicological data. In order to perform a risk assessment for migrating chemicals, both the toxicological and exposure data need to be combined. Since the generation of toxicological data is very resource and time consuming, a tiered approach is used for necessary data. Based on data from migration studies performed into food (simulants), a different amount of toxicological information must be provided as a minimum requirement. As a general principle, the greater the exposure through migration, the more toxicological information will be required. In case of:

a) High migration (i.e. > 5 mg/kg food), a full data set is needed, which comprises:
   – At least two in vitro genotoxicity tests, in line with the testing strategies of the EFSA Scientific Committee recommendations on genotoxicity testing strategies applicable to food and feed safety assessment:

i) A bacterial reverse mutation test.
ii) An *in vitro* mammalian cell micronucleus test.

- A 90-day oral toxicity study.
- Studies on absorption, distribution, metabolism and excretion.
- Studies on reproduction and developmental toxicity.
- Studies on long-term toxicity/carcinogenicity.

b) Migration between 0.05 and 5 mg/kg food, the following data are necessary (limited data set):

- At least two genotoxicity tests as indicated above.
- A 90-day oral toxicity study.
- Data to demonstrate the absence of bioaccumulation in human.

c) In case of low migration (i.e. < 0.05 mg/kg food), only two genotoxicity tests, at least, are needed.

Once the application is submitted, Unit 74 'Safety of Food Contact Materials' checks the compliance of the dossier with the requirements and assesses the scientific information with particular regard to possible consumer exposure. Studies on genotoxicity and, if necessary, on toxic effects after repeated dose (carcinogenicity, reproductive toxicity, neuro toxicity, immune toxicity and endocrine disruptor properties) are taken into account, along with findings on absorption, distribution, metabolism and excretion (ADME). In this context, the fellow worked on the evaluation of genotoxicity and subchronic toxicity studies of two substances applied for inclusion into the BfR recommendations. Afterwards, the evaluated dossiers were further discussed in the 'Toxicology' and 'Applications' subcommittee groups of the BfR Committee for Consumer Products (BeKo), staffed with external experts, which advise the BfR on the toxicological evaluation of the applied substances.

2.2.2. Hazard assessment of cyclic volatile methylsiloxanes (cVMS)

Owing to their elasticity, non-sticky surface, heat resistance and affordable price, silicone bakeware products are widely used in both industrial and consumer applications as alternative to metal or single-use paper bakeware. Silicone bakeware is often made of silicone elastomers, a rubber-like material obtained from fluid siloxanes by formation of cross-links between linear polymers during vulcanisation. However, unreacted cyclic volatile methylsiloxanes (cVMS), used in the starting materials or resulting from side reactions during the polymerisation process, can still be present in the final product and potentially migrate into foodstuff (Helling et al., 2012). cVMS (examples, see Table 1 and Figure 1) are man-made chemicals and consist of [(CH3)2SiO]n units arranged as cyclic structures. The Si–O atoms are singly bonded to form a ring and generally expressed as Dn, with n = number of Si atoms in the ring. In a recent opinion, ECHA listed three cVMS, namely octamethylcyclotetrasiloxane (D4), decamethylcyclo-pentasiloxane (D5) and dodecamethylcyclohexasiloxane (D6), in the 'Candidate List of Substances of Very High Concern' (SVHC list) for authorisation, according to the Article 57 of the Regulation (EC) No 1907/2006 (REACH Regulation), due to their properties as persistent, bioaccumulative and toxic (PBT) and very persistent and very bioaccumulative (vPvB). In addition, D4 is classified as toxic to reproduction (cat. 2) according to Regulation (EC) No 1272/2008 (CLP Regulation). Over the past decades, several scientific publications demonstrated that cVMS could migrate from silicone FCMs into food and food simulants, raising some concerns on potential adverse health effects resulting from the oral intake of cVMS (Meuwly et al., 2007; Helling et al., 2009; Fromme et al., 2019; Liu et al., 2021). In February 2021, a German official food control laboratory tested various silicone bakeware products for the potential release of D3, D4, D5, D6 and D7 (Table 1) into food simulants. For some of the tested silicone FCMs, migration into vegetable oil (food simulant D2) and poly(2,6-diphenyl-p-phenylene oxide (MPPO)) (food simulant E) exceeded 5 mg/kg food significantly. At the present, a comprehensive risk assessment on cVMS migrating from FCMs into food (simulants) does not exist yet. Due to the high uncertainties in the human exposure estimation, such as the occurrence of cVMS during repeated use, and the transferability of the migration data in food simulants to real food, a preliminary hazard assessment was performed to evaluate the nature of the potential adverse health effects associated with the oral intake of cVMS. First, a comprehensive literature review was carried out. Regulatory and authoritative reviews, together with peer-reviewed key publications, were consulted to identify potential critical endpoints relevant for human health. Once the critical endpoints were identified, the benchmark dose (BMD) approach was applied to establish a

point of departure (PoD). The BMD is a dose level, estimated from a fitted dose-response curve, associated with a specified change in response, the benchmark response (BMR) (EFSA Scientific Committee, 2017). The tool PROAST was used to calculate the BMD levels and the respective lower confidence bound (BMDL) and upper confidence bound (BMDU). The BMDL of the selected critical endpoint was used as PoD. Since the majority of the studies available were based on inhalation exposure studies, inhalation to oral extrapolation of the BMDL was conducted by applying a default physiological parameter, according to the ECHA 'Guidance on information requirements and chemical safety assessment' (ECHA, 2012). Differences between oral and inhalation uptake observed in absorption studies in animals were taken into account. The extrapolated oral BMDL was used to calculate a temporary tolerable daily intake (tTDI). Due to missing toxicological data, a read-across approach was applied to include higher molecular weight cyclic methylsiloxanes within the derived tTDI. From the tTDI, the acceptable migration into food was estimated, assuming a body weight (bw) of 60 kg and food consumption of 1 kg food/day. Based on the assessment studies performed, a tTDI was established for a group of cyclic methylsiloxanes, in order to set the basis for future risk assessment.

Table 1: Name and characteristics of five cyclic volatile methylsiloxanes

Name	Abbreviation	CASNR	Molecular formula	Molecular weight
Hexamethylcyclotrisiloxane	D3	541-05-9	C_6-H_{18}-O_3-Si_3	222.46
Octamethylcyclotetrasiloxane	D4	556-67-2	C_8-H_{24}-O_4-Si_4	296.64
Decamethylcyclopentasiloxane	D5	541-02-6	C_{10}-H_{30}-O_5-Si_5	370.80
Dodecamethylcyclohexasiloxane	D6	540-97-6	C_{12}-H_{36}-O_6-Si_6	444.93
Tetradecamethylcycloheptasiloxane	D7	107-50-6	C_{14}-H_{42}-O_7-Si_7	519.07

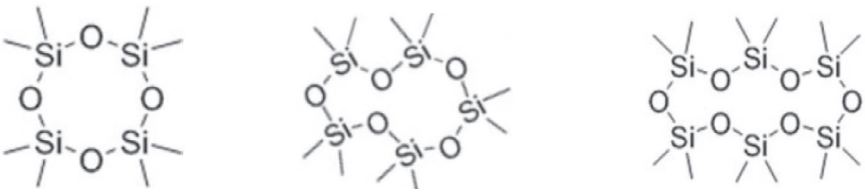

Figure 1: Chemical structures of D4, D5 and D6

2.2.3. *In vitro* metabolic stability study of cyclic methylsiloxanes using S9 fraction

In literature, *in vivo* metabolism of D4 and D5 was elucidated. According to Franzen et al. (2017) and Varaprath et al. (1999), the metabolite profiles reported in blood, tissues and excreta of rats following exposure to D4 suggest that D4 is initially oxidised to a hydroxylated derivative, presumable by cytochrome P450. The initial metabolite appears to rearrange and further hydrolysis leads to the formation of short-chain linear siloxanes, which are excreted via urine (Figure 2). The same metabolic pathway was proposed for the D5 (Dekant and Klaunig, 2016). However, beside D4 and D5, no data are available for higher molecular weight cyclic methylsiloxanes. The aim of this study was to develop a working protocol for the investigation of the metabolic stability *in vitro* of cyclic methylsiloxanes, in the presence of S9 fraction. The ability of cytochromes P450 (CYPs) enzymes to bind and metabolise higher molecular weight cyclic methylsiloxanes (e.g. Dn, $n \geq 6$) should be investigated, and possible metabolites should be identified. Due to the lipophilic properties of the cyclic methylsiloxanes (e.g. D4 logPoW = 6.98), several solubility tests were performed, taking into consideration the solvent compatibility with the S9-mix. The cyclic methylsiloxanes were incubated with S9-mix extracted from rat liver for 4 h at 37°C. The samples were extracted with a suitable organic solvent at the beginning of the experiment (t = 0 h) and after incubation at 37°C for 4 h (t = 4 h) in order to investigate if any decrease in cyclic methylsiloxane or increase in metabolites concentration occurs during the incubation period. The extracts were analysed by LC-GC coupled online to a triple quadrupole mass spectrometer for quantification of siloxanes in selected ion mode. All samples were prepared in duplicate, with and without S9-mix.

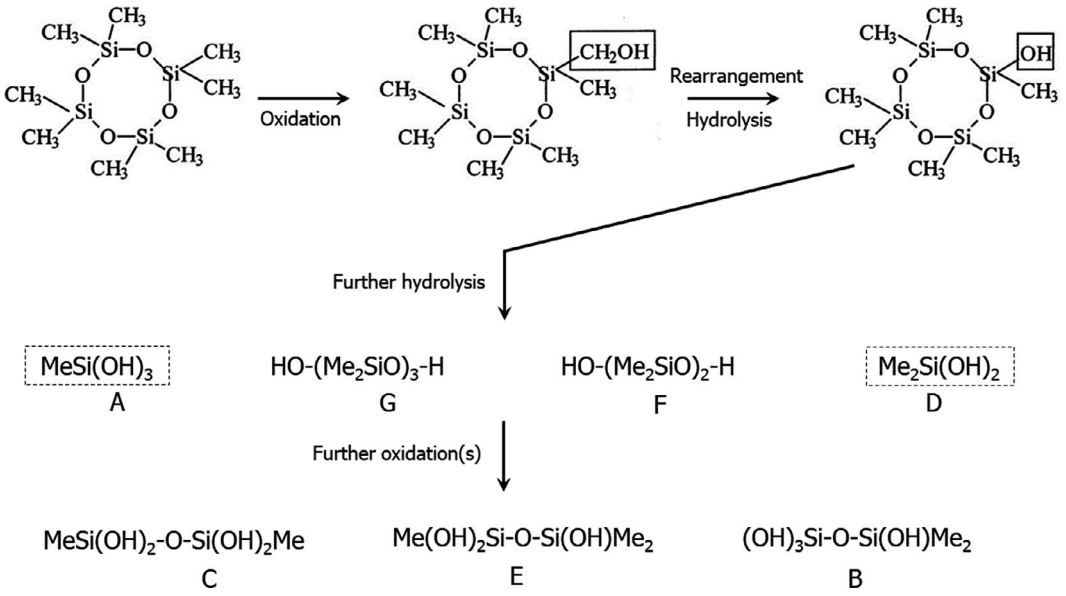

Figure 2: Adapted from Franzen et al. (2017) – Possible pathways for formation of D4 metabolites. A and D were considered major metabolites. Metabolites C, E and B can be produced by multiple pathways (Varaprath et al., 1999)

3. Conclusion

The EU-FORA programme allowed the fellow to gain deeper insight into risk assessment of food contact materials and to acquire relevant knowledge of different risk assessment tools. At the Bundesinstitut für Risikobewertung, the fellow worked side-by-side with the experts of Unit 74 'Safety of Food Contact Materials' on the scientific evaluation of two dossiers for the inclusion of new substances in the 'BfR Recommendations on Food Contact Materials', including the communication with the applicants concerning the occurring scientific questions. In a separate project, the fellow undertook the first steps to a risk assessment of cVMS migrating from silicone FCMs into food. Based on toxicological studies published in the literature or evaluated by other competent authorities, a hazard assessment for cVMS was performed, leading to a temporary tolerable daily intake value for these substances. In addition, in course of a laboratory work project in the German national reference laboratory for food contact materials, the fellow worked on the set-up of a test protocol for the *in vitro* metabolism study on cyclic methylsiloxanes. Moreover, beside the scientific knowledge, the EU-FORA fellowship was a great opportunity for the fellow to build a strong scientific network, to meet international experts and fellows and to be part of an international community of risk assessment professionals.

4. Disclaimer

The results of the hazard assessment of cVMS are intended to be published in a peer-reviewed journal. In order to avoid copyright claims, they were not included in the technical report.

References

CAC (Codex Alimentarius Commission), 2015. Procedural Manual. 24th edition. Joint FAO/WHO Food Standards Programme. Available online: https://www.fao.org/3/i5079e/i5079e.pdf

Campanella G, Ghaani M, Quetti G and Farris S, 2015. On the origin of primary aromatic amines in food packaging materials. Trends in Food Science & Technology, 46, 137–143. https://doi.org/10.1016/j.tifs.2015.09.002

Dekant W and Klaunig JE, 2016. Toxicology of decamethylcyclopentasiloxane (D5). Regulatory Toxicology and Pharmacology, 74, S67–S76. https://doi.org/10.1016/j.yrtph.2015.06.011

ECHA (European Chemicals Agency), 2012. Guidance on information requirements and chemical safety assessment Chapter R.8: Characterisation of dose [concentration]-response for human health. Available online: https://echa.europa.eu/documents/10162/17224/information_requirements_r8_en.pdf/e153243a-03f0-44c5-8808-88af66223258?t=1353935239897

EFSA (European Food Safety Authority), 2009. EFSA statement on the presence of 4-methylbenzophenone found in breakfast cereals. EFSA Journal 2009;7(3):243r, 45 pp. https://doi.org/10.2903/j.efsa.2009.243r

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2008. 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 Journal 2008;6(7):21r, 41 pp. https://doi.org/10.2903/j.efsa.2008.21r

EFSA Scientific Committee, Hardy A, Benford D, Halldorsson T, Jeger MJ, Knutsen KH, More S, Mortensen A, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Silano V, Solecki R, Turck D, Aerts M, Bodin L, Davis A, Edler L, Gundert-Remy U, Sand S, Slob W, Bottex B, Abrahantes JC, Marques DC, Kass G and Schlatter JR, 2017. Update: Guidance on the use of the benchmark dose approach in risk assessment. EFSA Journal 2017;15 (1):4658, 41 pp. https://doi.org/10.2903/j.efsa.2017.4658

European Commission, 2004. Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC. Available online: https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:32004R1935

European Commission, 2006. Commission Regulation (EC) No 2023/2006 of 22 December 2006 on good manufacturing practice for materials and articles intended to come into contact with food. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02006R2023-20080417

Franzen A, Greene T, Van Landingham C and Gentry R, 2017. Toxicology of octamethylcyclotetrasiloxane (D4). Toxicology Letters, 279, 2–22. https://doi.org/10.1016/j.toxlet.2017.06.007

Fromme H, Witte M, Fembacher L, Gruber L, Hagl T, Smolic S, Schober W, 2019. Siloxane in baking moulds, emission to indoor air and migration to food during baking with an electric oven. Environment International, 126, 145–152. https://doi.org/10.1016/j.envint.2019.01.081

Grob K, Biedermann M, Scherbaum E, Roth M and Rieger K, 2006. Food contamination with organic materials in perspective: packaging materials as the largest and least controlled source? A view focusing on the European situation. Critical Reviews in Food Science and Nutrition, 46, 529–535. https://doi.org/10.1080/10408390500295490

Groh KJ, Geueke B, Martin O, Maffini M and Muncke J, 2021. Overview of intentionally used food contact chemicals and their hazards. Environment International, 150, 106225. https://doi.org/10.1016/j.envint.2020.106225

Helling R, Mieth A, Altmann S and Simat TJ, 2009. Determination of the overall migration from silicone baking moulds into simulants and food using 1H-NMR techniques. Food Additives & Contaminants: Part A, 26, 395–407. https://doi.org/10.1080/02652030802520852

Helling R, Seifried P, Fritzsche D and Simat TJ, 2012. Characterisation and migration properties of silicone materials during typical long-term commercial and household use applications: a combined case study. Food Additives & Contaminants: Part A, 29:9, 1489–1500. https://doi.org/10.1080/19440049.2012.694374

IPCS (International Programme on Chemical Safety), 2004. IPCS risk assessment terminology. World Health Organization. Available online: https://apps.who.int/iris/handle/10665/42908

Liu Y-Q, Wrona M, Su Q-Z, Vera P, Nerín C and Hu C-Y, 2021. Influence of cooking conditions on the migration of silicone oligomers from silicone rubber baking molds to food simulants. Food Chemistry, 347, 128964. https://doi.org/10.1016/j.foodchem.2020.128964

Meuwly RD, Sager F, Brunner K and Dudler V, 2007. Migration of siloxane oligomers in foodstuffs from silicone baking moulds. Deutsche Lebensmittel-rundschau, 103, 561–568.

Morlock G and Schwack W, 2006. Determination of isopropylthioxanthone (ITX) in milk, yoghurt and fat by HPTLC-FLD. HPTLC-ESI/MS and HPTLC-DART/MS. Anal Bioanal Chem, 385, 586–595. https://doi.org/10.1007/s00216-006-0430-5

Trier X, Okholm B, Foverskov A, Binderup ML and Petersen JH, 2010. Primary aromatic amines (PAAs) in black nylon and other food-contact materials, 2004–2009. Food Additives & Contaminants: Part A, 27, 1325–1335. https://doi.org/10.1080/19440049.2010.487500

Varaprath S, Salyers KL, Plotzke KP and Nanavati S, 1999. Identification of metabolites of octamethylcyclotetrasiloxane (D4) in rat urine. Drug Metabolism and Disposition, 27, 1267–1273.

WHO (World Health Organization & Food and Agriculture Organization of the United Nations), 2021. Human health risk assessment toolkit: chemical hazards, 2nd Edition. Geneva: World. Health Organization; (IPCS harmonization project document, no. 8).

Yavuz O, Valzacchi S, Hoekstra E and Simoneau C, 2016. Determination of primary aromatic amines in cold water extract of coloured paper napkin samples by liquid chromatography-tandem mass spectrometry. Food Additives & Contaminants: Part A, 33, 1072–1079. https://doi.org/10.1080/19440049.2016.1184493

Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, Vom Saal FS, 2012. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. Endocrinology, 153, 4097–4110. https://doi.org/10.1210/en.2012-1422

Abbreviations

ADME	absorption, distribution, metabolism and excretion
BeKo	BfR Committee for Consumer Products
BfR	Bundesintitut Für Risikoberwertung
BMD	Benchmark Dose Modelling
cVMS	Cyclic Volatile Methylsiloxanes
EU-FOR A	European Food Risk Assessment Fellowship Programme
FCMs	Food Contact Materials
NRL-FCM	German National Reference Laboratory for Materials in Contact with Food
PoD	Point of Departure
RA	Risk Assessment

Appendix A – Training activities

Event	Title	Contribution	Location	Date
Webinar	Food Packaging Forum: Addressing endocrine disrupting chemicals (EDCs) and mixture	Attendance	Online	15.4.2021
Meeting	Toxicological subcommittee meeting BfR unit 74	Oral presentation	BfR	20.4.2021
Conference	Genetic Toxicology Association (GTA) Annual Meeting	Attendance	Online	3–6.05.2021
Webinar	Food Packaging Forum: Responding to hazardous chemicals in FCMs: substitution and simplification	Attendance	Online	13.5.2021
Conference	International Akademie Fresenius Online Conference "Residues of Food Contact Materials in Food"	Attendance	Online	24–26.6.2021
Summer School	BfR-Summer Academy 2021: Lecture Series	Attendance	BfR/Online	16–20.8.2021
Summer School	Parma Summer School 2021	Attendance	Online	28–30.9.2021
Meeting	Toxicological subcommittee meeting BfR unit 74	Oral presentation	BfR	9.11.2021
Webinar	Evidence-based Toxicology Collaboration (EBTC) 10th Anniversary Celebration	Attendance	Online	11.11.2021
Webinar	Food Packaging Forum: Is current phthalate regulation fit for purpose?	Attendance	Online	19.11.2021

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Emergent marine toxins risk assessment using molecular and chemical approaches

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Abstract

Cyanobacteria harmful blooms represent a deviation to the normal equilibrium in planktonic communities involving a rapid and uncontrolled growth. Owing to the capacity to produce toxins as secondary metabolites, cyanobacteria may cause huge economic losses in the fishing and aquaculture industries and poisoning incidents to humans due to their accumulation in the food chain. The conditions which promote toxic blooms have not yet been fully understood, but climate change and anthropogenic intervention are pointed as significant factors. For the detection of toxins in edible marine organisms, the establishment of international regulations and compulsory surveillance has been probed as exceptionally effective. However, not regulation nor monitoring have been settled concerning emergent marine toxins. In the light of this scenario, it becomes essential to apply fast and reliable surveillance methodologies for the early detection of cyanobacterial blooms as well as the occurrence of emergent marine toxins. Shotgun metagenomic sequencing has potential to become a powerful diagnostic tool in the fields of food safety and One Health surveillance. This culture-independent approach overcomes limitations of traditional microbiological techniques; it allows a quick and accurate assessment of a complex microbial community, including quantitative identification and functional characterisation, in a single experiment. In the framework of the EU-FORA fellowship, with the final goal of evaluate metagenomics as a promising risk assessment tool, the fellow worked on the development of an innovative workflow through state-of-the-art molecular and chemical analytical procedures. This work programme aims to evaluate the occurrence of emergent marine toxins and the producing organisms in Cabo Verde coastal cyanobacteria blooms. Our results show the outstanding potential of a holistic metagenomic approach for the risk assessment of emergent marine toxins and the producing organisms. Additionally, we have also highlighted its value for the identification and evaluation of secondary metabolites as natural bioactive compounds with biotechnological and industrial interest.

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Keywords: metagenomics, shotgun sequencing, bioinformatics, emergent marine toxins, cyanobacteria, harmful algal blooms, surveillance and risk assessment

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1. Introduction

Cyanobacteria, also known as blue-green algae, are a diverse group of Gram-negative photosynthetic prokaryotes with a great ecological importance. They colonise a wide heterogenous range of ecosystems and are a major component of the phytoplankton (Moreira et al., 2013). Their ability to produce toxins as secondary metabolites may lead to the development of harmful blooms causing huge economic losses in the fishing and aquaculture industries and poisoning incidents to humans due to their accumulation in the food-chain (Anderson et al., 2002). Nevertheless, cyanobacteria have also attracted an increasing interest owing to the production of a large number of natural bioactive compounds (e.g. alkaloids, non-ribosomal peptides, polyketides) with promising biotechnological and biomedical applications (Leão et al., 2012).

Harmful algal blooms (HABs) are unpredictable due to their erratic nature. Episodes of human poisoning caused by HABs have been commonly recorded in the last century mostly because of the lack of regulated monitoring programs. The establishment of international regulations and compulsory surveillance led to the detection of these toxins in edible marine organisms and today the cases of human poisonings in Europe are sporadic, usually because of violated health authorities' regulations imposing the closure of harvesting areas and seafood commercialisation (Regulation (EC) No 853/ 2004; 854/2004; 15/2011, 786/2013).

However, the situation is different when we refer to emergent marine toxins (e.g. ciguatera fish poisoning, cyclic imines, pufferfish poisoning, neurotoxic sellfish poisoning), which are not yet regulated nor monitored regularly in Europe. Climate change combined with human intervention in the ecosystem aid the migration and establishment of new toxic species typical from warmer waters into more temperate areas such as the European Union (EU), where they have been already reported (Otero et al., 2010; García-Altares et al., 2014). This fact highlights the essential need of a European surveillance plan to monitor and track these emergent toxins and toxin-producing organisms in marine ecosystems. A systematic compilation of data will provide the input needed to perform knowledge-based risk assessment which allow the regulatory authorities stablish measures to protect the consumers and advise the fish production sector.

At this regard, the present work programme aims to develop an innovative holistic approach to sample and analyse emergent marine toxins and their producing organisms both on marine ecosystems and food matrixes. With its power to comprehensively detect and analyse entire microbial communities in a single experiment, metagenomics-based methods stand out in addressing this need (Josić et al., 2017; Campos et al., 2020). This culture-independent methodology has the potential to overcomes limitations of the classical microbiology techniques which are time-consuming and target only specific subsets of microbes. Therefore, shotgun metagenomic sequencing and the subsequent computational analysis of the sequences represent a powerful tool capable of provide an exhaustive quantitative picture of the genetic and metabolic diversity encoded in these complex samples (Sharpton, 2014). Additionally, it has the potential to open access to untapped genetic resources for the screening and identification of genes encoding new toxins and bioactive compounds (Quince et al., 2017).

Cabo Verde is a diverse group of volcanic islands located in the Tropical Eastern Atlantic, in the Sahel region of Africa. The environmental characteristics of this archipelago provide niche conditions for a wide variety of cyanobacteria. Due to the scarce information existing at the date in what concerns cyanobacteria, their toxins and natural products, these underexplored habitats are an interesting and promising location for the development of this work programme (Semedo-Aguiar et al., 2018). Environmental samples have been collected and analysed following an integrated metagenomic approach which allows to assess the taxonomic biodiversity of the samples and the presence of genes of interest (including cyanotoxins and secondary metabolites with biotechnological potential). The final objective was obtaining results that will allow gathering new data on the distribution of emergent marine toxins in the region, detect new vectors, new toxin producing species and map the current state of this problem, essential steps all of them for a risk assessment analysis which study the potential food-chain implications of these cyanobacteria.

2. Description of work programme

The work programme proposed by CIIMAR to the EU-FORA fellow was framing in the context of the project EMERTOX, funded by EU Horizon 2020 Framework Research Programme. This project focus on the study of emergent marine toxins and the producing organisms in the North Atlantic and Mediterranean, with the aim of mapping the actual situation, developing new approaches to assess

their occurrence and predicting the possible future scenarios based on molecular data (routes of dispersion) and modelling in the framework of global environmental changes.

Regarding the inexistent legislation on emergent marine toxins and the producing organisms, the implementation of this project and development of a global surveillance and monitoring system, may provide insights into the mechanisms of emerging and spread of marine toxins and help identify hotspots and novel vectors. Thus, food risk assessment and knowledge-based interventions could be developed to reduce the spread and dispersal of marine toxins throughout the food chain.

With the objective of contributing to the data gathering and innovation of monitoring tools, the fellow worked on the development of an innovative workflow through state-of-the-art molecular and chemical analytical procedures.

2.1. Aims

The aims of the work programme were:

i) to broad the proficiency of the fellow with the food and feed safety risk assessment and surveillance methodologies, including hazard identification, hazard characterisation, exposure assessment and risk characterisation.
ii) to gain first-hand experience in the execution of qualitative and quantitative risk assessment and epidemiological surveillance based on molecular and chemical approaches in relation to emergent marine toxins and the producing organisms.
iii) to develop and validate a novel workflow (including collection, normalisation and analysis of data) for the identification and characterisation of emergent marine toxins and their producing organisms in environmental samples based on the potential of next-generation sequencing (NGS), shotgun metagenomic sequencing and computational analysis for One Health surveillance and food safety risk assessment.
iv) to contribute to the raise of awareness across Europe regarding emerging marine toxins and their producing organisms in the food chain.

2.2. Activities and methodology

The activities and methodology described below were in line with the aims of the work programme proposed by CIIMAR, rooted in the improvement of systematic collection and evaluation of data to facilitate the food risk assessment of emergent marine toxins and their producing organisms. As study model, the research project is dedicated to examine the biodiversity, toxicity and biotechnological potential of cyanobacteria from Cabo Verde islands following a molecular approximation relied on shotgun metagenomic sequencing and computational bioinformatic analysis.

2.2.1. Sampling, DNA extraction and shotgun metagenomic sequencing

Sampling points were stablished at specific locations in São Vicente and Santo Antão islands (Cabo Verde archipelago). Two millilitres of samples of cyanobacterial mat growing on sediment were collected in each sampling point using sterile plastic syringes. Care was taken to target only the mat, with sediment found embedded in the mat matrix. For preservation, cyanobacterial mat samples were snap-frozen in tubes containing an aqueous, nontoxic reagent that rapidly permeates tissue to stabilise and protect molecular integrity (RNA*later*, Thermo Fisher Scientific). Upon arrival to the laboratory, samples were stored at −80°C until processing.

A total of 10 metagenomic samples from Cabo Verde were studied during this work programme. After checking different approximations and optimisation of the DNA extraction procedure, a spin column silica-based methodology which requires no phenol or chloroform extraction was selected. DNA was extracted from 200 mg wet weight of each homogenised sample, using the NZY Plant/Fungi gDNA Isolation Kit (NZYtech) according to manufacturer's instructions, including a first step of cell lysis and RNA removal, followed by a clarification step of the crude lysate and the final genomic DNA (gDNA) purification.

The extracted gDNA quality was evaluated by agarose gel electrophoresis (1% agarose, 150V, 40min), NanoDrop Spectrophotometer and Qubit Fluorometer (Qubit dsDNA BR Assay Kit, Thermo Fisher Scientific) to determine DNA integrity, concentration and potential contaminations.

Shotgun sequencing was performed on a DNBseq™ sequencing platform by BGI (Beijing Genomics Institute). This system is powered by combinatorial Probe-Anchor Synthesis (cPAS), that combines linear isothermal Rolling-Circle Amplification (RCA) and DNA Nanoball (DNB) technology with stepwise

sequencing using DNA polymerase on patterned array flow cells, followed by fluorescent high-resolution digital imaging analysis (Goodwin et al., 2016; Natarajan et al., 2019). After gDNA fragmentation, library preparation and sequencing, short paired-end reads (150 bp) were generated with a deep of 5Gb (~ 40 million reads) per sample.

2.2.2. Computational data analysis: bioinformatics and statistics

Quality control

After sequencing, the raw reads were filtered to obtain clean reads. Data filtering includes removing adaptor sequences, contamination and low-quality reads as follow:

1) Filter adapter: delete the entire read if more than 25% match the adapter sequence.
2) Filter low-quality data: delete the entire read if there are more than 50% bases having a quality value lower than 20 and if the read is shorter than 150 bp.
3) Remove N: delete the entire read if there are more than 3% N in the read.
4) Filter out duplication.

Adapters were removed with the bioinformatic tool Cutadapt (version 3.5) (Martin, 2011). Reads were quality trimmed to a minimum quality score threshold of 20 and reads shorter than 150 bp were removed using Trimmomatic software (version 0.38) (Bolger et al., 2014). The final quality of the cleaned reads was assessed with the tool optimised for high throughput sequence data FastQC (version 0.11.9) (Andrews, n.d.).

Assembly-free taxonomic profiling

In order to determine the phylogenetic diversity of the microbial community present in each sample, the cleaned set of reads obtained after quality trimming were analysed under the pipeline MetaPhIAN (version 3.0.13) (Beghini et al., 2021). The minimum total nucleotide length for the markers in a clade for estimating the abundance without considering sub-clade abundance was set in 2000. The minimum mapping quality value (MAPQ) was set in 5 and the mapping was performed under the 'very sensitive' parameter with BowTie2, an ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences.

The taxonomically annotated reads allowed as to obtain an accurate estimation of organismal relative abundance (in terms of cells) at species-level resolution.

Assembly-free profiling of functional potential

With the aim of describing the metabolic potential of the microbial communities present in our samples, we utilised the tool HUMAnN2 (version 0.11.1) (Abubucker et al., 2012) with default parameters, a query coverage threshold for nucleotide alignments of 90 and using the UniRef90 cluster database. Paired-end reads were concatenated prior to their use in this pipeline because HUMAnN does not support paired end data. Gene families were clustered with the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database into functional orthologs.

We obtained the functional profiling and the abundance of genes and microbial pathways from our shotgun metagenomic sequencing data.

***De novo* assembly**

To improve the detection of toxins and secondary metabolites in our samples, the high-quality cleaned paired-end reads from each sample were undependably assembled *de novo* into contigs, following by scaffolding of these generated contigs, applying the pipeline metaSPAdes (version 3.15.3) (Nurk et al., 2017) with K-mer sizes of 33, 55, 77, 99 and 127. After assembly, only scaffolds exceeding 1 kb in length were retained for downstream analysis in order to avoid binning errors and ambiguous gene annotation from shorter contigs.

The assembled contiguous genome fragments were clustered into different linages (bins) using depth-of-coverage, nucleotide composition, and marker genes with the pipeline MaxBin2 (version 2.2.4) (Wu et al., 2016). The resulting draft Metagenome Assembled Genomes (MAGs) were quality-checked using QUAST (version 4.4) (Gurevich et al., 2013) to generate and verify assembly statistics, compare results from different workflows and preclude the inclusion of misassembles from our analysis.

MAGs taxonomic profiling and search for genes of interest

Taxonomic assignment to each draft MAG was done using GTDB-tk (version 1.7.0) (Chaumeil et al., 2020) and annotated using RASTtk (version 1.073) (Aziz et al., 2008; Overbeek et al., 2014; Brettin et al., 2015).

MAGs were additionally explored for the identification, annotation and analysis of secondary metabolite biosynthetic gene clusters (BGCs) using the antiSMASH web server pipeline (version 6.0.1) (Blin et al., 2021) under strict setting. To determine the genetic novelty of the BGCs identified, we performed homology searches against the NCBInt database using NCBI BLAST+.

3. Conclusions

3.1. Conclusions of the research study

Shotgun metagenomic sequencing has been scrutinised as a promising technology for food risk assessment and One Health surveillance. This methodology allows to explore and link the phylogenetic and metabolic complexity encoded in uncultured microbial communities from environmental, agricultural and clinical settings. While there are still technical limitations and challenges that need to be solved, such as the detection of low abundance microorganisms, metagenomics has the potential to address with precision manifold issues concerning the global food supply chain, including pathogen detection, monitoring of toxins and antimicrobial resistance determinants, detection of fraudulent products and quality control. Considering the active development and evolution of this field, together with the rapid establishment of WGS in the food and feed safety risk assessment during the recent few years, we envisage a near future where omics-based tools will become an indispensable part of the food safety surveillance and risk assessment.

Applying this state-of-the-art sequencing technology and computational bioinformatic analysis, we successfully determined the taxonomic biodiversity in our samples, the presence of genes of interest and explore the production of toxins and secondary metabolites with biotechnological potential. Our samples from Cabo Verde Islands exhibited a broad compositional biodiversity and interesting production of novel BGCs. Altogether, this study evidences the potential for the discovery of novel bioactive compounds with forthcoming biotechnological and biomedical implementations, but it also highlights the plausible risks of the appearance of HABs and new emergent marine toxins in the Cabo Verde coastal marine areas. Taking into account the food chain implications of these HABs and the toxins produced, our results will grant the evaluation of occurrence, toxicity and exposure to HABs toxins in order to accomplish an evidence-based risk assessment covering the food and feed chains.

3.2. Conclusions of the EU-FORA Fellowship experience

The fellow was hosted by the Blue Biotechnology and Ecotoxicology team at CIIMAR. Her integration on such a multidisciplinary research group has provided her a unique opportunity to interact and create synergies with scientific experts in the fields of Global Changes and Ecosystems Services, Marine Biotechnology and Biology, Aquaculture and Seafood Quality.

The proposed work programme was an excellent fit with the experience and expectations of the fellow. It has been an opportunity to consolidate and broaden her knowledge on microbiological and chemical risk assessment from a One Health perspective, enabling her to gain valuable first-hand experience on shotgun metagenomics analysis, bioinformatic tools and *in silico* evaluation at all stages of the food safety assessment.

Throughout the year, the fellow had the opportunity to participate in diverse scientific discussions and seminars. Additionally, she attended several complementary trainings to acquire further skills needed for the accurate performance of the research project: *Foundations of One Health* (University of Calgary), *Introduction to Bioinformatics* (University of Calgary) and *Metagenomics applied to surveillance of pathogens and antimicrobial resistance* (Technical University of Denmark).

The preliminary results of this project have been presented with a format of oral communication during the 19th International Conference on Harmful Algae (ICHA) in Mexico. A full description of the tools, methodology and results will be reported in a manuscript currently under preparation.

The work programme together with these supplementary activities, and the high-level set of trainings provided by the EU-FORA programme (covering in detail all the different areas of food safety risk assessment, risk management, risk and crisis communication), have expanded the fellow expertise

selecting and applying risk assessment methodologies, collecting and analysing relevant data, using computer models in risk assessment, and providing effective risk and crisis communication.

In short, both the fellow and the supervisor value positively the capacity building opportunity bring by the fellowship programme. We agree that EU-FORA provides an exceptional framework for the building of scientific networks. It is a win-win scenario for the development of cooperation, exchange of high-level knowledge and professional experiences with the final goal of achieve a major harmonisation of risk assessment methodologies and practices in the farm-to-folk chain across Europe.

4. Disclaimer

The results obtained during the research study are intended to be published in scientific peer review journals. In order to avoid copyright claims, they have not been included in the present technical report.

References

Abubucker S, Segata N, Goll J, Schubert AM, Izard J, Cantarel BL, Rodriguez-Mueller B, Zucker J, Thiagarajan M, Henrissat B, White O, Kelley ST, Methé B, Schloss PD, Gevers D, Mitreva M and Huttenhower C, 2012. Metabolic reconstruction for metagenomic data and its application to the human microbiome. PLoS Computational Biology, 8.

Anderson DM, Glibert PM and Burkholder JM, 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries, 25, 704–726.

Andrews S, (n.d.). FastQC A Quality Control tool for High Throughput Sequence Data. Available online: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A and Zagnitko O, 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics, 9.

Beghini F, McIver LJ, Blanco-Míguez A, Dubois L, Asnicar F, Maharjan S, Mailyan A, Manghi P, Scholz M, Thomas AM, Valles-Colomer M, Weingart G, Zhang Y, Zolfo M, Huttenhower C, Franzosa EA and Segata N, 2021. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3, Elife, 10.

Blin K, Shaw S, Kloosterman AM, Charlop-Powers Z, Weezel GP, Medema MH and Weber T, 2021. antiSMASH 6.0: improving cluster detection and comparison capabilities. Nucleic Acids Research, 49, 29–35.

Bolger AM, Lohse M and Usadel B, 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics, 30, 2114–2120.

Brettin T, Davis JJ, Disz T, Edwards RA, Gerdes S, Olsen GJ, Olson R, Overbeek R, Parrello B, Pusch GD, Shukla M, Thomason JA, Stevens R, Vonstein V, Wattam AR and Xia F, 2015. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Scientific Reports, 5.

Campos A, Freitas M, Almeida AM, Martins JC, Domínguez-Pérez D, Osório H, Vasconcelos V and Costa PR, 2020. OMICs approaches in diarrhetic shellfish toxins research. Toxins, 12, 493.

Chaumeil P, Mussig AJ, Hugenholtz P and Parks DH, 2020. GTDB-Tk: a toolkit to classify genomes with the genome taxonomy database. Bioinformatics, 36, 1925–1927.

García-Altares M, Casanova A, Bane V, Diogène J, Furey A and Iglesia P, 2014. Confirmation of pinnatoxins and spirolides in shellfish and passive samplers from Catalonia (Spain) by liquid chromatography coupled with triple quadrupole and high-resolution hybrid tandem mass spectrometry. Marine Drugs, 12, 3706–3732.

Goodwin S, McPherson JD and McCombie WR, 2016. Coming of age: ten years of next-generation sequencing technologies. Nature Reviews Genetics, 17, 333–351.

Gurevich A, Saveliev V, Vyahhi N and Tesler G, 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics, 29, 1072–1075.

Josić D, Peršurić Ž, Rešetar D, Martinović T, Saftić L and Pavelić SK, 2017. Use of foodomics for control of food processing and assessing of food safety. Advances in Food and Nutrition Research, 81, 187–229.

Leão PN, Engene N, Antunes A, Gerwick WH and Vasconcelos V, 2012. The chemical ecology of cyanobacteria. Natural Product Reports, 29, 372–391.

Martin M, 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal, 17, 10.

Moreira C, Vasconcelos V and Antunes A, 2013. Phylogeny and biogeography of cyanobacteria and their produced toxins. Marine Drugs, 11, 4350–4369.

Natarajan KN, Miao Z, Jiang M, Huang X, Zhou H, Xie J, Wang C, Qin S, Zhao Z, Wu L, Yang N, Li B, Hou Y, Liu S and Teichmann SA, 2019. Comparative analysis of sequencing technologies for single-cell transcriptomics. Genome Biology, 20.

Nurk S, Meleshko D, Korobeynikov A and Pevzner PA, 2017. metaSPAdes: a new versatile metagenomic assembler. Genome Research, 27, 824–834.
Otero P, Pérez S, Alfonso A, Vale C, Rodríguez P, Gouveia NN, Gouveia N, Delgado J, Vale P, Hirama M, Ishihara Y, Molgó J and Botana LM, 2010. First toxin profile of ciguateric fish in Madeira Arquipelago (Europe). Analytical Chemistry, 82, 6032–6039.
Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F and Stevens R, 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Research, 42, 206–214.
Quince C, Walker AW, Simpson JT, Loman NJ and Segata N, 2017. Shotgun metagenomics, from sampling to analysis. Nature Biotechnology, 35, 833–844.
Semedo-Aguiar AP, Pereira-Leal JB and Leite RB, 2018. Microbial diversity and toxin risk in tropical freshwater reservoirs of cape verde. Toxins, 10, 186.
Sharpton TJ, 2014. An introduction to the analysis of shotgun metagenomic data. Frontiers in Plant Science, 5, 209.
Wu Y, Simmons BA and Singer SW, 2016. MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. Bioinformatics, 32, 605–607.

Abbreviations

BGC	Biosynthetic Genome Cluster
BLAST	Basic Local Alignment Search Tool
BR	broad range
CIIMAR	Interdisciplinary Centre of Marine and Environmental Research (Portugal)
cPAS	Combinational Probe-Anchor Synthesis
DNB	DNA Nanoball
dsDNA	double-stranded DNA
EU-FORA	European Food Risk Assessment Fellowship Programme
gDNA	genomic DNA
HABs	harmful algal blooms
ICHA	International Conference on Harmful Algae
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAG	Metagenome-Assembled Genome
MAPQ	mapping quality
NGS	next generation sequencing
RCA	rolling-circle amplification

EU-FORA SERIES 4

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Risk–benefit assessment of seaweed Allergenicity risk assessment of novel protein

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Abstract

As the world population rapidly grows, there is a clear need for alternative food sources, particularly for the provision of protein. Seaweed is one such alternative source of protein that requires greater investigation. In this context, a working programme within the European Food Risk Assessment (EU-FORA) Fellowship Programme framework was developed at National Food Institute – Technical University of Denmark. This Programme is an initiative of the EFSA with the aim to build a European risk assessment community. The purpose of this technical report is to describe the activities in which the fellow was involved. As part of the Research Group for Risk–Benefit, the fellow performed a risk–benefit assessment of seaweed *Palmaria palmata* gaining an in-depth expertise in all the steps. The health impact of *Palmaria palmata* consumption was estimated, considering its high nutritional value but also highlighting concerns towards some components. Simultaneous to the work on the risk–benefit, the fellow also worked within the Research Group for Food Allergy, specifically on the allergenicity risk assessment of a plant-based novel protein (seaweed protein) using different laboratory assays. Seaweed protein digestibility was assessed, and its digestion products were characterised and assessed for immunogenicity. Finally, the fellow collaborated with the Research Group for Microbial Biotechnology and Biorefining in the development of a novel food (alfalfa protein) application dossier to be submitted to EFSA, gaining expertise in the risk assessment of a novel food. 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 seaweed, novel foods and the safety assessment of novel proteins.

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Keywords: risk–benefit, allergenicity risk assessment, seaweed, novel food, novel protein

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1. Introduction

European Food Risk Assessment (EU-FORA) Fellowship Programme is an initiative of European Food Safety Authority (EFSA) with the aim to build European risk assessment capacity and knowledge community. This technical report describes the activities of the programme conducted at National Food Institute – Technical University of Denmark, as follows: (i) Risk–benefit assessment (RBA) of seaweed, (ii) allergenicity risk assessment of a plant-based novel protein (seaweed) and (iii) risk assessment of novel food (alfalfa protein).

1.1. Seaweed

The world's population is continuously growing and there are studies concluding that total food production should be increased by at least 60% to feed a world of more than 9 billion people by 2050.[1] One of the challenges will be rising food production to meet the protein demand, showing the clear need to find alternative protein sources and to develop means of production that have less negative environmental impacts (European Commission, 2021). Seaweed is considered an alternative source of protein (Leandro et al., 2020).

Seaweed has a high nutritional value due to its content of several dietary components (Leandro et al., 2020) and has been found to be a source of potential bioactive compounds (Holdt and Kraan, 2011). Seaweed is characterised by a high level of protein (up to 47% of dry weight in red species) (Mouritsen et al., 2013), low fat content (0.3–3.8% dry weight) and relatively high concentration of omega-3, omega-6 fatty acids and dietary fibre (Leandro et al., 2020). Seaweed is a source of minerals and vitamins (Leandro et al., 2020), particularly iodine, which is present in significant high concentrations in some species (Roleda et al., 2018) and could be of health concern. Furthermore, seaweeds could also be a source of potential harmful contaminants such as cadmium, mercury, inorganic arsenic and lead, due to their cell wall structure conferring them high absorption properties (Banach et al., 2020). Seaweed allergenicity has not been widely studied, but there are some reports in literature describing allergic reactions to it (Thomas et al., 2019). This toxicological profile has raised concern from several food authorities regarding the exposure to excessive levels of these contaminants upon seaweed consumption (ANSES, 2018; Duinker et al., 2020). EFSA identified seaweed as a potential emerging risk (EFSA, 2017). Therefore, it is a food which requires further investigation.

1.2. Risk–benefit assessment

A risk–benefit assessment (RBA) is an evaluation of combined adverse and beneficial health effects associated with food in order to inform food safety and public health strategies (Verhagen et al., 2012). It is necessary to perform a RBA for seaweed in order to evaluate its health impact, considering its high nutritional value but also the concerns regarding some of its components. A risk–benefit balance for different intake levels of red seaweed *Palmaria palmata* (L.) Weber & Mohr had not been assessed previously, and therefore, it was included in this project under the EU-FORA Fellowship Programme.

1.3. Allergenicity risk assessment of novel proteins

Food allergy is considered as an adverse immune response to food (Bøgh and Madsen, 2016). Immunoglobulin E (IgE)-mediated immune reaction (type I), the most common one in food allergy, consists of two phases: (i) sensitisation, whereby the immune system develops hyper-reactivity to the allergen upon exposure without symptoms, (ii) elicitation, clinical manifestations of immune response occur after re-exposure to the allergen, which can be gastrointestinal, respiratory or on skin (Fernandez et al., 2013).

Taking into account that seaweed is considered an alternative source of protein, and there is little information on the potential allergenicity of seaweed protein, it was considered relevant to include a project investigating the allergenicity of seaweed protein within the EU-FORA Programme.

1.4. Risk assessment of novel foods

Novel foods are defined as foods that had not been consumed to a significant degree by humans in the European Union before 15 May 1997. Their safety for consumers has to be assessed in terms of nutritional composition, microbiology, toxicology and allergenicity (EFSA NDA Panel, 2021). In the

[1] https://www.fao.org/news/story/en/item/35571/icode/

specific case of alfalfa, it is widely consumed as food, but not as isolated alfalfa protein. Therefore, an application will be submitted to EFSA in order to evaluate its safety for consumers as novel food. The fellow collaborated on the development of this dossier as part of the EU-FORA Programme.

2. Description of work programme

2.1. Aims

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

1) Gaining expertise in performing RBA of food. This includes working on a specific RBA case on seaweed as part of the Risk–Benefit Group.
2) Gaining expertise in allergenicity risk assessment of a plant-based novel protein (seaweed) within the Research Group for Food Allergy Group. Introduction to novel proteins allergenicity risk assessment and experimental work were included.
3) Learning about risk assessment of novel foods (alfalfa protein) in collaboration with the Research Group for Microbial Biotechnology and Biorefining, including thorough examination of all aspects of a novel food risk assessment.

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

2.2. Activities/Methods

The fellow's main project was to perform the RBA of seaweed, gaining experience in searching scientific databases, performing systematic data extraction from scientific publications and its evaluation, generation of data using dose–response modelling, discussion of results and their presentation and drafting the final output with results and conclusions in an harmonised format. The fellow collaborated with the Research Group for Food Allergy for a period, gaining practical experience in allergenicity assessment techniques. Finally, the fellow collaborated in the development of a novel food application, gaining experience in data extraction and collation and resolving practical issues in the preparation of an application.

2.2.1. Risk–Benefit Assessment of seaweed

Initially, the fellow conducted a literature search including risk–benefit methodology together with previously performed risk–benefit cases in order to understand different steps of the assessment. Following this, the fellow was involved in the development of a specific risk–benefit assessment case on *Palmaria palmata* in collaboration with other members of the Research Group for Risk–Benefit. The overall RBA was based on five stages (Figure 1).

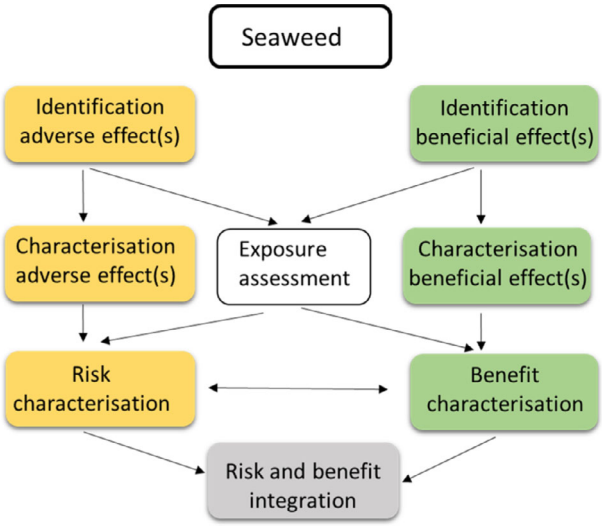

Figure 1: Risk–benefit assessment paradigm. Adapted from EFSA Scientific Committee (2010)

First, the risk–benefit objective was formulated: to quantify the overall health impact in terms of Disability-Adjusted Life-Years (DALYs) of the consumption of seaweed *Palmaria palmata* in the adult Danish population. In order to identify its nutrients and contaminants and their concentration levels, a literature search was performed. The identification of potential health effects was based on assessments from international expert reports, including publications from the Food and Agriculture Organisation (FAO), World Health Organisation (WHO), regulatory agencies (EFSA; French Agency for Food, Environmental and Occupational Health and Safety (ANSES); Food Safety Authority of Ireland (FSAI)), Rapid Alert System for Food and Feed (RASFF) and scientific papers. For hazards and benefits characterisation, a literature search was also carried out to identify systematic reviews for epidemiological studies, including meta-analyses, in order to develop dose–response models. These literature searches were performed in PubMed, ScienceDirect and Web of Science including articles up to September 2021. *Palmaria palmata* nutrients and chemical contaminants and their health effects are summarised in Appendix A.

Three consumption scenarios were investigated, each one compared to zero intake of seaweed (reference scenario). Concentration data of different components were collected from the literature and internal databases, including seaweed samples from different European countries, years and seasons and the Danish food monitoring programme. An average value was calculated for each component taking into consideration the wide variation in their levels.

Mean daily exposure to nutrients and chemical contaminants in each scenario was calculated considering mean daily intake of *Palmaria palmata* and mean concentration of each component in *Palmaria palmata*. For contaminants, it was expressed in terms of μg per unit body weight (70 kg as default), whereas for nutrients, it was expressed in absolute terms. Background dietary exposure was also taken into consideration when dose–response models applied were not considered linear. Data were taken from the Danish National Survey of Diet and Physical Activity 2011–2013 and National Food Institute. Population statistics used in the models were obtained from Statistics Denmark.

The calculated daily exposure to nutrients and contaminants was combined with dose–response models to estimate the size of a given health effect associated with *Palmaria palmata* consumption in the three scenarios. The health effects were expressed and integrated in the composite health metric DALYs. DALY allows for the comparison of different health effects as it integrates the morbidity and mortality of a health effect in a single number, and one DALY is equal to one healthy life year lost (Devleesschauwer et al., 2014). Depending on the availability of data, 'Top-down' or 'Bottom-up' approaches were applied to estimate incidence and DALY of the different health effects (Gibb et al., 2015) (Appendix A). The last step was the integration of risks and benefits expressed in DALYs for each scenario and the comparison between different scenarios, through the calculation of the difference between alternative and reference scenarios (in ΔDALYs). $\Delta DALY > 0$ implied a health loss due to *Palmaria palmata* intake, whereas $\Delta DALY < 0$ meant a health gain. All modelling and calculations were performed using Microsoft Excel.

A manuscript, currently under preparation, includes a comprehensive description of the risk–benefit assessment, details on the risk and benefit characterisation and dose–response models, calculations and results of the assessment. It is expected to be submitted for publication in a peer-reviewed journal.

To be able to carry out this project, the fellow had regular meetings and discussions with the supervisor as well as other members of the group experienced in risk–benefit. The fellow also scheduled meetings with different groups of the Division of Risk Assessment and Nutrition at National Food Institute in order to gather and discuss data such as seaweed composition, consumption, health effects, etc.

2.2.2. Allergenicity risk assessment of seaweed protein

Initially, the fellow performed a literature review including novel protein allergenicity risk assessment in order to understand the approach to be followed. There are no validated methods to assess novel protein allergenicity at present. *In vitro* digestibility tests can provide information on the susceptibility of a protein to digestion, which can simulate its digestibility in the human gastrointestinal system and subsequent presentation to the host's immune system (Foster et al., 2013). Historically, pepsin resistance test has been considered an assay for allergenic potential assessment within a weight-of-evidence approach (Astwood et al., 1996; Codex Alimentarius Commission, 2009; EFSA GMO Panel, 2017). However, the predictability value of this test has been controversial because there is evidence showing that digestion may cancel, reduce, increase or not induce an effect on the

allergenicity of food allergens (Bøgh and Madsen, 2016). The main limitation of the pepsin resistance test is the difficulty to mimic human gastric digestion physiological conditions. Therefore, EFSA recommends using *in vitro* digestibility methods where human digestion process conditions can be simulated (gastric and intestinal digestion) (EFSA GMO Panel, 2017). Nevertheless, the classical pepsin resistance test provides information on stability of proteins including biochemical and physico-chemical properties contributing to the overall safety assessment of the novel protein (EFSA GMO Panel, 2021).

These limitations highlight the necessity of immunological tests (enzyme-linked immunosorbent assay (ELISA), immunoblots) in order to assess allergenic potential of food allergens and their digested products (Bøgh and Madsen, 2016). In order to be considered a complete allergen (in type I food allergy), a protein has to fulfil these properties: IgE-binding ability, allergic reaction elicitation and *de novo* sensitisation capacity, although not all allergens are complete allergens (Aalberse, 2000). This means the allergenic potential of digested products needs to be assessed on those levels in order to evaluate the effect of digestion on allergenicity (Verhoeckx et al., 2019).

In this context, the fellow carried out a 'hands-on' project on allergenicity assessment of plant-based protein from different red and brown seaweed species. Previously, the fellow performed the Chemical Risk Assessment relevant to the assays to be carried out. This project required writing of protocols for each assay, assay optimisation and presenting the results in the Research Group for Food Allergy meetings.

With the purpose to assess resistance to enzymes as a parameter in allergenicity assessment of novel proteins, the fellow performed *in vitro* digestibility tests with red and brown seaweed simulating gastrointestinal conditions. The protein profiles of intact protein and digestion products were characterised using electrophoretic techniques, which allowed them to separate peptides according to their size. Afterwards, immunoblots were carried out in order to assess the immunoreactivity of seaweed protein and its digestion products, and ELISA to evaluate the levels of animal antibodies reactive to these proteins (Figure 2). Results showed differences in protein digestibility of different seaweed species. Limited methods and results are presented in this report in order not to compromise the future scientific publication under preparation.

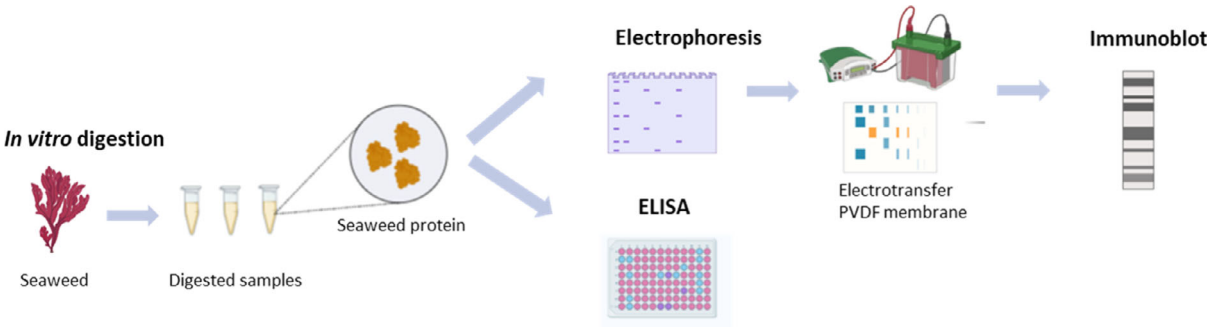

Figure 2: Stepwise approach followed in seaweed protein allergenicity assessment: seaweed protein *in vitro* digestion, protein characterisation throughout electrophoretic techniques and immunochemical techniques (ELISA and immunoblot). Pictures obtained from BioRender

2.2.3. Novel food application

The fellow collaborated with the Research Group for Microbial Biotechnology and Biorefining in the development of a plant-based novel food (alfalfa protein) application to be submitted to EFSA. Initial literature searches were performed including 'EFSA Guidance on the preparation and submission of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283' (EFSA NDA Panel, 2021) and scientific outputs (Ververis et al., 2020). After gathering all data requirements for the submission of novel food applications, several meetings took place where the fellow provided support and assistance guiding the responsible members of the project through the data requirements. Different practical aspects and the approach to be taken were also addressed. This task also included the exchange of knowledge with the supervisor, the professor and the PhD student in charge of the project.

2.2.4. Additional activities

During the EU-FORA fellowship, the fellow had the opportunity to take part in the following activities:

- Preparation of a manuscript including results from the RBA of seaweed and collaboration in the drafting of a manuscript on allergenicity risk assessment of seaweed.
- Scientific division seminars: lectures including a wide range of projects within the area of food research.
- Weekly group meetings: discussion of ongoing projects and presentation of results.
- EUROTOX Virtual Congress 2021.
- 9th BfR-Summer Academy 2021: online training course on risk assessment and risk communication in the area of food safety provided by The German Federal Institute for Risk Assessment.
- WHO webinar: Burden of foodborne diseases: how can we estimate it and why do we need it?
- 'Bridging European Science III' event in Copenhagen: international forum for scientific interactions between universities, companies and the public sector in the Nordic countries and Spain.
- National Food Institute - Technical University of Denmark Conference: digitalisation.

3. Conclusions

The work programme carried out at National Food Institute – Technical University of Denmark provided the fellow with expertise in RBA, novel foods and allergenicity risk assessment of novel proteins. Through participation in the research group for risk–benefit, the fellow gained broad 'hands-on' experience in performing a RBA and obtained deep knowledge through collaboration with colleagues experienced in different areas. Through collaboration with the research group for food allergy, the fellow was challenged to learn laboratory assays in the area of novel protein allergenicity assessment and to apply them in the assessment of specific seaweed protein samples. The outputs of the risk–benefit assessment and allergenicity assessment will be published in relevant peer-reviewed journals. At the same time, the fellow worked on the development of a novel food application. During this work, the fellow expanded the knowledge in plant-based novel foods and proteins, and got a thorough insight of the requirements for their risk assessment.

In addition to these projects and activities developed at the hosting site, the trainings provided by the different institutions throughout the programme were of great value in the learning process of the fellow. Therefore, EU-FORA programme was an excellent opportunity to promote knowledge exchange and future collaboration opportunities. The National Food Institute – Technical University of Denmark made it possible for the fellow to gain a broader perspective and contribute to food safety assessment, in a multicultural and pleasant atmosphere.

References

Aalberse RC, 2000. Structural biology of allergens. Journal of Allergy and Clinical Immunology, 106, 228–238. https://doi.org/10.1067/mai.2000.108434

ANSES, French Agency for Food, Environmental and Occupational Health & Safety, 2018. OPINION of the French Agency for Food, Environmental and Occupational Health & Safety on the risk of excess iodine intake from the consumption of seaweed in foodstuffs. Request No 2017- SA-0086. Available online: https://www.anses.fr/en/system/files/NUT2017SA0086EN.pdf

Astwood JD, Leach JN and Fuchs RL, 1996. Stability of food allergens to digestion *in Vitro*. Nature Biotechnology, 14, 1269–1273. https://doi.org/10.1038/nbt1096-1269

Banach JL, Hoek-van den Hil EF and van der Fels-Klerx HJ, 2020. Food safety hazards in the European Seaweed Chain. Comprehensive Reviews in Food Science and Food Safety, 19, 332–364. https://doi.org/10.1111/1541-4337.12523

Barendregt JJ and Veerma JL, 2010. Categorical versus continuous risk factors and the calculation of potential impact fractions. Journal of Epidemiology and Community Health, 64, 209–212. https://doi.org/10.1136/jech.2009.090274

Bøgh KL and Madsen CB, 2016. Food allergens: is there a correlation between stability to digestion and allergenicity? Critical Reviews in Food Science and Nutrition, 56, 1545–1567. https://doi.org/10.1080/10408398.2013.779569

Codex Alimentarius Commission, 2009. Foods Derived from Modern Biotechnology. 2nd edition. Food and Agriculture Organization of the United Nations and World Health Organization, Rome. Available online: https://www.fao.org/3/a1554e/a1554e.pdf

Devleesschauwer B, Havelaar AH, Maertens De Noordhout C, Haagsma JA, Praet N, Dorny P, Duchateau L, Torgerson PR, Van Oyen H and Speybroeck N, 2014. Calculating disability-adjusted life years to quantify burden of disease. International Journal of Public Health, 59, 565–569. https://doi.org/10.1007/s00038-014-0552-z

Duinker A, Kleppe M, Fjære E, Biancarosa I, Heldal HE, Dahl L and Lunestad BT, 2020. Knowledge update on macroalgae food and feed safety based on data generated in the period 2014-2019 by the Institute of Rapport Fra Havforskningen, 44, 28. Avaliable online: https://hdl.handle.net/11250/2712528

EFSA (European Food Safety Authority), Afonso A, García Matas R, Maggiore A, Merten C and Robinson T, 2017. Technical report on EFSA's Activities on Emerging Risks in 2016. EFSA Supporting Publication 2017;EN-1336, 59 pp. https://doi.org/10.2903/sp.efsa.2017.EN-1336

EFSA CONTAM Panel (EFSA Panel on Contaminants in the food chain), 2009a. Scientific Opinion on Arsenic in Food. EFSA Journal 2009;7(10):1351, 199 pp. https://doi.org/10.2903/j.efsa.2009.1351

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2009b. Cadmium in food - Scientific opinion of the Panel on Contaminants in the Food Chain. EFSA Journal 2009:980, 139 pp. https://doi.org/10/ggthsn

EFSA CONTAM Panel (EFSA Panel on Contaminants in the food chain), 2010. Scientific Opinion on Lead in Food. EFSA Journal 2010;8(4):1570, 151 pp. https://doi.org/10.2903/j.efsa.2010.1570

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2012. Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. EFSA Journal 2012;10(12):2985, 241 pp. https://doi.org/10.2903/j.efsa.2012.2985

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2017. Guidance on allergenicity assessment of genetically modified plants. EFSA Journal 2017;15(5):4862, 49 pp. https://doi.org/10.2903/j.efsa.2017.4862

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2021. Statement on in vitro protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants. EFSA Journal, 2021;19(1):6350, 16 pp. https://doi.org/10.2903/j.efsa.2021.6350

EFSA NDA Panel (EFSA Panel on Panel on Dietetic Products Nutrition and Allergies), 2014. Scientific Opinion on Dietary Reference Values for iodine. EFSA Journal 2014;12(5):3660, 57 pp. https://doi.org/10.2903/j.efsa.2014.3660

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2021. Guidance on the preparation and submission of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283 (Revision 1). EFSA Journal 2021;19(3):6555, 27 pp. https://doi.org/10.2903/j.efsa.2021.6555

EFSA Scientific Committee, 2010. Guidance on human health risk-benefit assessment of food. EFSA Journal 2010;8(7):1673, 41 pp. https://doi.org/10.2093/j.efsa.2010.1673

European Commission, 2021. Food 2030. European Research&Innovation for Food & Nutrition Security. https://doi.org/10.2777/069319

Fernandez A, Mills ENC, Lovik M, Spoek A, Germini A, Mikalsen A and Wal JM, 2013. Endogenous allergens and compositional analysis in the allergenicity assessment of genetically modified plants. Food and Chemical Toxicology, 62, 1–6. https://doi.org/10.1016/j.fct.2013.08.023

Foster ES, Kimber I and Dearman RJ, 2013. Relationship between protein digestibility and allergenicity: Comparisons of pepsin and cathepsin. Toxicology, 309, 30–38. https://doi.org/10.1016/j.tox.2013.04.011

Franceschi S, Preston-Martin S, Dal Maso L, Negri E, La Vecchia C, Mack WJ, McTiernan A, Kolonel L, Mark SD, Mabuchi K, Jin F, Wingren G, Galanti R, Hallquist A, Glattre E, Lund E, Levi F, Linos D and Ron E, 1999. A pooled analysis of case-control studies of thyroid cancer. IV. Benign Thyroid diseases. Cancer Causes & Control, 10, 583–595. https://doi.org/10.1023/a:1008907227706

Gibb H, Devleesschauwer B, Bolger PM, Wu F, Ezendam J, Cliff J, Zeilmaker M, Verger P, Pitt J, Baines J, Adegoke G, Afshari R, Liu Y, Bokkers B, van Loveren H, Mengelers M, Brandon E, Havelaar AH and Bellinger D, 2015. World Health Organization Estimates of the Global and Regional Disease Burden of Four Foodborne Chemical Toxins, 2010: A Data Synthesis. F1000Research, 4, 1–14. https://doi.org/10.12688/f1000research.7340.1

Holdt SL and Kraan S, 2011. Bioactive compounds in seaweed: functional food applications and legislation. Journal of Applied Phycology, 23, 543–597. https://doi.org/10.1007/s10811-010-9632-5

Jørgensen K and Olesen PT, 2018. Kainic Acid in the Seaweed *Palmaria Palmata* (Dulse). Food Additives and Contaminants: Part B Surveillance, 11, 198–200. https://doi.org/10.1080/19393210.2018.1462258

Leandro A, Pacheco D, Cotas J, Marques JC, Pereira L and Gonçalves AMM, 2020. Seaweed's bioactive candidate compounds to food industry and global food security. Life, 10, 140. https://doi.org/10.3390/life10080140

Mouritsen OG, Dawczynski C, Duelund L, Jahreis G, Vetter W and Schröder M, 2013. On the human consumption of the red seaweed dulse (*Palmaria palmata* (L.) Weber & Mohr). Journal of Applied Phycology, 25, 1777–1791. https://doi.org/10.1007/s10811-013-0014-7

Mozzafarian D and Rimm EB, 2006. Fish Intake, Contaminants, and Human Health. Evaluating the risks and the benefits. JAMA, 296, 1885–1899. https://doi.org/10.1001/jama.296.15.1885

Murray CJL, Ezzati M, Lopez AD, Rodgers A and Vander Hoorn S, 2003. Comparative quantification of health risks: Conceptual framework and methodological issues. Population Health Metrics, 1, 1–20. https://doi.org/10.1186/1478-7954-1-1

Nauta MJ, Andersen R, Pilegaard K, Pires SM, Ravn-Haren G, Tetens I and Poulsen M, 2018. Meeting the challenges in the development of risk-benefit assessment of foods. Trends in Food Science and Technology, 76, 90–100. https://doi.org/10.1016/j.tifs.2018.04.004

Reynolds A, Mann J, Cummings J, Winter N, Mete E and Morenga LT, 2019. Carbohydrate quality and human health: a series of systematic reviews and meta-analyses. The Lancet, 393, 434–445. https://doi.org/10.1016/S0140-6736(18)31809-9

Roleda MY, Skjermo J, Marfaing H, Jónsdóttir R, Rebours C, Gietl A, Stengel DB and Nitschke U, 2018. Iodine content in bulk biomass of wild-harvested and cultivated edible seaweeds: inherent variations determine species-specific daily allowable consumption. Food Chemistry, 254, 333–339. https://doi.org/10.1016/j.foodchem.2018.02.024

Thomas I, Siew LQC, Watts TJ and Haque R, 2019. Seaweed allergy. Journal of Allergy and Clinical Immunology. In Practice, 7, 714–715. https://doi.org/10.1016/j.jaip.2018.11.009

Thomsen ST, Pires SM, Devleesschauwer B, Poulsen M, Fagt S, Ygil KH and Andersen R, 2018. Investigating the risk-benefit balance of substituting red and processed meat with fish in a danish diet. Food and Chemical Toxicology, 120, 50–63. https://doi.org/10.1016/j.fct.2018.06.063

Verhagen H, Tijhuis MJ, Gunnlaugsdóttir H, Kalogeras N, Leino O, Luteijn JM, Magnússon SH, Odekerken G, Pohjola MV, Tuomisto JT, Ueland Ø, White BC and Holm F, 2012. State of the art in benefit-risk analysis: introduction. Food and Chemical Toxicology, 50, 2–4. https://doi.org/10.1016/j.fct.2011.06.007

Verhoeckx K, Bøgh KL, Dupont D, Egger L, Gadermaier G, Larré C, Mackie A, Menard O, Adel-Patient K, Picariello G, Portmann R, Smit J, Turner P, Untersmayr E and Epstein MM, 2019. The relevance of a digestibility evaluation in the allergenicity risk assessment of novel proteins. Opinion of a joint initiative of COST action ImpARAS and COST action INFOGEST. Food and Chemical Toxicology, 129, 405–423. https://doi.org/10.1016/j.fct.2019.04.052

Ververis E, Ackerl R, Azzollini D, Colombo PA, de Sesmaisons A, Dumas C, Fernandez-Dumont A, Ferreira da Costa L, Germini A, Goumperis T, Kouloura E, Matijevic L, Precup G, Roldan-Torres R, Rossi A, Svejstil R, Turla E and Gelbman W, 2020. Novel Foods in the European Union: Scientific Requirements and Challenges of the Risk Assessment Process by the European Food Safety Authority. Food Research International, 137, 109515. https://doi.org/10.1016/j.foodres.2020.109515

Yu X, Fan C, Shan Z, Teng X, Guan H, Li Y, Teng D, Jin Y, Chong W, Yang F, Dai H, Yu Y, Li J, Chen Y, Zhao D, Shi X, Hu F, Mao J, Gu X, Yang R, Tong Y, Wang W, Gao T, Li C and Teng W, 2008. A five-year follow-up study of goiter and thyroid nodules in three regions with different iodine intakes in China. Journal of Endocrinological Investigation, 31, 243–250. https://doi.org/10.1007/BF03345597

Abbreviations

ANSES	French Agency for Food, Environmental and Occupational Health & Safety
DALY	Disability-Adjusted Life Year
DHA	Docosahexaenoic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
EU-FORA	European Food Risk Assessment
FAO	Food and Agriculture Organization
FSAI	Food Safety Authority of Ireland
IgE	Immunoglobulin E
IQ	Intelligence Quotient
PIF	Population Impact Fraction
RASFF	Rapid Alert System for Food and Feed
RBA	Risk-Benefit Assessment
RR	Relative risk
WHO	World Health Organisation
YLDs	Years lived with disability
YLLs	Years of life lost

Appendix A – Risk-Benefit assessment of seaweed

A.1. Components and health effects associated

Table A.1: *Palmaria palmata* nutrients and chemical contaminants of interest included in the RBA

Component	Health effect	Reference
Lead	↑ Intellectual disability (lowered IQ)	EFSA CONTAM Panel (2010)
Cadmium	↑ Chronic kidney disease	EFSA CONTAM Panel (2009a,b)
Methylmercury	↑ Intellectual disability (lowered IQ)	EFSA CONTAM Panel (2012)
Inorganic arsenic	↑ Lung, bladder and skin cancer	EFSA CONTAM Panel (2009a,b)
Iodine	↑ Intellectual disability (lowered IQ)	EFSA NDA Panel (2014)
	↑/↓ Goiter	Yu et al. (2008)
	↑/↓ Thyroid cancer	Franceschi et al. (1999)
Kainic acid	↑ Neurotoxicity	Jørgensen and Olesen (2018)
Docosahexaenoic acid, (DHA), Eicosapentaenoic acid (EPA)	↓ Fatal coronary heart disease	Mozzafarian and Rimm (2006)
Dietary fibre	↓ Fatal coronary heart disease	Reynolds et al. (2019)
	↓ Type 2 diabetes mellitus	
	↓ Colorectal cancer	

↑: increased effect (adverse effect of the component and associated health outcome); ↓**:** decreased effect (beneficial effect of the component and associated health outcome); IQ: intelligence quotient.

A.2. Disability-adjusted life years (DALY) calculation

A.2.1. 'Bottom-up' approach

It was applied when estimates of current incident cases or DALY of a given health effect irrespective of risk factors were not available. Incidence of disease was estimated due to the exposure using dose–response models (Nauta et al., 2018). The disease burden of each health effect was quantified in DALYs, composed of years lived with disability (YLDs) and years of life lost due to premature mortality (YLLs). YLDs are calculated as follows:

$$YLD_{d,s,a} = AC_{d,s,a} \times D_d \times DW_d,$$

where $AC_{d,s,a}$ is the annual number of incident cases for health outcome d for sex s and age a, D_d is the duration of health outcome d and DW_d is the disability weight for health outcome d. The disability weight can be interpreted as a measure of good health, ranging from zero (perfect health) to one (death) (Devleesschauwer et al., 2014). YLLs are calculated as follows:

$$YLL_{d,s,a} = AD_{d,s,a} \times SEYLL_{s,a},$$

where $AD_{d,s,a}$ is the annual number of deaths due to the health outcome d for sex s and age a, and $SEYLL_{s,a}$ is the standard expected years of life lost for a death for sex s and at age a (Thomsen et al., 2018) (WHO, 2020). DALYs were calculated as the sum of YLD and YLL:

$$DALY_d = \sum_s \sum_a (YLD_{d,s,a} + YLL_{d,s,a}).$$

A.2.2. 'Top-down' approach

It combines epidemiological and incidence data and estimates the number of attributable cases of a certain health outcome due to exposure to *Palmaria palmata* (Nauta et al., 2018). It was applied when disease or DALY envelopes were available.

Dose–response models already available in literature were applied with modifications. When relative risk (RR) estimates were available in literature, dose–response functions were derived following these equations (Barendregt and Veerman, 2010):

$$\beta = \ln(RR_{literature})/dose$$
$$RR = \exp(\beta \times exposure),$$

where $RR_{literature}$ is the relative risk for each health outcome obtained from literature, RR is the relative risk for each scenario and exposure is the intake of each component in each scenario.

In order to calculate the change in DALYs for each scenario, population impact fraction (PIF) was used to estimate the proportion of health outcome cases that could be prevented by the change of the exposure to different components of *Palmaria palmata* from the reference to alternative scenarios (Murray et al., 2003). RR shift methodology was applied, assuming the changes in the RR of scenarios describe the change in exposure (Barendregt and Veerman, 2010), according to the following equation:

$$PIF_d = (RR_{d,ref} - RR_{d,alt})/RR_{d,ref},$$

where $RR_{d,ref}$ is the relative risk of reference scenario for health outcome d and $RR_{d,alt}$ is relative risk of alternative scenario for health outcome d.

The health effect in DALY for each component and health outcome was calculated using the following formula:

$$DALY = PIF_d \times DALY_{GBD},$$

where PIF_d is the population impact fraction for health outcome d and $DALY_{GBD}$ is DALY value obtained from GBD Results tool.

EU-FORA SERIES 4

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Seafood safety and food-borne zoonoses from fish

Examining the risk of *Anisakis* in the Portuguese Population and Consumer Risk Perceptions of Fish Consumption

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Abstract

Parasitic nematodes of the genus *Anisakis* are the causative agent of anisakiosis, an important fish-borne zoonosis. Humans are infected through consumption of raw or undercooked fish, contaminated with the parasite. Infection can result in both gastrointestinal and allergic symptoms. There are few reports of anisakiosis in Portugal, but evidence of *Anisakis* allergy exists, indicating that exposure is occurring in the population. The European Food Risk Assessment Fellowship Programme (EU-FORA) work programme, entitled: 'Food safety of fish and zoonoses: fish consumption and microbiological risk assessment and perception, from fisherman to final consumers in Portugal' was hosted by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), in Porto, Portugal. It aimed to gather information on risk perception and attitudes in the Portuguese population to contamination of fish with *Anisakis* spp. and on their knowledge of methods to prevent infection. In addition, it aimed to examine the risk of anisakiosis in the Portuguese population.

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Keywords: *Anisakis*, fish parasite, food safety, Zoonoses

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1. Introduction

1.1. European Food Risk Assessment Fellowship Programme (EU-FORA)

The European Food Risk Assessment Fellowship Programme (EU-FORA) is an initiative of the European Food Safety Authority (EFSA). It aims to build the European Union's (EU) scientific assessment capacity and knowledge community, in line with the key objectives outlined in EFSA's strategy 2020. It provides scientists working in food safety organisations across Europe with the opportunity to increase their knowledge and gain experience in food risk assessment, taking a 'learning by doing' approach to training. (Bronzwaer et al., 2016). The fellow was hosted by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), in Porto, Portugal. The work programme was entitled: Food safety of fish and zoonoses: fish consumption and microbiological risk assessment and perception, from fisherman to final consumers in Portugal. The programme included a 3-week induction training course and four 1-week modules, each focused on different aspects of risk assessment and risk communication. These modules would usually be held in different food safety institutes across the EU; however due to the Covid-19 pandemic, they were conducted on an online platform.

1.2. Background to Risk Assessment

Each year, almost one in 10 people fall ill from eating contaminated food. The World Health Organization has estimated that food-borne disease resulted in 420,000 deaths in 2010, with a global burden of 33 million Disability Adjusted Life Years (DALYs) (WHO, 2015). Food-borne zoonoses from fish and shellfish products are of significant public health concern, with high numbers of people at risk worldwide in both developed and developing countries (Lima dos Santos and Howgate, 2011). Anisakiosis is a fish-borne parasitic zoonosis caused by nematodes of the genus *Anisakis,* Dujardin 1845. These parasites have an indirect life cycle, using marine mammals, usually cetaceans as their definitive host. Invertebrates such as fish or squid are intermediate or paratenic hosts and crustaceans act as first intermediate hosts (Klimpel et al., 2004; EFSA BIOHAZ Panel, 2010; Smith and Wootten, 1978). Humans are infected with *Anisakis* spp. through consumption of the third-stage larval (L_3) form of the parasite in raw, smoked, marinated, salted or undercooked fish or squid (Audicana et al., 2002; Caldeira et al., 2021). They are accidental hosts for the parasite, so it does not mature, but on reaching the gastrointestinal tract, the larvae can cause disease (anisakiosis) (Mattiucci et al., 2018).

Van Thiel first described gastric anisakiosis in the 1960s (Van Thiel, 1962; Van Thiel and Van Houten, 1966; Buchmann and Mehrdana, 2016), the symptoms include nausea, vomiting and abdominal pain, and are caused by the larvae penetrating the gastrointestinal tract tissues (Caramello et al., 2003; Buchmann and Mehrdana, 2016). Patients can experience both abdominal and hypersensitivity symptoms and this condition is described as gastroallergic anisakiosis (Daschner et al., 2000). The other condition associated with *Anisakis* infection is an allergic response to fish products that contain parasite allergens. In these cases, live parasites may not be necessary to induce an allergic reaction, although it is generally believed that an initial *Anisakis* infection must occur to sensitise individuals to parasite antigens. However, it has not been possible to definitively rule out the occurrence of sensitisation through exposure to antigen alone (Audicana et al., 2002; EFSA BIOHAZ Panel, 2010).

There has been an increase in notifications of anisakiosis over the past few decades. This is probably due to increased inspection measures for fishery products, higher detection rates of contaminated seafood and of human infections due to improved detection and diagnostic techniques, along with the increasing popularity of raw or lightly cooked fish products (McCarthy and Moore, 2000; Chai et al., 2005; Audicana and Kennedy, 2008; Caldeira et al., 2021). The economic consequences of this parasite can be substantial, caused by increased rejection rates of contaminated products and decreased consumer confidence due to the negative aesthetic effects of visible nematode larvae in seafood products (D'amico et al., 2014; Llarena-Reino et al., 2015). *Anisakis* was ranked fourth of 14 food-borne parasites within the category of international trade importance in a report from the FAO/WHO (2014).

There are very few reported cases of anisakiosis in Portugal (Santos et al., 2022). The first case was reported in 2017 by Carmo et al. (2017). A case–control study by Falcão et al. (2008) found that 7% of the control group in the study population were sensitised to *Anisakis simplex,* while 22.5% of the patients in the case group, suffering from acute, relapsing urticaria, were sensitised. An earlier study by Nunes and Ladeira (2003) demonstrated that 8% of a population tested in a coastal region of

Portugal were allergic to *Anisakis* spp. indicating that exposure to the parasite is occurring in the population. Portugal does not have a tradition of eating raw fish, but this may change with the global trend towards consumption of raw or lightly processed foods (McCarthy and Moore, 2000; Käferstein, 2003). Also, grilling is a common method of cooking fish and this does not always result in sufficiently high temperatures throughout the fish to inactivate larvae. Furthermore, dead *Anisakis* larvae may be allergenic to previously sensitised individuals. Hake gonads are a popular Portuguese dish and this area of the fish is frequently parasitised by *Anisakis* (Santos et al., 2022). The factors outlined above indicate that the Portuguese population is at risk of exposure to this parasite.

2. Description of work programme

2.1. Aims

The work programme had three main aims:

i) Use survey data to characterise the situation in Portugal with regard to the zoonotic fish parasite *Anisakis*. The survey gathered information on general fish consumption, raw fish consumption and on consumer risk perception and attitudes to contamination of fish with *Anisakis* spp. Also, on consumer knowledge of methods of preventing infection.
ii) Examine the risk of anisakiosis in the Portuguese population from consumption of raw or undercooked fish.
iii) Determine the prevalence of *Anisakis* spp. in European hake (*Merluccius merluccius*), captured in North-East Atlantic Portuguese waters.

2.2. Activities/Methods

2.2.1. Assessing raw fish consumption trends and sociodemographic and health characteristics of raw fish consumers

A survey had been carried out prior to the start of the work programme consisting of 33 questions that gathered information on demographics, general health, fish consumption habits for both cooked and raw/undercooked products and knowledge of *Anisakis*. Data from questions regarding raw fish consumption were analysed in conjunction with data from sociodemographic and health-based questions, including those relating to allergy. This allowed analysis of the subset of respondents that consumed raw fish in relation to the type and volume of raw fish consumed, their sociodemographic profile, perceived health status and frequency of allergies.

2.2.2. Assessing consumer risk perception, awareness of *Anisakis* and 'willingness to pay' for *Anisakis*-free fish

Survey respondents were asked to describe what hazards they associated with fish consumption, to indicate if they had any prior knowledge of *Anisakis* and if this had affected their purchasing habits in the past. They were also asked if they were aware of methods to prevent infection with this parasite. In the final section of the survey, respondents were provided with information on *Anisakis* and also presented with a hypothetical scenario regarding a treatment that could remove all parasites and parasite antigens from the fish without affecting the quality. They were then asked if they would be willing to pay extra for such a product and given options of price ranges to choose from, for those that were willing to pay extra. This scenario and question were included to facilitate a contingent valuation study as an additional analysis of the survey data. Contingent valuation (CV) studies are used to assess the monetary trade-offs that consumers would be willing to make for a good or service (Carson, 2012). This approach was used by Bao et al. (2018) to ascertain the value that Spanish consumers would place on *Anisakis*-free fish.

2.2.3. Examine the risk of anisakiosis in the Portuguese population from consumption of raw or undercooked fish

The initial plan for this part of the project had been to carry out a quantitative risk assessment regarding the risk of anisakiosis posed to the Portuguese population from consumption of a given species of fish, raw or undercooked. One of the objectives of assessing raw fish consumption trends (see Section 2.2.1) was to identify a suitable hazard vehicle for this analysis. However, following

analysis of these data, no suitable hazard vehicle was identified. It was decided to focus on the five species most frequently eaten raw or undercooked in Portugal, identified by our survey, and examine the risks that they might pose to the population.

2.2.4. Determining the prevalence and distribution of *Anisakis* spp. larvae in a sample of European hake

2.2.4.1. UV-Press analysis

Forty-five European hake were examined for *Anisakis* L_3. The UV-Press method was used to estimate the number of parasites in the muscle and viscera of the fish. This method was first described by Karl and Leinemann (1993). *Anisakis* larvae fluoresce under UV light after freezing, allowing them to be identified and their location marked on the plastic bag. The larvae were removed from the samples, washed in 0.9% saline solution and examined using a stereoscope to distinguish between Type I and Type II *Anisakis* L_3.

This was the first time that the UV-Press method had been used in the laboratory, so some work was required to optimise the method such as establishing the most suitable light source to obtain the best visualisation of the larvae.

2.2.4.2. Statistical analysis of UV-Press results

From the findings of the UV-Press analysis, the intensity and abundance of infection per fish were calculated, as defined by Bush et al. (1997), as well as the intensity and abundance of infection in the muscle and viscera of each fish. The density of infection in the muscle and viscera was also calculated (Bush et al., 1997). The Mann–Whitney U test was used to establish if measurements such as infection abundance differed significantly between heavier and lighter fish, and Spearman's rank-order correlation was used to evaluate some host–parasite correlations, such as fish weight and length with intensity and density of infection in the muscle and viscera. SPSS version 27 was used for this analysis.

2.2.5. Molecular analysis of *Anisakis* L_3 obtained from a range of fish species

Anisakis L_3 isolated from a sample of 16 fish of the subclass Elasmobranchii and 14 gurnards were selected for identification using conventional PCR. DNA was extracted and amplified by PCR, using primers for Internal Transcribed Spacer rDNA (ITS) (Gasser et al., 1993; Gasser and Hoste, 1995), Mitochondrial-encoded cytochrome c oxidase subunit 2 (COX-2) (Nadler and Hudspeth, 2000) and elongation factor 1 alpha 1 (Mattiucci et al., 2016). PCR products were analysed by electrophoresis on a 1% agarose gel and submitted for forward and reverse Sanger sequencing with an external company. The sequences obtained were analysed using MEGA software and compared with sequences deposited on GenBank.

3. Conclusions

The survey analysis provided the fellow with hands-on training in data analysis and an understanding of how contingent valuation studies are conducted. It resulted in a data set with useful insights into the fish consumption habits of the Portuguese population, their knowledge and perception of risks associated with the parasite *Anisakis* and their awareness of methods of preventing infection.

Through working on the UV press method, the fellow also gained experience in introducing a new method to the host institute laboratory and this allowed collection of data regarding prevalence and distribution of *Anisakis* larvae in European hake from Portuguese waters. In addition, the fellow received training in molecular techniques to allow speciation of *Anisakis* larvae.

Preparation for the quantitative risk assessment that was planned, allowed the fellow to go through the process of gathering and organising data for such an assessment. When it became apparent from analysis of survey data, that no suitable hazard vehicles could be identified, the fellow worked on examining the risks of anisakiosis posed to the Portuguese population from the species of fish most commonly consumed raw in Portugal. This consolidated the knowledge of the topic already gained over the course of the programme, and provided an opportunity to acquire a broader understanding of this food safety issue, through a comprehensive review of the relevant literature.

The fellow also had the opportunity to attend regular on-line seminars at the host institute, learning about a wide variety of research projects conducted there. The EU-FORA modules provided training in a broad range of topics related to risk assessment, along with the opportunity to network with other scientists in this field.

Results of the fellow's project were presented in two posters at the international conference of the European Association of Fish Pathologists 2021 (Annex A and B), and will be published in four articles in peer-reviewed journals.

References

Audicana MT, Ansotegui IJ, De Corres LF and Kennedy MW, 2002. Anisakis simplex: dangerous - dead and alive? Trends in Parasitology, 18, 20–25. https://doi.org/10.1016/S1471-4922(01)02152-3

Audicana MT and Kennedy MW, 2008. Anisakis simplex: from obscure infectious worm to inducer of immune hypersensitivity. Clinical Microbiology Reviews, 21, 360–379. https://doi.org/10.1128/CMR.00012-07

Bao M, Pierce GJ, Strachan NJC, Martínez C, Fernández R and Theodossiou I, 2018. Consumers' attitudes and willingness to pay for Anisakis-free fish in Spain. Fisheries Research, 202, 149–160. https://doi.org/10.1016/j.fishres.2017.06.018

Bronzwaer S, Le Gourierec N and Koulouris S, 2016. Editorial: The European Food Risk Assessment Fellowship Programme (EU-FORA). EFSA Journal 2016;14(11):e14111, 2 pp. https://doi.org/10.2903/j.efsa.2016.e14111

Buchmann K and Mehrdana F, 2016. Effects of anisakid nematodes Anisakis simplex (s.l.), Pseudoterranova decipiens (s.l.) and Contracaecum osculatum (s.l.) on fish and consumer health. Food and Waterborne Parasitology, 4, 13–22. https://doi.org/10.1016/j.fawpar.2016.07.003

Bush A, Lafferty K, Lotz J and Shostak A, 1997. Parasitology meets ecology on its own terms: Margolis et al revisited. Journal of Parasitology, 83, 575–583. Available online: https://www.ncbi.nlm.nih.gov/pubmed/9267395

Caldeira AJR, Alves CPP and Santos MJ, 2021. Anisakis notification in fish: an assessment of the cases reported in the European Union Rapid Alert System for Food and Feed (RASFF) database. Food Control, 124, 107913. https://doi.org/10.1016/j.foodcont.2021.107913

Caramello P, Vitali A, Canta F, Caldana A, Santi F, Caputo A, Lipani F and Balbiano R, 2003. Intestinal localization of anisakiasis manifested as acute abdomen. Clinical Microbiology and Infection, 9, 734–737. https://doi.org/10.1046/j.1469-0691.2003.00660.x

Carmo J, Marques S, Bispo M and Serra D, 2017. Anisakiasis: a growing cause of abdominal pain! BMJ Case Reports, 2017, 2016–2017. https://doi.org/10.1136/bcr-2016-218857

Carson RT, 2012. Contingent valuation: a practical alternative when prices aren't available. Journal of Economic Perspectives, 26, 27–42. https://doi.org/10.1257/jep.26.4.27

Chai JY, Murrell KD and Lymbery AJ, 2005. Fish-borne parasitic zoonoses: status and issues. International Journal for Parasitology, 35, 1233–1254. https://doi.org/10.1016/j.ijpara.2005.07.013

D'amico P, Malandra R, Costanzo F, Castigliego L, Guidi A, Gianfaldoni D and Armani A, 2014. Evolution of the Anisakis risk management in the European and Italian context. Food Research International, 64, 348–362. https://doi.org/10.1016/j.foodres.2014.06.038

Daschner A, Alonso-Gómez A, Cabañas R, Suarez-de-Parga JM and López-Serrano MC, 2000. Gastroallergic anisakiasis: borderline between food allergy and parasitic disease - Clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by Anisakis simplex. Journal of Allergy and Clinical Immunology, 105, 176–181. https://doi.org/10.1016/s0091-6749(00)90194-5

EFSA BIOHAZ Panel, 2010. Scientific Opinion on risk assessment of parasites in fishery products. EFSA Journal 2010;8(4):1543, 45 pp. https://doi.org/10.2903/j.efsa.2010.1543

Falcão H, Lunet N, Neves E, Iglésias I and Barros H, 2008. Anisakis simplex as a risk factor for relapsing acute urticaria: a case-control study. Journal of Epidemiology and Community Health, 62, 634–637. https://doi.org/10.1136/jech.2007.061572

FAO/WHO, 2014. Multicriteria-based ranking for risk management of food-borne parasites. In W. H. Organization (Ed.), Microbiological Risk Assessment Series, Food and Agriculture Organization of the United Nations/World Health Organization (Issue No.23). World Health Organization. Available online: https://www.who.int/iris/handle/10665/112672

Gasser RB, Chilton NB, Hoste H and Beveridge I, 1993. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. Nucleic Acids Research, 21, 2525–2526. https://doi.org/10.1093/nar/21.10.2525

Gasser RB and Hoste H, 1995. Genetic markers for closely-related parasitic nematodes. Molecular and Cellular Probes, 9, 315–319. https://doi.org/10.1016/S0890-8508(95)91588-5

Käferstein FK, 2003. Actions to reverse the upward curve of foodborne illness. Food Control, 14, 101–109. https://doi.org/10.1016/S0956-7135(02)00017-8

Karl H and Leineman M, 1993. A fast and quantitative detection method for nematodes in fish fillets and fishery products. Archiv Fur Lebensmittelhygiene, 44, 124–125.

Klimpel S, Palm HW, Rückert S and Piatkowski U, 2004. The life cycle of Anisakis simplex in the Norwegian Deep (northern North Sea). Parasitology Research, 94, 1–9. https://doi.org/10.1007/s00436-004-1154-0

Lima dos Santos CAM and Howgate P, 2011. Fishborne zoonotic parasites and aquaculture: a review. Aquaculture, 318, 253–261. https://doi.org/10.1016/j.aquaculture.2011.05.046

Llarena-Reino M, Abollo E, Regueira M, Rodríguez H and Pascual S, 2015. Horizon scanning for management of emerging parasitic infections in fishery products. Food Control, 49, 49–58.

Mattiucci S, Acerra V, Paoletti M, Cipriani P, Levsen A, Webb SC, Canestrelli D and Nascetti G, 2016. No more time to stay 'single' in the detection of Anisakis pegreffii, A. simplex (s. s.) and hybridization events between them: a multi-marker nuclear genotyping approach. Parasitology, 143, 998–1011. https://doi.org/10.1017/S0031182016000330

Mattiucci S, Cipriani P, Levsen A, Paoletti M and Nascetti G, 2018. Molecular Epidemiology of Anisakis and Anisakiasis: An Ecological and Evolutionary Road Map. In Advances in Parasitology (1st ed., Vol. 99). Elsevier Ltd. https://doi.org/10.1016/bs.apar.2017.12.001

McCarthy J and Moore TA, 2000. Emerging helminth zoonoses. International Journal for Parasitology, 30, 1351–1359. https://doi.org/10.1016/S0020-7519(00)00122-3

Nadler SA and Hudspeth DSS, 2000. Phylogeny of the ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. Journal of Parasitology, 86, 380–393. https://doi.org/10.1645/0022-3395(2000)086[0380:POTANA]2.0.CO;2

Nunes C and Ladeira S, 2003. Allergy to Anisakis simplex in the Portuguese Population. Journal of Allergy and Clinical Immunology, 111, 255.

Santos MJ, Matos M, Guardone L, Golden O, Armani A, Caldeira AJR and Vieira-pinto M, 2022. Preliminary Data on the Occurrence of Anisakis spp. in European Hake (Merluccius merluccius) Caught off the Portuguese Coast and on Reports of Human Anisakiosis in Portugal. Microorganisms, 10, 331.

Smith JW and Wootten R, 1978. Anisakis and Anisakiasis. Advances in Parasitology, 16, 93–163.

Van Thiel PH, 1962. Anisakiasis. [Abstract]. Parasitology, 52, 16P–17P.

Van Thiel PH and Van Houten H, 1966. The Herring Worm Anisakis marina as a Human Parasite outside the Wall of the Gastro-Intestinal Tract. [Abstract]. Nederlands Tijdschrift Voor Geneeskunde, 110, 1524–1528. Available online: https://www.cabdirect.org/cabdirect/abstract/19672900175

WHO, 2015. WHO estimates of the global burden of foodborne diseases. In World Health Organisation. https://doi.org/10.1007/978-3-642-27769-6_3884-1

Abbreviations

CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
CV	Contingent Valuation
DALY	Disability Adjusted Life Years
FAO	Food and Agriculture Organization
L3	Stage-three parasite larva
PCR	Polymerase Chain Reaction
QMRA	Quantitative Microbiological Risk Assessment
UV	Ultraviolet
WHO	World Health Organisation

Annex A – Poster on UV-Press method results presented at the European Association of Fish Pathologists 20th international conference (virtual) on diseases of fish and shellfish 20–23 September 2021

Anisakis spp. in European hake (*Merluccius merluccius*) – a risk to Portuguese consumers?

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INTRODUCTION

Anisakiosis is a fish-borne zoonosis, caused by parasitic nematodes of the genus *Anisakis.* These marine parasites have an indirect life cycle, using marine mammals, usually cetaceans, as their definitive host. Humans are accidental hosts for *Anisakis* spp; the larvae do not mature, but on reaching the gastrointestinal tract can cause disease.

Portugal has one of the highest levels of fish consumption in the world and the European hake is one of the most popular fish in Portugal.

AIMS

Establish the level of contamination of European hake with *Anisakis* spp., in a sample caught in Portuguese waters. And characterise the distribution of infection between the viscera and muscle of infected fish.

MATERIAL & METHODS

Forty-five European hake of mixed age were measured, weighed and the viscera and muscle were examined for *Anisakis* spp. larvae using the UV-Press Method.

RESULTS

The fish analysed had a mean±SD length of 31.6±3.7 cm and weight of 212.6±85.7 g.

A total of 473 Anisakis stage 3 larvae were found, with a prevalence of 95.6% (95% CI 89.5-100%).

Three fish were fully eviscerated and these were excluded when calculating the intensity, abundance and density per gram of viscera.

A significantly higher number of larvae were found in the viscera and the muscle of the larger fish (Mann-Whitney test, Z = -2.21, and p<0.03). However, the muscle density values were not significantly different between large and small fish (Mann-Whitney test, Z =-0.07, and p=0.95).

Fish length was significantly, positively correlated with the total larval abundance per fish (Spearman correlation coefficient: ρ =0.64, p<0.01), the visceral abundance (ρ = 0.68, p<0.01) and the muscle abundance (ρ =0.393, p<0.01). Also with the density of parasites in the viscera (ρ = 0.49, p<0.01).
Fish weight was significantly, positively correlated with the total larval abundance per fish (ρ = 0.63, p<0.01), the visceral abundance (ρ = 0.67 , p<0.01) and the muscle abundance (ρ =0.38, p<0.01). And with the density of parasites in the viscera (ρ = 0.45, p<0.01).
The larval abundance in the viscera was significantly correlated with the larval abundance in the muscle (ρ = 0.44, p<0.01).

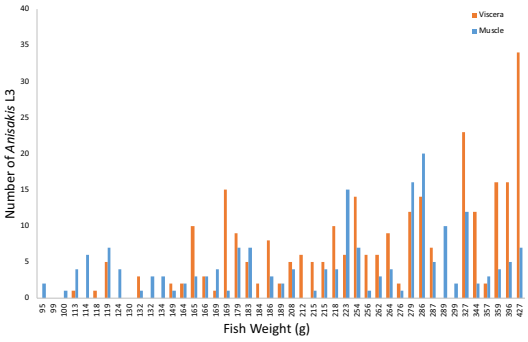

Figure 1. *Anisakis* spp. Larval Stage 3 Abundance in Muscle and Viscera

	Mean	Standard Deviation	Range
Intensity	11.3	9.7	1-41
Total Abundance	11	9.8	0-41
Visceral Abundance	6.6	7.1	0-34
Muscle Abundance	4.4	4.4	0-20
Density per gram of viscera	0.54	0.5	0-2.53
Density per gram of muscle (n=45)	0.05	0.04	0-0.16

Table 1. Summary of results for forty-two European hake caught in Portuguese waters in April 2021. Fish were examined for stage 3 *Anisakis* spp. larvae using the UV-press method.

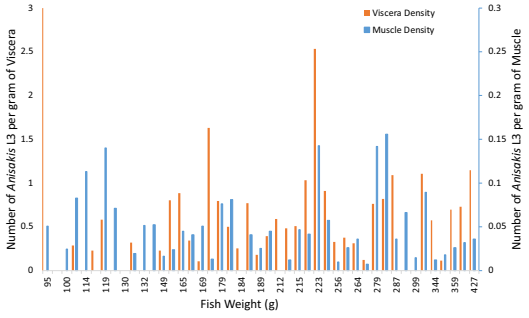

Figure 2. *Anisakis* spp. Larval Stage 3 Density per Gram of Viscera and Muscle

CONCLUSIONS

There is not a tradition of consuming raw or undercooked fish in Portugal, however these products are becoming increasingly popular, and common cooking methods such as grilling do not always reach sufficient temperatures to ensure death of parasite larvae. Although there are few reports of anisakiosis in Portugal, studies in other, neighbouring, countries have highlighted that this is a highly under-reported disease due to the non-specific symptoms and lack of awareness of the condition. Our findings form a basis from which we can establish a more accurate estimate of the risk posed to Portuguese consumers from the consumption of hake. The high prevalence of *Anisakis* larvae in our sample highlights a need for consumer education about this parasite.

REFERENCES

Almeida, C., Karadzic, V., & Vaz, S. (2015). The seafood market in Portugal: Driving forces and consequences. Marine Policy, 61(November), 87–94. https://doi.org/10.1016/j.marpol.2015.07.012
Bao, M., Pierce, G. J., Pascual, S., González-Muñiz, M., Mattiucci, S., Mladineo, I., Cipriani, P., Bušelić, I., & Strachan, N. J. C. (2017). Assessing the risk of an emerging zoonosis of worldwide concern: Anisakiasis. Scientific Reports, 7, 1–17. https://doi.org/10.1038/srep43699
EUMOFA. (2021). The EU Fishmarket. https://www.eumofa.eu/en/portugal.
Karl,H., & Leineman, M. (1993). A fast and quantitative detection method for nematodes in fish fillets and fishery products. Archiv Fur Lebensmittelhygiene, 44(5), 124-125
Mattiucci, S., Cipriani, P., Levsen, A., Paoletti, M., & Nascetti, G. (2018). Molecular Epidemiology of Anisakis and Anisakiasis: An Ecological and Evolutionary Road Map. In Advances in Parasitology (1st ed., Vol. 99). Elsevier Ltd. https://doi.org/10.1016/bs.apar.2017.12.001
Silva, M. E. R., & Eiras, J. C. (2003). Occurrence of Anisakis sp. in fishes off the Portuguese West coast and evaluation of its zoonotic potential. Bulletin of the European Association of Fish Pathologists, 23(1), 13–17.
Smith, J. W. and, & Wootten, R. (1978). Anisakis and Anisakiasis. Advances in Parasitology, 16, 93–163.

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Annex B – Poster on survey results presented at the European Association of Fish Pathologists 20th international conference (virtual) on diseases of fish and shellfish 20–23 September 2021

Anisakis spp. and the potential risks to human health; an assessment among consumers in Portugal

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INTRODUCTION

Anisakiosis is a zoonosis resulting from the accidental ingestion of viable *Anisakis* spp. larvae in raw or undercooked fish products. Portugal has one of the highest levels of fish consumption in the world and although raw fish is not part of the traditional Portuguese diet, the growth of international trade and increasing popularity of lightly cooked or raw food means that consumers may be at increased risk of exposure to this parasite.

AIMS

To assess the level of knowledge within the Portuguese population of *Anisakis* spp. and their potential risks; also, to assess awareness of methods to prevent infection.

MATERIALS & METHODS

A questionnaire consisting of 33 questions was circulated online. There were 746 respondents. Information was collated on socio-demographic factors and fish-consumption habits. Data on consumers' attitudes to contamination of fish with *Anisakis* spp., risk perception and knowledge of prevention methods were also analysed.

RESULTS

The majority of respondents were aged between 30 and 49 (55%) and were female (72%). About 67% had post-graduate degrees, 39% of respondents worked in the field of Biological and Health Sciences.

Most respondents (86%) cited "transmission of parasites" as a risk associated with the consumption of raw fish. Most respondents had not heard of *Anisakis* spp. (66%) or prevention methods (79%). Of those that stated they were aware of prevention methods, the majority cited "cooking thoroughly" and "freezing" as the most important.
Only 7% of people have avoided buying or eating fish due to the presence of worms. In these cases, the fish most commonly avoided were cod (29%), anchovies (26%), salmon or trout (22%).

Thirty-five per cent of respondents would be willing to pay between € 1 and € 2.5 extra for a fish product that was treated to remove *Anisakis* larvae and allergens. Thirteen percent of respondents stated that they would not buy this *Anisakis*-free product and 25% would not pay any extra for it. In these two cases, the main reason given was that they should not have to pay extra to have access to safe food.

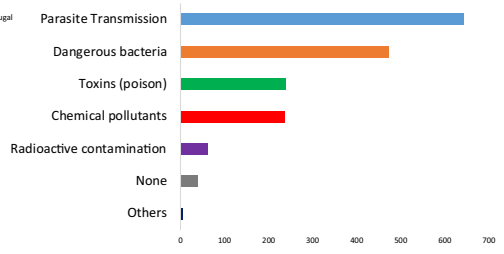

Figure 1. Survey responses regarding hazards associated with fish consumption

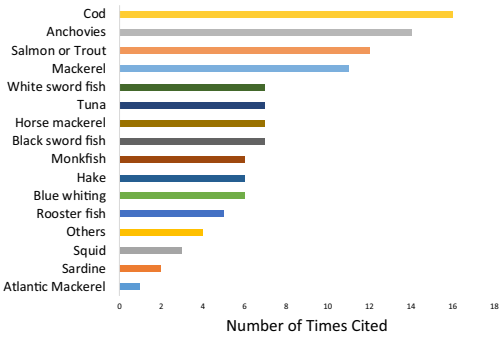

Figure 2. Types of fish avoided by the **55** respondents that have avoided buying or eating fish due to concerns about *Anisakis* spp.

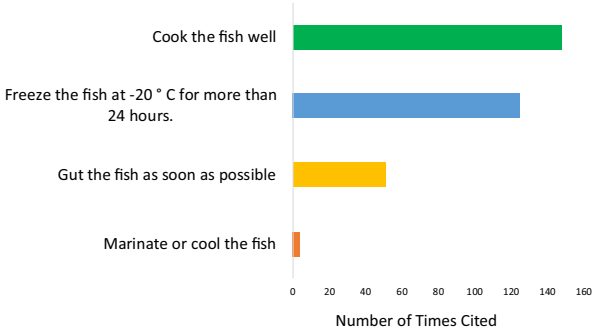

Figure 3. Prevention methods cited by the **156** respondents that stated they had both heard of *Anisakis* spp. and were aware of prevention methods.

CONCLUSIONS

A significant number of participants had little or no knowledge of *Anisakis* spp., the risks associated with them and prevention methods. The majority of respondents had a post-graduate degree, mostfrequently in th e area of Biological or Health Sciences. Itis clear thatthere is a need to raise awareness ofthis parasite, so that consumers know the risks assoc iated with eating raw or lightly processed fish, and are familiar with effective methods to preventinfection. This information should be inclu ded in consumer awareness programs regarding safe food.

REFERENCES

Almeida, C., Karadzic, V., & Vaz, S. (2015). The seafood marketin Portugal: Driving forces and consequences. Marine Policy, 61(November), 87–94. https://doi.org/10.1016/j.marpol.2015.07.012
EFSA BIOHAZ. (2010). Scientific Opinion on risk assessment of parasites in fishery products. EFSA Journal, 8(4). https://doi.org/10.2903/j.efsa.2010.1543
Mattiucci, S., Cipriani, P., Levsen, A., Paoletti, M., & Nascetti, G. (2018). Molecular Epidemiology of Anisakis and Anisakiasis:An Ecological and Evolutionary Road Map. In Advances in Parasitology (1st ed., Vol. 99). Elsevier Ltd. https://doi.org/10.1016/bs.apar.2017.12.001
Silva, M. E. R., & Eiras, J. C. (2003). Occurrence of Anisakis sp.in fishes off the Portuguese West coast and evaluation ofits zoonoti c potential. Bulletin ofthe European Association of Fish Pathologists, 23(1), 13–17.
Smith, J. W. and, & Wootten, R. (1978). Anisakis and Anisakiasis. Advances in Parasitology, 16, 93–163

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EU-FORA SERIES 4

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Risk assessment of rare earth elements, antimony, barium, boron, lithium, tellurium, thallium and vanadium in teas

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Abstract

In recent years, a great intensification in the use of various elements especially in modern technology can be observed. However, the anthropogenic activities, including industrialisation, urbanisation or intensive agriculture, have led to the release of many of the elements into the environment. The consequence of the accumulation of the elements both in soil and water systems is their presence in the food chain. Inhalation and consumption of the contaminated food and beverages have been indicated as the main pathways of the exposure to many elements. Due to the fact, that tea is considered the second most popular beverage worldwide and its consumption is constantly increasing, it is crucial to evaluate the safety of the product, especially for toxic elements contamination. Thus, the aim of the project was to evaluate the contamination levels of rare earth elements (REEs) including lanthanides, scandium (Sc) and yttrium (Y) and also antimony (Sb), barium (Ba), boron (B), lithium (Li), tellurium (Te), thallium (Tl) and vanadium (V) in teas. Subsequently, the risk assessment was carried out. Additionally, the Fellowship provided hands-on training on the evaluation of applications of new biocides and participation in the science-based advises given to the Danish Food and Veterinary Administration, Danish Environment Protection Agency and Danish Medical Agency.

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Keywords: tea, rare earth elements, ICP-MS, toxic elements, risk assessment

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1. Introduction

In the last few decades, there has been an intensive increase in technological development, which has involved the use of many chemical elements. Anthropogenic activities, including industrialisation, urbanisation or intensive agriculture, have already altered the natural occurrence state of many of the elements (Fedele et al., 2008). Release to the environment, and subsequent accumulation in soil, water and organisms has consequently led to the presence of many potentially toxic elements in the food chain, which may affect animal and human health.

Until now, some of the elements including lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As) or aluminium (Al) have been widely known and studied, especially as food contaminants (Pearson and Ashmore, 2020). However, it can be expected that humans, through consumption of various foods can be exposed to several other elements, which may reveal toxic potential. Still, there is a wide group of elements that has been poorly studied, both toxicologically and as food contaminants. Especially, little is known about the dietary exposure to a group of elements called rare earth elements (REEs).

REEs include 17 elements out of which 15 are named lanthanides, including lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium(Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium(Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and lutetium (Lu). Two additional elements have been classified as REEs, namely yttrium (Y) and scandium (Sc) (Gwenzi et al., 2018). REEs are chemically uniform with similar physical and chemical properties. Due to their specific physical properties, they found a broad application in the modern technology e.g. catalysis, electronics, mobile communication, LED light bulbs, wind turbines, electric cars, fuel cells and fuel additives (Gwenzi et al., 2018; Squadrone et al., 2018; Doulgeridou et al., 2020). REEs has also been used in the production of fertilisers, especially in China (Wang et al., 2003; Gwenzi et al., 2018; Squadrone et al., 2018; Doulgeridou et al., 2020), and it was estimated that 5200 tons of REE-enriched fertilisers, used as growth promoters, were released into the cultivated soil in China only in 2002 (Li et al., 2013). Due to the broad use of REEs and a potential release to the environment, they began to be perceived as emerging contaminants (Squadrone et al., 2018).

Similarly to REEs, lithium (Li) has numerous industrial and commercial applications e.g. as catalyst of chemical reactors, component of batteries or as sanitising agent for swimming pools, hot tubs and spas (EPA, 2008). Some additional elements including tellurium (Te) and thallium (Tl), despite their high toxicity, are also widely used. Thallium is used in semiconductor materials, photocells, infrared measuring devices and as a catalyst in various organic synthesis procedures. In some countries, it is also used for the production of pesticides (Willner et al., 2021). Tellurium is mainly applied in the production of cadmium telluride thin-film solar cells, followed by thermo-electrics (Willner et al., 2021). In the case of barium (Ba), it has been already evaluated that the industrial (e.g. petroleum industry, steel industry, production of semiconductors) and medicinal application more than doubled during the last 40 years (Kravchenko et al., 2014).

Industrial intensification and increasing presence of electronic waste worldwide are becoming a paramount problem leading to contamination of soil and water. It can be therefore expected that elevated concentrations of many elements will be present in various plants and consequently contribute to an increased consumer exposure to REEs.

Due to the fact that tea is considered the second most popular beverage worldwide (Wang et al., 2020) and its consumption is constantly increasing (Vieux et al., 2019), it is crucial to evaluate the safety of the product, especially for toxic elements contamination.

Aside of the essential trace elements such as potassium (K), manganese (Mn), selenium (Se), zinc (Zn), strontium (Sr) and copper (Cu), teas can contain various chemical elements that can be harmful and which cannot be eliminated while processing or tea infusions preparation (Zhang et al., 2018). Depending on tea origin, accumulation of various elements can occur naturally or result from manufacturing and agronomic processes (de Oliveira et al., 2018).

As tea has the ability to accumulate REEs in a higher degree than other major food crops (Wang et al., 2020), and fertilisers containing REEs have been also used for tea production (Wang et al., 2003, 2020), it can be expected that tea may contain elevated concentrations of REEs compared to other plants (Wang et al., 2020). As many other elements can also be present in tea plants, besides REEs, a set of elements including antimony (Sb), barium, boron (B), lithium, tellurium, thallium and vanadium (V) was also investigated.

2. Description of work programme

2.1. Aims

The aim of the project was to estimate the exposure of the adult Danish population to REEs and other selected elements, including Sb, B, Ba, Li, Te, Tl, V resulting from tea consumption. Finally, for each element, the risk assessment was performed. In the case of REEs, due to their similar properties and lack of toxicological data on all of the elements, the exposure and risk assessments were carried out for the sum of the analysed elements.

2.2. Activities/Methods

2.2.1. First part included a literature search (PubMed, Scopus, Science Direct) for the elements, besides REEs that could be incorporated into the scope of the project. The next step regarded identification of the most important dietary sources of REEs and other elements included in the study.

2.2.2. Analysis of a selection of dry teas (black, green – *Camellia sinensis*, and rooibos – *Aspalathus linearis*) for their content of REEs, Sb, B, Ba, Li, Ta, Tl and V with inductively coupled plasma triple quad mass spectrometry (TQ ICP-MS, Thermo Scientific). Samples of teas were grinded and homogenised before the analysis, and subsequently, a test portion (0,3 g) was subjected to acidic digestion with the use of microwave oven. Afterwards, samples were analysed by iCAP™ TQ ICP-MS with Standard, Kinetic Energy Discrimination and Oxygen Reaction modes. The quality of the analytical methods was assured by simultaneous analysis of a certified reference material for REEs (BCR-670), and adherence to European standard methods EN 13805:2014 and EN 15763:2009.

Since teas are consumed as an infusion, to evaluate the real ingestion of the investigated elements, the transfer rates of the elements to the infusion were also measured.

2.2.3. Exposure assessment combined data on REEs from the present study with consumption data from a consumer survey among Danish citizens. Only adult (> 18 years old) consumers were included. A consumer was defined as a person consuming min. one cup of tea per week. The estimated average consumption was 350 mL per day, and the high consumption (1,084 mL per day) was represented by 95th percentile (P95). For the purpose of risk assessment, an average body weight of 70 kg was adopted. Exposure was estimated using the average concentrations corrected with the transfer rates and the average consumption (scenario 1). However, as regards tea, one can expect the phenomenon called 'brand loyalty', which in the case of high contamination of the product can lead to long-term exposure to elevated concentrations of the hazards, additional exposure scenarios were taken into consideration. One, representing the case of the average consumption with exposure to high concentration (P95) (scenario 2), and the other, representing high consumption (P95) and high contamination (P95) of the products (scenario 3).

2.2.4. Risk assessment of the dietary exposure to REEs, Sb, B, Ba, Li, Ta, Tl and V resulting from tea consumption by Danish consumers.

3. Conclusions

3.1. Risk assessment

Rare Earth Elements: The group of REEs in the study consisted of 16 elements (La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Gd, Tb, Dy, Tm, Yb, Y, Sc). A key toxicological characteristic of the REEs is their common ability to displace calcium from calcium-binding sites in living systems, resulting in enzyme inhibition or other biochemical dysfunctions (Palasz and Czekaj, 2000). Some other potential human health effects include dysfunctional neurological disorders e.g. reduced intelligence quotient (IQ) in children, associated mainly with La (Gwenzi et al., 2018; Wang et al., 2019) and bone alteration (Zaichick et al., 2011). Additionally, genotoxicity and fibrotic tissue injury associated with several REEs were observed (Gwenzi et al., 2018). In the case of La, multiple adverse effects on various organs including liver, kidney and lung as well as the nervous system of animals were reported. The effects were related to the oxidative stress, disturbance of the homoeostasis of essential elements and enzymes as well as histopathological changes (Liu et al., 2010).

Since the interest in REEs is only recently increasing, there is no sufficient toxicological data on all individual elements. Consequently, there are no health-based guidance values for REEs derived by any of the authorities. For the purpose of risk assessment, we have adopted the values from toxicity studies on some of the REEs.

Ninety-day studies conducted according to the guidance of the Organization for Economic Co-operation and Development (OECD) with the established no observed adverse effect levels (NOAEL) were available for La, Ce, Y. Based on the NOAELs, tolerable daily intake (TDI) values were derived for La, Ce and Y. For La, 51.3 μg/kg body weight (bw) per day for a decreased body weight was derived (Fang et al., 2018). In the case of Ce, TDI was set at 161.5 μg/kg bw per day from NOAEL for induced weight loss, decreased erythrocyte, albumin, total bilirubin and phosphocreatine kinase as well as increased leukocytes in animals (Wu et al., 2019). For yttrium, the TDI of 145.5 μg/kg bw per day was proposed, based on NOAEL for multiple changes in mortality, clinical signs, daily food consumption and weekly body weights, urinalysis, haematology, blood coagulation, clinical biochemistry and histopathology of all the main organs/tissues except lung (Wang et al., 2017). The lowest TDI, derived for La of 51.3 μg/kg bw per day, was adopted as the reference vale for the sum of all REEs. The decision was based on the fact that La was one of the predominant elements in all analysed teas, as it was determined at one of the highest concentrations together with Ce and Y, thus had a major contribution to the overall contamination.

High uncertainty is associated with the adaptation of TDI derived for La, for the sum of REEs, as there is insufficient toxicological data on other elements. It is possible that other elements could have higher toxicity comparing to La, and adapted TDI could be insufficiently protective, and risk could have been underestimated.

The evaluated exposure to REEs (Table A.1, Appendix A) ranged from 7.57 to 58.06 ng/kg bw, depending on the tea type. However, as the highest exposure would constitute only 0.1% of the TDI, it can be said that the risk of adverse effects caused by REEs is rather not expected from the prolonged ingestion of teas.

Antimony: In the case of Sb, oral exposure predominantly affects the gastrointestinal system resulting with burning stomach pains, colic, nausea and vomiting (Sundar and Chakravarty, 2010). The health effects observed in animals orally exposed to higher doses of Sb included hepatocellular vacuolisation, haematological alterations such as decreases in red blood cell counts and haemoglobin levels and histological alterations in the thyroid (Atsdr, 2019). For Sb, the suggested NOAEL in the subchronic drinking water study in rats was established as 6.0 mg/kg bw per day based on decreased body weight gain and reduced food and water intake. By the application of an uncertainty factor of 1,000 (100 for intra- and interspecies variation and 10 for the use of a subchronic study), a TDI of 6 μg/kg bw was determined (WHO, 2003). Taking into the consideration the evaluated exposure to Sb, the daily intake from tea consumption would represent only 0.03% of a TDI, confirming the negligible risk to the health of the consumers.

Barium: Human and animal high-dose exposure to soluble Ba compounds results in a number of effects including electrocardiogram abnormalities, ventricular tachycardia, hypertension and/or and hypotension, muscle weakness and paralysis (SCHER, 2012). However, kidney effects are considered the most sensitive health effect associated with long-term ingestion of Ba (Kravchenko et al., 2014). Due to the marked severity of nephropathy, the lower confidence limit of the benchmark dose for a 5% response ($BMDL_{05}$) was selected over the typically 10% incidence as a point of departure. By applying an assessment factor of 300, a TDI of 0.2 mg/kg bw per day was derived (SCHER, 2012). In the case of the highest exposure resulting from black tea consumption, the TDI would be covered only in 0.3%, meaning that there is a negligible risk of the adverse health effect caused by Ba ingestion with tea.

Boron is not an essential nutrient for humans and any specific biochemical functions have not been identified. There is however, some evidence that, in humans, B may influence the metabolism and utilisation of other nutrients, especially calcium, and may have a beneficial effect on bone calcification and maintenance (EFSA, 2004a; Zioła-Frankowska et al., 2014). Symptoms related to B intoxication includes gastrointestinal disturbances, granular degeneration of tubular cells, exfoliate dermatitis, epilepsy, cardio-circulatory collapse. Congestion of the brain, hair loss, lethargy, anorexia and mental confusion were other identified effects (EFSA, 2006). The most sensitive endpoint of toxicity of B was, however, a developmental toxicity (Murray and Schlekat, 2004). A tolerable upper intake level (UL) was based on the decreased fetal body weight in rats resulting from maternal boron intake during pregnancy. The NOAEL for this effect (9.6 mg/kg bw per day) was extrapolated to humans by application of uncertainty factor of 60 (including intra- and interspecies variability) to give an UL of 0.16 mg/kg bw per day (EFSA, 2004a).

Consumption of rooibos tea would lead to the highest exposure to B compared to consumption of the other analysed teas. However, it would represent only 2.6% of the allowed UL. Based on the outcome, it can be said that the risk for the tea consumers related to the B would be very low.

Lithium is used as a treatment in the bipolar affective disorder; hence, most of the toxicological studies are based on clinical investigations of the patient subjected to Li treatment. The element has been identified as having an adverse renal effect, with the most common being nephrogenic diabetes insipidus. However, some additional adverse effects on thyroid function, primarily asymptomatic hypothyroidism have been observed in patients treated with Li (McKnight et al., 2012). In the case of lithium, the provisional subchronic and chronic reference dose (p-RfD) was derived from the lowest observed adverse effect level (LOAEL) of 2.1 mg/kg per day for adverse effects in several organs and systems. The LOAEL was divided by an assessment factor of 1,000, yielding a subchronic and chronic p-RfD of 2 μg/kg per day (EPA, 2008). The highest exposure from tea consumption was evaluated for rooibos. However, it would constitute only 8.14% of p-RfD, reflecting low risk to the health of the tea consumers.

Tellurium is an element with chemical properties resembling those of non-metals, such as sulfur, however, if the toxicity is concerned, the properties are closer to the effects caused by selenium (Health Council of the Netherlands, 2014). The clinical manifestation of the ingestion of substantial concentrations of Te includes vomiting, nausea, metallic taste, black discoloration of the oral mucosa and skin, corrosive gastrointestinal tract injury and a characteristic garlic-like odour of the breath (Vávrová et al., 2021). In long-term drinking water studies in rats and mice, no evidence of carcinogenic effects were found (Greim, 2005) .

Concentrations of Te determined in teas were very low and often below the limit of quantification of the method; in some samples, the element was not determined (data not shown). Due to the absence of the elements or very low determined concentration, tellurium was not included in the exposure assessment and in the general risk assessment.

Thallium: In the case of thallium, it is known that its salts can cause a wide spectrum of adverse effects in humans and animals, and thallium is considered a cumulative poison (EPA, 2009). Acute thallium poisoning is usually accompanied by gastrointestinal symptoms, while neurological findings (sensory and motor changes) predominate in chronic exposure. Other symptoms include polyneuritis, encephalopathy, tachycardia and degenerative changes of the heart, liver and kidneys (Cvjetko et al., 2010). P-RfD values for thallium of 1×10^{-5} mg/kg per day (for hair follicle atrophy) were derived by Environmental Protection Agency (EPA) (EPA, 2009, 2012). Taking into account the highest estimated exposure of 1.48 ng/kg bw, resulting from drinking black tea, it can be said that the risk for the human health is low, as the exposure covers 14.8% of the p-RfD.

Vanadium has not been shown to be essential for humans or possess any nutritional value. The most common non-occupational sources of vanadium exposure are contaminated food and drinking water (Rodríguez-Mercado et al., 2011; Crebelli and Leopardi, 2012). High concentrations of V may cause irreversible damage to the kidneys (EFSA, 2004b). However, vanadium in mammalian species can accumulate in the liver, kidneys, bones, lungs and spleen (Rodríguez-Mercado et al., 2011; Crebelli and Leopardi, 2012). Vanadium compounds may initiate some gastrointestinal problems such as diarrhoea, vomiting, general dehydration with weight reduction, intestinal inflammation and a characteristic green tongue (Wilk et al. 2017). In the case of V, the reference dose (RfD) of 7 μg/kg bw per day was derived by the EPA. The dose was based on gastrointestinal disturbance (intestinal cramping and diarrhoea) observed in human studies (EPA, 2006). The exposure to V through consumption of teas is low, ranging from 0.1 to 2.6 ng/kg bw, contributing only to 0.04% of RfD in the case of the highest exposure. Thus, the risk of adverse effects is not expected from the prolonged ingestion of teas.

Due to the lack of substantial information on the toxicity of most of the elements from the REEs group, the related uncertainty should be evaluated as high. Some of the REEs may reveal higher toxicity than this established for lanthanum. Thus, the applied TDI would not be enough protective. Consequently, the assessment would lead to the underestimation of the risk. However, due to the fact that lanthanum, cerium and yttrium constituted 60% of total REEs contamination, the contribution from other elements is significantly lower, and performed risk assessment should provide a reliable outcome. An additional factor contributing to the uncertainty of the assessment is the fact that some of the consumers can brew tea longer than 3 min that was used in the transfer rate study. Therefore, higher rates of the elements can be leached into infusions and thus contribute to higher exposure. As the exposure to most of the elements was contributing to a small per cent of the tolerable daily intakes, the whole uncertainty of the risk assessment could be evaluated as low, and moderate in the case of REEs.

3.2. Final conclusions

The analysis of tea samples from the Danish market for the determination of REEs, Sb, B, Ba, Li, Te, Tl, V was carried out. The aim of the analysis was to determine the contamination levels and subsequently evaluate the risk related to the exposure of adult consumers of tea to these elements. The risk assessment revealed that exposure to all investigated elements through consumption of tea poses a negligible risk to the consumers and no adverse effects are expected even for high consumers.

Overall, the work programme allowed the fellow to gain knowledge and practical skills on risk assessment. Additionally, the fellow gained a practical knowledge on ICP-MS analysis and sample preparation for elemental analysis. Results of the project were presented as a EUROTOX conference poster and are planned to be published as a scientific paper in a peer-reviewed scientific journal.

References

Atsdr, 2019. Toxicological Profile for Antimony and Compounds. Available online: https://www.atsdr.cdc.gov/toxprofiles/tp23.pdf

Crebelli R and Leopardi P, 2012. Long-term risks of metal contaminants in drinking water: a critical appraisal of guideline values for arsenic and vanadium. Annali Dell'istituto Superiore Di Sanita, 48, 354–361. https://doi.org/10.4415/ANN_12_04_03

Cvjetko P, Cvjetko I and Pavlica M, 2010. Thallium toxicity in humans. Arhiv Za Higijenu Rada I Toksikologiju, 61, 111–119. https://doi.org/10.2478/10004-1254-61-2010-1976

Doulgeridou A, Amlund H, Sloth JJ and Hansen M, 2020. Review of potentially toxic rare earth elements, thallium and tellurium in plant-based foods. EFSA Journal 2020;18(S1):e181101, 11 pp. https://doi.org/10.2903/j.efsa.2020.e181101

EFSA (European Food Safety Authority), 2004a. Opinion of the Scientific Panel on Dietetic products, nutrition and allergies [NDA] related to the Tolerable Upper Intake Level of Boron (Sodium Borate and Boric Acid). EFSA Journal 2004;2(8):80, 22 pp. https://doi.org/10.2903/j.efsa.2004.80

EFSA (European Food Safety Authority), 2004b. Opinion of the Scientific Panel on Dietetic products, nutrition and allergies [NDA] related to the Tolerable Upper Intake Level of Vanadium. EFSA Journal 2004;2(3):33, 45 pp. https://doi.org/10.2903/j.efsa.2004.33

EFSA (European Food Safety Authority), 2006. Scientific Committee on Food Scientific Panel on Dietetic Products, Nutrition and Allergies. Tolerable upper intake levels for vitamins and minerals. https://www.efsa.europa.eu/sites/default/files/efsa_rep/blobserver_assets/ndatolerableuil.pdf

EPA (Environmental Protection Agency), 2006. Inorganic Contaminant Accumulation in Potable Water Distribution Systems. https://www.epa.gov/sites/default/files/2021-05/documents/issuepaper_tcr_inorganicaccumulation_posted.pdf

EPA (Environmental Protection Agency), 2008. Provisional Peer Reviewed Toxicity Values for Lithium (CASRN 7439-93-2). EPA/690/R-08/016F.

EPA (Environmental Protection Agency), 2009. Toxicological Review of Thallium and compounds (CAS No. 7440-28-0). EPA/635/R-08/001F. Available online: www.epa.gov/iris

EPA (Environmental Protection Agency), 2012. Provisional Peer-Reviewed Toxicity Values for Thalliumand Compounds: Metallic Thallium (7440-28-0), Thallium (I) acetate (563-68-8), Thallium (I) carbonate (6533-73-9), Thallium (I) chloride (7791-12-0), Thallium (I) nitrate (10102-45-1), and Thallium (I) sulfate (7446-18-6).

Fang HQ, Yu Z, Zhi Y, Fang J, Li CX, Wang YM, Peng SQ and Jia XD, 2018. Subchronic oral toxicity evaluation of lanthanum: a 90-day, repeated dose study in rats. Biomedical and Environmental Sciences, 31, 363–375. https://doi.org/10.3967/bes2018.047

Fedele L, Plant JA, de Vivo B and Lima A, 2008. The rare earth element distribution over Europe: Geogenic and anthropogenic sources. In: Geochemistry: Exploration, Environment, Analysis, 8, Geological Society of London, 3–18. https://doi.org/10.1144/1467-7873/07-150

Greim H, 2005. Deutsche Forschungsgemeinschaft., Deutsche Forschungsgemeinschaft. Kommission zur Prüfung Gesundheitsschädlicher Arbeitsstoffe. Wiley-VCH. MAK value documentations.

Gwenzi W, Mangori L, Danha C, Chaukura N, Dunjana N and Sanganyado E, 2018. Sources, behaviour, and environmental and human health risks of high-technology rare earth elements as emerging contaminants. Science of the Total Environment, 636, 299–313. https://doi.org/10.1016/j.scitotenv.2018.04.235

Health Council of the Netherlands, 2014. Tellurium - Evaluation of the effects on reproduction, recommendation for classification. The Hague: Health Council of the Netherlands, publication no. 2014/07.

Kravchenko J, Darrah TH, Miller RK, Lyerly HK and Vengosh A, 2014. A review of the health impacts of barium from natural and anthropogenic exposure. Environmental Geochemistry and Health, 36, 797–814. https://doi.org/10.1007/s10653-014-9622-7

Li X, Zhibiao C, Zhiqiang C and Zhang Y, 2013. A human health risk assessment of rare earth elements in soil and vegetables from a mining area in Fujian Province, Southeast China. Chemosphere, 93, 1240–1246. https://doi.org/10.1016/j.chemosphere.2013.06.085

Liu J, Li N, Ma L, Duan Y, Wang J, Zhao X, Wang S, Wang H and Hong F, 2010. Oxidative injury in the mouse spleen caused by lanthanides. Journal of Alloys and Compounds, 489, 708–713. https://doi.org/10.1016/j.jallcom.2009.09.158

McKnight RF, Adida M, Budge K, Stockton S, Goodwin GM and Geddes JR, 2012. Lithium toxicity profile: a systematic review and meta-analysis. Lancet, 379, 721–749. https://doi.org/10.1016/S0140

Murray FJ and Schlekat CE, 2004. Comparison of risk assessments of boron: alternate approaches to chemical-specific adjustment factors. Human and Ecological Risk Assessment, 10, 57–68. https://doi.org/10.1080/10807030490280954

de Oliveira LM, Das S, da Silva EB, Gao P, Gress J, Liu Y and Ma LQ, 2018. Metal concentrations in traditional and herbal teas and their potential risks to human health. Science of the Total Environment, 633, 649–657. https://doi.org/10.1016/j.scitotenv.2018.03.215

Palasz A and Czekaj P, 2000. Toxicological and cytophysiological aspects of lanthanides action. Acta Biochimica Polonica, 47, 1107–1114. https://doi.org/10.18388/abp.2000_3963

Pearson AJ and Ashmore E, 2020. Risk assessment of antimony, barium, beryllium, boron, bromine, lithium, nickel, strontium, thallium and uranium concentrations in the New Zealand diet. Food Additives and Contaminants - Part A, 37, 451–464. https://doi.org/10.1080/19440049.2019.1704445

Rodríguez-Mercado JJ, Mateos-Nava RA and Altamirano-Lozano MA, 2011. DNA damage induction in human cells exposed to vanadium oxides in vitro. Toxicology in Vitro, 25, 1996–2002. https://doi.org/10.1016/j.tiv.2011.07.009

SCHER (Scientific Committee on Health and Environmental Risks), 2012. Assessment of the tolerable daily intake of barium. [place unknown]: European Commission, 22 March 2012.

Squadrone S, Stella C, Brizio P and Abete MC, 2018. A baseline study of the occurrence of rare earth elements in animal feed. Water, Air, and Soil Pollution, 229, 1–7. https://doi.org/10.1007/s11270-018-3825-y

Sundar S and Chakravarty J, 2010. Antimony toxicity. International Journal of Environmental Research and Public Health, 7, 4267–4277. https://doi.org/10.3390/ijerph7124267

Vávrová S, Struhárňanská E, Turňa J and Stuchlík S, 2021. Tellurium: a rare element with influence on prokaryotic and eukaryotic biological systems. International Journal of Molecular Sciences, 22, 1–15. https://doi.org/10.3390/ijms22115924

Vieux F, Maillot M, Rehm CD and Drewnowski A, 2019. Tea consumption patterns in relation to diet quality among children and adults in the United States: analyses of NHANES 2011–2016 data. Nutrients, 11, 1–17. https://doi.org/10.3390/nu11112635

Wang D, Wang C, Ye S, Qi H and Zhao G, 2003. Effects of spraying rare earths on contents of rare earth elements and effective components in tea. Journal of Agricultural and Food Chemistry, 51, 6731–6735. https://doi.org/10.1021/jf0303417

Wang H, Chen X, Ye J, Jia X, Zhang Q and He H, 2020. Analysis of the absorption and accumulation characteristics of rare earth elements in Chinese tea. Journal of the Science of Food and Agriculture, 100, 3360–3369. https://doi.org/10.1002/jsfa.10369

Wang Y, Wang D, Huang Yi N, Sheng P and Yang B, 2019. Relationship between rare earth elements, lead and intelligence of children aged 6 to 16 years: a bayesian structural equation modelling method. International Archives of Nursing and Health Care, 5, 1–9. https://doi.org/10.23937/2469-5823/1510123

Wang Y-M, Yu Z, Zhao Z-M, Jia LI, Fang H-Q, Zhang T-F, Yuan X-Y, Shu Y-L, He J, Peng H, Li L-Z, Zhao J, Jia X-D and Peng S-Q, 2017. Subchronic toxicity study of yttrium nitrate by 90-day repeated oral exposure in rats. Regulatory Toxicology and Pharmacology, 90, 116–125. https://doi.org/10.1016/j.yrtph.2017.08.020

WHO (World Health Organization), 2003. Antimony in Drinking-water Background document for development of WHO Guidelines for Drinking-water Quality. WHO/SDE/WSH/03.04/74.

Wilk A, Szypulska-Koziarska D and Wiszniewska B, 2017. The toxicity of vanadium on gastrointestinal, urinary and reproductive system, and its influence on fertility and fetuses malformations. Postępy Higieny i Medycyny Doświadczalnej (Online), 71, 850–859. https://doi.org/10.5604/01.3001.0010.4783

Willner J, Fornalczyk A, Jablonska-Czapla M, Grygoyc K and Rachwal M, 2021. Studies on the content of selected technology critical elements (Germanium, tellurium and thallium) in electronic waste. Materials, 14, 1–16. https://doi.org/10.3390/ma14133722

Wu Y, Tang X, Yang W, Fan J, Tang L, Wang C, Yu Z, Jia XD and Fan B, 2019. Subchronic toxicity of cerium nitrate by 90-day oral exposure in wistar rats. Regulatory Toxicology and Pharmacology, 108, 1044742. https://doi.org/10.1016/j.yrtph.2019.104474

Zaichick S, Zaichick V, Karandashev V and Nosenko S, 2011. Accumulation of rare earth elements in human bone within the lifespan. Metallomics, 3, 186–194. https://doi.org/10.1039/c0mt00069h

Zhang J, Yang R, Chen R, Peng Y, Wen X and Gao L, 2018. Accumulation of heavy metals in tea leaves and potential health risk assessment: a case study from Puan County, Guizhou Province, China. International Journal of Environmental Research and Public Health, 15, 1–22. https://doi.org/10.3390/ijerph15010133

Zioła-Frankowska A, Frankowski M, Novotny K and Kanicky V, 2014. Comparison of the level of boron concentrations in black teas with fruit teas available on the polish market. Scientific World Journal, 98425, 1–8. https://doi.org/10.1155/2014/89842

Abbreviations

$BMDL_{05}$	Lower confidence limit of the benchmark dose for a 5% response
Bw	Body weight
DTU	Technical University of Denmark
EPA	Environmental Protection Agency
ICP-MS	Inductively coupled plasma mass spectrometry
LOAEL	Lowest observed adverse effect level
NOAEL	No observed adverse effect level
p-RfD	Provisional reference dose
REEs	Rare earth elements
RfD	Reference dose
TDI	Tolerable daily intake
UL	Tolerable upper intake level

Appendix A – Exposure assessment

Table A.1: Estimated exposure to the investigated elements resulting from the consumption of black, green and rooibos tea, expressed in ng/kg bw per day

	REEs	B	Ba	Li	Sb	Tl	V
Black tea							
Scenario 1	9	482	132	2	0.4	0.2	0.1
Scenario 2	18	578	196	5	0.4	0.5	0.3
Scenario 3	56	1,789	606	16	1	1.5	0.8
Green tea							
Scenario 1	8	636	109	2	0.3	0.03	0.5
Scenario 2	9	714	133	5	0.5	0.6	0.8
Scenario 3	27	2,211	412	14	2	0.2	2.5
Rooibos tea							
Scenario 1	15	991	44	43	0.05	0.01	0.6
Scenario 2	19	1,345	64	53	0.07	0.02	0.8
Scenario 3	58	4,167	199	163	0.2	0.04	2.6

Appendix B – Secondary activities

1) Webinar on ICP-MS. 'Heavy Metals in Baby Food' (28 April 2021).
2) Participation in 9th BfR-Summer Academy 2021: Lecture Series on Risk analysis in Food safety (23–27 August 2021).
3) Participation in the postgraduate course 'Risk Analysis in Food Safety' consisting of two main modules: first focusing on microbiological, and a second on chemical risk assessment. Each module consisted of 12 submodules, including case studies intended to the elaboration of a risk assessment on a specific microbiological/chemical hazard, finalised with the preparation of the reports and poster presentations (31 August to 3 December 2021).
4) Participation with a poster presentation in the 56th Congress of the European Society of Toxicology – EUROTOX 2021. The poster presentation: 'Rare earth element as the emerging contaminants in black tea – risk assessment resulting from the dietary exposure' was related to the project on risk assessment carried out by the fellow at DTU (27 September to 1 October 2021).
5) Webinar on the application procedure for active substances in pesticides and maximum residue levels (28 October 2021).
6) Hands-on training on the evaluation of applications and requests related to biocides products, mainly destined to be used as disinfectant/cleaning agents.
7) Taking part in advice-giving to the Danish Food and Veterinary Administration, the Danish Environment Protection Agency and the Danish Medical Agency.
8) Participation in division meetings.

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Appraising diet–disease associations to be used in risk assessment, including an insight in nutritional epidemiology

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Abstract

High consumption of red meat, in particular of its processed products, has been linked to the development of various chronic diseases, and the need to reduce consumption levels of these products has been identified as a public health priority in Europe. Among the potential alternatives, pulses have gained a prominent position in recent years. Stemming from the broader context of the substitution of red meat in the diet with alternative plant-based protein sources, this work programme was developed to address the need of improving the current understanding on the public health impact of potential substitutes. From a training perspective, the main goal was to advance the fellow's background knowledge in the principles of nutritional epidemiology, while contributing to the harmonisation of food risk assessment practices across Europe. The activities developed comprised a comprehensive learning experience in the different components of the risk assessment framework, giving particular focus to the appraisal of epidemiological evidence within this context (hazard identification and characterisation). The fellow had the opportunity to gain hands-on experience with EFSA's databases among other relevant national and international data sources. The association between the consumption of legumes and risk of cardiovascular disease in particular was extensively explored and described by summarising the available evidence through dose–response meta-analyses. The one-stage approach method was used to explore the shape of the associations, including studies with as few as two levels of exposure, in a weighted mixed-effects model. The analysis was also performed taking into account the potential risk of bias of included studies, assessed using the ROBINS-I tool. Upon the completion of the activities carried out, the fellow made significant progress towards the learning outcomes expected to be achieved throughout the duration of the EU-FORA fellowship period.

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Keywords: risk assessment, plant foods, legumes, pulses, dose–response association, risk of bias

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1. Introduction

The consumption of red meat has significantly increased during the last five decades. Despite being an important source of several macro- and micronutrients, high levels of red meat in the diet, especially of its processed products, have been linked with various chronic diseases. Hence, a reduction in the consumption of red meat has been identified as a public health priority in Europe. Reasons such as consumers' increasing concern and preference for health promoting foods have also led to the emergence of products that can replace red meat. Among the potential alternatives, pulses have gained a prominent position. Cultivation and consumption of pulses have a long tradition in almost all regions of the world and, for centuries, this food group has played a fundamental role in the functioning of traditional agricultural systems and dietary patterns of populations. Pulses are extremely nutritious, with high levels of protein, dietary fibre and with a very low fat content. Moreover, they offer agricultural and ecological benefits in terms of nitrogen fixation in the soil and have a low water and carbon footprint. Despite their nutritional and environmental benefits, in the recent decades, pulses have not received deserved attention as an important food component in the diet of populations worldwide. More recently, higher concerns with health, nutrition and the environment seem to be leading to significant changes in food consumption patterns and driving a shift to more plant-based inclusive diets. This work programme was developed within the broader context of the substitution of red meat in the diet with alternative plant-based protein sources, addressing the need to better understand the impact of potential substitutes on public health. This report describes the knowledge and experience acquired by the fellow during the EU-FORA fellowship programme based at the Dept. of Hygiene, Epidemiology and Medical Statistics of the School of Medicine, National and Kapodistrian University of Athens, as well as a synthesis of results achieved.

2. Description of work programme

2.1. Aims

Using the *Leguminosae* family species as a case study, the programme's main goal was to advance the fellow's background knowledge in the principles of nutritional epidemiology, while contributing to the harmonisation of food risk assessment practices across Europe, namely those related to the appraisal of epidemiological evidence, which are inextricably related to hazard identification and characterisation. The specific learning objectives for this work programme were (1) to increase the fellow's expertise in the utilisation of food consumption data, (2) to develop the fellow's knowledge on systematic review and meta-analysis methodologies and (3) to develop the fellow's capacity on how to weigh scientific evidence and to assess uncertainties of epidemiological research to be used under the risk assessment framework.

2.2. Activities/Methods

2.2.1. Identification of the subject matter and patterns of consumption of pulses in Greece and beyond

A single food is often known under several common names, whereas the use of similar names for different commodities is not uncommon. The foods belonging to the *Leguminosae* family (i.e. *fabaceae*, commonly known as the legume or pea family) are a good example of such inconsistencies, with terms such as 'pulses', 'legumes', 'dried vegetables' or 'grain legumes' being often used interchangeably by the scientific community. However, these terms characterise food items with different nutritional profiles, have distinct roles in eating habits of populations and have different implications for human and animal health, as well as for the environment. In the first module of this work programme, and to address *Problem formulation*, the fellow has explored, identified and characterised the definitions and inconsistencies among these terms through the consultation of selected sources (EFSA FoodEx2 and other food classification systems at the global or European level, food composition databases and dietary guidelines) that included food items belonging to the *Leguminosae* family in sufficient detail. In order to evaluate consumption patterns and trends for this food group, the fellow further consulted and worked with food intake data available for the global and European contexts. Food consumption data from Greece was used more extensively due to its traditional mediterranean eating pattern, of which pulses are an integral component, serving as a case study for a detailed insight into specific trends of consumption, types of pulses with higher

contributions to the overall intake and main characteristics of consumers. For these purposes, three levels of available data were considered: FAO Food Balance Sheets (FBS), compiling yearly country-level data on the available national food supply; Household Budget Surveys, providing information on food availability at the household level; and studies assessing food consumption at the individual level, in particular those following the EU Menu methodology, available at the EFSA Comprehensive European Food Consumption Database, further complemented by other data collections.

2.2.1.1. Definition of the exposure

The FoodEx2 – EFSA Food classification and description system for exposure assessment – is a comprehensive food classification system developed by EFSA (EFSA, 2015), including different grouping hierarchies, depending on the food domain of interest. The exposure-oriented hierarchy is designed to facilitate the grouping of food items for exposure calculations and the preferred hierarchy for reporting consumption data, being thus considered under the scope of this module. The EFSA Catalogue Browser was used as the main source to describe the specific terms and categorisation for the items under the *Leguminoase* family in FoodEx2. In this classification system, this food group is comprised within two major categories (hierarchy terms): 'Legumes, nuts, oilseeds and spices' and 'Vegetables and vegetable products'. The first is further divided into two hierarchy terms: 'Legumes', comprising two generic terms depending on whether the items are in the form of fresh ('Legumes fresh seeds') or dried seeds ('Pulses (dried legume seeds)'), with both categories including similar groups of several species of beans (including soyabeans), peas, lentils, lupins and other legumes (fresh seeds of horse gram, peanut, kersting's and bambara groundnut); and 'Processed legumes, nuts, oilseeds and spices', which is further divided into 'Canned or jarred legumes', including canned/jarred beans, peas, lentils and chickpeas, and 'Pulses flour', along with other core items pertaining to nuts, oilseeds and spices. On the other hand, the latter hierarchy term ('Vegetables and vegetable products') comprises the generic term of 'Legumes with pods', which is further divided into three core terms – 'Beans (with pods)', 'Lentils (with pods)' and 'Lentils (young pods)', with each term also including several species, along with other categories of vegetables and vegetable products. The major distinguishing aspect between these two main terms is that under the 'Legumes, nuts, oilseeds and spices', the fresh seeds of legumes without pods are considered; whereas in the category of 'Vegetables and vegetable products', only the fresh leguminous seeds within pods are included.

In order to further explore the definitions of this food group, other sources of food classification and description were consulted. A detailed comparison between the categorisation of FoodEx2 and other food classification systems for the species in the *Leguminosae* family is described in Appendix A. Briefly, FoodEx2, along with the FAO Definition and Classification of Commodities and FAO/WHO Codex classification are the most descriptive and comprehensive among the included classifications, describing a similar listing of species under the pulses category, with the exception of soyabeans, which are explicitly excluded from the second and are found under the category of oil-bearing crops. Additionally, it seems consensual among the selected sources that when the term pulses is used it refers exclusively to the dried legume seeds. In some cases, the category 'legumes' includes both the fresh and dried seeds and in other comprises specifically the fresh leguminous seeds, thus excluding pulses. The most evident discrepancies are related to the categorisation of the fresh legume seeds and processed legumes. Unlike in FoodEx2, other food classification systems do not distinguish legumes within pods from those in the shelled form. This might however be relevant from a chemical exposure perspective, since pods are fully exposed to pesticides, whereas the seeds are protected within the pod. In a similar way, processed legumes are described as a specific group in FoodEx2, an important distinction from a nutritional standpoint. However, it should be noted that different classification systems were developed to fill different needs and the inconsistencies identified might be a reflection of those rather than actual inconsistencies or disagreements.

2.2.1.2. Assessment of trends in the consumption of pulses worldwide, in Europe and Greece

According to the OECD-FAO Agricultural Outlook 2020–2029 (OECD/FAO, 2020) , about 22 g/day/per capita of pulses were available for consumption in 2020, globally. The values were highest in Africa and Latin America (about 30 g/day/per capita), followed by Asia (19 g/day/per capita) and North America (16 g/day/per capita) and lowest in Europe (8 g/day/per capita) and Oceania (5 g/day/per capita). The overall supply of pulses had a slow but steady decline in both high- and low-income regions since the early 1960s, reaching a plateau between the early 1990s and 2000s. No major changes are foreseen in the global per capita availability of pulses for the next decade (OECD/FAO,

2020). However, pulses are expected to regain importance in the diets and farming systems of Europe as a whole and in the European Union in particular (OECD/FAO, 2020). After consulting the available data from FAO FBS (FAOSTAT, 2021), in 2010, the per capita supply of pulses in Europe was estimated at 6.8 g/day, with a slow but steady increase since then. This value is expected to increase to 9.5 g/day per capita in 2029. Between 1961 and 2018, peas were the most available pulse in Europe, with an average of 2.5 g/day per capita in 2018, followed by beans with 1.7 g/day per capita in the same year. However, the supply available for consumption of both of these types of pulses has been slowly decreasing throughout time, while for other pulses (including broad beans, horse beans, chickpeas, cowpeas, pigeon peas, lentils, bambara beans, vetches, lupins, flour and bran of pulses), it has been steadily increasing. Due to the nature of categorisation of data in FAO FBS, it was not possible to understand if this is due to higher levels in the supply of a single or more types of pulses.

Focusing on a National context, data available at the FAO FBS for Greece, the Greek Household budget surveys and original studies on food consumption at the individual level were consulted and used. Table 1 shows the trends in the average per capita supply (g/day) in Greece by decade and the respective average share in the total caloric and protein supply for that time period, according to FAO FBS. Similarly to the observed for the global context, the per capita supply of pulses in the country has been steadily declining since the early 1960s from 21.5 g/day to 13.4 g/day in the 2010s. In the 1960s, pulses represented 5.1% of the total protein supply in Greece and 2.5% of the total supply of calories intake (Table 1). Six decades later, in the 2010s, these values are about half of those previously reported. These trends were also in line with those described using data from the Greek Household budget surveys.

Table 1: Average per capita per day supply of pulses and its contribution to the supply of protein and calories in Greece (1961–2018)

	1961–1970	1971–1980	1981–1990	1991–2000	2001–2010	2011–2018
Average consumption (g)	21.5	18.6	14.4	13.9	13.0	13.4
% of total protein supply	5.1	3.8	2.8	2.6	2.5	2.7
% of total energy supply	2.5	1.9	1.4	1.3	1.2	1.3

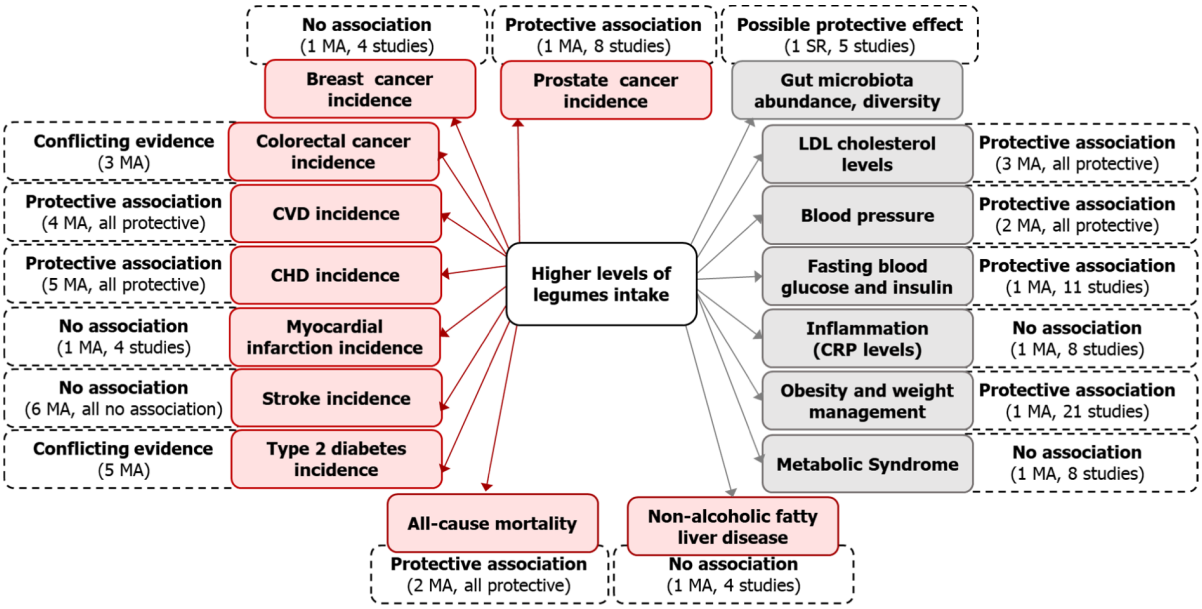
All estimates were calculated using data available at the FAO Food Balance Sheets for Greece (FAOSTAT, 2021).

At the individual level, Greek food consumption data available at the EFSA Comprehensive Food consumption database were retrieved. The number of individuals reporting any level of intake of pulses was relatively low in all age groups. For the pulses' food group as a whole, the percentage of consumers was 18.2% in adolescents, 15.8% in adults and 23.7% in the elderly in 2015. The average consumption was higher among the elderly [mean (sd): 6.73 (15.08) g/day] and lower among adolescents [mean (sd): 4.72 (14.13) g/day]. Lentils were the most consumed pulse among adolescents (9.1%), while beans were reported by a higher number of adult and elderly consumers (10.4% and 11.3%, respectively). Taking into account the Greek Dietary Guidelines (Kastorini et al., 2019), which recommend an amount of at least 450 g of pulses per week for adolescents and adults, the results provided in this study show that regardless of the age group, the Greek population is currently characterised by low levels of intake of pulses, with an average of about 33 g/week among adolescents, 34 g/week among adults and 47 g/week among the elderly.

2.2.2. Understanding of the current state of the art: health outcomes associated with the consumption of legumes

The second module of the work programme consisted in conducting of a comprehensive literature search to describe the health outcomes associated with the consumption of pulses (hazard identification) and to identify existing dose–response meta-analyses (hazard characterisation). For this purpose, the fellow searched PubMed and other literature sources in order to identify systematic reviews and meta-analyses addressing the association between the dietary intake of pulses and health outcomes, considering either the whole food group or specific types. Despite the main interest of the present work programme being legumes in their dried form, i.e. pulses, after a preliminary search it was evident that in the majority of studies there was no distinction between subtypes, with the food group of legumes being assessed as an aggregated term, including fresh and dried seeds. For this reason, the term legumes was used in the sections of this report aiming to address the epidemiological evidence on the association between this food group and health outcomes (Sections 2.2.2 and 2.2.3).

In addition, studies focusing exclusively on the health effects of the corresponding nutrients and other compounds (e.g. proteins, fibre, isoflavones) were excluded, in an attempt to capture interactions among food constituents and approach holistically the association between food intake and disease risk. Another aim of this module was to gather the necessary evidence to allow the selection of a health outcome to be studied in the context of a dose–response meta-analysis in module 3. For this reason, studies assessing exclusively the health effects of soyabeans and soy products were also excluded, since these have already been extensively reviewed elsewhere (46 systematic reviews and meta-analyses identified in a preliminary search). For the association between the intake of legumes and pulses (excluding soy) and health outcomes, including hard clinical outcomes and potential intermediate factors (surrogate endpoints), 44 systematic reviews and/or meta-analyses were identified. An overview of the conclusions resulting from these studies is described in Figure 1.



The associations with hard clinical endpoints are highlighted in red and with intermediate factors in gray. For each outcome, the direction of the association with the intake of legumes is indicated. For those cases where only one meta-analysis was performed, the number of included studies is presented in parenthesis, whereas when two or more meta-analyses were available for the same association, the agreement or disagreement in their conclusions is noted also in parenthesis. MA: meta-analysis; SR: systematic review; CVD: cardiovascular disease; CHD: coronary heart disease; CRP: C-Reactive Protein.

Figure 1: Consumption of legumes and related health outcomes, according to the results from the identified systematic reviews and meta-analyses

Through the literature search performed, 11 publications with a dose–response meta-analysis on the association between the intake of legumes and one or more hard clinical endpoints were identified. Considering the most recent meta-analyses conducted for each outcome, a statistically significant dose–response association was reported for Type 2 diabetes and CVD only, albeit with a limited number of included studies. The strengths and limitations of these studies were assessed and discussed with the supervisor and other team members, aiming to increase the fellow's capacity to critically appraise the available evidence and to inform the next steps of the work programme.

In order to enhance the relevance and interpretation of findings in terms of health impact, the fellow has consulted the Global Burden of Disease Study (GBD, 2019) to identify estimates of disease burden associated with each hard clinical endpoint identified in Figure 1. Disability adjusted life years (DALYs), combining years of potential life lost due to premature mortality and years lived with a disability of specified severity and duration, reported in the Global Burden of Disease Study 2019 (GBD, 2019) were used as the estimate of interest. In 2019, CVD was responsible for 393 million (95% UI 368–417) DALYs and was the overall leading cause of disease burden – specifically for CVD subtypes, coronary heart disease reached a total 182 million (95% UI 170–194) DALYs and stroke a total of 143 million (95% UI 133–153). Type 2 diabetes was second with 66.3 million (95% UI 55.5–79.0) global DALYs in 2019, followed by colorectal cancer with 24.3 million (22.6–25.7) DALYs.

The final step of this module consisted in selecting the health outcome to be studied in the context of a dose–response meta-analysis. For this purpose, five criteria were defined and applied: (1) hard clinical endpoints with a plausible association with the intake of legumes; (2) outcomes with no previous or recent dose–response meta-analysis exploring their association with the intake of legumes; (3) outcomes with available estimates of disease burden (i.e. DALYs); (4) outcomes with an established positive association with the intake of red meat and red meat products, considering the substitution of red meat with plant-based protein sources as the wider scope of this work programme; (5) availability of several publications for the association between the health outcome and the intake of legumes, so as to increase the precision of the effect measures. For all the identified hard clinical endpoints with a plausible association with the consumption of legumes (Figure 1) (criterion 1), a relatively recent dose–response meta-analysis was performed (criterion 2) and with the exception of all-cause mortality, all the identified outcomes had an associated estimate of disease burden (criterion 3). Colorectal cancer and CVD have an established positive association with the intake of red meat and a recent dose-response meta-analysis has also linked the intake of red meat to the development of type 2 diabetes (Yang et al., 2020) (criterion 4). However, two dose-response meta-analyses on this association were published in 2020 and 2021, limiting the need to conduct a new synthesis of findings. CVD and colorectal cancer were therefore regarded as the most suitable outcomes for the investigation proposed within the context of the present work plan. Considering the availability of publications for the association between these health outcomes and the intake of legumes (criterion 5), preliminary literature searches were conducted. For colorectal cancer, the preliminary search retrieved 141 papers published after the most recent update (Schwingshackl et al., 2018), all with a cross-sectional or retrospective design. On the other hand, a preliminary search on the association between the intake of legumes and CVD after the most recent update (Viguiliouk et al., 2019) retrieved 557 publications, with original research addressing the association of interest including prospective and retrospective study designs. Prospective studies are generally considered to rank higher than retrospective approaches in the hierarchy of evidence, thus improving the strength and robustness of pooled effect measures. For this reason, overall CVD and its different subtypes were selected as the health outcomes to be explored within the context of a dose–response meta-analysis.

2.2.3. Assessment of dose–response associations between the consumption of legumes and the selected health outcome

The third module of the work programme consisted in the evaluation of dose–response associations between the intake of legumes and the health outcomes selected in module 2 – CVD and subtypes – in the context of a systematic review and meta-analysis. In an initial stage, the fellow consulted the guidelines from the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2019) and the EFSA guidance document on the application of systematic review methodology to food and feed safety assessments (EFSA, 2010), along with other relevant literature, to support the methodological design and procedures of the study. After a thorough discussion with the supervisor and the supporting team on methods and major aspects to be considered, the study protocol was prepared following the PRISMA-P guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses protocols) (Moher et al., 2015) and registered in PROSPERO (International prospective register of systematic reviews), an open access online database of systematic review protocols in health and social care, so as to ensure transparency and to enable *a posteriori* comparisons of reported methods with those initially planned (PROSPERO registration ID: CRD42021247565).

2.2.3.1. Literature search and selection of studies

In an initial step, several literature databases were considered for literature search. After assessing their scope and characteristics, taking into account comprehensiveness and complementarity as criteria, PubMed, Scopus and Web of Science were considered and searched since inception in order to retrieve potential eligible studies. For this purpose, several search expressions were developed and tested, in accordance to each database format and specific controlled vocabulary thesaurus. Terms designating the species belonging to the *Leguminosae* family were included, using both their common and scientific names. The final search expression was designed so as to ensure an adequate balance between specificity and sensitivity, however favouring a higher sensitivity in order to capture the highest number of eligible studies. After performing several sensitivity analyses, broader terms such as 'dietary protein', 'plant protein' and 'Mediterranean diet' were included, resulting in a total of 14 374 publications for reviewing, after the removal of duplicates. Study selection was then developed in three

stages: (1) title/abstract review, (2) full-text review and (3) evaluation of data in the format required for synthesis, applying the selection criteria defined at the protocol stage, and performed independently by the fellow and other member of the scientific supporting team. Lastly, a back and forward citation tracking of eligible studies was conducted, in order to ensure the completeness of study selection.

2.2.3.2. Data extraction and dose calculation

A data extraction template was created to ensure a standardised approach to data extraction, including all the relevant variables to allow a robust analysis. The data were extracted independently by the fellow and another member of the scientific supporting team for validation purposes. For each exposure category, the median or mean intake level was extracted, depending on the availability of the data provided by the authors. In cases where this information was reported in intervals of consumption, the midpoint of each exposure strata was used and when the highest and lowest exposure category intervals were open-ended, a value that was respectively 20% higher and lower than the closest cut point was used as boundary. In cases where the intake levels were reported only as number of servings, a standard portion size was assigned. Finally, all the dose levels were converted into a standard unit, in order to allow comparability between estimates for the dose-response assessment.

2.2.3.3. Risk of bias assessment

For the assessment of the risk of bias in observational research, the use of two possible frameworks was initially considered – the Risk of Bias in Non-randomised Studies of Interventions (ROBINS-I) and the Risk of Bias in Non-randomised Studies of Exposures (ROBINS-E) – and discussed, comparing advantages and limitations. Both are based on the Cochrane risk of bias (RoB) tool for randomised trials and follow the premise that an observational study should be compared to a hypothetical randomised trial to identify potential biases, comprising similar domains and signalling questions. However, ROBINS-E, even if apparently more suited for the nature of the included research in the present study, is still currently under development and lacks an accompanying guidance document. On the contrary, ROBINS-I is a well-established tool with a detailed and comprehensive guidance document published (Sterne et al., 2016). For this reason, ROBINS-I was selected for the RoB assessment. In accordance with the guidance document, the tool was tailored to address the specific research question accommodating the nature and methodology of studies selected to be considered in the analysis. Table 2 summarises the assumptions considered in the RoB assessment across the seven domains covered in ROBINS-I. Each domain was classified as having a low, moderate, serious or critical risk of bias, with a derived overall assessment for each study. The appraisal was performed in parallel by the fellow and another researcher using a standardised template specifically created for this purpose, in order to ensure the repeatability of the process and ultimately the comparability of the assessments.

Table 2: Segments of the protocol adopted for the RoB assessment

ROBINS-I domains	Criteria
Bias due to confounding	Factors mandatory to be considered in the analysis so as to judge a study at low RoB were sex, age, energy intake and BMI; mandatory factors for a moderate RoB judgement were age, sex, BMI and measures of dietary factors/patterns (in the absence of energy intake) or age, sex, energy intake, physical activity and clinical CVD risk factors (in the absence of BMI).
Bias in selection of participants	Selection of eligible participants must not be related to either legumes intake or to CVD.
Bias in classification of interventions	Studies were judged to be at a low RoB for exposure misclassification if using a validated dietary assessment method and quantifying the dose of exposure (i.e. portion consumed). If one of these conditions was not satisfied, the study would be judged to be at moderate RoB.
Bias due to deviations from intended interventions	There should be no concern about departure from intended exposure due to the long-term stability of dietary patterns. A threshold of > 10 years of mean follow-up was considered at moderate RoB for possible change in exposure since the beginning of the study, if suggested that factors conditioning the change were not at random.

ROBINS-I domains	Criteria
Bias due to missing data	Studies with less than 10% of missing data in the exposure, outcome or covariates were considered at low risk of bias. If more than 10% of the data was missing for one of these domains, the studies were judged to be at moderate RoB.
Bias in outcome measurement	Studies were judged to be at low risk of bias if using valid methods for the outcome assessment (e.g. medical records, self-reported diagnosis of CVD with external validation, death registries/autopsies). Studies only partially validating the outcome assessment were judged to be at moderate RoB.
Bias in selection of the reported results	Suggestion that published results have not been selected and clear reporting of statistical methods. If the analysis described in the methods section does not correspond entirely to what is presented in the results section, but unlikely to mean selective reporting on the basis of the results, the study was judged to be at moderate RoB.
Overall RoB judgement	If all domains were at low risk of bias, the overall risk was considered low; if at least one domain was found at moderate risk of bias, the overall risk was considered moderate. The same approach was applied for serious and critical risk of bias.

2.2.3.4. Dose–response analysis

Data were analysed following two approaches: firstly, a traditional meta-analysis was conducted comparing highest to lowest intakes. A random effects model was applied and the heterogeneity across included studies was assessed using the I^2 statistic. In a second step, the dose–response analysis was performed. The methodology developed by Orsini et al. (2012) and Crippa et al. (2019) was used to explore the shape of the relationship between the intake of legumes and CVD (overall and separately for CHD and stroke). The advantage of the dose–response model applied (one-stage approach) is that studies with as few as two levels of exposure can be considered through the application of a weighted mixed-effects model. The one-stage approach was carried out, using a restricted cubic spline model with three knots at fixed percentiles (10, 50 and 90%), under no *a priori* assumption on the shape of the curve. The estimates were pooled using the restricted maximum likelihood method in mixed-effects models. The possible presence of publication bias was verified through visual inspection of funnel plots. Several sensitivity and subgroup analyses were conducted to understand if the associations varied with different study characteristics. Finally, a manuscript was prepared following the PRISMA guidelines to be submitted for publication.

2.2.4. Additional activities

2.2.4.1. Preliminary literature review on anti-nutrients present in pulses

There is a general consensus that a diet rich in plant-based foods has an important role in the prevention and reduction of disease. However, despite being rich sources of micro- and macronutrients, plant-based foods contain significant concentrations of other compounds for which the health effects are not yet fully understood. These compounds are often referred to as antinutrients (also bioactive compounds or phytochemicals), as they are thought to restrict bioavailability of key nutrients. The fellow has completed a preliminary narrative review on the presence of antinutrients in pulses, aiming to identify potential differences between species and possible impact in human nutrition, as well as to acknowledge current research gaps. Briefly, pulses are generally a rich source of antinutrients, including lectins, enzyme inhibitors, phytates, oxalates, phytoestrogens, saponins, alkaloids and oligosaccharides, and their content in these factors may widely vary between different subtypes, as well as between different species within the same subtype (Alcázar-Valle et al., 2020; Mayer Labba et al., 2021). The evidence on the potential health effects of antinutrients in pulses is mixed, with some studies showing they may condition the adequate absorption of nutrients and have other negative health effects (Fredlund et al., 2006; Petroski and Minich, 2020), while others demonstrate their potential in disease prevention, in particular for phytoestrogens, lectins and phytates (Gautam et al., 2020; Yamagata and Yamori, 2021; Silva and Bracarense, 2016). However, the concentration of these compounds in pulses is significantly reduced through traditional processing such as soaking, sprouting, fermenting, boiling and autoclaving (Luo and Xie, 2013; Bento et al., 2021), and there is yet not sufficient evidence showing their potential effects on human health (Petroski and Minich, 2020). The interaction between antinutrients and other constituents in food matrixes is also not well established (Petroski and Minich, 2020).

Other additional activities completed during the fellowship period are reported in Appendix B.

3. Conclusions

The work programme at the Dept. of Hygiene, Epidemiology and Medical Statistics of the School of Medicine, National and Kapodistrian University of Athens provided the fellow the opportunity to develop important skills within critical aspects of the risk assessment framework. Using the *Leguminosae* family as a case study, the fellow has become familiar with a different range of food classification systems, food composition tables and dietary guidelines and has significantly improved her knowledge in the utilisation of these tools for identifying inconsistencies across the different sources and establishing a definition for the exposure of interest (an important step in **hazard identification**). The fellow has extensively explored the EFSA Comprehensive European Food Consumption Database and has improved her knowledge on the FAO Food Balance Sheets and the Household Budget Surveys databases in order to assess temporal trends in the consumption of pulses worldwide, in Europe and Greece. This has allowed the fellow to substantially advance her skills in the utilisation of food consumption databases for the assessment and monitoring of habitual dietary intakes at the individual, household and country level. The differences and complementary aspects across these dietary assessment methods were also analysed taking into account sources of potential bias, in order to improve the fellow's knowledge and skills on the critical appraisal of available food consumption data (a critical aspect of **exposure assessment**). In order to advance the skills of the fellow on **hazard characterisation**, the fellow has conducted a literature search to identify and summarise the evidence that investigated the association between different health outcomes and the consumption of legumes. This has enhanced the fellow's knowledge on how to identify and assess epidemiological evidence addressing the complex relationship between diet and disease. The fellow has also increased her expertise on systematic review and meta-analysis methodologies, by preparing a systematic review and a dose–response meta-analysis in accordance with the guidelines from Cochrane and EFSA. The fellow has further acquired knowledge in the appraisal of risk of bias in observational research and improved expertise on weighing of scientific evidence in nutritional epidemiology studies related to hazard characterisation. The EU-FORA fellowship programme and the stay at the University of Athens was an extremely rewarding experience for the fellow, both in terms of professional and personal development.

References

Alcázar-Valle M, Lugo-Cervantes E, Mojica L, Morales-Hernández N, Reyes-Ramírez H, Enríquez-Vara JN and García-Morales S, 2020. Bioactive compounds, antioxidant activity, and antinutritional content of legumes: a comparison between four phaseolus species. Molecules, 1, 25, 3528.

Bento JAC, Ribeiro PRV, Alexandre e Silva LM, Alves Filho EG, Bassinello PZ, de Brito ES, Caliari M and Soares Júnior MS, 2021. Chemical profile of colorful bean (Phaseolus vulgaris L) flours: changes influenced by the cooking method. Food Chemistry, 15, 129718.

Crippa A, Discacciati A, Bottai M, Spiegelman D and Orsini N, 2019. One-stage dose-response meta-analysis for aggregated data. Statistical Methods in Medical Research, 28, 1579–1596.

EFSA (European Food Safety Authority), 2010. Application of systematic review methodology to food and feed safety assessments to support decision making. EFSA Journal 2010;8(6):1637, 90 pp. https://doi.org/10.2903/j.efsa.2010.1637

EFSA (European Food Safety Authority), 2015. The food classification and description system FoodEx2 (revision 2). EFSA Supporting Publication 2015;12(5):EN-804, 90 pp. https://doi.org/10.2903/j.efsa.2015.en-804

FAOSTAT, 2021. Food balance sheets. Available online: https://www.fao.org/faostat/en/#data/FBS

Fredlund K, Isaksson M, Rossander-Hulthén L, Almgren A and Sandberg A-S, 2006. Absorption of zinc and retention of calcium: dose-dependent inhibition by phytate. Journal of Trace Elements in Medicine and Biology, 20, 49–57.

Gautam AK, Sharma D, Sharma J and Saini KC, 2020. Legume lectins: potential use as a diagnostics and therapeutics against the cancer. International Journal of Biological Macromolecules, 1, 474–483.

GBD (Global burden of disease), 2019. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet, 396, 1204–1222.

Higgins JPT, Thomas J, Chandler J, Cumpston M, Li T, Page MJ and Welch VA, 2019. Cochrane Handbook for Systematic Reviews of Interventions, 2nd Edition. John Wiley & Sons, Chichester (UK).

Kastorini C-M, Critselis E, Zota D, Coritsidis AL, Nagarajan MK, Papadimitriou E, Belogianni K, Benetou V and Linos A, 2019. National Dietary Guidelines of Greece for children and adolescents: a tool for promoting healthy eating habits. Public Health Nutrition, 22, 2688–2699.

Luo YW and Xie WH, 2013. Effect of different processing methods on certain antinutritional factors and protein digestibility in green and white faba bean (Vicia faba L.). CyTA-J Food, 11, 43–49.
Mayer Labba I-C, Frøkiær H and Sandberg A-S, 2021. Nutritional and antinutritional composition of fava bean (Vicia faba L., var. minor) cultivars. Food Research International, 140.
Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, Shekelle P and Stewart LA, 2015. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Systematic Reviews, 4, 1.
OECD/FAO, 2020. OECD-FAO Agricultural Outlook 2020–2029. Rome/OECD Publishing, Paris, FAO.
Orsini N, Li R, Wolk A, Khudyakov P and Spiegelman D, 2012. Meta-analysis for linear and nonlinear dose-response relations: examples, an evaluation of approximations, and software. American Journal of Epidemiology, 175, 66–73.
Petroski W and Minich DM, 2020. Is there such a thing as "anti-nutrients"? A narrative review of perceived problematic plant compounds. Nutrients, 12, 2929.
Schwingshackl L, Schwedhelm C, Hoffmann G, Knüppel S, Laure Preterre A, Iqbal K, Bechthold A, De Henauw S, Michels N, Devleesschauwer B, Boeing H and Schlesinger S, 2018. Food groups and risk of colorectal cancer. International Journal of Cancer, 142, 1748–1758.
Silva EO and Bracarense AP, 2016. Phytic acid: from antinutritional to multiple protection factor of organic systems. Journal of Food Science, 81, R1357–R1362.
Sterne JAC, Higgins JPT, Elbers RG, Reeves BC, 2016. Risk Of Bias In Non-randomized Studies of Interventions (ROBINS-I): detailed guidance, updated 12 October 2016.
Viguiliouk E, Glenn AJ, Nishi SK, Chiavaroli L, Seider M, Khan T, Bonaccio M, Iacoviello L, Mejia SB, Jenkins DJA, Kendall CWC, Kahleová H, Rahelić D, Salas-Salvadó J and Sievenpiper JL, 2019. Associations between dietary pulses alone or with other legumes and cardiometabolic disease outcomes: an umbrella review and updated systematic review and meta-analysis of prospective cohort studies. Advances in Nutrition, 10(Suppl. 4), S308–S319.
Yamagata K and Yamori Y, 2021. Potential effects of soy isoflavones on the prevention of metabolic syndrome. Molecules, 26, 5863.
Yang X, Li Y, Wang C, Mao Z, Zhou W, Zhang L, Fan M, Cui S and Li L, 2020. Meat and fish intake and type 2 diabetes: sose-response meta-analysis of prospective cohort studies. Diabetes Metabolism, 46, 345–352.

Abbreviations

CVD	Cardiovascular disease
DAFNE	Data Food Networking
DALYs	Disability-adjusted Life Years
EU-FORA	European Food Risk Assessment Fellowship Programme
FAO	Food and Agriculture Organization
FBS	Food balance sheets
OECD	Organisation for Economic Co-operation and Development
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROSPERO	International prospective register of systematic reviews
RoB	Risk of bias
ROBINS-I	Risk of Bias in Non-randomised Studies of Interventions

Appendix A – Comparison between EFSA FoodEx21 and other food classification systems for the food group of species in the Leguminosae family

Food classification system	FoodEx2 hierarchy term	FoodEx2 generic term	Terms and description used in the respective food classification system for equivalent categories of the FoodEx2* classification system
FAO Definition and Classification of Commodities[2]	Legumes	Pulses (dried legume seeds)	Pulses and derived products: annual leguminous crops yielding from one to 12 grains or seeds of variable size, shape and colour within a pod. The term 'pulses' is limited to crops harvested solely for dry grain. Also includes processed pulses like flour and bran of pulses and explicitly excludes soyabeans.
	Vegetables and vegetable products	Legumes with pods	Vegetables and Derived Products: pulses belong to this group when harvested green; the list includes green beans, green peas (edible-podded peas or sugar peas), green broad beans and string beans, mostly for shelling.
FAO/WHO Codex Classification of Foods and Feeds[3]	Legumes	Pulses (dried legume seeds)	Pulses: derived from the mature seeds, naturally or artificially dried of leguminous plants, consumed after processing or household cooking. Included under the broader category of vegetables. Includes soyabeans.
	Vegetables and vegetable products	Legumes with pods	Legume vegetables: derived from the succulent seed and immature pods of leguminous plants; may be consumed as whole pods or as the shelled product. Included under the broader category of vegetables. Includes soyabeans.
WCO Harmonized Commodity Description and Coding System[4]	Legumes	Pulses (dried legume seeds)	Dried leguminous vegetables: includes only shelled, whether or not skinned or split, dried seeds of leguminous plants. Covers the same subgroups and roughly the same species, with some exceptions (e.g. lupins and vetches not mentioned). Soyabeans are not mentioned.
	Vegetables and vegetable products	Legumes with pods	Leguminous vegetables: includes species shelled or unshelled, in their fresh or chilled form. Only specifies generic species of beans and peas. Soyabeans are not mentioned.
DAFNE Food Classification System[5]†	Legumes	Pulses (dried legume seeds)	Pulses: the nomenclature varies with country, but most use the term 'dried' peas/beans/pulses or 'dried vegetables'. Presents limited detail on included specific species. Some countries include canned pulses in this category (France and Italy).
	Processed legumes, nuts, oilseeds and spices	Canned/ jarred legumes	Processed vegetables: including canned, frozen, boiled/cooked food items from the leguminosae family. The reporting of this category varies widely according to the country. Presents limited detail on included specific species.
	Vegetables and vegetable products	Legumes with pods	Fresh vegetables: generally, only the fresh forms of the leguminous plants seeds or pods are included. Presents limited detail on included specific species.

1: EFSA (European Food Safety Authority), 2015. The food classification and description system FoodEx2 (revision 2). EFSA supporting publication 2015;EN-804, 90 pp.
2: FAO (Food and Agriculture Organization of the United Nations), 1994. Definition and classification of commodities, 4. Pulses and derived products.
3: Codex Alimentarius Commission, 1993. Codex Classification of Foods and Animal Feeds. Codex Alimentarius, 2.
4: WCO (World Customs Organization), 2017. The Harmonized Commodity Description and Coding Systems (HS).

5: European Commission, DG SANCO, 2005. The DAFNE food classification system. Operationalisation in 16 European countries. Services of the European Commission, Luxembourg.
*: FoodEx2 categories without correspondence in the designated food classification system are omitted.
†: Classification system of data collected from the National Household Budget Surveys in Europe.

Appendix B – Additional activities

Next to the training provided in the context of the EU-FORA fellowship programme, including the five EU-FORA modules organised by EFSA (Italy), AGES (Austria), BfR (Germany) and EFET (Greece), the fellow also had the opportunity to attend and participate in other training activities covering a wide range of topics, as described below.

	Description of activity/training	Tutor	Date
1	Observer and commentator on presentations of the students' on dietary survey data available in Greece, in the context of the Nutritional Epidemiology course of the MSc in Epidemiology – Research methodology in biomedical sciences, clinical practice and public health of the School of Medicine, National and Kapodistrian University of Athens (NKUA)	–	March 2021
2	Attendance to the WHO/Europe NCD Office Seminar for Early Career Researchers – 'Global health career development'	Dr Gauden Galea	26 March 2021
3	Lecture 'Analysing nutrient and food data' to the students of the MSc in Epidemiology – Research methodology in biomedical sciences, clinical practice and public health of the School of Medicine of the National and Kapodistrian University of Athens	–	1 April 2021
4	Attendance to the seminar 'Tackling Non-communicable diseases and promoting Public Health in WHO/Europe', organised by the DeHMS/NKUA	Dr João Breda	12 April 2021
5	Attendance to the WHO/Europe NCD Office Seminar for Early Career Researchers – 'Novel opportunities of interdisciplinary approaches'	Dr Afton Halloran	21 April 2021
6	Attendance to the WHO/Europe NCD Office Seminar for Early Career Researchers – 'Alcohol and Cancer: Evidence for Action'	Prof. Linda Bauld	25 May 2021
7	Attendance to the seminar 'From epidemiologic evidence to formulation and implementation of nutrition policy: the case of trans-fatty acids', within the scope of the Nutritional Epidemiology Course organised by the DeHMS/NKUA	Dr Theodora Mouratidou/ Dr Sandra Caldeira	27 May 2021
8	Attendance to the WHO/Europe NCD Office Seminar for Early Career Researchers – 'Corporate political activity as a commercial determinant of health: a case study and methodological considerations'	Dr Kathrin Lauber	28 June 2021
9	Attendance to the Conference 'Future steps to tackle obesity – digital innovations into policy and actions', organised by the Portuguese Ministry of Health in the context of the Portugal's Presidency of the Council of the EU	–	29 June 2021
10	Attendance to the 'International workshop on risk assessment of combined exposure to multiple chemicals', promoted by EFSA	–	18-20 October 2021
11	Attendance to the Conference of the WHO Collaborating Centre for Nutrition and Childhood Obesity by the National Institute of Health Dr Ricardo Jorge, Portugal	–	19 October 2021
12	Attendance to the 9th Virtual Panhellenic Conference of Greek Lipid Forum 'Current trends in the field of Lipids'	–	22 October 2021
13	Completion of the Cochrane Interactive Learning Course: 'Conducting an intervention review', promoted by Cochrane through the People Services Department	–	November 2021
14	Attendance to the WHO seminar 'Improving digital food environments: why do we need to act now?'	–	7 December 2021

EU-FORA SERIES 4

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Study of the different evaluation areas in the pesticide risk assessment process

Focus on pesticides based on microorganisms

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Abstract

Both chemical and microbial active substances can currently be approved as pesticides in the EU, the provisions of their approval being set under Regulation (EC) No 1107/2009. Although sharing the same legal framework, chemicals and microorganisms used as pesticides have different risk profiles especially because once released into the environment, microbial active substances may produce secondary metabolites, multiply, spread and possibly genetically adapt or transfer antimicrobial resistance genes to other microorganisms. Consequently, the risk assessment process must adjust to the specificities ensuing from the chemical or microbial nature of the active substance. This specific programme focused on the risk assessment of microorganisms used as pesticides, especially on the low-risk criteria linked to antimicrobial resistance and the risk assessment of secondary metabolites. The use of microorganisms in integrated pest management (IPM) programmes was also investigated. In 2020, the recently adopted Farm to Fork Strategy and the Biodiversity Strategy for 2030, two important action plans of the European Green Deal, called for a 50% reduction in the use of and risk from chemical and more hazardous pesticides. Many microorganisms are likely to be approved as low-risk active substances, thus representing important tools to achieve this goal. Given the central role that microbial active substances could play towards a more sustainable food system, a need for information regarding the actual production of secondary metabolites by the microorganisms of interest and projects investigating IPM programmes at national and EU levels was identified.

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Keywords: active substance, integrated pest management, low risk, microorganisms, pesticides, plant protection product, risk assessment

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1. Introduction

The main objective of the European Food Risk Assessment Fellowship Programme (EU-FORA) is to allow early to mid-career professionals from European Union (EU) and EFTA countries to widen their knowledge and gain hands-on experience in food safety risk assessment. The work was performed at the Spanish National Institute for Agricultural and Food Research and Technology (INIA – Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria), in the Plant Protection Products Unit (Unidad de Productos Fitosanitarios). This unit acts in Spain as the Independent Evaluation Institution authorised by the Spanish Ministry of Agriculture, Fisheries and Food (Ministerio de Agricultura, Pesca y Alimentación - MAPA) to conduct the risk assessment of pesticides in the following areas: identity and physico-chemical properties, analytical methods, efficacy, metabolism and residues, fate and behaviour in the environment and ecotoxicology; that is to say all aspects of the pesticide risk assessment except human toxicology, whose evaluation is carried out by the Spanish Ministry of Health, Consumer Affairs and Social Welfare.

Both chemical and microbial active substances can be authorised as pesticides in the EU. Regardless of the type of active substance, the placing of plant protection products (PPPs) on the market must comply with Regulation (EC) No 1107/2009 that defines microorganisms as '*any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material*' (European Commission, 2009a) . Although sharing the same legal framework, chemicals and microorganisms used as pesticides have different risk profiles mainly because once released into the environment, microorganisms can produce and release toxic metabolites, multiply, spread and possibly genetically adapt or transfer antimicrobial resistance genes to other microorganisms. Consequently, the risk assessment process must adjust to the specificities ensuing from the chemical or microbial nature of the active substance.

This specific programme focused on the risk assessment of microorganisms used as pesticides, especially on the low-risk criteria linked to antimicrobial resistance and the risk assessment of secondary metabolites. The fellow also investigated the use of microorganisms in integrated pest management (IPM) programmes.

1.1. Low-risk active substances

There is currently no simple regulatory definition of what a low-risk active substance is in the EU. Instead, the following exclusion criteria were set in Annex II, point 5 of Regulation (EC) No 1107/ 2009, regardless of the nature of the active substance considered:

'An active substance shall not be considered of low risk where it is or has to be classified in accordance with Regulation (EC) No 1272/2008 as at least one of the following:

- *carcinogenic,*
- *mutagenic,*
- *toxic to reproduction,*
- *sensitising chemicals,*
- *very toxic or toxic,*
- *explosive,*
- *corrosive*.

It shall also not be considered as of low risk if:

- *persistent (half-life in soil is more than 60 days),*
- *bioconcentration factor is higher than 100,*
- *it is deemed to be an endocrine disruptor, or*
- *it has neurotoxic or immunotoxic effects'*.

However, the necessity to make a distinction between chemical active substances and microorganisms was identified thereafter and the low-risk criteria were amended by Regulation (EU) 2017/1432 to '*reflect the current state of scientific and technical knowledge'*. Two exclusion criteria were then established for microorganisms: demonstrated adverse effects on non-target insects for baculoviruses and demonstrated multiresistance to antimicrobials used in human or veterinary medicine for other microorganisms. Additionally, since microorganisms are evaluated at strain level, compliance with the low-risk criteria should be assessed at this same level, especially as the antimicrobial resistance mechanisms of different strains belonging to the same species can vary greatly.

Appendix A displays the current exclusion criteria of the different types of active substances.

1.2. Secondary metabolites

Microorganisms are known to produce metabolites. These metabolites are of two types: primary and secondary metabolites. Primary metabolites are directly involved in general metabolic processes such as the growth, development or reproduction of the microorganism and are not considered of potential concern. Secondary metabolites are biosynthesised from primary metabolites and show biological activities often relating to survival and ecological functions of the organism, such as competition against other micro- and macroorganisms, parasitism, symbiosis and transport of substances (OECD, 2018).

Although most of them are of non-concern, some secondary metabolites might produce undesirable adverse effects and threaten human and animal health or the environment. Consequently, the assessment of the potential risk caused by the production of these metabolites is part of the risk assessment performed in the framework of the approval of a microorganism as legally provided in EU data requirements (European Commission, 2013) and Uniform principles for the evaluation and authorisation of plant protection products (European Commission, 2011). However, various feedbacks revealed a need for guidance in interpreting the specific provisions on metabolites, materialising in a guidance document finalised by the Standing Committee on Plants, Animals, Food and Feed (ScoPAFF) at the end of 2020 (European Commission, 2020a). Applicable from 1 November 2021 onwards, this document proposed a practical stepwise approach to assess the risk related to the production of metabolites by microorganisms (intended to be) used as active substances that can be summed up as follows. After determining the type of assessment required, information on the microorganism and its metabolites is collected through literature searches and experimental data to establish a list of metabolites of potential concern. Then, based on data concerning the actual production of the metabolites by the strain under assessment, the possible routes of exposure and qualitative risk assessment, the list is refined to a list of metabolites of concern. Finally, a quantitative risk assessment is performed for each identified metabolite of concern.

1.3. Integrated Pest Management

According to the FAO, Integrated Pest Management is 'an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimize the use of pesticides'. According to the European Commission, it implies the considerations of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of PPPs and other forms of interventions to levels that are economically and ecologically justified, while reducing or minimising risks to human health and the environment.

In May 2020, the European Commission presented the Farm to Fork Strategy, a road map towards a fair, healthy and environmental-friendly EU food system. This action plan includes two pesticide-related goals that should be achieved by 2030: the reduction by 50% of the overall use and risk of chemical pesticides and the reduction by 50% of the use of more hazardous pesticides. By encouraging the use of alternative control techniques such as appropriate cultivation techniques and crop rotation, IPM is one of the main tools in reaching these targets.

The general principles of IPM are set in Annex III to Directive 2009/128/EC and can be summed up as follows:

1) Prevention and/or suppression of harmful organisms by adequate cultivation techniques.
2) Monitoring of harmful organisms with adequate methods and tools.
3) Application of protection measures based on the monitoring of harmful organisms and threshold levels.
4) Preference to biological, physical and non-chemical methods over chemical methods if they provide satisfactory pest control.
5) Preference to specific pesticides with the least side effects on human health, non-target organisms and the environment.
6) Keeping the use of pesticides and other forms of interventions to levels that are necessary.
7) Prevention of resistance development.
8) Checking the success of the applied plant protection measures.

By fitting with several of these principles, microbial pest control products (MPCPs, that is, PPPs using microbial active substances) are an important component of IPM. Firstly, as indicated in principle

4, when providing a sufficient level of pest control, they should be preferred over chemical pesticides. Additionally, MPCPs are usually specific to a particular pest and, as microorganisms are organic matter, they tend to break down more easily and be less persistent than chemical pesticides (Principle 5). Furthermore, microorganisms' modes of action differ from those of chemical pesticides. Consequently, if used with chemical pesticides or other means of protection, they can be a valuable tool in the resistance prevention strategy (Principle 7). All these principles rely on reducing pesticide applications or using lower doses of chemicals, in other words, reducing dependence on chemical control. Thus, microorganisms used as pesticides play a crucial role in reducing resort to chemical active substances (De Cal y Cortina et al., 2020).

2. Description of the work programme

2.1. Aims

As defined in the final work programme, the main aim of this work programme was to share expertise and develop training activities regarding risk assessment of active substances and PPPs. As agreed with the fellow upon arrival at the hosting sites, the work programme was adapted to focus on microorganisms used as active substances. This slight amendment of the work programme seemed particularly appropriate since two new SANCO guidance documents had been published shortly before the beginning of the fellowship. One deals with the approval and low-risk criteria linked to 'antimicrobial resistance' (European Commission, 2020a), while the other addresses the risk assessment of metabolites produced by microorganisms used as plant protection active substances (European Commission, 2020b). Additionally, as a former scientific risk assessor in the Residues and Consumer Safety Unit of Anses (French National Agency for Food, Environmental and Occupational Health Safety), the fellow already had good knowledge of the general concepts regarding the risk assessment of active substances and PPPs at European Level as well as a 6-year experience in the evaluation of the Residues and Metabolism section. Thus, the training sessions provided by INIA-CSIC related mainly to the efficacy section and the environmental fate and behaviours aspects assessed in a dossier.

As previously mentioned, although both chemicals and microorganisms used as active substances fall under the same regulations, the risk assessment process varies depending on the nature of the active substances. During her experience as an assessor of the Residues and Metabolism section, the fellow only evaluated chemical active substances. Thus, to get a comprehensive view of the specificities of the risk assessment of microbial active substances, the fellow had the opportunity to follow a 1-week training course on the risk assessment of microorganisms used as pesticides or biocides organised by the BTSF Academy. The course was very complete and addressed the regulatory framework and all the different scientific aspects (efficacy, toxicology, environmental fate and behaviour, ecotoxicology, sampling techniques) of the risk assessment of microorganisms used as active substances.

In parallel to the training activities, the fellow had to draw up a picture of the situation regarding the approval of microorganisms as active substances in the European Union, with particular attention to the low-risk status and the available information regarding the production of secondary metabolites. This task was then followed by a preliminary assessment of the applicability of the new SANCO Guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substances (European Commission, 2020b). As for every new guidance document, experience and feedback based on concrete case studies are key elements to assess its suitability. The objective of this assessment was to identify recurrent data gaps and critical points that would require further attention once the guidance document will be applicable for the approval of microorganisms as active substances.

Finally, following the training session on efficacy, the use of microorganisms in IPM programmes was investigated.

2.2. Activities

As a first activity, an Excel database compiling general information on all microbial active substances registered in the EU Pesticide database was created to provide an overview of the use of microorganisms as pesticides in the European Union. The type, approval and low-risk status, and, when available, the reference of the related EFSA conclusion, compliance to low-risk criteria, production of metabolites in the active substance, the product or *in situ*, known microbial resistances, data gaps related to secondary metabolites and high concerns were made available for 112 microbial active substances. The scientific data available in the database came from the EU Pesticides database

and the consultation of the EFSA Conclusions published at the time of the review. Appendix B lists the EFSA Conclusions consulted to populate the database.

Since numerous data gaps related to the possible production of secondary metabolites, a second database compiling all the metabolites mentioned in the EFSA conclusions of microorganisms used as active substances together with information regarding the production of these metabolites, when available. One of the aims of this task was also to capture the amount of data on secondary metabolites currently available in the monographs. Consequently, EFSA conclusions were also systematically reviewed for data on analytical methods, toxicity or environmental fate. One hundred and twenty secondary metabolites were registered in the database but specific analytical, toxicology and fate data could be reported for only 6, 20 and 5 of them, respectively.

Based on the experience of the UPF members, *Bacillus amyloliquefaciens* and *Beauveria bassiana* were chosen as case studies to assess the applicability of the new guidance document (European Commission, 2020b). As some of the oldest species approved as pesticide active substances in the European Union, sufficient data were presumed to be available in the literature to carry out the case studies. However, since neither the fellow nor members of the UPF team had experience in toxicology, only the first steps of the guidance document were investigated. Indeed, after a few steps, an assessment of the available toxicological data is required to refine the list of metabolites of potential concern that should be further assessed. Consequently, the task mainly consisted in systematic literature reviews on the two microorganism species to identify the secondary metabolites of potential concern. As recommended in the guidance document, the systematic literature reviews were conducted according to EFSA guidance on the submission of scientific peer-reviewed open literature (EFSA, 2011). Taking advantage of INIA's subscriptions, the literature reviews were performed using Scopus, PubMed and Web of Science databases.

In order to acquire knowledge and deepen her understanding of the efficacy section, the fellow was provided with a lot of documentation and received personal training. This training helped the fellow understand the structure and the different aspects studied under the efficacy section that in reality goes way beyond assessing the sole effectiveness of the product since it also covers aspects like adverse effects and phytotoxicity on target plants, development of resistance, effects on yield and quality of plants or transformation process and other undesirable and unintended side effects.

Stand-alone MPCPs tend to have lower efficacy than chemical PPPs. Nonetheless, since these products tend to be generally less persistent, less harmful to the environment and the non-target organisms (NTOs) and intended as components of IPM programmes, reduced data packages and demonstrated efficacy are usually accepted. However, is the inclusion of MPCPs in IPM programmes used by the applicants to defend the authorisation of their product? To answer this question, the fellow reviewed the efficacy section of various registration reports available on the collaborative platform of the European Commission, CIRCABC. This review was retrained to the MPCPs using a strain of *Bacillus* as an active substance, assuming that this sample of products would permit an overview of the current situation. To complete the picture, the fellow also looked into the national guides for IPM, the *'Guías de Gestión Integrada de Plagas'*, developed by the Spanish Ministry of Agriculture, Fisheries and Food (Ministerio de Agricultura, Pesca y Alimentación) to analyse the proposed recommendations regarding the use of MPCPs.

Finally, the fellow received training in environmental fate and behaviour, especially in the different steps and software programmes of FOCUS used to determine the predicted environmental concentrations (PECs) of active substances and degradation products in surface and groundwater. As an application of this training, calculations of PEC_{SW} and PEC_{sed} using software programme STEPS1–2 were performed for crystal proteins, some secondary metabolites produced by Bacillus thuringiensis and the only metabolites for which some basic fate data (DT_{50}, K_{OC}) were available. However, due to the lack of specific fate data, the assessment could not be further refined using STEP 3 calculations. A summary of the calculations is available in Appendix C.

3. Conclusions

3.1. Low-risk active substances

Since low-risk active substances have less-negative effects on human and animal health and the environment, their use should be promoted, according to Directive 2009/128/EC of the European Parliament and the Council on the sustainable use of pesticides (European Commission, 2009b). In particular, as provided in Article 12 of this directive, Member States shall consider in the first place

the use of low-risk pesticides, that is to say, pesticides containing only low-risk substances and for which no specific mitigation measures are required. However, some active substances were authorised in the European Union under Council Directive 91/414/EEC (European Commission, 1991), thus before the legal provisions for the approval of active substances as low-risk. Consequently, although some of these active substances would probably be approved nowadays as low risk, it is not until the renewal of their approval that they will be considered as such. Meanwhile, to help the EU Member States comply with Article 12 of the Commission Directive, the European Commission drew up a list of potentially low-risk active substances approved for use in plant protection (European Commission, 2018).

Of the 65 microorganisms authorised to date as active substances in the European Union, 20 are currently considered low risk. Although representing only 15% of the active substances approved in the European Union (65 of 448), microorganisms represent more than 60% of the low-risk active substances. However, when considering the list of potentially low-risk active substances established by the European Commission, an additional 35 microorganisms are expected to be of low risk, the number of potential low-risk microorganisms adding up to 55 of 65, that is, 85% of the microbial active substances. These numbers outline the central role that microorganisms play as low-risk active substances, thus their key importance in achieving the challenges of the Farm to Fork strategy.

3.2. Applicability of the new SANCO Guidance

Systematic literature reviews were performed to identify the secondary metabolites and toxins produced by the authorised strains of *Bacillus amyloliquefaciens* and *Beauvaria bassiana*. Thanks to the technological advances and important cost reductions of the last decade in whole-genome sequencing (WGS), it is nowadays relatively simple to obtain the genomic profile of a microorganism. Consequently, information was available regarding genes encoding for metabolites that could be produced by these microorganisms. However, little information was found regarding the conditions in which such genes would be expressed, leading to the synthesis of the metabolite. When such information was found, it mainly concerned metabolites already known to be produced by the strain in certain conditions.

The relevant articles captured in the search can be divided into two groups:

- Articles in which a complete genotyping of a specific strain is performed and whose outcome is a very long list of secondary metabolites that could be produced by the microorganism since encoded in its genome.
- Articles investigating the conditions of production of secondary metabolites known for a long time to be produced by a specific species of microorganisms.

The outcomes of the literature searches were thus very exhaustive lists of metabolites encoded in the genome of the strains with no specific information regarding their synthesis in the conditions of production of the Microbial Pest Control Agent (MPCA) or *in situ*, except for already well-known metabolites such as beauvericin, a toxin produced by *Beauvaria bassiana*. In other words, too much information at the genomic level and too little at the proteomic one. Yet, when conducting a risk assessment of the secondary metabolites produced by a given strain, information regarding the production of the metabolites is essential to estimate the exposure component of the risk.

According to the guidance document, once the list of metabolites of potential concern is established, an additional literature search should be performed for each identified metabolite to determine if there is an indication for antimicrobial activity or hazardousness. When ending up with an exhaustive list of all the possible metabolites encoded in the genome of the microorganism, this represents a tremendous amount of work, especially since it implies searching for hazardous effects on human and non-target organisms (NTOs).

Following the SANCO guidance document, a few steps after establishing a list of metabolites of potential concern comes the question of the actual production of the metabolite. If it cannot be excluded, a risk assessment should be performed requiring the determination of toxicity reference values and ecotoxicity testing. It can therefore be foreseen that without information regarding the production of secondary metabolites, a significant amount of (eco-)toxicity data will have to be generated to avoid numerous data gaps.

3.3. Integrated Pest Management

As previously mentioned (see Section 2.2), data provided in the efficacy section for the assessment of MPCPs can be scarce. In particular, although MPCPs are an important component of IPM programmes and permit to reduce the use of chemical active substances, this aspect, which compensates for their sometimes lower effectiveness as stand-alone products, is not always mentioned by the applicants. Thus, out of the 31 reviewed Registration Reports of MPCPs using a strain of Bacillus as an active substance, only 71% of the documents included specific IPM data or repeated mentions of the possibility to use the product in IPM strategies. Additionally, despite the dedicated IPM section of the Registration Reports template, IPM was not mentioned at all in 10% of the cases.

Mentioned under Article 14 of Directive 2009/128/EC, integrated pest management is a key tool to ensure sustainable use of pesticides, which is one of the objectives of the European Green Deal. According to the regulation, Member States *'shall ensure that professional users have at their disposal information and tools for pest monitoring and decision making, as well as advisory services on integrated pest management'* and *'establish appropriate incentives to encourage professional users to implement crop or sector-specific guidelines for integrated pest management on a voluntary basis'* (European Commission, 2009b). To comply with these provisions, Spain developed and implemented 39 crop-type-specific guides for IPM. However, although several of the guides recommend using products containing microorganisms as a general measure, specific species of microorganisms are rarely mentioned and no recommendation about specific strains to be used to control specific pests was found. It could be argued that the Member States are required to respect the principle of free competition and thus cannot recommend a specific species or strain over another when several microbial active substances are authorised to control a given pest. However, this is probably more likely due to the lack of knowledge and scientific projects investigating crop-specific IPM programmes.

An interesting example of scientific projects to promote is the Operational Group FITOSCEREZO project conducted by INIA-CSIC from 2019 to 2021. The project aimed at designing and developing an IPM programme for cherry trees in two regions of Spain, Aragón and Extremadura. The outcome of the project was a new IPM programme allowing the control of six plant diseases[1] and six plant pests[2] (De Cal y Cortina et al., 2021).

In conclusion, to overcome the need for concrete IPM programmes, more projects should be conducted at national and EU levels to provide suitable sector-specific guidelines to professional users.

3.4. Fellowship experience

Apart from the personal enrichment that always brings an experience in a foreign country, the working programme has been the opportunity to gain experience in different areas of pesticide risk assessment, especially regarding efficacy and environmental fate and behaviour. The fellow also acquired a lot of knowledge about microorganisms, especially in the context of pesticide risk assessment. Finally, having learned to perform systematic literature reviews according to EFSA standards will serve the fellow's future professional experiences for sure.

References

De Cal y Cortina A, Guijarro Díaz-Otero B, Larena Nistal I, Melgarejo P, 2020. Which Biocontrol Strategies Best Fit with Other IPM System Components? In: De Cal y Cortina DE, Melgarejo P and Magan N (Eds.)., How Research Can Stimulate the Development of Commercial Biological Control Against Plant Diseases. Progress in Biological Control, vol. 21, Springer, Cham. https://doi.org/10.1007/978-3-030-53238-3_14

De Cal y Cortina DE, Larena Nistal I, González-Nuñez M, Sánchez-Ramos I, Seris Barrallo E, López Goti C, Sandín-España P, Alonso Prados JL, 2021. FITOSCEREZO: programa de control integrado de plagas y enfermedades para el uso racional de productos fitosanitarios en el cultivo del cerezo, PHYTOMA-España, 330. Junio-Julio, 330, 58–64.

EFSA (European Food Safety Authority), 2011. Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (OJ L 309, 24.11.2009, p. 1-50). EFSA Journal 2011;9(2):2092, 49 pp. https://doi.org/10.2903/j.efsa.2011.2092

[1] Brown rot, caused by *Monilinia spp*., shot hole, caused by *Stigmina carpophila*, cherry leaf spot (CLS), caused by *Blumeriella jaapii*, cherry leaf scorch caused by *Apiognomonia erythrostoma*, bacterial canker, caused by *Pseudomonas syringae*, and crown gall, caused by *Agrobacterium tumefaciens*.

[2] *Myzus cerasi*, *Rhagoletis cerasi*, *Drosophila suzukii*, *Tetranychus urticae*, *Frankiniella occidentalis* and *Thrips tabaci*.

European Commission, 1991. Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market, OJ L 230, 19.8.1991, p. 1–32.
European Commission, 2009a. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC, OJ. L 309, 24.11.2009, p. 1–50.
European Commission, 2009b. Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. OJ L 309, 24.11.2009, p. 71–86.
European Commission, 2011. Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products, OJ L 155, 11.6.2011, p. 127–175.
European Commission, 2013. Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, OJ L 93, 3.4.2013, p. 1–84.
European Commission, 2018. Commission notice concerning a list of potentially low-risk active substances approved for use in plant protection. Official Journal of the European Union, C 265, 27.7.2018, 2018/C 265/02, p. 8–11.
European Commission, 2020a. Guidance on the approval and low-risk criteria linked to "antimicrobial resistance" applicable to microorganisms used for plant protection in accordance with Regulation (EC) No 1107/2009, SANTE/2020/12260, 23 October 2020.
European Commission, 2020b. Guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substances in accordance with Article 77 of Regulation (EC) No 1107/2009, SANTE/2020/12258, 23 October 2020.
OECD (Organisation for Economic Co-operation and Development), 2018. Series on Pesticides No. 98, Working document on the risk assessment of secondary metabolites of microbial biocontrol agents. ENV\JM\MONO\(2018)33.

Abbreviations

Anses	Agence Nationale de la Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail - French Agency for Food, Environmental and Occupational Health & Safety
EU-FORA	EUropean FOod Risk Assessment
INIA	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria – Spanish National Institute for Agricultural and Food Research and Technology
IPM	Integrated Pest Management
MPCA	Microbial Pest Control Agent
MPCP	Microbial Pest Control Product
NTO	Non-Target Organism
PEC	Predicted Environmental Concentration
PPP	Plant Protection Product
RR	Registration Report
SCoPAFF	Standing Committee on Plants, Animals, Food and Feed
WGS	Whole Genome Sequencing

Appendix A – Exclusion criteria applying for the approval of low-risk active substances in the European Union (consolidated version of Regulation (EC) No 1107/2009)

Exclusion/Cut-off criteria applying for the approval as low-risk active substance	Chemical active substances			Microorganisms including viruses	
	Synthetical	Naturally occurring	Emitted and used by plants, animals and other organisms for communication	Baculoviruses	Other microorganisms and viruses
(a) classified in accordance with Regulation (EC) No 1272/2008 as any of the following: *— carcinogenic category 1A, 1B or 2,* *— mutagenic category 1A, 1B or 2,* *— toxic to reproduction category 1A, 1B or 2,* *— skin sensitiser category 1,* *— serious damage to eye category 1,* *— respiratory sensitiser category 1,* *— acute toxicity category 1, 2 or 3,* *— specific Target Organ Toxicant, category 1 or 2,* *— toxic to aquatic life of acute and chronic category 1 on the basis of appropriate standard tests,* *— explosive,* *— skin corrosive, category 1A, 1B or 1C;*	X	X	X		
(b) identified as priority substance under Directive 2000/60/EC;	X	X	X		
(c) deemed to be an endocrine disruptor;	X	X	X		
(d) neurotoxic or immunotoxic effects	X	X	X		
persistent (half-life in soil is more than 60 days) or its bio-concentration factor is higher than 100.	X				
at strain level, demonstrated multiple resistance to anti-microbials used in human or veterinary medicine					X
at strain level, demonstrated adverse effects on non-target insects.				X	

N.B.: Information highlighted in green corresponds to the precisions and additional criteria listed under the Annex of Reg. (EU) 2017/1432, compared to Annex II, Point 5 of Reg. (EC) No 1107/2009.

Appendix B – References of the EFSA Conclusions used to populate the databases

EFSA Question Number	Microorganism	Reference
EFSA-Q-2009-00324	Adoxophyes orana GV strain BV-0001	EFSA Journal 2012;10(4):2654
EFSA-Q-2017-00055	Akanthomyces muscarius Ve6 (formerly Lecanicillium muscarium strain Ve6)	EFSA Journal 2020;18(6):6121
EFSA-Q-2015-00021	Ampelomyces quisqualis strain AQ10	EFSA Journal 2017;15(12):5078
EFSA-Q-2011-01200	Aureobasidium pullulans (strains DSM 14940 and DSM 14941)	EFSA Journal 2013;11(4):3183
EFSA-Q-2016-00172	Bacillus amyloliquefaciens (former subtilis) str. QST 713	EFSA Journal 2021;19(1):6381
EFSA-Q-2015-00614	Bacillus amyloliquefaciens AH2	EFSA Journal 2020;18(7):6156
EFSA-Q-2014-00323	Bacillus amyloliquefaciens MBI 600	EFSA Journal 2016;14(1):4359
EFSA-Q-2014-00322	Bacillus amyloliquefaciens strain FZB24	EFSA Journal 2016;14(6):4494
EFSA-Q-2013-00038	Bacillus amyloliquefaciens subsp. plantarum D747	EFSA Journal 2014;12(4):3624
EFSA-Q-2011-00999	Bacillus firmus I-1582	EFSA Journal 2012;10(10):2868
EFSA-Q-2012-00776	Bacillus pumilus QST 2808	EFSA Journal 2013;11(8):3346
EFSA-Q-2015-00389	Bacillus subtilis strain IAB/BS03	EFSA Journal 2018;16(6):5261
EFSA-Q-2016-00696	Bacillus thuringiensis subsp. Aizawai strain ABTS-1857	EFSA Journal 2020;18(10):6294
EFSA-Q-2016-00698	Bacillus thuringiensis subsp. Aizawai strain GC-91	EFSA Journal 2020;18(11):6293
EFSA-Q-2016-00699	Bacillus thuringiensis subsp. Israeliensis (serotype H-14) strain AM65-52	EFSA Journal 2020;18(12):6317
EFSA-Q-2016-00697	Bacillus thuringiensis subsp. Kurstaki strain ABTS 351	EFSA Journal 2021;19(10):6879
EFSA-Q-2017-00131	Bacillus thuringiensis subsp. Kurstaki strain EG 2348	EFSA Journal 2021;19(4):6495
EFSA-Q-2017-00133	Bacillus thuringiensis subsp. Kurstaki strain PB 54	EFSA Journal 2021;19(4):6496
EFSA-Q-2017-00132	Bacillus thuringiensis subsp. Kurstaki strain SA-11	EFSA Journal 2020;18(10):6261
EFSA-Q-2016-00700	Bacillus thuringiensis subsp. Kurstaki strain SA-12	EFSA Journal 2020;18(10):6262
EFSA-Q-2009-00250	Bacillus thuringiensis subsp. Tenebrionis strain NB 176 (TM 14 1)	EFSA Journal 2013;11(1):3024
EFSA-Q-2017-00593	Beauveria bassiana 203	EFSA Journal 2020;18(11):6295
EFSA-Q-2015-00362	Beauveria bassiana IMI389521	EFSA Journal 2017;15(9):4831
EFSA-Q-2015-00361	Beauveria bassiana PPRI 5339	EFSA Journal 2018;16(4):5230
EFSA-Q-2014-00324	Beauveria bassiana strain 147	EFSA Journal 2015;13(10):4261
EFSA-Q-2009-00251	Beauveria bassiana strain ATCC 74040	EFSA Journal 2013;11(1):3031
EFSA-Q-2009-00252	Beauveria bassiana strain GHA	EFSA Journal 2013;11(1):3031
EFSA-Q-2014-00327	Beauveria bassiana strain NPP111B005	EFSA Journal 2015;13(10):4264
EFSA-Q-2009-00338	Candida oleophila strain O	EFSA Journal 2012;10(11):2944
EFSA-Q-2013-00548	Cerevisane	EFSA Journal 2014;12(6):3583
EFSA-Q-2015-00582	Clonostachys rosea strain J1446 (Gliocladium catenulatum strain J1446)	EFSA Journal 2017;15(7):4905
EFSA-Q-2014-00656	Coniothyrium minitans Strain CON/M/91-08 (DSM 9660)	EFSA Journal 2016;14(7):4517
EFSA-Q-2009-00254	Cydia pomonella Granulovirus (CpGV)	EFSA Journal 2012;10(4):2655
EFSA-Q-2009-00341	Helicoverpa armigera nucleopolyhedrovirus (HearNPV)	EFSA Journal 2012;10(9):2865
EFSA-Q-2013-00833	Isaria fumosorosea Apopka strain 97 (formerly Paecilomyces fumosoroseus)	EFSA Journal 2014;12(5):3679
EFSA-Q-2017-00139	Metarhizium anisopliae var. anisopliae strain BIPESCO 5/F52	EFSA Journal 2020;18(10):6274
EFSA-Q-2015-00546	Metschnikowia fructicola	EFSA Journal 2017;15(12):5084
EFSA-Q-2014-00474	Mild Pepino Mosaic Virus isolate VC 1	EFSA Journal 2017;15(1):4651
EFSA-Q-2014-00472	Mild Pepino Mosaic Virus isolate VX 1	EFSA Journal 2017;15(1):4650
EFSA-Q-2009-00323	Paecilomyces fumosoroseus strain Fe9901	EFSA Journal 2012;10(9):2869
EFSA-Q-2015-00405	Pasteuria nishizawae Pn1	EFSA Journal 2018;16(2):5159

EFSA Question Number	Microorganism	Reference
EFSA-Q-2018-00110	Pepino mosaic virus (PepMV) Chilean (CH2) strain, mild isolate Abp2 (PEPMVO)	EFSA Journal 2021;19(1):6388
EFSA-Q-2018-00111	Pepino mosaic virus (PepMV) European (EU) strain, mild isolate Abp1 (PEPMVO)	EFSA Journal 2021;19(1):6388
EFSA-Q-2014-00054	Pepino mosaic virus strain CH2 isolate 1906	EFSA Journal 2015;13(1):3977
EFSA-Q-2017-00140	Phlebiopsis gigantea strain FOC PG 410.3	EFSA Journal 2016;17(10):5820
EFSA-Q-2017-00140	Phlebiopsis gigantea strain VRA 1835	EFSA Journal 2016;17(10):5820
EFSA-Q-2017-00140	Phlebiopsis gigantea strain VRA 1984	EFSA Journal 2016;17(10):5820
EFSA-Q-2015-00814	Pseudomonas chlororaphis strain MA342	EFSA Journal 2017;15(1):4668
EFSA-Q-2011-01198	Pseudomonas sp. Strain DSMZ 13134	EFSA Journal 2012;10(12):2954
EFSA-Q-2009-00315	Pseudozyma flocculosa	EFSA Journal 2015;13(9):4250
EFSA-Q-2015-00520	Purpureocillium lilacinum strain 251 (former Paecilomyces lilacinus strain 251)	EFSA Journal 2020;18(9):6238
EFSA-Q-2017-00141	Pythium oligandrum M1	EFSA Journal 2020;18(11):6296
EFSA-Q-2014-00333	Saccharomyces cerevisiae strain LAS02	EFSA Journal 2015;13(12):4322
EFSA-Q-2009-00507	Spodoptera littoralis nucleopolyhedrovirus (SpliNPV)	EFSA Journal 2012;10(9):2864
EFSA-Q-2017-00142	Streptomyces K61 (formerly S. griseoviridis)	EFSA Journal 2020;18(7):6182
EFSA-Q-2012-00775	Streptomyces lydicus WYEC 108	EFSA Journal 2013;11(11):3425
EFSA-Q-2009-00300	Trichoderma asperellum (formerly T. harzianum) strain ICC012	EFSA Journal 2013;11(1):3036
EFSA-Q-2009-00300	Trichoderma asperellum (formerly T. harzianum) strain T25	EFSA Journal 2013;11(1):3036
EFSA-Q-2009-00300	Trichoderma asperellum (formerly T. harzianum) strain TV1	EFSA Journal 2013;11(1):3036
EFSA-Q-2011-00899	Trichoderma asperellum strain T34	EFSA Journal 2012;10(5):2666
EFSA-Q-2009-00297	Trichoderma atroviride (formerly T. harzianum) strain IMI 206040	EFSA Journal 2015;13(5):3056
EFSA-Q-2009-00297	Trichoderma atroviride (formerly T. harzianum) strain T11	EFSA Journal 2015;13(5):3056
cf. each strain	Trichoderma atroviride (formerly T. harzianum) strain T11 and IMI 206040	*EFSA Journal 2015;13(5):3056*
EFSA-Q-2011-00900	Trichoderma atroviride strain I-1237	EFSA Journal 2012;10(10):2706
EFSA-Q-2014-00334	Trichoderma atroviride strain SC1	EFSA Journal 2015;13(4):4092
EFSA-Q-2012-00424	Trichoderma gamsii (formerly T. viride) strain ICC080	EFSA Journal 2013;11(1):3062
EFSA-Q-2009-00298	Trichoderma harzianum strain ITEM 908	EFSA Journal 2013;11(10):3055
EFSA-Q-2009-00298	Trichoderma harzianum strain T-22	EFSA Journal 2013;11(10):3055
EFSA-Q-2009-00299	Trichoderma polysporum strain IMI 206039	EFSA Journal 2013;11(1):3035
EFSA-Q-2017-00296	Verticillium albo-atrum (formerly Verticillium dahliae) strain WCS850	EFSA Journal 2019;17(1):5575
EFSA-Q-2009-00346	Zucchini yellow mosaic virus – weak strain	EFSA Journal 2012;10(6):2754

Appendix C – Summary of PECSW and PECsed calculations using software programme STEPS1-2

Parameters considered for the calculations – based on data available for different crystal proteins (protoxin, Cry1Aa, Cry1Ab, Cry1Ac & Cry3Bb1)

Water solubility (mg.L^{-1})	1
DT_{50} in soil (days)	41.3
DT_{50} in sediment/water system (days)	28
DT_{50} in water (days)	110.7
DT_{50} in sediment (days)	96
Kd_{OC} (L.kg^{-1})	1,000

Microorganism	Most critical scenario	STEP 1		STEP 2	
		PEC_{SW} (µg/L)	PEC_{sed} (µg/kg)	PEC_{SW} (µg/L)	PEC_{sed} (µg/kg)
ABTS-1857	Pepper (vegetables, fruiting); 8 × 182* g/ha; 6-day interval No interception; Southern Europe, March-May	221.39	2,090	59.46	584.04
GC-91	Pome fruits, early application; 6 × 80* g/ha; 7-day interval Minimal crop interception; Southern Europe, March-May	115.29	864.26	32.36	289.51
AM65-52	*Not performed - only indoor uses*				
ABTS-351	Cabbage (Brassica (vegetables, root)); 8 × 64.8* g/ha; 7 day interval No interception; Southern Europe, March-May	78.82	742.4	20.18	198.2
EG-2348	Pome fruits, early application; 10 × 234.6* g/ha; 7-day interval Minimal crop interception; Southern Europe, March-May	563.46	4,220	135.8	1,210
PB-54	Ornamental trees (pome fruits, early application); 3 × 48* g/ha; 7-day interval Minimal crop interception; Southern Europe, March-May	34.59	259.28	11.16	100.08
SA-11	Pome fruits, early application; 6 × 100.3* g/ha; 7-day interval Average crop interception; Southern Europe, March-May	144.54	1,080	35.68	314.4
SA-12	Ornamental trees (pome fruits, early application); 6 × 411.4* g/ha; 7-day interval Minimal crop interception; Southern Europe, March-May	592.86	4,440	166.42	1,490

*: The concentrations of applied crystal proteins used in the calculations are based on the average concentration of total Cry-proteins in the technical active substance and the content of technical grade active substance in each representative product.

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Meta-analysis and systematic literature review of climate change effects on livestock welfare

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Abstract

Climate change is a phenomenon that includes different dramatic events that deeply affect the physiology of animal species both directly and indirectly with qualitative–quantitative impacts on livestock performances and health. The implications of climate change on animal welfare and on production demand are complex and call for a multidisciplinary approach which involved both animal science and economic sciences. The current technical report will describe the activities performed by the fellow while placed at the University of Foggia, Department of Agriculture, Natural Resources and Engineering, in Italy. Furthermore, the work programme offered by the hosting site consisted in performing a systematic literature review, following the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) Statement, and a quantitative synthesis of the literature on the impact of climate change events (e.g. heat stress) on livestock welfare and productivity and the effect of heat relieving strategies on the animals' performance.

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Keywords: climate change, heat stress, livestock welfare and production, systematic literature review, meta-analysis

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1. Introduction

1.1. Climate change and livestock welfare

Climate change is a complex phenomenon that includes different dramatic events, such as global warming, floods and droughts, and all these events deeply affect the physiology of animal species both directly and indirectly with semi-quantitative impacts on livestock performances and health. The implications of climate change on animal welfare and on production demand are complex and call for a multidisciplinary approach which involves both animal science and economic sciences.

According to Filipe et al. (2020), global warming is the main and strongest phenomenon associated with climate change. Furthermore, the Earth's temperature is increasing by 0.2°C per decade and it is estimated that by 2,100, it will be increased by 1.4–5.8°C. Also, most of the studies on climate change effects on immune system and, thus, animal's welfare and health, focus on the increasing temperatures worldwide. Heat stress has several negative consequences on livestock, with activation of mechanisms that either aim at reducing the production of heat due to metabolism or increase inflammatory responses or lead to immune-suppression, such as: (i) decrease in daily intake; (ii) increase in peripheral cortisol levels; (iii) production of heat shock proteins (HSPs).

To the best of our knowledge, this is the first meta-analyses performed on the impact of climate change (e.g. heat stress) on livestock welfare, particularly on the effects on ruminants' performance and health. We have put higher relevance on the temperature rise events (i.e. heat stress), due to the conspicuous number of studies published on the effects that temperature has on livestock (Rahbar et al., 2019; Ahmad Para et al., 2018) and, in consideration of the relevant influence that changes in temperature have on the immune response system of different species. This latter aspect has been rarely investigated.

According to Baumgard and Rhoads (2013), it undermines genetic, nutritional, pharmaceutical and management advances made by the animal agriculture industries.

When the ambient temperature and other environmental conditions create a situation that is either below or above the respective threshold values, the animal's efficiency is compromised mainly because nutrients are diverted to maintain euthermia, as preserving a safe body temperature becomes the highest priority, and product synthesis (e.g. milk or meat) is deemphasised.

Heat stress negatively impacts a variety of productive parameters including: (a) milk yield and composition, (b) growth, (c) reproduction and (d) carcass traits.

In addition, a heat load: (1) increases health care costs, and (2) animals can even succumb to severe thermal stress, especially lactating cows and animals without shade.

Livestock welfare can be evaluated considering the Welfare Quality Protocol, using four categories for all species. These are described as good feeding, good housing, good health and good behaviour (Welfare Quality®, 2009). Good feeding includes two criteria: absence of prolonged hunger and absence of prolonged thirst. Good housing includes three criteria: comfort around resting, thermal comfort and ease of movement. The first criterion is measured as time needed to lie down, animals colliding with housing equipment during lying down, number of animals lying partly or completely outside the lying area and cleanliness of different body parts (udders, flank/upper legs and lower legs). Moreover, good health includes three different criteria: absence of injuries (lameness), absence of disease and absence of pain induced by management procedures. Finally, good behaviour, although not represented in our study due to the type of experiments used in our systematic literature review, it encompasses four criteria: expression of social behaviours, expression of other behaviours, good human–animal relationship and positive emotional state.

1.2. Heat stress and Temperature–humidity index (THI)

Heat stress is caused by a combination of environmental factors (temperature, relative humidity, solar radiation, air movement and precipitation).

Thom (1959) introduced a temperature–humidity index (THI) to characterise the combined effect of the environmental climatic conditions (dry temperature, relative humidity, dew point). Both temperature and humidity can act together as stressors for animals. According to Correa-Calderon et al. (2004), many indices combining these different environmental factors could be used to measure the level of heat stress (Correa-Calderon et al., 2004). However, most studies on heat stress in livestock focused mainly on temperature and relative humidity due to data on temperature and humidity records being able to be obtained from a meteorological station located nearby, in contrast to

the publicly unavailable data regarding the amount of thermal radiation received by the animal, wind speed and rainfall. The parameter that describes heat load on animals and is a good indicator of stressful thermal climatic conditions is the THI, combining both temperature and humidity and measures animal comfort.

Furthermore, in relation to THIs measuring sensitivity, according to Habeeb et al. (2018), THI is better predictors of body temperature in heat-stressed cows than other measurements of environmental conditions. Also, THI serves as indicators to heat stress of climatic conditions with relation to production and reproduction of farm animals, which in our case, the accounted species were, besides bovine, ovine and caprine. THI indices can be placed into classes to indicate the degree of heat stress and the terms used to describe these classes and the ranges of THI used to define each class are arbitrary. In our study, we classified the degrees of heat stress according to Marai et al. (2007) and Marai et al. (2001) for small ruminants (ovine and caprine) (Marai, et al., 2007; Marai et al., 2001).

Depending on the study, THI may be calculated using different equations, for instance:

THI = 0.8 dbT + RH × (dbT − 14.4) + 46.4; where dbT is dry bulb temperature (°C) and RH is relative humidity in decimal form. A THI of 74 or less is considered normal, 75–78 is alert status, 79–83 is danger status and a THI equal to or above 84 is an emergency (Tom, 1959). In Fahrenheit, the THI is arrived at from a combination of wet and dry bulb air temperature for a particular day and expressed in a formula as follows:

$$\mathrm{THI} = 0.72(\mathrm{W^{\circ}C} + \mathrm{D^{\circ}C}) + 40.6,$$

where W°C = wet bulb and D°C = dry bulb. In this case, THI values of 70 or less are considered comfortable, 75–78 stressful, and values greater than 78 cause extreme distress and animals are unable to maintain thermoregulatory mechanisms or normal temperature (McDowell et al., 1976).

From Marai et al. (2007), the changes in THI mean values may also depend upon the equation used. For instance, when temperature is measured (°F, Fahrenheit), the equation to determine THI is as follows (LPHSI, 1990):

$$\mathrm{THI} = \mathrm{db^{\circ}F} - [(0.55 - 0.55\ \mathrm{RH})(\mathrm{db^{\circ}F} - 58)],$$

where db°F is the dry bulb temperature in °F and RH is the relative humidity (RH%)/100, for sheep and goats.

The obtained values indicate the following (LPHSI, 1990): THI < 82 (absence of heat stress); 82 to < 84 (moderate heat stress); 84 to < 86 (severe heat stress); and over 86 (extreme severe heat stress). However, when the temperature is expressed in °C, the equation changes as follows:

$$\mathrm{THI} = \mathrm{db^{\circ}C} - [(0.31 - 0.31\ \mathrm{RH})(\mathrm{db^{\circ}C} - 14.4)],$$

where db°C is the dry bulb temperature (°C) and RH is the relative humidity (RH%)/100. The values obtained indicate the following: THI < 22.2 (absence of heat stress); THI from 22.2 to < 23.3 (moderate heat stress); THI from 23.3 to < 25.6 (severe heat stress) and THI ≥ 25.6 (extreme severe heat stress).

This way, according to Johnson et al. (1989), THI is still the simplest and most practical index for measuring environmental warmth which causes heat stress in cattle, hence, being thoroughly used for estimation of the level of heat stress.

2. Description of work programme

2.1. Aims

As part of the EU-FORA fellowship, the study aims at involving the fellow in all the activities required for investigating the impact of climate change events (e.g. heat stress) on livestock welfare and productivity and the effect of heat-relieving strategies on the animals' performance, through a SLR, following the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) Statement, and a quantitative synthesis of the literature on the issue.

2.2. Activities/Methods

For development of the work programme, the priority of the hosting site was to provide the fellow with the basic theoretical background required to perform a meta-analysis with a systematic literature review. The fellow joined a working team from both animal and socio-economic sciences with proved expertise in the use of meta-analytic tools and received training on specific topics such as:

- Performing a meta-regression analysis (MRA);
- Handling of electronic databases (e.g. Scopus);
- Performing PRISMA guidelines for data screening on climate change and livestock welfare;
- Developing inclusion/exclusion criteria regarding climate change effects, particularly regarding heat stress, and livestock welfare, with access to the available databases on the subject;
- Overview of the animal welfare protocol used for Bovine (dairy and beef production), ovine and caprine species;
- Statistical analysis of the data extracted;
- Software tools that can be applied to meta-analysis, that were introduced to the fellow within the scope of course on 'Risk Analysis and Risk Management in Agriculture: Updates on Modelling and Applications' (e.g. STATA, R and R Studio).

2.2.1. Experience gained on meta-regression analysis (MRA): systematic literature review (SLR) and semi-quantitative analysis

In order to combine both climate change effects in livestock welfare and productivity, a review of the already performed meta-analytical studies on the subject of interest was conducted. Using the Scopus electronic database, the results, with refinement for category 'article' written in 'English' from peer-reviewed journals, and with criteria selected regarding only bovine, ovine and caprine species, for data screening and extraction were selected nine articles (Table 1).

Table 1: Search strings, rationale for inserting the systematic literature review, results I (without refinement) and II (with refinement) of the data screening, regarding possible already performed meta-analytical studies on climate change and livestock welfare

Editor	Tool[(a)]	Purpose	Search strings	Results I[(b)]	Results II[(c)]
Morgado JN et al.	Scopus	Investigate for already existing meta-analysis on the topic	TITLE-ABS-KEY ("Climate change" OR climat* OR "extreme weather" OR "heat stress" OR "Increased atmospheric carbon dioxide concentration" OR "precipitation variation" AND "animal welfare" OR "BCS" OR "Body weight" OR "Respiration rate" OR "conception rate" OR "rectal temperature" OR "milk yield" OR "carcass weight" OR "fat thickness" OR "behavioural alteration" AND "meta-analysis")	45	9

(a): Electronic database used for search string used.
(b): Number of results without refinement for category 'article' written only in 'English', from peer-reviewed journals.
(c): Number of results, with refinement, selected for screening regarding the criteria in study, including only bovine, ovine and caprine species.

After this first step, the research question and the scope of the SLR were thus defined and further developed.

Therefore, in this study, a higher relevance was put on the temperature rise events (i.e. heat stress), due to the conspicuous number of studies published on the effects that temperature has on livestock (Rahbar et al., 2019; Ahmad Para et al., 2018) and, in consideration of the relevant influence that changes in temperature have on the immune response and health system of different species.

The SLR was conducted using the Scopus database for data sourcing, following all the PRISMA guidelines for screening (Appendix A). The search keywords were selected under four interconnected categories: climate change (CC); animal welfare; species; management strategy.

To be included in the database, studies followed these parameters: category 'Article', written in 'English', from peer-reviewed journals; only bovine, caprine and ovine species; and, for each experimental group, the THI was reported.

For data extraction, the decision for inclusion relied on the following two criteria: experiments had to analyse the animals' performance under both heat stress (HS) and thermoneutral conditions (TN); and control (TN) and heat stress groups had to encompass a management strategy to compare and observe the different effects.

2.2.2. Semi-quantitative analysis

In the count noun sense, a descriptive statistic is a summary statistic that quantitatively describes or summarises features from a collection of information (Mann, 1995), with the aim to summarise a sample. Some measures that are commonly used to describe a data set are measures of central tendency and measures of variability or dispersion. In this analysis, the measures of central tendency included the mean and median, while measures of variability include the standard deviation, the minimum and maximum values of the variables.

The type of indicators found in the studies was from different categories, from which the following can be highlighted: body temperature (rectal temperature, respiration rate); blood parameters (glucose, insulin, BUN, NEFA, pH); milk production and composition (milk yield, protein yield, lactose yield); reproduction and conception rate.

The indicators were classified according to species either all together or individually and to the variation of the Delta THI (severe or otherwise).

In Table 2, an overview of the indicators' descriptive analysis is considered. Three main categories (cat.) were used to estimate the influence of the CC impacts on bovine, ovine and caprine (described as 'all species'), with or without applying management strategies (e.g., dietary interventions; bed treatment; use of fans and or sprinklers). These were defined as: category one (Cat.1), to estimate the impact in control conditions and without management strategies; category 2 (Cat.2), in treatment conditions and with management strategies; and category 3 (Cat. 3), for estimating the impact of adaptation strategies in animals' performances. The level/magnitude of impact that may or not influence the performance of all the species considered was also qualitatively estimated. Performance in our study may encompass milk production, conception rate and reproduction rate, growth rate and carcass traits.

Table 2: Overview of the indicators' descriptive analysis considering climate change categories 1, 2 and 3

Category (Cat.)	Climate change (CC)	CCs conditions' description	Species	Worst performance RF[(a)]	Changes in performance[(b)]
1	Impact (control)	Impact in control conditions comparing both TN and HS: control TN vs. control HS	All species[(1)]	0.26	+
2	Impact (with treatment[(2)])	Impact in treatment conditions comparing both TN and HS	All species	0.15	+++
3	Adaptation	Adaptation effects comparing both experiments in control in HS with treatment in HS	All species	0.14	++

(a): Relative frequency (%).

(b): Expressed qualitatively as the level/magnitude of impact (e.g. +, ++ or +++) that influences all species performance (e.g. worst or improved) for all species:+: climate change impacted for the worst the animals' performance, e.g. low impact on the improvement of the performance; +++: climate change impacted for an improved performance of the animals (highest improvement); ++: climate change impacted towards a better performance of the animals, putting into evidence the adaptive capacity of the animals (intermediate improvement).

(1): All species refer to bovine, ovine and caprine.

(2): Treatment refers to the strategy (e.g. nutritional, mechanical) applied to the animals during experiments to better understand the impact of heat stress and changes on performance of animals.

From the overview, Cat 3 estimates proved that the management (i.e. altered nutritional programs; mechanical, e.g. cooling) strategies applied in the experiments were useful adaptation strategies. In Appendix B statistical descriptive are presented for the different species, as well as the different impacts climate change events (e.g. heat stress) affected the performance (e.g. negatively, positively).

3. Conclusions

3.1. Conclusions from the meta-analytic study

To our knowledge, this was also the first study that classified and analysed indicators regarding direct/indirect effects on performance and animal welfare principles. Expert consultation was also used, thus considered a valid methodological approach for the CC effects on animal welfare and productivity.

According to Vlasova and Saif (2021), the growing world population (7.8 billion) exerts an increased pressure on the cattle industry amongst others. High yielding dairy cattle and their calves are more vulnerable to various diseases leading to shorter life expectancy and reduced environmental fitness. This indicates that improved understanding of cattle immune function is needed to provide optimal tools to combat the existing and future pathogens and improve food security.

From our study, insights were provided on the influence of different treatments/experiments under different climate conditions, and also for the need for adaptation strategies that may help improve animal's welfare as well as their productivity, foreseeing a need for future research on this subject.

3.2. Scientific activities of the fellowship

During the fellowship, the fellow developed the following scientific activities and awarded:

- Presentation online for the Scholar Programs webinar, organised by the Rector's delegate for Scholar Programs of the University of Foggia, on the topic 'EFSA: opportunities for PhD, Post-Doc and Senior scholars', on 22 June 2021;
- One-week Summer School course, organised by Wageningen School of Social Sciences (WASS) on the subject of 'Risk Analysis and Risk Management in Agriculture: Updates on Modelling and Applications'. Held online from 5 July 2021 until 9 July 2021;
- Abstract acceptance for presenting at the LVII Congress of Italian Society of Agrarian Economics or 'Società Italiana di Economia Agraria' (SIDEA) under the topics of 'Enterprises in between innovation, market and environment: the new frontiers of analysis of the agrarian and food companies' (L'impresa tra innovazione, mercato e ambiente: le nuove frontiere di analisi dell'impresa agraria e alimentare) from 16 to 17 September 2021, in Bologna, Italy;
- Abstract acceptance for presenting and attendance at the XXIX Congress of the Italian Society of Agro-food economics or 'Società Italian di Economia Agro-alimentare' (SIEA) under the topics of Brexit, The new Common Agricultural Policy (CAP), Covid-19: Italian agri-food restarts, from 30 September to 1 October 2021, in Verona, Italy.
- Poster presentation with this study's preliminary results to the ONE – Health, Environment, Society – Conference, 21–24 June 2022.

3.3. Conclusions from the participation in the fellowship programme

The main focus of the work programme was the development and application of a Systematic Literature Analysis and meta-analysis in order to estimate the climate change impacts on livestock welfare and productivity. The work plan provided training and knowledge on all the steps and tools required to perform a meta-analysis, taking as an example the CLimate change and Emerging risks for Food Safety (CLEFSA) project on emerging risks, in which animal health and welfare subjects are topics of interest. The fellow performed and was involved in all the activities of the meta-analysis process, from data collection, screening, extraction and analysis, establishing different scenarios of impact and future recommendations regarding adaptive strategies. The fellow had a very significant contribution during all the steps of the programme.

References

Ahmad Para I, Ahmad Dar P, Ahmad Malla B, Punetha M, Rautela A, Maqbool I, Mohd A, Ahmad Shah M, Ahmad War Z, Ishaaq R, Akram Malla W, Ahmad Sheikh A and Rayees M, 2018. Impact of heat stress on the reproduction of farm animals and strategies to ameliorate it. Biological Rhythm Research, https://doi.org/10.1080/09291016.2018.1548870

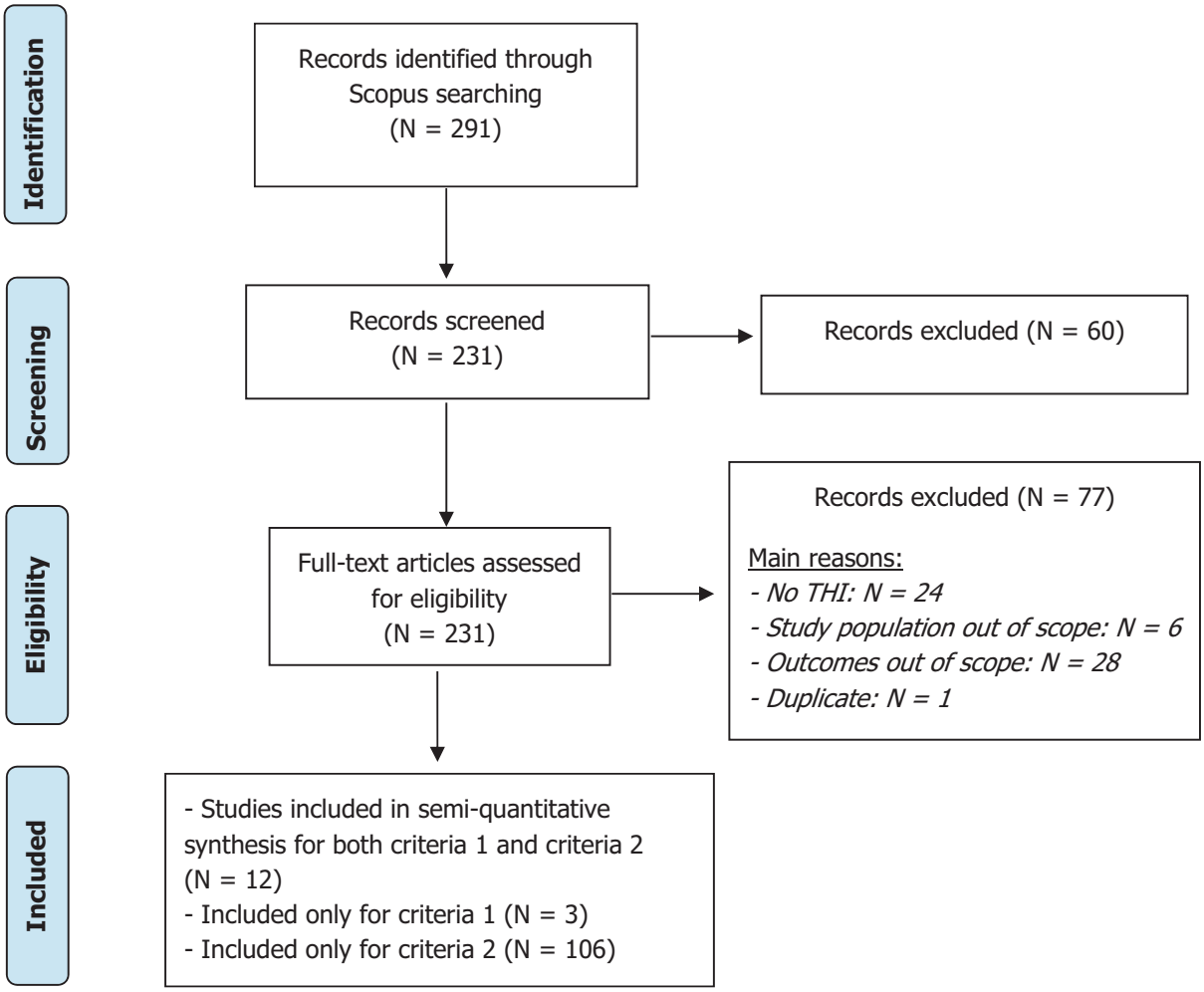
Baumgard LH and Rhoads Jr RP, 2013. Effects of heat stress on postabsorptive metabolism and energetics. Annual Review of Animal Biosciences, 1, 311–337. https://doi.org/10.1146/annurev-animal-031412-103644
Correa-Calderon A, Armstrong D, Ray D, DeNise S, Enns M and Howison C, 2004. Thermoregulatory responses of Holstein and Brown Swiss heat-stressed dairy cows to two different cooling systems. International Journal of Biometeorology, 48, 142–148. https://doi.org/10.1007/s00484-003-0194-y
Filipe JF, Herrera V, Curone G, Vigo D and Riva F, 2020. Floods, hurricanes, and other catastrophes: a challenge for the immune system of livestock and other animals. Frontiers in Veterinary Science, 7, 16. https://doi.org/10.3389/fvets.2020.00016
Habeeb AA, Gad AE and Atta MA, 2018. Temperature-humidity indices as indicators to heat stress of climatic conditions with relation to production and reproduction of farm animals. International Journal of Biotechnology and Recent Advances, 1, 35–50. https://doi.org/10.18689/ijbr-1000107
Johnson HD, Shanklin MD and Hahn L, 1989. Productive adaptability indices of Holstein cattle to environmental heat. Proceedings of Agriculture and Forest and Meteorology Conference, pp. 291–297.
LPHSI, 1990. Livestock and Poultry Heat Stress Indices Agriculture Engineering Technology Guide, Clemson University, Clemson, SC 29634, USA.
Mann PS, 1995. Introductory Statistics, 2nd Edition. Wiley. ISBN 0-471-31009-3.
Marai IFM, Ayyat MS and Abd El-Monem UM, 2001. Growth performance and reproductive traits at first parity of New Zealand White female rabbits as affected by heat stress and its alleviation, under Egyptian conditions. Tropical Animal Health and Production, 33, 457–462.
Marai I, El-Darawany AA, Fadiel A and Abdel-Hafez M 2007. Physiological traits as affected by heat stress in sheep—a review. Small Ruminant Research, 71, 1–12. https://doi.org/10.1016/j.smallrumres.2006.10.003
McDowell RE, Hooven NW and Camoens JK, 1976. Effect of climate on performance of Holsteins in first lactation. Journal of Dairy Science, 59, 965–973. https://doi.org/10.3168/jds.S0022-0302(76)84305-6
Rahbar R, Sadeghi-Sefidmazgi A, Abdullahpour R and Nejati-Javaremi A, 2019. Can post-milking insemination increase conception rate in high-producing Holstein cows under heat stress? A retrospective study. The Journal of Agricultural Science, 157, 254–259. https://doi.org/10.1017/S0021859619000510
Thom EC, 1959. The discomfort index. Weatherwise, 12, 57–59.
Vlasova AN and Saif LJ, 2021. Bovine immunology: implications for dairy cattle. Frontiers in Immunology, 12, 643206. https://doi.org/10.3389/fimmu.2021.643206
Welfare Quality®, 2009. Welfare Quality® assessment protocol for cattle. Welfare Quality® Consortium. Lelystad, Netherlands.

Abbreviations

BUN	Blood Urea Nitrogen
Cat	Category
CC	Climate Change
CLEFSA	CLimate change and Emerging risks for Food Safety
HS	Heat Stress conditions
HSPs	Heat shock proteins
MRA	Meta-regression analysis
NEFA	Non-esterified Fatty Acids
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
SLR	Systematic Literature Review
THI	Temperature–Humidity index
TN	Thermoneutral conditions

Appendix A – PRISMA flow chart

A.1. PRISMA Flow chart of the search of the influence of climate change events (e.g. heat stress) on livestock welfare, health and productivity



Appendix B – Descriptive analysis of climate change effects on livestock

B.1. Descriptive analysis of the impacts of climate change events (e.g. heat stress) on livestock performance for studies that included both criteria 1 and criteria 2

Category (Cat.)	Climate change (CC)	Species	Worst performance RF(1)(a)	Better performance RF(1)(b)	No change RF(1)(c)	NA RF(1)(d)
1	Impact (control)	All species(2)	0.26	0.04	0.31	0,39
		Bovine	0.20	0.03	0.25	0.52
		Caprine	0.36	0.02	0.62	0
		Ovine	0.79	0.17	0.04	0
2	Impact (with treatment)	All species	0.15	0.02	0.34	0.49
		Bovine	0.20	0.03	0.3	0.47
		Caprine	0	0	0.55	0.45
		Ovine	0	0	0	1
3	Adaptation	All species	0.14	0.29	0.44	0.13
		Bovine	0.17	0.25	0.39	0.19
		Caprine	0.02	0.36	0.62	0
		Ovine	0.42	0.42	0.17	0

(a): Climate change effect on different animals' indicators that influence negatively the species performance (e.g. worst performance) expressed as a relative frequency (%).
(b): Climate change effect on different animals' indicators that influence positively the species performance (e.g. better performance) expressed as a relative frequency (%).
(c): No change observed on performance.
(d): Not applicable.
(1): RF stands for relative frequency (%).
(2): All species refer to bovine, ovine and caprine.

EU-FORA SERIES 4

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Improving the risk assessment of antimicrobial resistance (AMR) along the food/feed chain and from environmental reservoirs using qMRA and probabilistic modelling

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Abstract

Efficient risk assessment of antimicrobial resistance (AMR) in environmental reservoirs, particularly agroecosystems, is critical for predicting threats to animal and human health due to infections unresponsive to antibiotic therapy. However, approaches currently employed for the risk assessment of AMR along the human food chain rarely rely on antibiotic resistance gene (ARG) environmental pathways connected to food production and related quantitative data. The present project aimed at improving the risk assessment related to the spread of AMR along the food/feed chain based on ARG quantification in agroecosystems and interconnected environments. The fellow received training and worked in close cooperation with the team on two ongoing research projects which involved: (i) the monitoring of ARGs in field soils and surface waters to identify and characterise food/feed chain-associated environmental reservoirs of AMR relevant at the national level; (ii) the evaluation of ARG dynamics in relation to agricultural practice within an international project assessing biodiversity as an ecological barrier for the spread of clinically relevant ARGs in the environment. ARG quantification was performed using single/multiplex real-time polymerase chain reaction (PCR) with tailor-made primers/probe sets according to in-house optimised and validated conditions. The assessment was completed by a comprehensive revision of available literature data for risk-ranking of ARGs along with a literature review exploring AMR quantitative knowledge gaps and the role of certain AMR determinants encoded on free extracellular DNA (exDNA) in their environmental spread.

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Keywords: antimicrobial resistance, antibiotic resistance genes, ARG, extracellular DNA, risk assessment, agroecosystem

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1. Introduction

1.1. General background on the risk assessment of AMR in agroecosystems

Agroecosystems are considered one of the reservoirs of antimicrobial resistance (AMR) due to risk to human health posed by agrochemicals as a factor contributing to the expression of antibiotic resistance genes (ARGs) in resident bacteria and to ARGs pre-existing in the animal gut (Ramakrishnan et al., 2019). Practice such as irrigation with reclaimed wastewaters and application of manure contribute to the contamination of agricultural soil and crops (Cerqueira et al., 2019a,b, 2020; Sorinolu et al., 2021), while surface runoff and water drainage systems are the primary pathways of ARG diffusion across ecosystem boundaries, in particular to adjacent waterbodies (Iwu et al., 2020). Although the highest concentrations of ARGs upon irrigation with water contaminated by antibiotics have been detected in soil and its surface (Pan and Chu, 2018), current wastewater treatment technologies only partially remove antibiotic residues, ARGs and ARB (Anthony et al., 2020).

Despite a large body of scientific literature addressing AMR, substantial knowledge gaps regarding the complexity of its evolution and spread decrease the accuracy of risk assessment for human health (Niegowska et al., 2021). Monitoring and surveillance of ARGs are the primary tools providing key information for accurate risk assessment of AMR dissemination, however available approaches addressing AMR spread in environmental settings are scarce (Claycamp and Hooberman, 2004; Ashbolt et al., 2013; Baker et al., 2016; Ben et al., 2019). In particular, dose–response models including the quantification of infection risks due to ARGs along the food/feed chain together with long-term holistic studies evaluating the accumulation and dissipation of ARGs related in agroecosystems are missing (FAO/OIE/WHO, 2020; Sorinolu et al., 2021). The development of high-quality risk assessment models requires quantitative data for relevant ARGs, including information on abundance and potential to confer resistance to various antibiotics belonging to distinct classes in pathogenic bacteria (Larsson et al., 2018). Additionally, few quantitative data are reported on the frequency of ARG transfer across ecosystem boundaries and the uptake rates of free extracellular DNA (exDNA) by potentially pathogenic bacteria.

1.2. Hosting site risk assessment projects addressing AMR

Investigation of AMR has been included under the One Health approach from an integrated perspective in which environmental, animal and human compartments are interconnected (WHO, 2017). The role of the environment, including agroecosystems, in halting or promoting the spread of AMR through pathogenic and non-pathogenic microorganisms is under investigation, as foreseen in the FAO action plan on AMR 2021–2025 (FAO, 2021). In this context, Austrian Agency for Health and Food Safety (AGES) is part of the ANTIVERSA project European Consortium which aims at studying biodiversity as an ecological barrier for the spread of clinically relevant antibiotic resistance in diverse terrestrial and aquatic ecosystems, focusing on the impact of ARGs and antibiotic resistant bacteria (ARB) on AMR diffusion in wastewater treatment plant (WWTP) effluents and soil fertilised with manure. The outcomes will help in developing strategies in support of water and soil quality.

Under assignment from the Austrian Ministry of Health and the Ministry of Agriculture, AGES is a leading part of the MARGINS project with the main objective to close quantitative knowledge gaps on ARG prevalence as naturally occurring background loads as well as on their modification and resilience to external inputs in the tested environmental reservoirs. To this end, a range of ARG targets are quantified in various agricultural environments allowing a future selection of few ARGs to be used as locally-specific AMR markers in a realistic scenario where routine monitoring of numerous resistance genes in parallel is not feasible for economic reasons. Additionally, the project foresees the development of optimised operating procedures for sample collection, analysis and data evaluation.

AGES is also partner in the One Health European Joint Programme (OHEJP) and is currently analysing the role of exDNA on the dissemination of AMR in agroecosystems in the framework of the FED-AMR project developed at the Division of Data, Statistics and Risk Assessment.

2. Description of work programme

The fellow was hosted by the Division of Data, Statistics and Risk Assessment at AGES. The hosting division has a long-standing experience in the risk assessment of toxic substances in food and related environments, and is a national unit of reference for the development of assessment and monitoring strategies for AMR.

2.1. Aims

The present work programme aimed primarily at improving the risk assessment of AMR of environmental origins and to evaluate the impact of clinically relevant antibiotic resistance genes identified in environmental compartments and along the food/feed chain on human and animal health. Narrowing known knowledge and data gaps which are still hampering a holistic AMR risk analysis as outlaid in the One Health concept was one primary target of this fellowship. Providing a sound training for and presenting the tools already available for AMR risk assessment to the fellow, who should be capable of disseminating the obtained expertise at the end of the fellowship, was another objective.

2.2. Activities/Methods

2.2.1. Comprehensive revision of available data

To meet the objectives of the work programme, the fellow assessed and identified exposure pathways of AMR and ARGs in agroecosystems and connected environments, including the identification of potential pathways of ARG dissemination from the environment to human and animal pathogens, mainly along the food chain, as well as collection of data on the current knowledge status relating to environmental AMR. The identification of knowledge gaps of major relevance in the risk assessment of AMR in the environmental context, with focus on AMR spread through exDNA and quantitative information on ARGs related to the food chain and agroecosystems was used in a predictive modelling approach and used to prepare a draft of a literature review, currently under refinement for a publication in a peer-review journal.

To provide an evidence-based section of ARG targets the quantitative monitoring of AMR dissemination in environmental compartments, research trends of recent years were evaluated focusing on agricultural soil, wastewater, manure and food/feed ecosystems. The literature review was performed taking into account experimental approaches based on quantitative PCR (qPCR) and using appropriate keywords. Resulting ARG ranking was subsequently used to verify the relevance of gene targets selected for the practical part of the work programme.

2.2.2. Practical work – quantification of ARGs in environmental samples

During the practical part integrative of the work programme, the fellow completed laboratory tasks which included optimisation and validation of detection protocols, in particular: (i) validation of TaqMan qPCRs for the quantification of ARGs with improved positive controls used for generating standard curves for absolute quantification; (ii) validation of ARGs in a duplex approach that allows to assess two targets simultaneously with a significant reduction of sample volume, reagents and time necessary for the assay; (iii) determination of an optimal sample dilution to avoid PCR inhibition for the qPCR assays based on 16S gene target; and (iv) quantification of core and accessory ARGs in environmental samples (agricultural soil, forest soil, surface water) by qPCR with a total of 26 gene targets assessed (Table 1) and over 10,000 assays performed.

A subsequent analysis of the obtained data executed by the fellow indicated trends in ARG prevalence in agricultural soils with reference to fertilisation practice and their background concentrations in naturally occurring ecosystems. The movement of ARGs between different environmental compartments was assessed along with the impact of environmental conditions. Also, in a field plot experiment where the field was separated into three single plots of the same size, the variability of qPCR results between single plots and a composite soil sample representative of the whole field was determined in a methodological approach.

Table 1: Target genes quantified in soil and/or surface water samples

Target genes	Resistance mechanism	Inactivated class of antibiotics
Core targets		
aph(3')-IIa	Antibiotic inactivation	Aminoglycosides
aph(3')-IIIa	Antibiotic inactivation	Aminoglycosides
bla_{TEM-1}	Antibiotic inactivation	β-lactams
ermB	Target alteration	Macrolides
sul1	Target replacement	Sulfonamides
tet(W)	Ribosomal protection	Tetracyclines
vanA	Target alteration	Glycopeptides
16S	–	–
Accessory I ARGs		
cmxA	Efflux	Amphenicols
ermF	Target alteration	Macrolides
mcr-1	Target alteration	Peptide antibiotics (colistin)
qacEΔ1	Efflux	Quaternary ammonium salts
qnrS(rt11A)	Target protection	Fluoroquinolones (ciprofloxacin)
tet(A)	Efflux	Tetracyclines
Accessory II ARGs		
bla_{OXA-10}	Antibiotic inactivation	β-lactams
dfrA1	Target replacement	Diaminopyrimidine antibiotics (trimethoprim)
sat-4	Antibiotic inactivation	Streptothricin
tet(M)	Target alteration	Tetracyclines
tet(O)	Target alteration	Tetracyclines
$bla_{CTX-M-1-15}$	Antibiotic inactivation	Cephalosporin
bla_{GES-1}	Antibiotic inactivation	β-lactams
bla_{KPC}	Antibiotic inactivation	Carbapenems
bla_{NDM-1}	Antibiotic inactivation	Carbapenems, penicillins
bla_{VEB-1}	Antibiotic inactivation	β-lactams
mecA	Antibiotic target replacement	Carbapenem, cephalosporin, penam, cephamycin, monobactam
aph(6)-Id (strB)	Antibiotic inactivation	Aminoglycosides

ARGs: antibiotic resistance genes.

2.2.3. Risk assessment training

In addition to training modules foreseen by the EU-FORA Fellowship, the fellow followed face-to-face workshops with the hosting site experts on general issues related to risk assessment activities including risk assessment in food/feed with focus on novel foods and chemical substances including pesticide residues, heavy metals and veterinary medicinal products, classification and management of monitoring and risk assessment data in the national system and their communication to EFSA, introduction to the use of geographic information systems (GIS) for data projection, evaluation of risks and dynamic trends.

The fellow participated in the regular weekly meetings on the current scientific work carried out by the department group and in the OHEJP Working Group meetings and conferences on ongoing international efforts to harmonise AMR risk assessment according to the One Health concept. Supporting activities provided by the hosting site during the EU-FORA fellowship are summarised in Table 2.

Table 2: Training and supporting activities provided by the hosting site

Type of activity	Title/subject	Date/frequency
Trainings and workshops	Characterisation of exposure pathways of antimicrobial ARGs in naturally occurring ecosystems (agricultural soils, surface, ground and wastewater and along the food/feed chain)	8 February 2021
	Modelling and simulation of the spreading of infectious diseases and antimicrobial resistance genes using probabilistic modelling	9 February 2021
	Quantitative microbiological risk assessment (qMRA) for evaluation of AMR dissemination in natural environments (soil, water; food/feed chain	10 February 2021
	Monitoring of AMR in the environment: case studies based on MARGINS and ANTIVERSA projects	15 and 16 February 2021
	Processing of laboratory and monitoring data for internal reporting and generation standardised reports for stakeholders (Data management)	11 and 19 March 2021
	Internal webinar on *C. difficile* - the environmental perspective	12 March 2021
	Compilation of risk maps using Geographical Information System	8 April 2021
	Introduction to risk assessments along the food/feed chain	19 and 27 April 2021
Meetings	Group/Department meetings	Weekly
	OHEJP FED-AMR Working Group meetings	Monthly
Other activities	Introduction to the Reference Laboratories of Molecular Biology and Method Development	4 May 2021
	Introduction to the Reference Laboratory of Microbiology	5 May 2021

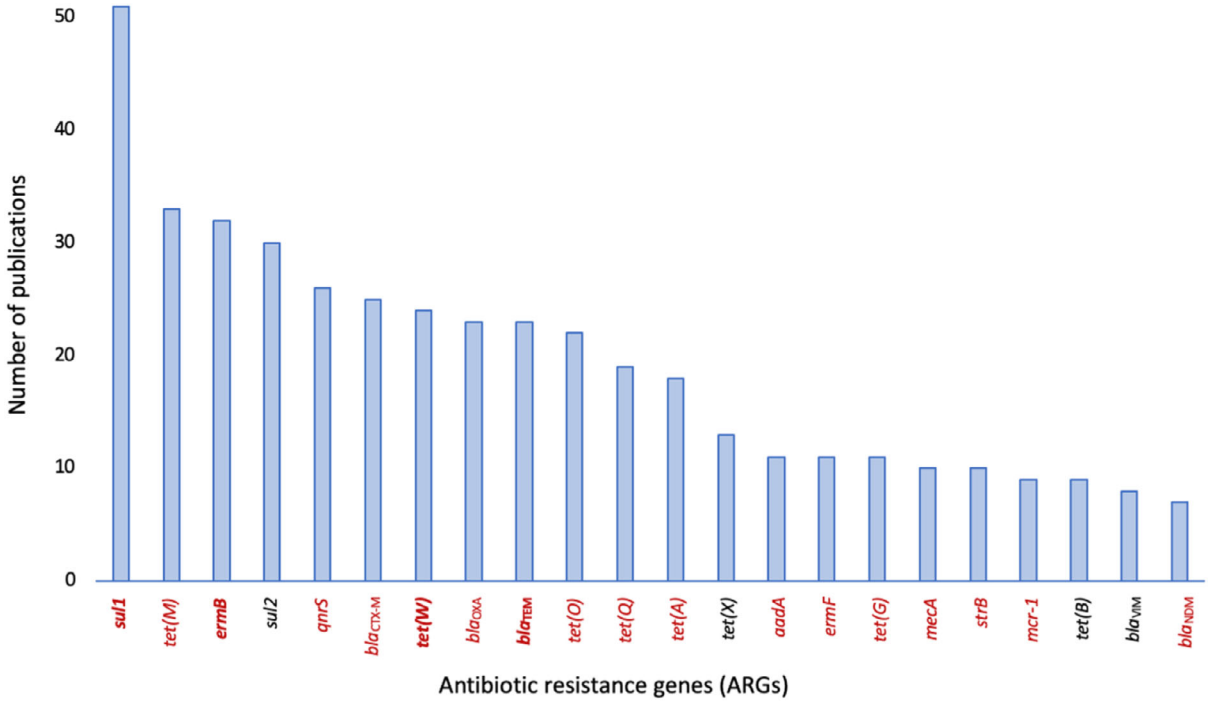
OHEJP: One Health European Joint Programme.

3. Results

The investigation of ARGs in natural ecosystems and those impacted by human activities is crucial due to uneven availability of data across environments and sources. In particular, occurrence and movement of ARGs from non-point sources to soil, plants and plant/animal food products, are less characterised (FAO, 2019). Although the mechanisms of AMR spread are widely described, the transfer of ARGs encoded on exDNA remains poorly evaluated and has been identified as the main knowledge gap in AMR assessment. The analysis of available literature data during the fellowship revealed that, besides background knowledge on exDNA behaviour in soil, little is known about the abundance of exDNA encoding ARGs in farmlands, its uptake by resident bacteria and persistence under various environmental conditions; even less is know when considering the food/feed chain and very few studies investigating exDNA-related ARGs in crops have been published so far.

One step ahead has been performed by assessing the transfer of ARGs via exDNA during cultivation of vegetables. A probabilistic modelling performed based on experimental data showed that the presence of exDNA in a cultivation system constitutes a key factor impacting the transformation rates of competent soil bacteria. Detailed description of the probabilistic model and outcomes will be published in a peer-review journal (in preparation).

The analysis of ARGs most frequently quantified using qPCR technique in farmlands, agricultural wastewater, manure and food/feed over the last years provided a ranking of gene targets relevant due to their frequency in the studies matrices, impact on human health (i.e. presence in pathogenic bacteria), determination of resistance covering various antibiotic classes occurring through different mechanisms. The ranking showed that 18 out of 22 most studied ARGs have been included in the qPCR analysis within the MARGINS projects, reflecting a widely recognised relevance of these targets for AMR assessment (Figure 1). Further analysis of ARG quantitative data generated during the practical part of the fellowship will show potential associations with agricultural practice, climate and site conditions as well as the extent to which AMR determinants spread across environments.



Red: accessory ARGs quantified within the MARGINS project. Black: ARGs non included in the MARGINS project.

Figure 1: The most frequently quantified ARGs in agroecosystems over 2017–2021 based on literature reviewRed bold: core ARGs quantified within the MARGINS project

4. Conclusions

Overall, the fellow acquired skills in defining the spreading pathways of AMR, ARGs and ARB along the environment–animals–humans axis. Increasing the conceptual knowledge underlying AMR development upon exposure to antibiotics with reference to natural conditions, being a primary objective of the work programme, introduced the fellow to further steps during the fellowship, such as definition of existing knowledge gaps in the current state-of-the-art, which particularly refer to very limited availability of quantitative data for specific ARGs present in environmental compartments.

The work programme allowed the fellow to gain practical knowledge in validation, optimisation and execution of analytical protocols for the quantification of clinically relevant ARGs in farmlands and connected environments. Through this activity, the fellow participated in the first environmental ARG quantification project at national level required for narrowing quantitative data gaps in the assessment of ARG dissemination pathways from the source to distanced environments. Also, by collecting data on the advancement of quantitative microbiological risk assessment (qMRA) models for AMR, the fellow acquired practical and theoretical basis for evaluating their suitability and applicability in environmental compartments as well as their overall potential for improvement of AMR risk assessment strategies.

5. Disclaimer

Detailed outcomes of the ARG quantification, data collection on the role of exDNA on AMR dissemination, ranking of ARGs for monitoring purposes and probabilistic modelling approach are intended to be published in peer-review journals (currently in preparation). In order to avoid copyright claims, they were not included in the present report.

References

Anthony ET, Ojemaye MO, Okoh OO and Okoh AI, 2020. A critical review on the occurrence of resistomes in the environment and their removal from wastewater using apposite treatment technologies: limitations, successes and future improvement. Environmental Pollution, 263, 113791.

Ashbolt NJ, Amézquita A, Backhaus T, Borriello P, Brandt KK, Collignon P, Coors A, Finley R, Gaze WH, Heberer T, Lawrence JR, Larsson DGJ, McEwen SA, Ryan JJ, Schönfeld J, Silley P, Snape JR, Van den Eede C and Topp E, 2013. Human Health Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environmental Health Perspectives, 121, 993–1001.

Baker M, Hobman JL, Dodd CER, Ramsden SJ and Stekel DJ, 2016. Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate. FEMS Microbiology Ecology, 92.

Ben Y, Fu C, Hu M, Liu L, Wong MH and Zheng C, 2019. Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: a review. Environmental Research, 169, 483–493.

Cerqueira F, Matamoros V, Bayona J, Elsinga G, Hornstra LM and Piña B, 2019a. Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (*Vicia faba* L.) grown under different watering regimes. Environmental Research, 170, 16–25.

Cerqueira F, Matamoros V, Bayona J and Piña B, 2019b. Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial *Lycopersicon esculentum* fields under different agricultural practices. Science of the Total Environment, 652, 660–670.

Checcucci A, Trevisi P, Luise D, Modesto M, Blasioli S, Braschi I and Mattarelli P, 2020. Exploring the animal waste resistome: the spread of antimicrobial resistance genes through the use of livestock manure. Frontiers in Microbiology, 11, 1416.

Claycamp HG and Hooberman BH, 2004. Antimicrobial resistance risk assessment in food safety. Journal of Food Protection, 67, 2063–2071.

FAO (Food and Agriculture Organisation of the United Nations), 2019. Antimicrobial movement from agricultural areas to the environment: the missing link. A fore for nuclear techniques. ISBN 978-92-5-131648-1.

FAO (Food and Agriculture Organisation of the United Nations), 2021. The FAO action plan on antimicrobial resistance 2021–2025. Supporting innovation and resilience in food and agriculture sectors. ISBN 978-92-5-134673-0.

FAO/OIE/WHO (Food and Agriculture Organisation of the United Nations, World Organisation for Animal Health and World Health Organisation), 2020. Technical brief on water, sanitation, hygiene and wastewater management to prevent infections and reduce the spread of antimicrobial resistance. ISBN 978-92-5-132641-1.

Iwu CD, Korsten L and Okoh AI, 2020. The incidence of antibiotic resistance within and beyond the agricultural ecosystem: a concern for public health. Microbiology Open, 9, e1035.

Larsson DGJ, Andremont A, Bengtsson-Palme J, Brandt KK, de Roda Husman AM, Fagerstedt P, Fick J, Flach C-F, Gaze WH, Kuroda M, Kvint K, Laxminarayan R, Manaia CM, Nielsen KM, Plant L, Ploy M-C, Segovia C, Simonet P, Smalla K, Snape J, Topp E, van Hengel AJ, Verner-Jeffreys DW, Virta MPJ, Wellington EM and Wernersson A-S, 2018. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environment International, 117, 132–138.

Niegowska M, Sanseverino I, Navarro A and Lettieri T, 2021. Knowledge gaps in the assessment of antimicrobial resistance in surface waters. FEMS Microbiology Ecology, 97.

Pan M and Chu LM, 2018. Occurrence of antibiotics and antibiotic resistance genes in soils from wastewater irrigation areas in the Pearl River Delta region, southern China. Science of the Total Environment, 624, 145–152.

Ramakrishnan B, Venkateswarlu K, Sethunathan N and Megharaj M, 2019. Local applications but global implications: can pesticides drive microorganisms to develop antimicrobial resistance? Science of the Total Environment, 654, 77–189.

Sorinolu AJ, Tyagi N, Kumar A and Munir M, 2021. Antibiotic resistance development and human health risks during wastewater reuse and biosolids application in agriculture. Chemosphere, 265, 129032.

WHO (World Health Organization), 2017. One Health. Questions and answers. Available online: https://www.who.int/news-room/questions-and-answers/item/one-health [Accessed: 17 January 2022].

Abbreviations

AGES	Austrian Agency for Health and Food Safety
AMR	antimicrobial Resistance
ARB	antibiotic-resistant bacteria
ARG	antibiotic resistance genes
exDNA	free extracellular DNA
GIS	geographic information system
OHEJP	*One Health European Joint Programme*
PCR	polymerase chain reaction
qMRA	quantitative microbiological risk assessment
qPCR	quantitative polymerase chain reaction
WWTP	wastewater treatment plant

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Assessment of endocrine disruptive properties of PFOS: EFSA/ECHA guidance case study utilising AOP networks and alternative methods

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Abstract

Endocrine disruptors (EDs) are chemical substances that interfere with the endocrine system, adversely affecting human health and environment. Legislation with aim to eliminate and ban EDs have been introduced in EU, but the identification of EDs remains challenging and crucial step towards regulation and risk management. A guidance for ED assessment has been recently established for pesticides and biocides in the EU, which heavily relies on traditional toxicological testing *in vivo*. Most notably lacking mechanistic methods for some ED modalities and not covering many other modalities that might be affected by EDs. In this project, we focus on the ED assessment according to the valid legislation and explore the possibility to employ alternative methods to bolster the mechanistic understanding of the ED effects and eventually decrease the need for *in vivo* testing. We selected a well-studied industrial chemical perfluorooctanesulfonic acid (PFOS) to be a model compound in a case study for ED assessment where the EU criteria were applied in the frame of human health risk assessment with focus on thyroid disruption and developmental neurotoxicity. A systematic literature review has been conducted for these effects (Scopus, Pubmed, Embase), and relevant studies were selected by title/abstract screening (RAYYAN) and full-text examination. Selected studies were assessed for reliability (SciRAP), and all relevant data were extracted into a database and assessed by Weight of Evidence (WoE) approach. The initial analysis showed potential endocrine adverse effects and endocrine activity, meeting the ED criteria. The use of mechanistic and alternative methods enhanced the outcomes of WoE assessment. Also, the study provides a great hands-on experience with the most up-to-date development in the area of risk assessment and EDs.

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Keywords: PFOS, endocrine disrupter, next generation risk assessment, AOP, NAM

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1. Introduction

The endocrine disruptive substances are one of the major challenges in current EU chemical regulation. There is legislation in place for substances with endocrine disrupting properties in the EU regulations for Plant Protection Products (PPP, Regulation, EC No 1107/2009) and Biocidal products (BP, Regulation, EU No 528/2012). Scientific criteria for identifying endocrine disrupting properties within the PPP and BP legislative frameworks were implemented in guidance developed by the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA) and published in 2018 (ECHA/EFSA, 2018). The European Commission stated the aim of developing a horizontal approach to identify endocrine disruptors (EDs) across different EU chemical legislations (EC Communication, 2018). The criteria for identification of EDs are based on the compounds' ability to cause an adverse effect, presence of an endocrine Mode of Action (MoA) and the adverse effect as a consequence of the endocrine MoA (ECHA/EFSA, 2018). The regulatory assessment of EDs thus requires extensive animal testing to identify toxicological effects, as well as a high level of understanding the toxicity mechanism. The PPP and BP regulations further state that the identification of an ED should be carried out by making use of all relevant data, using systematic review methodology and Weight of Evidence (WoE) approaches. The need for systematic and transparent approaches for collecting, evaluating and integrating toxicological data for health risk assessment of environmental factors, including chemicals, has been recognised during the last years (Whaley et al., 2016).

The focus of this project was to investigate the application of the ED criteria and guidance for PPPs and BPs in other regulatory frameworks, such as REACH. Important part was to explore systematic approaches that maximise the use of mechanistic data from non-animal tests and new approach methodologies (NAMs) to facilitate ED assessment. The main focus was on NAMs using modern *in vitro* methods and biomarker assays including omics studies, the links towards the apical effects *in vivo* and their application in hazard and risk assessment. The fellow did get familiar with the legislation related to the assessment of EDs as well as the most current developments in the field via conducting literature review. Based on the collected information a case study was designed where combination of *in vitro*, *in vivo* and mechanistic data (transcriptomics) is being utilised for an ED and risk assessment and which is currently being conducted.

1.1. ED Assessment

Despite differences in the regulatory requirements across legislations (REACH, cosmetics, plant protection products, biocides, etc.), the current ED assessment approach has largely been built around animal studies as adequate models for prediction of potential adverse effects in humans (Knight et al., 2021). However, it is known and accepted that animal studies alone may fail to predict some adverse effects (Takasuna et al., 2017). In addition, for a long time, there has been an ethical concern with the excessive or avoidable use of experimental animals. Directive 2010/63/EU of the EU on the protection of animals used for scientific purposes unambiguously fosters the application of the principle of the 3Rs (i.e. Replacement, Reduction and Refinement of animal testing) when considering the choice of methods to be used. The EU Cosmetics Regulation has gone furthest and banned animal testing of cosmetics altogether both as finished products and certain categories of regulated ingredients. Our work focuses on the assessment outlined in the ECHA/EFSA, (2018) guidance.

1.2. New approach methodologies

Recent regulatory and research activities emphasise the inclusion of modern mechanistic *in vivo* as well as *in vitro* assays in (eco)toxicological risk assessment, including ED assessment of chemicals. The current regulatory approach for identification of EDs focuses on so-called EATS (oestrogen, androgen, thyroid and steroidogenesis) pathways as targets of EDs and defined MoAs while other potentially relevant MoAs should be considered on a case-by-case basis, depending on available evidence (ECHA/EFSA, 2018). Furthermore, even for the EATS modalities, there is only a handful of validated alternative methods for screening and detection to recognise the endocrine activity, since generally the established methods largely rely on mammalian *in vivo* experiments and histopathology. The *in vivo* tests present ethical and economical concerns as well as scientific doubts since these methods are in many cases used mainly for historical reasons while there are more relevant methods available (Knight et al., 2021). There is a large interest from risk assessors, risk managers, researchers and NGOs for comprehensive assessment of applicability and utilisation of new methods, and the EU has an

expressed ambition to reduce the number of animals used for toxicity testing and research purposes (Directive, EC 2010/63/EU). However, the use of non-animal methods for assessment of chemicals in the regulatory setting requires that the mechanistic data generated from such methods can be reliably linked to the adverse health effect that is being predicted. The adverse outcome pathway (AOP) framework provides a means for increased mechanistic understanding and can be used as structured approach for causally linking early events on molecular and cellular levels to adverse health effects relevant for regulatory hazard and risk assessment of chemicals (Ankley et al., 2010).

There is consensus that the most sensitive window of exposure to EDs is during important periods of development, such as foetal development or infancy (Diamanti-Kandarakis et al., 2009). Exposure to EDs during these periods may cause permanent adverse effects later in life. It is also generally recognised that EDs can interfere with endocrine system in various ways. So far, the focus was mainly limited on a number of endocrine modalities, i.e. EATS. However, it has been shown that other aspects of the endocrine system and physiology can be sensitive to EDs as well (Grignard et al., 2020). There is also increasing evidence showing that EDs can work together to produce additive effects ('mixture effect') so that exposure to a combination of EDs may produce an adverse effect at concentrations at which individually no effect has been observed (Thrupp et al., 2018).

However, knowledge gaps still exist. These relate in particular to issues with the classification and assessment of the potential consequences that might results from exposure to EDs. These relate for example to unknown impact of exposure to EDs on disease development, wildlife and ecosystems. Also, there is the ongoing controversy whether and potentially how some basic toxicological principles such as 'safe threshold' are applicable to EDs (Knight et al., 2021). In this context, there is often only limited understanding of the specific contribution of chemical exposure and the way to separate it from other possible causes of the negative impacts being investigated. There is recognised need for better understanding of the mode of action of the endocrine disruption and need for new methods that will better address those needs (Pistollato et al., 2021).

Major efforts are being made and rapid development is seen in research towards new approach methodologies (NAMs) for chemical safety assessment largely driven by interest in regulatory needs (Moné et al., 2020). The ultimate goal of modernised next generation risk assessment (NGRA) is to develop a new approach in which adverse effects are inferred from upfront mechanistic understanding rather than using extensive animal studies (Luijten et al., 2020). Although animal models are currently the standard in predicting adverse human health effects, the correlation between animal models and human health effects is being questioned. Novel methods that would replace the traditional animal testing include batteries of *in silico* (QSARs, PBPK) and *in vitro* assays that would determine the MoA and allow accurate modelling of expected toxicity. However, lack of validated methods (and robust data to base those models on) as well as lack of funding hinder development of such models (Knight et al., 2021). Another driver for this transition is the long-term desire in general population to minimise animal testing. A better mechanistic understanding of toxicological MoA may provide *in vitro* testing methods that more closely represent human biology and accordingly give more accurate predictions (Krewski et al., 2020). To secure a mechanistic basis, the knowledge of toxicological mechanisms needs to be organised in a systematic and transparent manner. Furthermore, such organisation will reveal where appropriate tools and methods are lacking and further investments are needed.

1.3. Adverse Outcome Pathways

The global aim of shifting towards the development of new assessment methods require maximising the use of existing toxicological knowledge. The AOP framework summarise and makes available knowledge about toxicological pathways. Within the efforts of modernising the chemical risk assessment, the AOP framework has prominent place. It can be the major instrument to support the use and interpretation of non-animal and mechanistic data for drawing conclusions about potential health effects of chemicals, as well as for the identification and assessment of EDs. Essentially, AOPs are linear constructs describing biologically plausible chains of events linking a molecular initiating event (MIE), in which the stressor perturbs the biological system, to a series of intermediate key events (KEs) at different levels of biological organisation. The existing link between an upstream KE and a downstream KE in an AOP is called key event relationship (KER). At the other end, the AOP is anchored by an adverse outcome (AO) at the organism or population level (Knapen et al., 2020). The most promising development is formation of quantitative AOPs (qAOP) that provide detailed quantitative understanding of the relations between KEs which would provide ideal tool for connecting mechanistic information with adversity (Spinu et al., 2020).

2. Description of work programme

2.1. Aims

The main goal of the project was to apply the current regulatory rules for ED assessment in a case study with perfluorooctanesulfonic acid (PFOS) and expand the assessment for utilisation of alternative methods beyond classical mammalian models towards hazard and risk assessment in humans. The aim was to explore the possibility of utilisation of the mechanistic data produced by NAMs within the regulatory criteria for ED assessment and the potential of inferring adversity in humans with limited or no animal data.

2.2. Methods

In the presented case study, we focused on PFOS as a model compound. PFOS was selected because it is a well-studied compound for which we could reliably collect sufficient data on both classical studies (e.g. *in vivo* mammalian) as well as studies using NAMs. PFOS is also generally discussed as an ED in the research community but has not been officially assessed and identified as such according to the regulatory criteria. It was therefore of interest to explore to what extent PFOS fulfil the criteria laid out in the regulations while having sufficient data to explore the use of NAMs within the assessment. In our case study we follow the scientific criteria set in the EFSA/ECHA guidance for ED assessment (ECHA/EFSA, 2018) to collect and evaluate available data. We introduced several advancements compared to the guidance by limiting the focus on specific modality only (Thyroid modality only, to make the project manageable in the given time-frame) and including alternative methods in the assessment.

Vast amounts of literature are available on PFOS, covering many aspects of its toxicity in humans and wildlife. There are also several EFSA opinions available on the health risks of PFOS with comprehensive summary of available data and risk assessment for various toxicities with described effects on neurotoxicity, metabolic disruption, immunotoxicity, developmental toxicity (EFSA CONTAM Panel, 2018, 2020). However, the risk assessment predominantly focuses on mammalian toxicity with limited or no mechanistic data for most toxicities and with almost complete lack of any mechanistic insight for, e.g., neurotoxicity. There is an apparent gap in current risk assessment approaches that needs to be addressed. In recent years PFOS has remained of high interest and new studies exploring the mechanisms of its toxicity are being published continuously. Notably, there are several proposed mechanisms for the neurotoxicity and developmental neurotoxicity that would suggest effects in general populations and might be able to provide biologically plausible link between the effects on molecular level and effects observed in epidemiology studies relating to IQ and other neurological impairments.

2.2.1. AOP network

The search for relative information of PFOS, as well as selection and organisation of retrieved data, was supported by use of relevant available AOPs. As a first step, an AOP-wiki screening was conducted to collect the AOPs that provide information on EATS-mediated toxicity pathways. AOPs relevant for EATS were identified in the AOP Wiki by searching for specific toxicological effects and parameters listed as relevant for ED assessment in the ECHA/EFSA guidance (2018). The identified AOPs were manually sorted and combined into an AOP network at common KEs (details on network construction in Appendix A). For this case study, further refinement was made to focus only on AOPs relevant for thyroid hormone (TH) disruption and developmental neurotoxicity (DNT) and a subnetwork was constructed (Appendix A). The information from the TH and DNT AOP network was used as a basis for identifying relevant search terms and constructing a search strategy to identify relevant toxicity data from the scientific literature related to thyroid disruption and effects on neurodevelopment (list of the KEs and related AOPs in Table 1).

2.2.2. Systematic literature search

Search of available peer-reviewed literature was conducted to collect data to support the proposed endocrine disruption property of PFOS disrupting thyroid hormone balance and ultimately causing developmental neurotoxicity. Specific queries were constructed for individual databases based on information collected from the AOP network and initial literature information. The detailed queries for

individual databases are listed in Appendix B. The search was conducted in widely used scientific literature databases Scopus, PubMed and Embase. Two steps were applied for the studies selection from the search: (1) screening by title and abstract, and (2) full-text examination. Title and abstract screening was independently performed by the fellow and one more reviewer using the RAYYAN tool (https://rayyan.qcri.org/). Differences between the reviewers were resolved through discussion. The included and excluded studies were critically identified after defining the problem formulation (scope, scientific needs/objectives and feasibility and the eligibility (inclusion/exclusion) criteria, according the EFSA systematic review methodology (EFSA, 2010). Studies meeting the eligibility criteria were kept for next screening step. Studies clearly not relevant to the problem formulation or meeting the exclusion criteria were excluded. When exclusion could not be made based on the title/abstract, studies were kept for subsequent full-text examination performed by the fellow. A deep examination at full-text level was then performed by the fellow for the screened studies, where those considered that met the eligibility criteria were included into and classified into epidemiological (as supporting information), *in silico*, *in vitro*, *in vivo* mammalians, and *in vivo* non-mammalians.

Table 1: Table of terms extracted from the available AOPs and literature for the systematic search of information on PFOS and TH disruption and DNT (Detailed queries in the Appendix B)

Associated AOP ID	Event ID	Event type	Event name
[42, 54, 128, 134, 159, 175, 176, 188, 271]	277	KE	Decreased thyroid hormone synthesis
[42, 119, 159, 175, 271]	279	MIE	Thyroperoxidase inhibition
[8, 42, 54, 134, 152]	280	KE	Decreased thyroxine (T4) in neuronal tissue
[8, 42, 54, 134, 152, 159, 175, 176, 366, 367]	281	KE	Decreased thyroxine (T4) in serum
[54]	341	AO	Impairment of learning and memory
[54]	381	KE	Reduced levels of BDNF
[54]	385	KE	Decrease of synaptogenesis
[54]	386	KE	Decrease of neuronal network function
[42, 134, 152, 300]	402	AO	Decreased cognitive function
[8, 42, 134, 152, 300]	756	KE	Altered hippocampal gene expression
[8, 42, 134, 152, 300]	757	KE	Altered hippocampal anatomy
[8, 42, 134, 152, 300]	758	KE	Altered hippocampal physiology
[54]	851	KE	Decrease of GABAergic interneurons

2.2.3. Data collection and evaluation

According to the systematic search method, studies were assessed for relevance against inclusion criteria in two steps: (1) screening of titles and abstracts for relevance to the study question, and (2) full-text examination for the eligibility of studies (EFSA, 2010). Therefore, assessment of relevance at this stage was considered as a confirmation and only two categories (relevant and partially relevant) were included since the not relevant studies were excluded at the previous steps after the literature collection. The relevant studies were then assessed for reliability (inherent quality of the test method and level of reporting) by the online web-tool Science in Risk Assessment and Policy – SciRAP (https://www.scirap.org). SciRAP provides pre-defined criteria and a colour-coding tool aimed to promote structure and transparency in the evaluation toxicity (*in vitro* and *in vivo*) studies for hazard and risk assessment of chemicals. When a study contained both *in vitro* and *in vivo* individual SciRAP evaluations were performed for the endpoints. The SciRAP score was converted into Klimisch reliability criteria (reliable without restriction, reliable with restriction, not reliable and not assignable) which were then use for the purpose of the WoE assessment according to systematic approach previously described in Ingre-Khans et al. (2020).

2.2.4. Weight of evidence assessment

The extracted parameters along with the study quality assessment scores were assembled into lines of evidence for the groups (a) thyroid-related endocrine activity, (b) thyroid and nervous system adversity, and (c) general toxicity. Each group was subdivided into categories based on the nature of the data addressing specific endpoints or MoAs. Each individual line of evidence was assessed

considering the quantity and quality of both the studies and the included parameters, as well as their coherence dose/concentration–response, consistency among studies and repeatability for the line of evidence. Each line of evidence was assessed, and evidence was categorised into five groups: Strong, Moderate, Weak and No evidence for an effect and No evidence available. The evidence assembled was then used to draw conclusion whether sufficient evidence is available for endocrine disruptive effects for the proposed modality of thyroid disruptions and adversity of developmental neurotoxicity.

2.3. Activities

The fellow participated in regular group meeting during the placement at IMM, KI and engaged in discussion with multiple out of the team colleagues. Despite the challenging circumstances and limited time when personal meetings were possible, fellow also participated in following activities:

- Preparation and moderation of a IMM organised webinar Next Generation Approaches for Regulatory Assessment of Endocrine Disruptors, October 28, 2021
- Meetings and discussions with collaborators from University of Antwerp discussing the AOP networks and further applications, October 2021–January 2022
- Participation in webinars focusing on NAMs
    - Endocrine disruption as a mechanism of developmental Neurotoxicity (DNT), September 15, 2021, hosted by International Neurotoxicology Association, (virtual; https://www.neurotoxicology.org/ina-webinars/)
    - 10th Annual Meeting of the ASCCT, 'Practical applications of new tools in toxicology' October 12-14, 2021 (virtual; https://www.ascctox.org/annualmeeting)
- Planning with colleagues from unit about next generation risk assessment report that should be prepared in future.
- European commission Third Annual Forum on Endocrine Disruptors (https://ec.europa.eu/environment/events/third-annual-forum-endocrine-disruptors_en).
- Activities related to oversight and management of student projects of Sara Caccia (master project) and Linus Wiklund (PhD project) within the unit.
- Attending lectures on relevant topics within IMM.

3. Conclusions

Exploring and implementing innovative non-animal-based approaches requires a long-term and focused development effort that is complemented with well-planned and funded research. How to reach implementable outcome is not yet clear. To apply these approaches once developed, the relevant EU legislation and guidance as well as regulatory practice will have to be updated. In particular, combining NAMs with standard methods to strengthen the evidence for regulatory needs, i.e. read-across and WoE, and their potential use as screening and priority setting tools to identify compounds of regulatory interest. There is also the opportunity re-consider the safety evaluation in general to adopt predictive toxicology especially the costs, the ethics and the usefulness of animal studies, and to expand the role of monitoring exposure post market with the aim to achieve at least the same level of protection without cross validation to animal studies.

It should be possible to improve the identification of adverse effects that are not addressed by current validated toxicology studies and at the same time aim towards reducing the use of animals for toxicity testing. In this regard, the AOP concept is an important development. It should help to formalise toxicological base and evidence for development of a testing battery based on in silico and *in vitro* methods to allow predictive toxicology and inform on the adversity or at least help with prioritisation. The overall conclusion from predictive toxicology for a compound must be clear on how it was made and what is the associated level of uncertainty. A key challenge for new safety assessment approaches is therefore to agree on what constitutes adequate evidence to justify non-animal-based safety testing and assessment approaches. One of the main concepts is the acceptance of non-animal data as reliable predictors for health effects in humans. The key issues to promote that are to develop and employ standards for NAM data, including the biological relevance of the methods, and data integration approaches to conclude on the safety assessment. Additional challenge is adapting the training and skills of future risk assessors that will need better understanding of the new methods and concepts. That brings substantial requirements on the risk assessors in terms of expertise in very wide field from QSARs and *in vitro* assays to biomarkers and omics. It will require

further resources and coordinated approach to prepare the next generation risk assessors that will be a match to the next generation risk assessment.

References

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE and Villeneuve DL, 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environmental Toxicology and Chemistry, 29, 730–741. https://doi.org/10.1002/etc.34

Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT and Gore AC, 2009. Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocrine Reviews, 30, 293–342. https://doi.org/10.1210/er.2009-0002

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

ECHA/EFSA (European Chemicals Agency and European Food Safety Authority), 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA Journal 2018;16(6):5311, 45 pp. https://doi.org/10.2903/j.efsa.2018.5311

EFSA (European Food Safety Authority), 2010. Application of systematic review methodology to food and feed safety assessments to support decision making. EFSA Journal 2010;8(6):1637, 55 pp. https://doi.org/10.2903/j.efsa.2010.1637

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen HK, Alexander J, Barregard L, Bignami M, Bruschweiler B, Ceccatelli S, Cottrill B, Dinovi M, Edler L, Grasl-Kraupp B, Hogstrand C, Hoogenboom LR, Nebbia CS, Oswald IP, Petersen A, Rose M, Roudot A-C, Vleminckx C, Vollmer G, Wallace H, Bodin L, Cravedi J-P, Halldorsson TI, Haug LS, Johansson N, van Loveren H, Gergelova P, Mackay K, Levorato S, van Manen M and Schwerdtle T, 2018. Scientific Opinion on the risk to human health related to the presence of perfluorooctane sulfonic acid and perfluorooctanoic acid in food. EFSA Journal 2018;16(12):5194, 284 pp. https://doi.org/10.2903/j.efsa.2018.5194

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Schrenk D, Bignami M, Bodin L, Chipman JK, del Mazo J, Grasl-Kraupp B, Hogstrand C, Hoogenboom LR, Leblanc J-C, Nebbia CS, Nielsen E, Ntzani E, Petersen A, Sand S, Vleminckx C, Wallace H, Barregård L, Ceccatelli S, Cravedi J-P, Halldorsson TI, Haug LS, Johansson N, Knutsen HK, Rose M, Roudot A-C, Van Loveren H, Vollmer G, Mackay K, Riolo F and Schwerdtle T, 2020. Scientific Opinion on the risk to human health related to the presence of perfluoroalkyl substances in food. EFSA Journal 2020;18(9):6223, 391 pp. https://doi.org/10.2903/j.efsa.2020.6223

EU Communication, 2018. 734 final. Communication from the commission to the European parliament, the council, the European Economic and Social Committee and the Committee of The Regions. Towards a Comprehensive European Union Framework on Endocrine Disruptors. Available online: https://ec.europa.eu/transparency/regdoc/rep/1/2018/EN/COM-2018-734-F1-EN-MAIN-PART-1.PDF

European Commission, 2018. Towards a comprehensive European Union framework on endocrine disruptors EN. European Commission Communication. Available online: https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A52018DC0734

Grignard E, Håkansson H and Munn S, 2020. Regulatory needs and activities to address the retinoid system in the context of endocrine disruption: the European viewpoint. Reproductive Toxicology, 93, 250–258. https://doi.org/10.1016/j.reprotox.2020.03.002

Ingre-khans E, Ågerstrand M, Rudén C, Beronius A, Ågerstrand M, Rudén C, Beronius A, Ingre-khans E, Ruden C and Published B, 2020. Human and ecological risk assessment: an international improving structure and transparency in reliability evaluations of data under REACH: suggestions for a systematic method. Human Ecology Risk Assessments an International Journal, 26, 212–241. https://doi.org/10.1080/10807039.2018.1504275

Knapen D, Stinckens E, Cavallin JE, Ankley GT, Holbech H, Villeneuve DL and Vergauwen L, 2020. Toward an AOP network-based tiered testing strategy for the assessment of thyroid hormone disruption. Environmental Science and Technology, 54, 8491–8499. https://doi.org/10.1021/acs.est.9b07205

Knight DJ, Deluyker H, Chaudhry Q, Vidal J-M and de Boer A, 2021. A call for action on the development and implementation of new methodologies for safety assessment of chemical-based products in the EU – a short communication. Regulatory Toxicology and Pharmacology, 119. https://doi.org/10.1016/j.yrtph.2020.104837

Krewski D, Andersen ME, Tyshenko MG, Krishnan K, Hartung T, Boekelheide K, Wambaugh JF, Jones D, Whelan M, Thomas R, Yauk C, Barton-Maclaren T and Cote I, 2020. Toxicity testing in the 21st century: progress in the past decade and future perspectives. Archives of Toxicology, 94. https://doi.org/10.1007/s00204-019-02613-4

Luijten M, Rorije E, Sprong RC and Van Der Ven LTM, 2020. Practical application of next generation risk assessment of chemicals for human health. Chemical Research in Toxicology, 33, 693–694. https://doi.org/10.1021/acs.chemrestox.0c00074

Moné MJ, Pallocca G, Escher SE, Exner T, Herzler M, Bennekou SH, Kamp H, Kroese ED, Leist M, Steger-Hartmann T and van de Water B, 2020. Setting the stage for next-generation risk assessment with non-animal approaches: the EU-ToxRisk project experience. Archives of Toxicology, 94, 3581–3592. https://doi.org/10.1007/s00204-020-02866-4

Pistollato F, Madia F, Corvi R, Munn S, Grignard E, Paini A, Worth A, Bal-Price A, Prieto P, Casati S, Berggren E, Bopp SK and Zuang V, 2021. Current EU regulatory requirements for the assessment of chemicals and cosmetic products: challenges and opportunities for introducing new approach methodologies. Archives of Toxicology, https://doi.org/10.1007/s00204-021-03034-y

Pollesch NL, Villeneuve DL and O'Brien JM, 2019. Extracting and benchmarking emerging adverse outcome pathway knowledge. Toxicological Sciences, 168, 349–364. https://doi.org/10.1093/toxsci/kfz006

Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products.

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.

Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC.

Spinu N, Cronin MTD, Enoch SJ, Madden JC and Worth AP, 2020. Quantitative adverse outcome pathway (qAOP) models for toxicity prediction. Archives of Toxicology, 94, 1497–1510. https://doi.org/10.1007/s00204-020-02774-7

Takasuna K, Asakura K, Araki S, Ando H, Kazusa K, Kitaguchi T, Kunimatsu T, Suzuki S and Miyamoto N, 2017. Comprehensive in vitro cardiac safety assessment using human stem cell technology: overview of CSAHi HEART initiative. Journal of Pharmacological and Toxicological Methods, 83, 42–54. https://doi.org/10.1016/j.vascn.2016.09.004

Thrupp TJ, Runnalls TJ, Scholze M, Kugathas S, Kortenkamp A and Sumpter JP, 2018. Science of the Total Environment The consequences of exposure to mixtures of chemicals: something from 'nothing' and 'a lot from a little' when fi sh are exposed to steroid hormones. Science of the Total Environment, 619–620, 1482–1492. https://doi.org/10.1016/j.scitotenv.2017.11.081

Whaley P, Halsall C, Ägerstrand M, Aiassa E, Benford D, Bilotta G, Coggon D, Collins C, Dempsey C, Duarte-davidson R, Fitzgerald R, Galay-burgos M, Gee D, Hoffmann S, Lam J, Lasserson T, Levy L, Lipworth S, Mackenzie S, Martin O, Meads C, Meyer-baron M, Miller J, Pease C, Rooney A, Sapiets A, Stewart G and Taylor D, 2016. Implementing systematic review techniques in chemical risk assessment: challenges, opportunities and recommendations. Environment International, 92–93, 556–564. https://doi.org/10.1016/j.envint.2015.11.002

Abbreviations

AOP	adverse outcome pathway
DNT	developmental neurotoxicity
EATS	oestrogen, androgen, thyroid hormone and steroidogenesis
ED	endocrine disruptor
KE	key event
KER	key event relationship
MIE	molecular initiating event
MoA	mode of action
NAM	new approach method
NGRA	next generation risk assessment
PFOS	perfluorooctanesulfonic acid
qAOP	quantitative adverse outcome pathway
TH	thyroid
WoE	weight of evidence

Appendix A – AOP Network construction

AOPs from the OECD AOP-Wiki 2.4 were investigated manually to develop the derived AOP network. The full list of linear AOPs available in the AOP-Wiki database was the starting point for our search. The linear AOPs were collected April 9th, 2021, and the last check was performed in July 2021. The list of linear AOPs relevant to EATS modalities was manually extracted by assessment of the Abstract, the Background (if present) and the Overall Assessment sections for each extracted AOP as well as analysing their graphical representation displayed into the AOP-Wiki. An additional refining step was applied to isolate from the EATS-related AOPs list the ones that could be considered specifically Thyroid-related. At this purpose, another single-concept querying of the AOP-Wiki database was performed: the sorting procedure started from the previously collected AOPs retrieved typing the general key word 'Thyroid' into the AOP-Wiki database. A new systematic search in the AOP-Wiki followed employing only those parameters that in the ED GD are reported as indicative of thyroid modalities (Indicative of T modality) as search terms for developing a TH-related AOPs list. The list of linear AOPs manually extracted from the AOP-Wiki database applying the strategy was further inspected to exclude those AOPs that, even if retrieved through a single-concept querying based on TH-parameters, were erroneously included in the selection. This procedure was again performed by assessing the Abstract, the Background (if present) and the Overall Assessment sections for each extracted AOP as well as analysing their graphical representation displayed into the AOP-Wiki. Eventually, matches among the collected AOPs and the previously refined EATS-related AOPs list were highlighted obtaining the final refined TH-related AOPs list. The search was performed in the AOP-Wiki database on 26 May 2021, and the last check was performed in July 2021.

Cytoscape 3.8.2 (https://cytoscape.org/) was employed to model both the EATS-related and TH-related AOP-networks. This open-source software platform enables its users to generate a wide variety of networks either manually or importing data tables (e.g. Excel spreadsheets containing interaction-data between biological pathways); additionally, the program provides a basic set of features for data integration, analysis, and visualisation. The full content of the AOP-Wiki is available in an XML format (https://aopwiki.org/downloads/aop-wiki-xml.gz). Additional files with specific subsets of content are also accessible for users who don't wish to analyse the full XML documents; however, these files are daily updated and replaced with no permanent backups. In this project, for the generation of the EATS-related and TH-related AOPNs the download and use of the XML files was used in combination with information from tab-separated files (.tsv) that were downloaded from the AOP-Wiki platform (https://aopwiki.org/info_pages/5) on 4 July 2021. Since these files have no permanent backups in the AOP-Wiki platform, their original version will be conserved and made available for reproducibility purposes. For extraction of data from the xml file (downloaded on 4th July, 2021) modified R code from Pollesch et al. (2019) was used to extract information about biological level relevance and AOP status information.

The downloaded documents were processed employing KNIME Analytics Platform (https://www.knime.com/knime-analytics-platform), an open-source software that offers visual workflows for data analytics with an intuitive, drag and drop style graphical interface and for which no complex coding is required. Among all, KNIME enables to combine and handle data in simple text formats. Using KNIME's features, in both EATS- and TH-related tables a new attribute named 'Associated AOP Ids' was created and assigned to each listed KE and added to the downloaded tables in a dedicated column. This newly generated information lists in square brackets all the concatenated AOP Ids the referred KE belongs to and is essential to obtain an automatic AOPN mapping employing Cytoscape. Eventually, data coming from the three downloaded.tsv Files were merged in two distinct Excel tables (one for EATS-related and one for TH-related AOPs). Those tables were based on the Key Event Relationships File to which data from Key Events and Key Event Components File were attached (link-up was set for the Upstream Event Id in each KER and Event Id in the Key Events file; KEs without KERs were attached at the end of the table). Two additional data columns were added to the tables named 'Label' (combining the following attributes taken from Key Event file: Event Id, Event Type, Event Name and Associated AOP IDs) and 'Rel. Label' (combining the following attributes taken from KE Relationships File: Relationship Id, Associated AOP Ids). Importing in Cytoscape the unified Excel spreadsheets, two networks were automatically generated and graphically displayed using the program's default mapping features. These were promptly customised applying a series of graphical changes working manually on Cytoscape's Style interface. An accurate refinement of both networks was obtained through the following passages: first, a specific geometrical shape was addressed to each node according to its KE Type property as shown in Figure. An edge target arrow shape was then defined to enable the visual

understanding of the AOP of interest and facilitate the KEs along the path itself. The edges' look was further made to correspond to the KER's Adjacency applying a continuous and a dashed line fashion for adjacent and non-adjacent relationships respectively (Figure A.1).

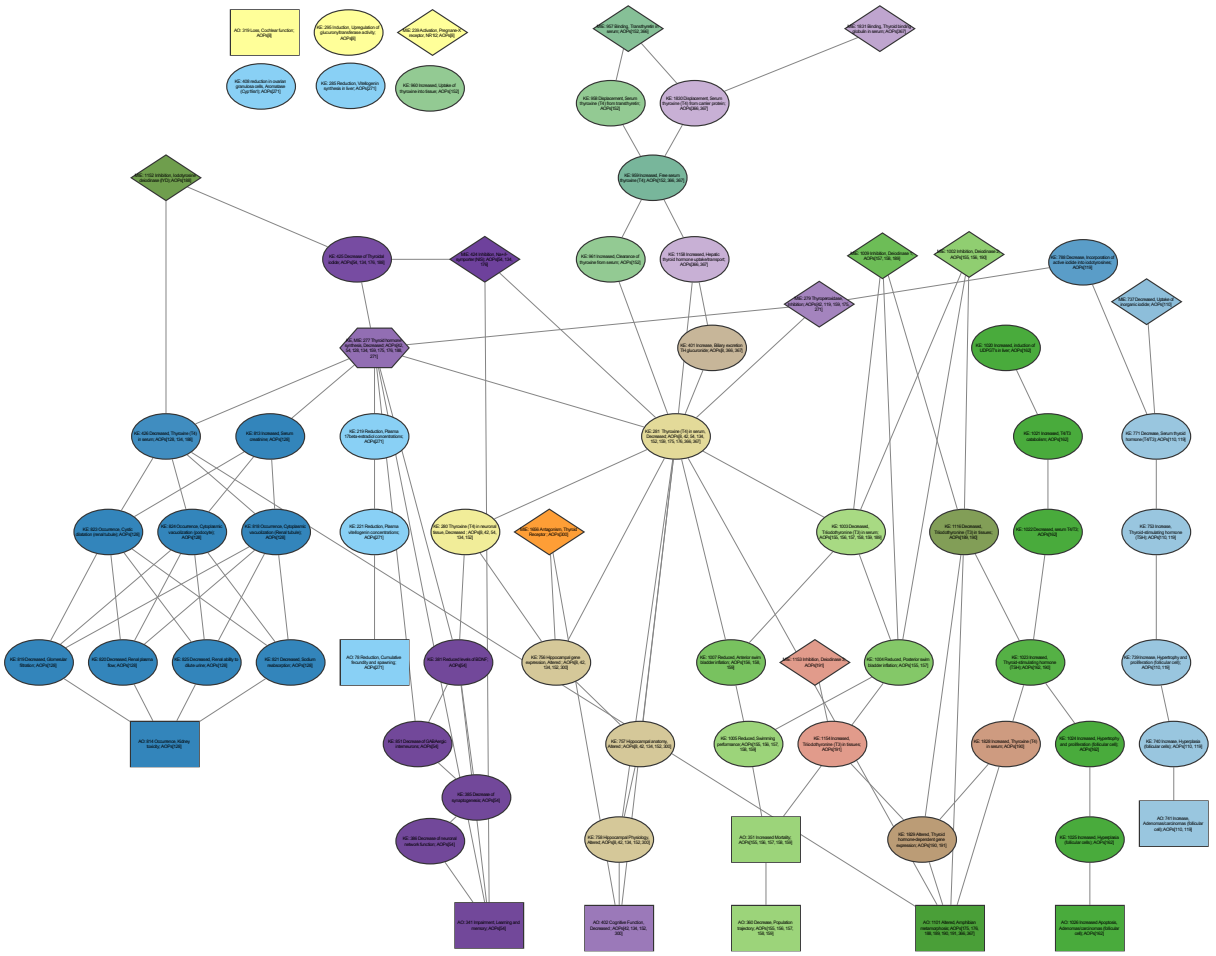

Figure A.1: Putative AOP Network of all KEs associated with AOPs that are related to Thyroid hormone disruption and Developmental neurotoxicity. The network was generated by using data from AOP-wiki (Accessed: 4 July 2021)

Appendix B – Systematic literature search

Table B.1: Basis of the search query derived from the selected AOPs

Database	Scopus	PubMed (Abstract Sifter)	Embase
CAS	CASREGNUMBER(1763-23-1)	–	–
Compound	ALL(PFOS OR "Perfluorooctanesulfonic acid" OR "Perfluorooctane sulfonic acid" OR "heptadecafluorooctane sulfonic acid" OR "Perfluorooctane sulfonate"))	("1763-23-1" OR PFOS OR "Perfluorooctanesulfonic acid" OR "Perfluorooctane sulfonic acid" OR "heptadecafluorooctane sulfonic acid" OR "Perfluorooctane sulfonate")	('1763-23-1':ti,ab,kw OR pfos:ti,ab,kw OR 'perfluorooctanesulfonic acid':ti,ab,kw OR 'perfluorooctane sulfonic acid':ti,ab,kw OR 'heptadecafluorooctane sulfonic acid':ti,ab,kw OR 'perfluorooctane sulfonate':ti,ab,kw)
TH terms	(TITLE-ABS(thyroid OR "thyroid hormone*" OR "thyroid gland" OR "thyroid peroxidase" OR "thyroperoxidase" OR "iodide peroxidase" OR "thyroxine")	((thyroid OR "thyroid hormone*" OR "thyroid gland" OR "thyroid peroxidase" OR "thyroperoxidase" OR iodide peroxidase OR "thyroxine")	(thyroid:ti,ab,kw OR 'thyroid hormone*':ti,ab,kw OR 'thyroid gland':ti,ab,kw OR 'thyroid peroxidase':ti,ab,kw OR 'thyroperoxidase':ti,ab,kw OR 'iodide peroxidase':ti,ab,kw OR 'thyroxine':ti,ab,kw
DNT terms	TITLE-ABS(hippocampus OR hippocampal OR synaptogenesis OR "neuronal network*" OR "cognitive" OR "GABAergic interneuron" OR "neuronal tissue*" OR learning OR memory OR bdnf OR "brain derived neurotrophic factor*"))	(hippocampus OR hippocampal OR synaptogenesis OR "neuronal network*" OR "cognitive" OR "GABAergic interneuron" OR "neuronal tissue*" OR learning OR memory OR bdnf OR "brain derived neurotrophic factor*"))	hippocampus:ti,ab,kw OR hippocampal:ti,ab,kw OR synaptogenesis:ti,ab,kw OR 'neuronal network*':ti,ab,kw OR 'cognitive':ti,ab,kw OR 'gabaergic interneuron':ti,ab,kw OR 'neuronal tissue*':ti,ab,kw OR learning:ti,ab,kw OR memory:ti,ab,kw OR bdnf:ti,ab,kw OR 'brain derived neurotrophic factor*':ti,ab,kw) NOT [medline]/lim

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Assessment of the possible health risks associated with the consumption of botanical preparations of *Mitragyna speciosa* (kratom)

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Abstract

The current report summarises the work performed in the context of the European Food Risk Assessment Fellowship Programme (EU-FORA), which included the evaluation of health risks associated with the consumption of botanical preparations of *Mitragyna speciosa* (kratom). *Mitragyna speciosa* is a tree native to Southeast Asia, where its leaves and preparations of the leaves have been used for centuries, among others, as a stimulant or as a traditional herbal medicine. Preparations of the plant have recently gained increasing popularity in other parts of the world, and are presently also accessible via online platforms, e.g. as food supplements. Kratom has been considered a botanical of possible health concern by the FDA and EFSA, which together with its increasing popularity, makes kratom a subject of international concern. Major alkaloids of the plant, mitragynine and 7-hydroxymitragynine, are agonists of the μ-opioid human receptor and are assumed to be mainly responsible for its psychoactive effects. The aim of the present project was to conduct an assessment of potential health risks associated with oral use of kratom-based preparations. The animal and human data that were evaluated in the course of the current assessment indicate that kratom consumption has the potential to not only lead to adverse neurological effects, including addiction and withdrawal syndrome, but also to elicit distinct organ toxicity with respect to e. g. liver and kidney as target organs. Nevertheless, actual risk characterisation is impeded by considerable uncertainties. Such uncertainties, based on the variability in composition of kratom preparations, insufficient information on dose–response relationships and on limited data on long-term use effects, currently do not allow the derivation of distinct health based guidance values for kratom/kratom preparations. Further information from well-designed studies, conducted with kratom preparations that have been clearly defined with respect to their composition, would be required to enable a more refined risk assessment of this botanical.

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Keywords: *Mitragyna speciosa*, kratom, addiction, withdrawal, herb-induced liver toxicity, food supplement, μ-opioid agonist

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1. Introduction

1.1. European Food Risk Assessment Fellowship Programme (EU-FORA)

The European Food Risk Assessment (EU-FORA) Fellowship Programme, supported by the European Food safety authority (EFSA), aims to train and increase the number of food safety risk assessment experts available in Europe. In this context, mid-career scientists are offered the opportunity to increase their knowledge and experience in food safety risk assessment, by both theoretical and practical training. The EU-FORA fellow participated in the general work programme entitled 'Risk assessment of botanical preparations used in food supplements and fortified foods', that was hosted by the German Federal Institute for Risk Assessment (BfR), Department of Food Safety, Unit of Nutritional Risks, Allergies and Novel Foods. The work programme comprised the elaboration of a detailed scientific monograph by the EU-FORA fellow that was to include the results of a safety assessment of a botanical and its preparations, taking into account the EFSA guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients of food supplements (EFSA, 2009). The final selection of *Mitragyna speciosa* (kratom) for the current safety assessment was made in agreement between the hosting unit and the EU-FORA fellow.

1.2. General background regarding the risk assessment of *Mitragyna speciosa*

Herbal products are often promoted as safe and effective alternatives to synthetic medicines. An increasing interest in products perceived as being natural and the general misperception that natural equals safe and is thus not associated with adverse effects, may lead to uncritical use of such products by consumers. However, in many cases, botanicals and botanical preparations contain active phytochemicals or complex mixtures of phytochemicals, respectively, that may potentially cause adverse effects which in certain cases may even be life-threatening (Di Lorenzo et al., 2015; Marcus and Grollman, 2015). Many of the products containing botanical preparations are sold as food supplements. According to the current regulatory framework in the EU and the EU General Food Law Regulation (EC) No 178/2002, food supplements are regulated as food. Food business operators, manufactures and suppliers placing the product on the market are responsible for the safety of these products.

Mitragyna speciosa, which is also called kratom, is a tree native to Southeast Asia. Its leaves have been used in the area for centuries as a traditional herbal medicine for the treatment of several illnesses and pain relief, but also by labour workers in order to increase their stamina. The most common traditional way of use is by chewing or by preparing an infusion of the leaves as tea. During the last decade, botanical preparations from *Mitragyna speciosa* have increasingly also been used in other parts of the world, such as in the United States and Europe. They are often being promoted as food supplements – most commonly in the form of powder, pills and capsules or leaf extracts – for the self-treatment of opioid withdrawal, the relief of withdrawal syndrome, for pain management or for recreational purposes. The major alkaloids of the plant, mitragynine and 7-hydroxymitragynine, are agonists of the μ-opioid human receptor and are assumed to be mainly responsible for the psychoactive effects of kratom. The botanical is listed in the EFSA Compendium of botanicals, reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements (EFSA, 2012) and it has also been considered a botanical of concern by the FDA (FDA, 2017). Although kratom use is illegal in some countries in Europe, it is easily available via Internet providers. This increasing popularity and the easy access to it makes kratom a subject of international health concern.

2. Description of work programme

2.1. Aims

The major aim of the work programme for the fellow was to become acquainted with the general principles of human health risk assessment for foods and in this context, to gain more detailed experience in the risk assessment of certain substances used in food supplements and fortified foods, i.e. substances other than vitamins and minerals, that are substances of plant origin (botanicals, botanical preparations and plant secondary constituents). The hands-on experience acquired by

specifically assessing the possible health risks associated with the consumption of *Mitragyna speciosa* and preparations thereof was further aimed to be the basis for the elaboration of a detailed monograph. Moreover, the programme aimed to create a network of food risk assessment experts by building professional connections between the hosting institution, the fellows of the programme and other experts.

2.2. Activities/Methods

2.2.1. Preparation of a monograph regarding the risk assessment of *Mitragyna speciosa*

The purpose of present scientific project conducted by the fellow was to retrieve, evaluate and summarise the current scientific data regarding health risks related to the consumption of kratom, taking into account the available published animal and human data. The risk assessment was performed based on the BfR-Guidance for health assessments (BfR, 2020) as well as on the EFSA 'Guidance on Safety assessment of botanicals and botanical preparations intended for use in food supplements' (EFSA, 2012). For the hazard characterisation, the relevant published toxicological animal studies and human data, including information from observational studies and case reports, were evaluated. To this end, a literature search was performed in PubMed and other scientific databases to identify relevant scientific publications. In addition, websites of acknowledged scientific bodies or national authorities were checked for information regarding kratom.

In behavioural and neurological studies in rodents involving oral subacute (28 days) administration of mitragynine, cognitive impairments of learning and memory function were observed already at a dose of 1 mg/kg bodyweight (Apryani et al., 2010; Yusoff et al., 2016; Suhaimi et al., 2021). Acute and subacute (28 days) oral administration of *Mitragyna speciosa* preparations (*Mitragyna speciosa* extracts or isolated mitragynine) caused hepatotoxic and mild nephrotoxic effects in rats and mice. Furthermore, neurotoxicity and pulmonary toxicity were observed in rats (28 days) at doses of $\geq$ 100 mg/kg bodyweight for a methanol extract or isolated mitragynine (Harizal et al., 2010; Kamal et al., 2012; Sabetghadam et al., 2013; Sakaran et al., 2014; Ilmie et al., 2015; Panjaitan and Liridah, 2021).

Currently, human intervention studies are not available and human observational studies in which adverse effects of kratom ingestion were investigated are limited. Following cessation of regular kratom use by humans, dependence and withdrawal syndrome have been observed. Other adverse effects associated with kratom use include liver toxicity and neurological symptoms such as dizziness and drowsiness (Suwanlert, 1975; Ahmad and Aziz, 2012; Saingam et al., 2013; Singh et al., 2018). A large number of human case reports have described signs of intoxication and even death following kratom ingestion. The documented adverse events include herb-induced liver injury, neurological effects (seizures, coma, central nervous system depression, altered mental status, hallucinations, confusion and drowsiness), cardiovascular effects (palpitations, tachycardia, cardiac arrest), withdrawal syndrome, respiratory dysfunction as well as cases of lethal outcome (Alsarraf et al., 2019; Schimmel and Dart, 2020).

2.2.2. EU-FORA Fellowship supporting programme

At the beginning 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 Nutritional Risks, Allergies and Novel Foods). The fellow obtained experience in the risk assessment of botanicals and botanical preparations by performing a risk assessment with respect to *Mitragyna speciosa* and by the elaboration of the respective monograph. The above work was completed under the guidance and effective supervision which was provided through regular meetings and on site communication with members of the hosting unit. Apart from the practical work at the BfR during the fellowship year, the fellow participated in the following activities described in Table 1. A considerable part of the fellowship programme was carried out remotely, due to the COVID-19 pandemic situation.

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

	Title	Date
Training modules provided by EFSA	Induction training – Microbiological and chemical risk assessment (virtual)	11–29 January 2021
	Training Module 1 – Risk communication, organised by the German Federal Institute for Risk Assessment (BfR) (virtual)	22–26 March 2021
	Training Module 2 – Emerging risks, organised by the Hellenic Food Authority (EFET) (virtual)	7–14 June 2021
	Training Module 3 – Data collection and reporting, organized by EFSA (virtual)	4–7 October, 2021
	Training Module 4 – Other risk assessments, organized by the Austrian Agency for Health and Food Safety (AGES) (virtual)	22–26 November, 2021
Other training/ workshops	BfR-Summer Academy (virtual event)	16–20 August 2021
	Workshop 'Risk Assessment and Risk Management of Genetically Modified Organisms (GMO)' (BfR)	9 November 2021
Scientific meetings	Poster presentation 'Evaluation of possible health risks associated with consumption of botanical preparations of *Mitragyna speciosa* (kratom)' at the EUROTOX 2021 Virtual Congress	27 September to 1 October 2021
Other activities	Regular meetings with the Unit of Nutritional Risks, Allergies and Novel Foods of the BfR (virtual and on site)	–
	Regular seminars organised by the Department of Food Safety of the BfR: Food safety related scientific presentations on current projects of the different department units (virtual)	Twice per month
	Presentation by the fellow of the EU-FORA programme of the results of *Mitragyna speciosa* risk assessment at the department seminar (BfR)	14 December 2021
	Participation in international socialising events organised by the International Affairs team of the BfR	–

3. Conclusions

3.1. Conclusions regarding *Mitragyna speciosa* (kratom) risk assessment

The available animal and human data that were evaluated by the fellow strongly indicate that kratom consumption has the potential to cause a number of adverse health effects, including addiction, associated withdrawal syndrome and other manifestations of toxicity, such as further aspects of neurotoxicity, but also liver toxicity and nephrotoxicity. However, the derivation of any health based guidance values for kratom preparations or constituents thereof and a subsequent concrete risk characterisation are severely impeded by considerable uncertainties, including the variability in composition of kratom preparations, insufficient information on dose–response relationships or on effects of long-term use. Further information, based on well-designed studies that cover different doses and periods of time and have been conducted with preparations clearly specified with respect to their composition, is required in order to reduce uncertainties and enable a more refined risk assessment.

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

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 substances and preparations of botanical origin used in food supplements, 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.

References

Ahmad K and Aziz Z, 2012. Mitragyna speciosa use in the northern states of Malaysia: a cross-sectional study. Journal of Ethnopharmacology, 141, 446–450. https://doi.org/10.1016/j.jep.2012.03.009

Alsarraf E, Myers J, Culbreth S and Fanikos J, 2019. Kratom from head to toe—case reviews of adverse events and toxicities. Current Emergency and Hospital Medicine Reports, 7, 141–168. https://doi.org/10.1007/s40138-019-00194-1

Apryani E, Taufik Hidayat M, Moklas MAA, Fakurazi S and Farah Idayu N, 2010. Effects of mitragynine from Mitragyna speciosa Korth leaves on working memory. Journal of Ethnopharmacology, 129, 357–360. https://doi.org/10.1016/j.jep.2010.03.036

BfR, 2020. Leitfaden für die Bewertung gesundheitlicher Risiken. Available online: https://www.bfr.bund.de/cm/350/leitfaden-fuer-gesundheitliche-bewertungen-bf.pdf

Di Lorenzo C, Ceschi A, Kupferschmidt H, Lüde S, De Souza Nascimento E, Dos Santos A, Colombo F, Frigerio G, Nørby K, Plumb J, Finglas P and Restani P, 2015. Adverse effects of plant food supplements and botanical preparations: a systematic review with critical evaluation of causality. British Journal of Clinical Pharmacology, 79, 578–592. https://doi.org/10.1111/bcp.12519

EFSA (European Food Safety Authority), 2009. Guidance on Safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements. EFSA Journal 2009;7(9):1249, 65 pp. https://doi.org/10.2903/j.efsa.2009.1249

EFSA (European Food Safety Authority), 2012. Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements. EFSA Journal 2012;10(5):2663, 21 pp. https://doi.org/10.2903/j.efsa.2012.2663

FDA, 2017. Statement from FDA Commissioner Scott Gottlieb, M.D. on FDA advisory about deadly risks associated with kratom. Available online: https://www.FDA.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-fda-advisory-about-deadly-risks-associated-kratom

Harizal SN, Mansor SM, Hasnan J, Tharakan JKJ and Abdullah J, 2010. Acute toxicity study of the standardized methanolic extract of Mitragyna speciosa Korth in Rodent. Journal of Ethnopharmacology, 131, 404–409. https://doi.org/10.1016/j.jep.2010.07.013

Ilmie MU, Jaafar H, Mansor SM and Abdullah JM, 2015. Subchronic toxicity study of standardized methanolic extract of Mitragyna speciosa Korth in Sprague-Dawley Rats. Frontiers in Neuroscience, 9, 189. https://doi.org/10.3389/fnins.2015.00189

Kamal MA, Ghazali R, Yahya N, Wasiman M and Ismail Z, 2012. Acute Toxicity Study of Standardized Mitragyna speciosa Korth Aqueous Extract in Sprague Dawley Rats. Journal of Plant Studies, 1. https://doi.org/10.5539/jps.v1n2p120

Marcus DM and Grollman AP, 2015. Toxicity of botanical medicines: an overlooked global health problem. American Journal of Public Health, 106, 16–17. https://doi.org/10.2105/AJPH.2015.302937

Panjaitan RGP and Liridah L, 2021. Liver Organ Impairment Due to the Consumption of Kratom Leaves (Mitragyna speciosa Korth.). Pharmacognosy Journal, 13, 179–184.

Sabetghadam A, Ramanathan S, Sasidharan S and Mansor SM, 2013. Subchronic exposure to mitragynine, the principal alkaloid of Mitragyna speciosa, in rats. Journal of Ethnopharmacology, 146, 815–823. https://doi.org/10.1016/j.jep.2013.02.008

Saingam D, Assanangkornchai S, Geater AF and Balthip Q, 2013. Pattern and consequences of krathom (Mitragyna speciosa Korth.) use among male villagers in southern Thailand: a qualitative study. International Journal of Drug Policy, 24, 351–358. https://doi.org/10.1016/j.drugpo.2012.09.004

Sakaran R, Othman F, Jantan I, Thent Z and Das S, 2014. Effect of subacute dose of Mitragyna Speciosa Korth crude Extract in Female Sprague Dawley Rats. Journal of Medical and Bioengineering, 3, 98–101. https://doi.org/10.12720/jomb.3.2.98-101

Schimmel J and Dart RC, 2020. Kratom (Mitragyna Speciosa) Liver Injury: a comprehensive review. Drugs, 80, 263–283. https://doi.org/10.1007/s40265-019-01242-6

Singh D, Müller CP, Murugaiyah V, Hamid SBS, Vicknasingam BK, Avery B, Chear NJY and Mansor SM, 2018. Evaluating the hematological and clinical-chemistry parameters of kratom (Mitragyna speciosa) users in Malaysia. Journal of Ethnopharmacology, 214, 197–206. https://doi.org/10.1016/j.jep.2017.12.017

Suhaimi FW, Hassan Z, Mansor SM and Müller CP, 2021. The effects of chronic mitragynine (Kratom) exposure on the EEG in rats. Neuroscience Letters, 745, 135632. https://doi.org/10.1016/j.neulet.2021.135632

Suwanlert S, 1975. A study of kratom eaters in Thailand. Bulletin on Narcotics, 27, 21–27.

Yusoff NHM, Suhaimi FW, Vadivelu RK, Hassan Z, Rümler A, Rotter A, Amato D, Dringenberg HC, Mansor SM, Navaratnam V and Müller CP, 2016. Abuse potential and adverse cognitive effects of mitragynine (kratom). Addiction Biology, 21, 98–110. https://doi.org/10.1111/adb.12185

Abbreviations

BfR	Bundesinstitut für Risikobewertung (German Federal Institute for Risk Assessment)
EU-FORA	European Food Risk Assessment Fellowship Programme
FDA	Food and Drug Administration

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Training in tools to develop quantitative risk assessment of fresh produce using water reuse systems in Mediterranean production

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Abstract

Water resources are increasingly coming under pressure specially around the Mediterranean area, leading to water scarcity and a deterioration in water quality. The use of treated wastewater represents an alternative source to enhance the demand for irrigation water. Water reuse in combination with the promotion of the use of water-efficient technologies in industry and water-saving irrigation techniques could lead to good qualitative and quantitative water status for surface and ground water bodies. Nevertheless, food-borne outbreaks linked to fresh produce irrigated with partially or untreated wastewater caused by bacteria, parasites and enteropathogenic viruses have been widely reported. In the absence of solid scientific understanding of the actual risks involved, consumers are likely less receptive to buy leafy greens irrigated with treated wastewater, also known as reclaimed water. In this study, we aimed to assess the microbiological risks of leafy green vegetables irrigated with treated wastewater in Spain using Norovirus as a model organism to facilitate the development of risk management strategies. A conceptual exposure model was designed to describe the virus fate and transport from the Wastewater treatment plant (WWTP) secondary effluent to the consumers' fork. This study is an example of the use of reclaimed water for irrigation of commercial fields producing leafy greens in the south-east of Spain and tries to assess potential microbiological risks to the consumers by establishing their safety.

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1. Introduction

The present work, dedicated to the training in tools to develop quantitative risk assessment of fresh produce using water reuse systems in Mediterranean production, was conducted in the context of the EFSA EU-FORA fellowship programme. This programme aims at early to mid-career professionals from European Union (EU) and European Free Trade Association (EFTA) countries, offering candidates the chance to widen their knowledge and hands-on experience of food safety risk assessment. EU-FORA fellowship programme provides competency based training and practical experience using the 'learning by doing' approach in acknowledged training sites across EU and European Economic Area (EEA) Member States.

Theofilos Papadopoulos is a Veterinary surgeon who has been working in the Directorate of Veterinary Centre of Thessaloniki. He graduated from the School of Veterinary Medicine, Aristoteles' University in Greece in 2003 and he received a Master of Science's degree in Aquatic Animal Health in 2008, and a Master of Science's degree in Public Health in 2011. The fellow completed his PhD in Molecular Microbiology in 2015, and he is EBVS European Veterinary Specialist in Veterinary Microbiology and a *de facto* member of the European College of the Veterinary Microbiology. Before EUFORA, he worked for nearly 10 years in the field of Surveillance of Zoonoses and Food Safety in Greece and in the field of Epidemiology in SCIENSANO - Belgium. In January 2021, he started the fellowship at the Polytechnic University of Cartagena (UPCT), joining the research group of Food Safety and Preservation in the Agronomic Engineering Department under the supervision of Professor Pablo Fernández Escámez. The fellowship was developed jointly by the UPCT and CEBAS-CSIC (Spain).

Water is a critical input for agricultural production and agricultural water has been identified as one of the main risk factors of microbial contamination for fresh produce. Agriculture consumes a significant amount of water resources in Europe, accounting for around 30% of total water use. In Europe, the main water sources are surface waters (rivers, lakes), reservoirs supplied by well or rain water, well water and potable quality water particularly in the case of hydroponics (EFSA BIOHAZ Panel, 2014). However, water is a limited resource, particularly for at least nine Mediterranean countries which are currently considered as water-stressed countries, related to pressure on the quantity and quality of water resources. This water stress situation is expected to be aggravated by the expected increasing water demand during the coming years and climate change (Mancuso et al., 2020). For this reason, alternative water sources have started to be used in an attempt to reduce the water stress. The use of reclaimed wastewater is a promising alternative water resource, particularly for agriculture, which is currently the main user of renewable water resources.

The use of reclaimed wastewater mainly allows to preserve the freshwater stock. However, it also represents a source of nutrients, namely nitrogen, phosphorus, and other salts, which are necessary for the physiological growth of crops (Mancuso et al., 2020). The wastewater treatment process usually includes primary (sedimentation) and secondary treatments (biological oxidation), as well as more advanced tertiary treatments such as chemical coagulation, filtration and/or chemical disinfection (EFSA BIOHAZ Panel, 2014). Wastewater cannot be used for irrigation, and reclaimed water needs to be of a particular quality (European Union, 2020). The use of reclaimed water for irrigation is beneficial in agriculture but if wastewater treatments are not well implemented, it may be associated with potential human health risks. One of the important challenges when using reclaimed water in agriculture is ensuring the safety of food products considering that if the water reuse systems are not properly implemented, human pathogens may be still present.

The consumption of leafy salads has increased internationally in recent years as promotion of healthier lifestyles. These foods are generally consumed fresh and are not subject to further processing to eliminate pathogenic microorganisms such as viruses (Callejón et al., 2015). Since leafy greens eaten raw as salads do not include any processing steps or control points which will ensure removal or inactivation of biological hazards, it is particularly important to consider risk factors and control options at the point of production (EFSA BIOHAZ Panel, 2014). Food-borne outbreaks linked to fresh produce irrigated with partially or untreated wastewater have been reported (Lynch et al., 2009; Berger et al., 2010; Gelting and Baloch, 2013), while contamination of crops with enteric viruses, faecal coliforms, and bacterial pathogens and parasites has also been evidenced (Hamilton et al., 2006; Sales-Ortells et al., 2015; Adegoke et al., 2018).

Food-borne outbreaks may be caused by bacteria, parasites and enteropathogenic viruses including rotaviruses, astroviruses, adenoviruses, noroviruses and caliciviruses (Domínguez et al., 2009). During 2019, norovirus was associated with 457 food-borne outbreaks and, most importantly, with 11,125 related illnesses (22.5% of total cases) meaning one in five of all outbreak-related illnesses in the EU.

During the same period, 51 outbreaks were associated with the consumption of food of non-animal origin (leafy green vegetables, olives, tomatoes, cucumbers and radish sprouts) with leading causes norovirus (14 outbreaks) and *Salmonella* (12 outbreaks) (EFSA and ECDC, 2021). Norovirus caused most of the outbreaks associated with the consumption of leafy vegetables in USA during 2014–2018 (CDC, 2021). The microbiological risks derived from irrigation of fresh produce with reclaimed water have been previously reported for enteric viruses (Hamilton et al., 2006; Sales-Ortells et al., 2015; López-Gálvez et al., 2016; Adegoke et al., 2018; Summerlin et al., 2021). Some studies have also focused on norovirus risks/presence in fresh produce (Mara and Sleigh, 2009; Barker, 2014; Sales-Ortells et al., 2015; Eregno et al., 2017; Torok et al., 2019; Emilse et al., 2021). Studies on the prevalence and infectivity of Norovirus are limited, and quantitative data on viral load are scarce making establishment of microbiological criteria for Norovirus on leafy greens difficult (EFSA BIOHAZ Panel, 2014).

Quantitative microbiological risk assessment (QMRA) is a methodology used to organise and analyse scientific information to estimate both the probability and severity of an adverse event as well as prioritise efforts to reduce the risk of food-borne pathogens (Habib et al., 2020). The development of QMRA involves a four-phase process to estimate the human health risk associated with exposure to the target pathogen. The microbial risk assessment process consists of four distinct steps: (i) the hazard identification; (ii) the hazard characterisation; (iii) the exposure assessment; and (iv) the risk characterisation (Lammerding and Fazil, 2000; Koutsoumanis and Aspridou, 2016). QMRA is based on a quantitative description of the microbial response to the different conditions encountered during each step of the field-to-fork chain of the product based on mathematical models.

The results of the quantitative model of pathogens associated to fresh produce using water reuse systems in Mediterranean production will provide an estimation of the burden of pathogenic microorganisms present in the plant products that are irrigated with reclaimed water considering the different scenarios and production practices. These data, combined with the existing data in the literature on the consumption of leafy vegetables and dose-response curves will provide an estimate of the possible risks associated with this practice.

2. Description of work programme

2.1. Aims

The work programme will focus on the QMRA of pathogens associated with fresh produce using water reuse systems in Mediterranean production. It will cover hazard identification, exposure assessment, hazard characterisation and risk characterisation, applying a robust statistical method. It will be developed in collaboration with CEBAS-CSIC, the partner that was included in the application. Main activities related to the Work Programme where the fellow is going to be involved in:

- Training of the fellow person on methodologies related to risk assessment routinely used by the supervisors and co-supervisors at UPCT and CEBAS-CSIC. This will include databases, different software available for predictive microbiology and risk assessment, statistical analysis and programming in R.
- Data selection to characterise the microbial response required for QMRA. Data available in both institutions will be used, as well as data from literature. If there are data gaps, they will be filled performing experimental work.
- Development of mathematical models to describe microbial behaviour along the food change (prevalence, growth, inactivation, acclimation, contamination, etc.), based on data gathered from the groups and from literature. The models will be validated so that they can be applied for a QMRA. Dose–response models will be selected from those already published. When possible, R statistical software (open access) and f.eg. Shiny app will be used.
- Estimation of the risk based on different scenarios. Health risks will be established on the basis of conditions included in the study using web-based tools (such as MicroHibro, FDA-iRisk, @Risk) and the data and models developed. This will allow the establishment of a risk ranking and the interpretation of the impact of variability and uncertainty on a QMRA.

2.2. Activities/Methods

2.2.1. Practical work, research project on QMRA of leafy greens irrigated with reclaimed water

As part of the fellowship, the priority of the hosting site was to provide the fellow with the basic theoretical background required to perform a QMRA. The fellow joined a working team based in the UPCT in collaboration with CEBAS-CSIC and with proved expertise in the use of risk assessment tools.

The fellow performed a comprehensive literature mining to collect published information and identify potential data gaps in performing QMRA. This also included training in handling of available databases (EFSA, FAO and ECDC). The fellow was also trained in growth and inactivation modelling (such as Combase or Bioinactivation FE, the latter developed in the group).

Together with his supervisors, the fellow agreed to work in a study project in QMRA of norovirus gastroenteritis associated with the consumption of leafy greens from commercial fields irrigated with reclaimed water. The QMRA application covered all the steps from the secondary effluent of a wastewater treatment plant to the consumer's fork.

A total of 570 samples (water and crop) were analysed across the water reuse system used to irrigate leafy greens in commercial growing fields. Reclaimed water originated from two WWTPs in Murcia region (Spain) using different wastewater treatment processes. Samples were tested for the presence of several microbiological hazards during 2017–2019. Sampling was performed in WWTP inlet (N = 100), WWTP outlet (N = 100), WWTP reservoir (N = 100), grower reservoir (N = 75), irrigation point ((N = 100) and crop (N = 95). A database, provided by the supervisors, was analysed including the prevalence data of several Norovirus I, Norovirus II, hepatitis virus and bacteriophages in leafy green primary production. After analysing the data, some laboratory work was planned to cover potential gaps for the QMRA application. However, due to the uncertainty of COVID-19 situation, and the lockdown at the university performing laboratory work was not feasible. Therefore, a more theoretical approach for the project had to be adapted.

The fellow received training in implementation of statistical analysis using Monte Carlo and Bayesian methods and risk ranking methodologies. He was trained in using various software tools specific for risk assessment (e.g. MicroHibro, @Risk, FDA-iRISK). He also gained experience in the separation between variability and uncertainty, the quantification of these terms and the incorporation in predictions from the point of view of experimental design and statistical analysis.

2.2.2. Training in risk assessment

During the fellowship, the fellow obtained general information on Risk assessment activities. Due to the COVID-19 pandemic, all courses were held as online modules. Initially, the fellow followed the 3-week induction training in microbiological and chemical risk assessment by EFSA (11–29 January 2021). He attended an additional 1-week training module focusing on risk communication and crisis response, organised by the BfR (22–26 March 2021) and the 6-day training module focusing on emerging risks, nanotechnology, omics, new concepts and tools in toxicology, risk ranking organised by the Hellenic Food Authority (7–14 June 2021). He also attended the 4-day training module on data collection and reporting (4-7 October 2021) and finally the 1-week in module 'module 4 training of the European Food Risk Assessment Fellowship Programme' (22–26 November 2021), both organised by EFSA.

The fellow joined a 2-day visit in the headquarters of the Spanish Agency for Food Safety and Nutrition (AESAN) on October 28, 2021, in Madrid and on 29 in Centro Nacional de Alimentación in Majadahonda. During this visit, he had the opportunity to meet in person all the other fellows hosted in Spanish Institutes and to receive information and in-hand training in risk assessment regarding biological and chemical risks, risk management, official control in foods and alerts, food contact materials, residues of veterinary drugs, microbiology, and antimicrobial resistance.

Moreover, the fellow presented and discussed the results of his project at the First Workshop of EUt+ (European University of Technology) Sustainability Lab on 14 June, 2021.

2.3. Secondary scientific activities during fellowship

Along with the scheduled activities, additional training and other opportunities were provided by the hosting and other organisations face to face or online. This helped the fellow further improve his general knowledge on risk assessment.

2.3.1. Participating in various conferences/webinars/meetings

1) 'Elicitation and practical use of disability weights for quantifying years lived with disability'. Webinar provided by Burden of Disease Network, January 2021 (online).
2) 'Basic Concepts in Epidemiology and Surveillance'. Training school provided by Animal Health Ireland, 1-2 February 2021 (online).
3) 'Learning R'. LinkedIn learning online course, March 2021 (online).
4) 'Risk communication' EPIET Alumni Network webinar, March 2021 (online).
5) 'Activities and achievements of the Italian Global Burden of Disease Initiative'. Webinar provided by Burden of Disease Network, April 2021 (online).
6) 'The ESCMID study group in Public Health Microbiology Virtual Meeting', May 2021, (online).
7) 'Burden of foodborne diseases: how can we estimate it and why do we need it'? Webinar provided by World Health Organization, June 2021, (online).
8) 'Forecasting the Global Burden of Disease Study'. Webinar provided by SCIENSANO, June 2021, (online).
9) 'First Workshop of EUt+ Sustainability Lab' June 2021, (online).
10) 'Basic pharmacokinetics and pharmacodynamics – focus on antibiotics'. Training School, School of Veterinary Medicine, University of Padova, Italy, August 2021
11) 'European Network for Optimization of Veterinary Antimicrobial Treatment working group meeting'. University of Padova, Italy, August 2021
12) 'Epidemiology of Methicillin Resistant *Staphylococcus aureus* in abattoirs and slaughtered ruminants', (25 days) Short Term Scientific Mission by Risk-based meat inspection and integrated meat safety assurance CA18105, Thessalonik'i, Greece, August 2021.
13) 'Animal Health economics'. Webinar provided by Standardizing output-based surveillance to control non-regulated Diseases of cattle in the EU (SOUND) COST action, September 2021 (online).
14) 'Innovative Dairy Science education material development, focused on Products, Processes, Quality, Safety & Entrepreneurship, using Information and Communication Technologies (ICTs) and Open Educational Resources (OER)' final meeting, Larissa, Greece, September 2021.
15) 'AGM meeting of the European College of Veterinary Microbiology, October 2021 (online).
16) 'Novel technologies for surveillance and characterization of Extended-spectrum β-lactamase and Carbapenemase producing Enterobacteriaceae, in humans and animals (CARBATECH)' seminar, Ioannina, Greece, November 2021
17) 'Virtual training module 4' Training module provided by the European Food Risk Assessment Fellowship Programme, 22–26 November 2021 (online).

2.3.2. Oral presentation

'Water reuse in agricultural irrigation of commercial fields producing leafy greens in South-east Spain'. Papadopoulos T., Allende A., Egea J.A., Gomez A.P., Fernandez P.S. Oral presentation at First Workshop of EUt+ Sustainability Lab June 2021, Cartagena, Spain (online) **(Annex)**.

3. Results

Leafy greens, eaten raw as salads, represent a minimally processed, ready-to-eat food. In order to provide an analysis of the risks associated with the consumption of this food item we considered all the production processes of leafy greens starting from the field, growing, harvesting, distribution, retail and handling in domestic environments. On top of this, we considered the irrigation of the leafy greens with reclaimed water and the risks associated with this practice using norovirus as the model organism (Figure 1).

One of the main pathways for the contamination at primary production is due to the microbial quality of the reclaimed water used for irrigation. Water treatment usually includes primary (sedimentation) and secondary (biological oxidation) treatments, as well as more advanced tertiary treatments such as chemical coagulation, filtration and/or chemical disinfection. Nevertheless, they can vary even if there are standards established at EU level for treatment of municipal wastewater to be used for irrigation (European Union, 2020). However, the water reuse system includes not only the water treatment process but also the distribution and storage of the water before the reclaimed water

is used in the delivery point at the grower field. Therefore, the microbiological quality of the reclaimed water not only depends on the good implementation of the water treatments but also on the management of all the steps of the water reuse system.

Contamination risk depends also on the irrigation strategies (mixing with other waters, irrigation system) and the use of manure as fertiliser and Norovirus (NoV) internalisation from irrigation or the soil. Irrigation with untreated or partially treated wastewater together with spraying prior to harvest and overhead irrigation that leads to wetting of edible parts are the main risk factors regarding this stage of production.

We considered, as basic scenario, leafy greens consumed after harvesting without any processing from the industry (washing, cutting, packaging, etc.) but washing by the consumer. As an alternative scenario we considered all these processes made by industry, leafy greens sold packed and eaten raw without any other process from the consumer.

The main factors for contamination of leafy greens during primary production is the use of untreated wastewater for irrigation and the irrigation system. During processing, the main factors are contamination or cross-contamination via equipment, water or by food handlers. Finally, at distribution in domestic and commercial environments, cross contamination by food handlers or equipment are very important factors, while at home and according to the basic scenario, washing practices before consumption play the most important role.

Figure represents a flow chart for the QMRA while table includes all the inputs used for QMRA application (Figure 1).

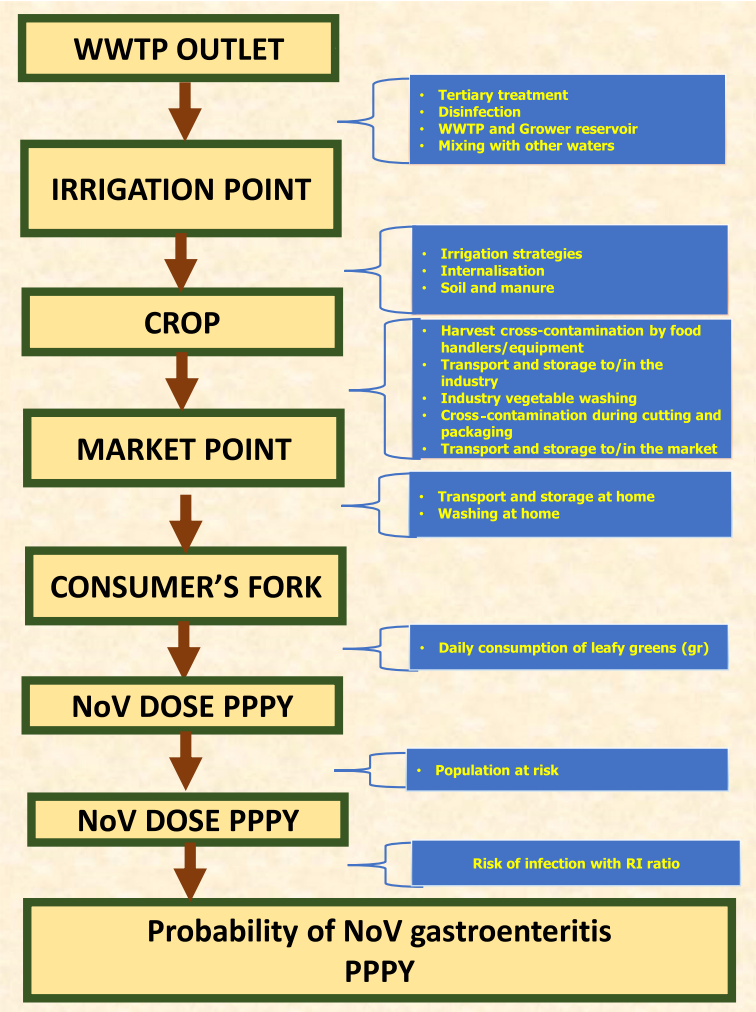

Figure 1: Flow chart of QMRA for predicting the probability of developing NoV acute gastroenteritis for an individual after consuming raw leafy greens

4. Conclusions from the participation in the EU-FORA programme

Participation in the EFSA EU-FORA work programme was a valuable opportunity for the fellow to obtain experience in tools used in quantitative microbial risk assessment. This was also a perfect chance to expand his knowledge and skills in food safety, particularly in the field of QMRA, by working in a professional environment according to European guidelines and standards. It also provided the hosting institutions, Universidad Politécnica de Cartagena and CEBAS-CSIC, an excellent opportunity to interact with the EU-FORA Programme through a motivated and highly capable fellow to improve our training capacity in QMRA. A multidisciplinary biological risk assessment was developed.

The fellow learned many new skills through the modules, and had opportunities to broaden his understanding of the risk assessment methodology in a wider range of hazards including infectious diseases. Besides all the learning-by-doing, he had the chance to further develop his network of professionals by attending the EU-FORA training modules, conferences, and meetings.

The EU-FORA programme provided a great environment to build a strong professional and personal network that will be used for future collaborations between the sending and both the hosting institutions. Finally, fellow's training in risk assessment methodology was a great added value also for the sending institute and will help both fellow and the Institute to apply risk assessment methodology in practice.

References

Adegoke AA, Amoah ID, Stenström TA, Verbyla ME and Mihelcic JR, 2018. Epidemiological evidence and health risks associated with agricultural reuse of partially treated and untreated wastewater: a review. Frontiers Public Health, 6. https://doi.org/10.3389/fpubh.2018.00337

Barker SF, 2014. Risk of norovirus gastroenteritis from consumption of vegetables irrigated with highly treated municipal wastewater—evaluation of methods to estimate sewage quality. Risk Analysis, 34, 803–817. https://doi.org/10.1111/risa.12138

Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P and Frankel G, 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environmental Microbiology, 12, 2385–2397. https://doi.org/10.1111/j.1462-2920.2010.02297.x

Callejón RM, Rodríguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC and Troncoso AM, 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. Foodborne Pathog Dis, 12, 32–38. https://doi.org/10.1089/fpd.2014.1821

CDC (Centers for Disease Control and Prevention), 2021. Lettuce, Other Leafy Greens, and Food Safety. Available online: https://www.cdc.gov/foodsafety/communication/leafy-greens.html

Domínguez A, Godoy P, Torner N, Cardeñosa N and Martínez A, 2009. The viral gastroenteritis: a public health problem. Revista Espanola De Salud Publica, 83, 679–687. https://doi.org/10.1590/s1135-57272009000500009

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2021. The European Union One Health 2019 Zoonoses Report. EFSA Journal 2021;19(2):e06406, 45 pp. https://doi.org/10.2903/j.efsa.2021.6406

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (Salmonella and Norovirus in leafy greens eaten raw as salads). EFSA Journal 2014;12(3):3600, 118 pp. https://doi.org/10.2903/j.efsa.2014.3600

Emilse PV, Matías V, Cecilia ML, Oscar GM, Gisela M, Guadalupe DiCola, Elizabeth RV, Victorio PJ, Rodney C, Viviana NS and Angélica BP, 2021. Enteric virus presence in green vegetables and associated irrigation waters in a rural area from Argentina. A Quantitative Microbial Risk Assessment. LWT, 144. https://doi.org/10.1016/j.lwt.2021.111201

Eregno FE, Moges ME and Heistad A, 2017. Treated greywater reuse for hydroponic lettuce production in a green wall system: quantitative health risk assessment. Water (Switzerland), 9. https://doi.org/10.3390/w9070454

European Union, 2020. Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on minimum requirements for water reuse, Official Journal of the European Union, L 177/132.

Gelting RJ and Baloch M, 2013. A systems analysis of irrigation water quality in environmental assessments related to foodborne outbreaks. Aquatic Procedia, 1, 130–137. https://doi.org/10.1016/j.aqpro.2013.07.011

Habib I, Coles J, Fallows M and Goodchild S, 2020. Human campylobacteriosis related to cross-contamination during handling of raw chicken meat: application of quantitative risk assessment to guide intervention scenarios analysis in the Australian context. International Journal of Food Microbiology, 332, 108775. https://doi.org/10.1016/j.ijfoodmicro.2020.108775

Hamilton AJ, Stagnitti FS, Premier R and Boland A-M, 2006. Is the risk of illness through consuming vegetables irrigated with reclaimed wastewater different for different population groups? Water Science and Technology, 54, 379–386. https://doi.org/10.2166/wst.2006.714

Koutsoumanis KP and Aspridou Z, 2016. Moving towards a risk-based food safety management. Current Opinion in Food Science, 12, 36–41. https://doi.org/10.1016/j.cofs.2016.06.008

Lammerding AM and Fazil A, 2000. Hazard identification and exposure assessment for microbial food safety risk assessment. International Journal of Food Microbiology, 58, 147–157. https://doi.org/10.1016/S0168-1605(00)00269-5

López-Gálvez F, Truchado P, Sánchez G, Aznar R, Gil MI and Allende A, 2016. Occurrence of enteric viruses in reclaimed and surface irrigation water: relationship with microbiological and physicochemical indicators. Journal of Applied Microbiology, 121, 1180–1188. https://doi.org/10.1111/jam.13224

Lynch MF, Tauxe RV and Hedberg CW, 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiology and Infection, 137, 307–315. https://doi.org/10.1017/s0950268808001969

Mancuso G, Lavrnić S and Toscano A, 2020. Chapter three - reclaimed water to face agricultural water scarcity in the Mediterranean area: an overview using Sustainable Development Goals preliminary data. In: Verlicchi P (ed.). Advances in Chemical Pollution, Environmental Management and Protection (Vol. 5, pp. 113–143): Elsevier.

Mara D and Sleigh A, 2009. Estimation of norovirus infection risks to consumers of wastewater-irrigated food crops eaten raw. Journal of Water and Health, 8, 39–43. https://doi.org/10.2166/wh.2009.140

Sales-Ortells H, Fernandez-Cassi X, Timoneda N, Dürig W, Girones R and Medema G, 2015. Health risks derived from consumption of lettuces irrigated with tertiary effluent containing norovirus. Food Research International, 68, 70–77. https://doi.org/10.1016/j.foodres.2014.08.018

Summerlin HN, Pola CC, Chamakura KR, Young RY, Gentry T, McLamore ES, Karthikeyan R and Gomes CL, 2021. Fate of enteric viruses during leafy greens (romaine lettuce) production using treated municipal wastewater and AP205 bacteriophage as a surrogate. Journal of Environmental Science and Health, Part A, 1–7. https://doi.org/10.1080/10934529.2021.1968231

Torok VA, Hodgson KR, Jolley J, Turnbull A and McLeod C, 2019. Estimating risk associated with human norovirus and hepatitis A virus in fresh Australian leafy greens and berries at retail. International Journal of Food Microbiology, 309. https://doi.org/10.1016/j.ijfoodmicro.2019.108327

Abbreviations

EEA	European Economic Area
EFTA	European Free Trade Association
QMRA	quantitative microbiological risk assessment
UPCT	Polytechnic University of Cartagena
WWTP	Wastewater treatment plant

Annex A – Oral presentation at First Workshop of EUt+ Sustainability Lab June 2021

Water reuse in agricultural irrigation of commercial fields producing leafy greens in South-east Spain

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- Water scarcity and deterioration in water quality in the Mediterranean area due to climate change
- Use of reclaimed water is alternative water source for irrigation water.
  - Promote sustainability of the water in surface and ground bodies
  - Way of restoring nutrients to the ground
  - Water transfers and desalination have significant costs

Agricultural products irrigated with reclaimed water should ensure safety across EU Ms.

EU legislation=Wastewater treatment plants operators have the main responsibility

BUT

End-users have responsibility for the microbiological quality of the water to produce safe food

E. coli for irrigation water is 2 log CFU/100 ml

What is the risk for the consumers of leafy greens irrigated with reclaimed water?

Study objectives:

- Give an example of the use of reclaimed water for irrigation of leafy green fields in the S. Spain
- Monitor indicator and pathogenic microorganisms across leafy green production
- Identify potential microbiological risks to the consumers
- Perform microbiological risk assessment- quantify this risk to the consumers

2017-2019, growing cycles of leafy greens in Murcia-Spain (570 samples)

2 WWTPs with tertiary treatments for water disinfection

Different sampling points: WWTP inlet, WWTP outlet, WWTP reservoirs, grower reservoirs, irrigation (3 types) and leafy greens

Microorganisms: *E. coli,* Norovirus I and II, Bacteriophages, HVA, non-O157:H7 Shiga-toxigenic *E. coli* (STEC), *E. coli* O157:H7 and *Salmonella* spp.

Modelling the microbial behaviour from WWTTP to the crop: concentration in effluent, removal by tertiary treatment, inactivation in the field and during storage and transport, transfer through irrigation.

Estimating the health risks to the consumers for different scenarios: washing removal, leafy greens consumption, dose, food poisoning in different populations, burden of disease.

Universidad Politécnica de Cartagena — EUROPEAN UNIVERSITY OF TECHNOLOGY "Think human first"

Workshop of the EUt+ Sustainability Lab 14 & 15 June, 2021

EU-FORA SERIES 4

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Integration of genomics in surveillance and risk assessment for outbreak investigation

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Abstract

Keeping food safe is a challenge that needs continuous surveillance for the sake of consumers' health. The main issue when a food-borne pathogen outbreak occurs is represented by the identification of the source(s) of contamination. Delivering this information in a timely manner helps to control the problem, with positive outcomes for everyone, especially for the consumers, whose health is in this way preserved, and for the stakeholders involved in food production and distribution, who could face enormous economic losses if recalls or legal issues occur. Whole genome sequencing (WGS) is a tool recently implemented for the characterisation of isolates and the study of outbreaks because of its higher efficiency and faster results, when compared to traditional typing methods. Lower sequencing costs and the development of many bioinformatic tools helped its spread, and much more attention has been given to its use for outbreak investigation. It is important to reach a certain level of standardisation, though, for ensuring result reproducibility and interoperability. Moreover, nowadays it is possible, if not mandatory for Open Science Practices, to share WGS data in publicly available databases, where raw reads, assembled genomes and their corresponding metadata can be easily found and downloaded. The scope of this Fellowship was to provide the Fellow all the training necessary for successfully integrating genomics to surveillance and risk assessment of food-borne pathogens from farm to fork.

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Keywords: whole genome sequencing, outbreak, antimicrobial resistance, food safety, surveillance, risk assessment, farm to fork

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1. Introduction

Whole genome sequencing (WGS) is becoming a main tool for outbreak investigation because it has higher efficiency in identification and characterisation of food-borne microorganisms than other traditional typing methods (Koutsoumanis et al., 2019). Compared to these traditional methods, such as pulsed field gel electrophoresis (PFGE), multiple locus variable number tandem repeat Analysis (MLVA), random amplified polymorphic DNA (RAPD) analysis, variable number tandem repeat (VNTR) analysis or multilocus sequence typing (MLST), WGS delivers outputs at higher resolution and in a shorter time. In addition, depending on the bioinformatic application(s) used and on the performance of the machine, it can provide the results of multiple tests in one single assay (e.g. identification of virulence and antimicrobial resistance (AMR) genes, and other phenotype predictions based on genotype), which speeds up the emergence response in case of an outbreak. Recently, WGS has been used for the detection of pathogens in several outbreaks with great impact for public health. For example, WGS was used to identify the likely source of the largest known outbreak of *Listeria monocytogenes,* which happened in South Africa between 2017 and 2018 (Smith et al., 2019) and in the EU multi-country outbreak of *L. monocytogenes* ST6 linked to frozen corn (EFSA and ECDC, 2018). Another concern is the possibility of dissemination of AMR- and virulence-related genes via the food chain. WGS is also helpful in this case, allowing to rapidly characterise biological determinants related to AMR, virulence, mobile genetic elements (MGEs), and their dissemination patterns, which contributes to the protection of public health with respect to food-borne diseases. The 'One Health' approach is nowadays widely recognised for investigating AMR and how it spreads across all sectors, for example in hospitals or through animal farming (Hernando-Amado et al., 2019). In addition, dose–response models would benefit from a better understanding on the virulence potential of certain strains. Several recent studies have demonstrated the promise of routine WGS of bacterial pathogens for epidemiological surveillance, outbreak detection, and infection control. For example, Neuert et al. (2018) used WGS to identify genetic traits responsible for phenotypic AMR in 3,491 non-typhoidal *Salmonella enterica* isolates. In addition, WGS can be used to track the occurrence and distribution of these genetic traits leading to AMR or virulence in different environments, including foods, food-related environments and clinical specimens, facilitating source attribution. If integrated with metadata gathered from food or clinical samples, genomic data can be implemented within quantitative risk assessment frameworks by including statistical analyses and mathematical modelling of resistance and virulence determinants occurrence and dissemination. A cross-sectorial platform developed in an EFSA funded project (INNUENDO project) is available and allows to identify flaws and needs in data flow during outbreak investigation and routine implementation of WGS in molecular epidemiology of food-borne pathogens, providing information to solve outbreaks and enhancing scientific cooperation between the food, veterinary and human health sectors. WGS can thus provide to national and international regulatory agencies and researchers a framework for the evaluation and communication of risks linked to foods. Furthermore, it facilitates the investigation of outbreaks and the actualisation of measures for risk reduction. Thanks to the application of open science practices, all the WGS data published in the scientific literature are publicly available in online databases. These data can be used for many different additional investigations, such as to further characterise isolates responsible for outbreaks, or to study the occurrence of virulence and antimicrobial genes in specific regions or in a specific time frame.

Strengthening global surveillance of food-borne pathogens and their related characteristics (e.g. virulence or AMR potential), is critical as it sets the basis for developing global strategies, monitoring the effectiveness of public health interventions and detecting new trends and emerging threats.

2. Description of work programme

2.1. Aims

The aim of this fellowship was to prepare the fellow on exploring the potential of next-generation sequencing (in particular, of WGS) methodologies as a tool for surveillance of food-borne pathogens, AMR and virulence genes. The core of the work was related to the study of publicly available WGS data of some of the most common food-borne pathogens, to explore the characteristics of their resistome and associate the presence of AMR genes to metadata, such as geographical data, isolation source and temporal distribution.

2.2. Activities/Methods

To achieve the objectives mentioned in Section 2.1, the fellow received training on the most common practices and protocols used for WGS and whole metagenome sequencing (WMS) by experienced scientists. Furthermore, an outbreak simulation was performed to assess the fellow's acquired skills on an outbreak investigation, and training on some of the most common software used for risk assessment was delivered. The activities related to each objective of this fellowship are described below.

Objective 1: Training of the fellow on general risk assessment methodologies routinely used by the mentor and other collaborators at the host institution. Extensive training was arranged about the functionalities of the software *Oracle Crystal Ball*, used by the host institution for performing Monte Carlo simulations and predictive modelling. The training and the analyses performed consisted in four phases: variable definition, model development, simulation and uncertainty analysis (Figure 1). Practical examples were explored by the fellow and discussed with the tutors, e.g. to predict the reductions in microbial counts following the pasteurisation of milk, or to assess the exposure of European consumers to extended-spectrum β-lactamase (ESBL)- producing *Escherichia coli* through the consumption of pork meat.

Objective 2: Training of the fellow on different integrated platforms for the use of genomics in food-borne pathogen surveillance and outbreak investigation. An intensive hands-on training was delivered to the fellow on the use of the most common applications for the analysis of WGS data. In particular, the fellow learnt how to use Python- and Pearl-based software (e.g. *StarAMR*, *ResFinder*, *PointFinder*, *PlasmidFinder*, *PlasFlow*, *MLST*, *dRep*, *Tormes*) launched with command lines and adapted Ruby scripts for parallel analyses on multiple genomes, using Linux and Unix environments. The fellow was also trained on the use of a server owned by the hosting site for analyses that required high-performing computing. Eventually, a training on the use of specific R packages for statistical analyses and charts preparation (*dplyr*, *ggplot2*, *pheatmap*, *ggpubr*, *vegan*, *tidyr*) was given to the fellow.

Objective 3: *In silico* analysis of genomes from food-borne pathogens.

The fellow analysed about 30,000 *Staphylococcus aureus* genomes and their metadata downloaded from publicly available repositories describing the distribution of antimicrobial resistance genes (ARGs) among clonal complexes (CCs), geographical regions, isolation sources and time periods. The resistome of *S. aureus* was described and trends highlighted. Furthermore, the location of ARGs on MGEs (plasmids) was described and analysed. The workflow of this analysis is summarised in Figure 2.

A similar analysis was also conducted on about 3,000 *Salmonella enterica* serovar Enteritidis and 3,000 *Salmonella enterica* serovar Typhimurium genomes.

Two manuscripts reporting the main results of these activities are currently in preparation.

Objective 4: Assessment of the potential of WGS in outbreak investigation.

A case study of an outbreak was prepared by the tutors and investigated by the fellow, using WGS data and metadata about the sources of samples, to determine the source(s) of the simulated outbreak (Appendix A).

Objective 5: Collaboration of the fellow in dissemination and outreach activities.

The fellow has worked on a literature review on the use of WGS for improving food safety which has been submitted to the Food Microbiology section of *Current Opinion in Food Science*. A semi-systematic literature review on the use of WGS for outbreak investigation has also been prepared. Furthermore, a manuscript on the *S. aureus* resistome analysis has been already prepared and it is ready for being submitted for peer review. One more manuscript on the analysis of *Salmonella* Enteritidis and *S.* Typhimurium is also in preparation.

The fellow will also present the *S. aureus* resistome analysis at the next ONE – Health, Environment, Society – Conference, in Brussels, 21–24 June 2022.

Additional activities were also undertaken by the fellow during this year at the host institution. For example, the fellow attended online courses to improve his skills on Python and Linux commands and applied for the Marie Skłodowska-Curie Actions (MSCA) Postdoctoral Fellowships 2021. The fellow was fully integrated into the working team of his host institution and participated to lab meetings in which he presented his work to other colleagues. The fellow also received an invitation for a visit, with the other EU-FORA fellows hosted in Spain, to the Spanish Food Safety and Nutrition Agency (AESAN) in Madrid and Majadahonda which took place at the end of October 2021.

3. Conclusions

This fellowship transferred to the fellow's new skills on the use of WGS for the study of outbreaks and the characterisation of AMR in food-borne pathogens. Outputs derived from this fellowship will be published in peer-reviewed journals, which will include a literature review on the use of WGS for food safety, a semi-systematic review on the use of WGS for outbreak investigation, and two research papers, one on the analysis of the resistome of *S. aureus* and one on the analysis of the resistome of *S*. Enteritidis and *S*. Typhimurium. The fellow is actively working to finalise the remaining manuscripts on a timely manner. Furthermore, the fellow had the opportunity to familiarise with tools for Risk assessment and Monte Carlo simulations.

3.1. Future goals

The cooperation between the fellow and the hosting site will be maintained in the future. A Marie Curie fellowship application has been already submitted by the fellow and the host organisation, and hopefully more projects will be shared in the future to maintain this fruitful collaboration.

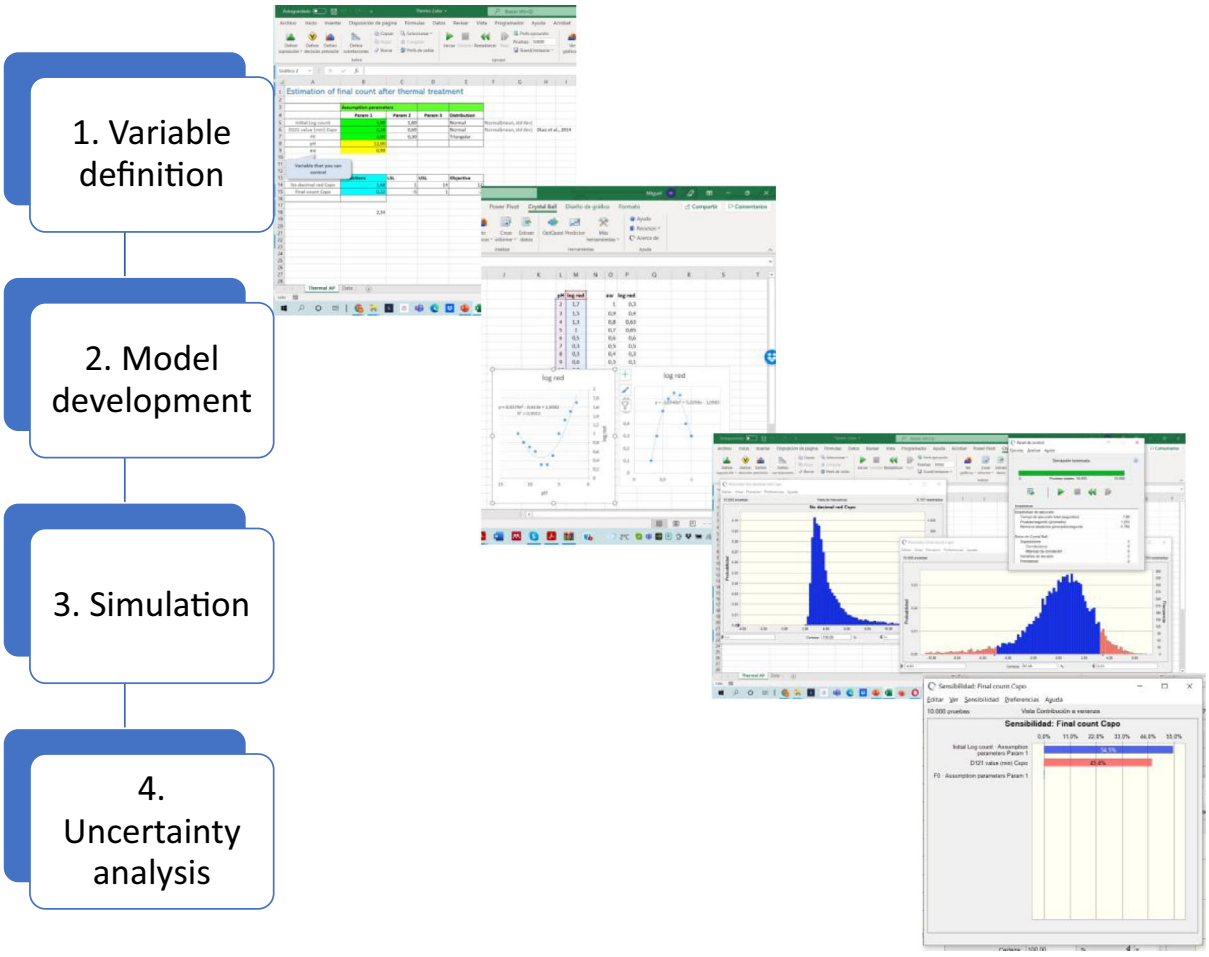

Figure 1: Workflow used for Monte Carlo simulation and predictive modelling with *Oracle Crystal Ball*

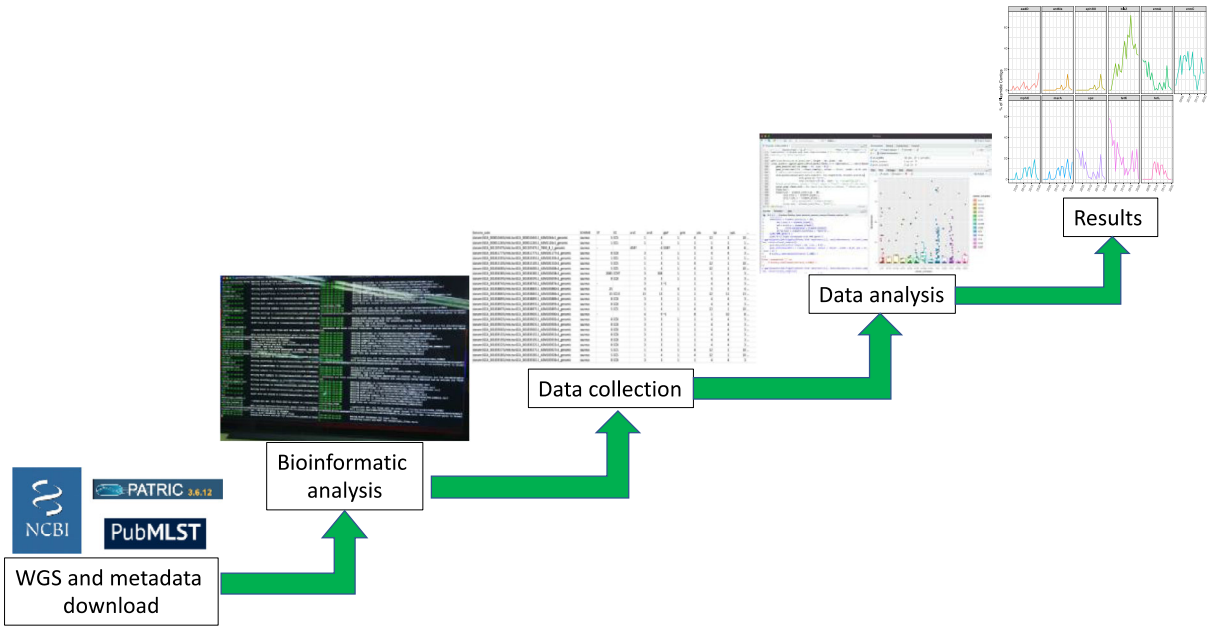

Figure 2: Workflow of the analyses performed in Objective 3

References

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2018. Multi-country outbreak of *Listeria monocytogenes* serogroup IVb, multi-locus sequence type 6, infections linked to frozen corn and possibly to other frozen vegetables – first update. EFSA Supporting Publications 2018;15(7): en-1448, 55 pp. https://doi.org/10.2903/sp.efsa.2018.EN-1448

Hernando-Amado S, Coque TM, Baquero F and Martínez JL, 2019. Defining and combating antibiotic resistance from One Health and Global Health perspectives. In Nature Microbiology (Vol. 4, Issue 9, pp. 1432–1442). Nature Publishing Group. https://doi.org/10.1038/s41564-019-0503-9

Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, Davies R, De Cesare A, Hilbert F, Lindqvist R, Nauta M, Peixe L, Ru G, Simmons M, Skandamis P, Suffredini E, Jenkins C, Malorny B, Ribeiro Duarte AS, Torpdahl M, da Silva Felício MT, Guerra B, Rossi M and Herman L, 2019. Whole genome sequencing and metagenomics for outbreak investigation, source attribution and risk assessment of food-borne microorganisms. EFSA Journal, 17. https://doi.org/10.2903/j.efsa.2019.5898

Neuert S, Nair S, Day MR, Doumith M, Ashton PM, Mellor KC, Jenkins C, Hopkins KL, Woodford N, de Pinna E, Godbole G and Dallman TJ, 2018. Prediction of phenotypic antimicrobial resistance profiles from whole genome sequences of non-typhoidal *Salmonella enterica*. Frontiers in Microbiology, 9. https://doi.org/10.3389/fmicb.2018.00592

Smith AM, Tau NP, Smouse SL, Allam M, Ismail A, Ramalwa NR, Disenyeng B, Ngomane M and Thomas J, 2019. Outbreak of *Listeria monocytogenes* in South Africa, 2017–2018: laboratory activities and experiences associated with whole-genome sequencing analysis of isolates. Foodborne Pathogens and Disease, 16, 524–530. https://doi.org/10.1089/fpd.2018.2586

Abbreviations

AESAN	Agencia Española de Seguridad Alimentaria y Nutrición
AMR	antimicrobial resistance
ARG	antimicrobial resistance gene
CC	clonal complex
ESBL	extended-spectrum beta-lactamases
MGE	mobile genetic element
MLST	multilocus sequence typing
MLVA	multiple locus variable number tandem repeat Analysis
MSCA	Marie Skłodowska-Curie Actions
PFGE	pulsed-field gel electrophoresis
RAPD	random amplified polymorphic DNA analysis
ST	sequence type

VNTR	variable number tandem repeat analysis
WGS	whole genome sequencing
WMS	whole metagenome sequencing

Appendix A – Listeriosis outbreak simulation

Case: Listeriosis outbreak in León (Simulation)

A recent increase of Listeriosis cases has been reported in the Hospital of León, with 10 patients presenting associated symptoms during the last week. *Listeria monocytogenes* isolates were obtained from all of them, and whole genome sequencing data were obtained. After a questionnaire filled by patients, the main suspects of origin of infection were identified and are summarised in Table A.1. The genomic analysis showed that all the isolates belonged to ST9, excluding those from patient01 (ST121), patient03 (ST155) and patient04 (ST14). The virulence and antimicrobial resistance profiles were very similar between the ST9 samples 02, 05, 06, 07, 08, 09 and 10. Average Nucleotide Identity (ANI) analysis showed that, considering the accessory genes, the samples were distributed in two main clusters: Cluster 1, with similarity of 100%, formed by the samples 02, 06, 08 and 09 and Cluster 2, with similarity of 99.9%, including isolates 05, 07 and 09 (Figure A.1). According to the metadata, the common sources identified in Cluster 1 were 'sausage table (embutidos)' and 'Commercial burger', while in Cluster 2 'Homemade burger' was the common source, while 'Commercial burger' was a possible source in 2/3 of the isolates. Regarding the other two samples, patient03 and patient04, the two possible sources were still 'Commercial burger' and 'Homemade burger', however, different strains of *L. monocytogenes* might have contaminated the suspect foods. Additional samples were obtained and are listed in Table A.2. The MLST assignation gave these results: food02 and producer03 belonged to unknown ST; food12 and patient03 belonged to ST155; all the other isolates were ST9, excluding food04 (ST37), patient01 (ST121) and patient04 (ST14). The results of the ANI analysis are shown in Figure A.1. In particular:

- the clinical isolates forming Cluster 1 clustered with food03 and producer02;
- the clinical isolates forming Cluster 2 clustered with two isolates of the Commercial burger;
- patient03 clustered with another strain found on lettuce in the Commercial burger;
- producer03 provided contaminated food (lomo) to the restaurant;
- patient01 and patient04 were infected by isolates not related to any other in this study;

In conclusion, four patients were traced back to producer02 and three patients to the Commercial burger.

Table A.1: Suspected origins of infection

Code	Suspect 1	Suspect 2	Suspect 3
patient01	Commercial burger	Raw milk	Fresh cheese
patient02	Sausage table (embutidos)	Smoked salmon	Commercial burger
patient03	Commercial burger	Fresh cheese	Smoked salmon
patient04	Raw milk	Homemade burger	Smoked salmon
patient05	Homemade burger	Commercial burger	Melon
patient06	Commercial burger	Melon	Sausage table (embutidos)
patient07	Homemade burger	Fresh cheese	Melon
patient08	Sausage table (embutidos)	Smoked salmon	Commercial burger
patient09	Commercial burger	Sausage table (embutidos)	Raw milk
patient10	Homemade burger	Commercial burger	Fresh cheese

Table A.2: Additional samples from foods and producers

N	Sample	Product	Origin	N	Sample
01	food01	Fresh cheese	Commercial burger	13	producer01
02	food02	Lomo	restaurant	14	producer02
03	food03	Chorizo	restaurant	15	producer03
04	food04	Salchichón	restaurant	16	producer04
05	food05	Cecina	restaurant		
06	food06	Cooked ham	Commercial burger		
07	food07	Lettuce	Commercial burger		

N	Sample	Product	Origin	N	Sample
08	food08	Cooked ham	Commercial burger		
09	food09	Chorizo	restaurant		
10	food10	Fresh cheese	Commercial burger		
11	food11	Salchichón	restaurant		
12	food12	Lettuce	Commercial burger		

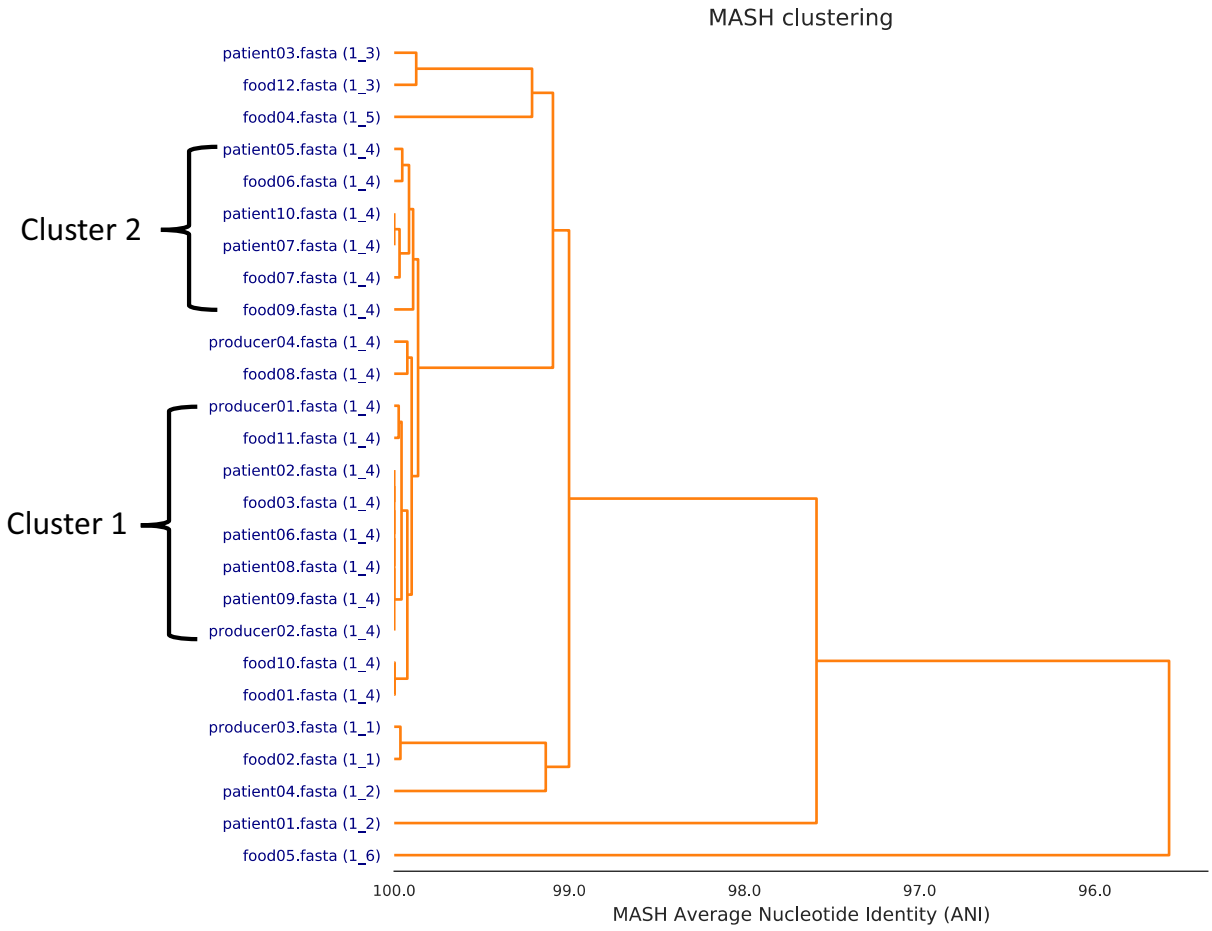

Figure A.1: ANI tree of all the isolates, performed by *dRep* software

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Monitoring of pesticide amount in fruit and vegetables by a fluorescence-based sensor

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Abstract

Faster, sensitive and real-time methods for detecting organophosphate (OP) pesticides are urged for *in situ* monitoring of these widely spread contaminants. For this reason, several efforts have been addressed for the development of performant biosensors. The thermostable enzyme esterase-2 from *Alicyclobacillus acidocaldarius* (EST2), with a lipase-like Ser-His-Asp catalytic triad with a high affinity to OPs, is a promising candidate as a bioreceptor for biosensor development. Within this EU-FORA fellowship project, two different components of the biosensor were evaluated: (i) the use of the enzymatic bioreceptor in solution or immobilised in a solid membrane; (ii) the measurement of fluorescence quenching by direct measurement of the fluorescence probe intensity signal or by fluorescence resonance energy transfer (FRET) from the tryptophans located in the catalytic site of the enzyme to a binded fluorescence probe. Fluorescence spectroscopy is among the most used techniques in analytical chemistry laboratories, mainly due to its high sensitivity and simplicity. To this aim, the developed IAEDANS-labelled EST2-S35C mutant has been used. Fluorometric measurements with both methods showed linearity with increased EST2-S35C concentrations. No significant interference on FRET measurements was observed due to changes in medium pH or due to the addition of other organic components (glucose, ascorbic acid, yeast extract). Both methods presented similar sensitivity towards detecting OPs, with fluorescence quenching due to the presence of paraoxon at environmentally relevant concentrations from 0.09 μM. The obtained results are of high relevance to further development of biosensors for the pesticide monitoring that: (i) decrease the expenses of the analysis; (ii) simplify the procedures for pesticide detection; (iii) reduce the time of response. Furthermore, the use of biosensors for pesticides real-time and *in situ* detection of pesticides promises to increase the number of samples analysed, providing a larger amount of data for food safety risk assessment.

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Keywords: organophosphate pesticides, enzymatic bioreceptor, thermophilic esterase, fluorescence-based methods, environmental monitoring

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Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

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1. Introduction

1.1. European Food Risk Assessment Fellowship Programme (EU-FORA)

The present work focusing on the development of fluorescence-based sensors to monitor pesticides in food products was developed in the context of the EFSA EU-FORA fellowship programme. EFSA aims to invest in methodology development, harmonisation activities and capacity building to maintain excellence in performing risk assessment. In this sense, EFSA EU-FORA programme was created to provide practical training to scientists from EU authorities or other Article 36 organisations, increasing and/or updating their knowledge and expertise in food safety risk assessment (Bronzwaer et al., 2016). The fellow was hosted by the Institute of Biochemistry and Cell Biology at the Italian National Research Council (CNR).

1.2. Biosensors as new tools for pesticide monitoring

Synthetic chemical pesticides are essential in modern agriculture and industry practices to increase the overall productivity of crops, cattle farms and other industries by controlling and eradicating pests and related diseases. Nevertheless, the over-application of pesticides in urban and agricultural areas primarily increased pesticides contamination of water bodies and food products, paving the way towards terrestrial and aquatic ecosystems impacts. Thus, pesticides might be present on fruit and vegetables and contaminate their derivates, such as fruit juice and animal meat. Therefore, there is a need for continuous monitoring and report of pesticides residue levels in food. In the European Union, the recent report by EFSA summarised the big picture of pesticides use in 2019, with 96.1% of samples complying with the maximum residue levels (MRLs), but 3.9% exceeded the MRLs, and 2.3% were classified as non-compliant (EFSA, 2021). Although no acute or chronic risk to consumer health through dietary exposure to pesticide residues was estimated, the need to increase and improve the control systems is highlighted (EFSA, 2021). Therefore, establishing a capillary network of sensors to monitor toxic chemicals in food should be considered one of the most critical challenges.

Biosensors appeared as promising tools to develop easy to use, low-cost and rapid-sensing techniques, and increased research has been developed in the last years. Several approaches using enzymes, cell receptors, nanomaterials, among others, have been developed, reaching a good sensitivity, comparable to LS-GC–MS methodology, and reducing the number of false positives (Bhattu et al., 2021; Umapathi et al., 2021).

The research group at the IBBC has been investigating enzymatic-based biosensors for organophosphate pesticides (OP) detection. The carboxylesterase esterase 2 from *Alicyclobacillus acidocaldarius* (EST2) structure has been solved at 2.6 Å resolution (De Simone et al., 2000), allowing the modelling of the structure *in silico* and molecular docking predictions. The EST2 stability, sensitivity and selectivity towards phosphoryl OPs, such as paraoxon and methyl paraoxon, being irreversibly inhibited (Febbraio et al., 2011; Carullo et al., 2015), make it a good candidate to be used as a bioreceptor in biosensors for OPs detection.

Nevertheless, the complexity of food samples can present several challenges to fluorescence-based methods since other organic molecules present in the sample can emit fluorescence at similar wavelengths or interfere with the catalytic site of the enzymatic bioreceptor. Therefore, two fluorescence-based solutions were investigated during this work programme to overcome the mentioned issues: (i) the use of the bioreceptor in liquid solution was evaluated using fluorescence energy transfer (FRET) method; (ii) the immobilisation of the bioreceptor on a solid membrane, and the respective adapter and holder designed and optimised, to be used on different fluorometers, commonly available on research laboratories.

2. Description of work programme

2.1. Aims

The main aim of the present work programme was to develop fast, cheap and user-friendly enzymatic based biosensors to be used in real-time to detect organophosphate pesticides in food samples. Subsequent activities were defined to achieve such goal, starting with the enzyme preparation in free and immobilised form, which was then used as a bioreceptor for two fluorescence-based biosensors. Then, the bioreceptor was validated in the two different operative conditions (in solution and immobilised in a membrane) to detect OP presence in solution, complex mixtures and

fruit washing waters. This work plan allowed the fellow to apply microbiology, molecular biology, biochemistry, biotechnology, and chemical risk assessment knowledge.

2.2. Activities/Methods

2.2.1. Overexpression and purification of EST2-S35C

The fellow performed the over-expression of a mutant of a thermostable carboxylesterase esterase-2 (EST2), to which a serine was replaced with a cysteine near the catalytic site (EST2-S35C), in the mesophilic host *Escherichia coli* strain BL21 (DE3, already available in the laboratory). *E. coli* were grown in an appropriate medium, and the protein overexpression was induced (Carullo et al., 2018). The recovery of the biomass was made by centrifugation, and the protein extract was obtained after a sonication step. The purification of EST2-S35C was achieved by thermoprecipitation followed by ultracentrifugation to remove the host proteins, as described in detail in Rodrigues et al (Rodrigues et al., 2021). Finally, a gel filtration (Se-phadex G-25 column, GE Healthcare Bio-Sciences AB, Sweden) was performed, achieving > 95% purity of the enzyme. The final amount of protein was estimated following the Bradford method (Bradford, 1976).

2.2.2. EST2-S35C labelling

The fellow accomplished the labelling of cysteine in the active site of purified EST2-S35C incubating the protein in the presence of the fluorescent probe 1,5-IAEDANS (5-((((2-iodoacetyl)amino)ethyl) amino) naphthalene-1-sulphonic acid). Different enzyme to probe ratios (1:10 to 1:200) were evaluated to assess the ratio that retrieved the best fluorescence signal. Incubation occurred overnight at room temperature. The excess of the probe was then removed at room temperature in the dark using a QuixSep micro dialyser (Creative Biomart inc., NY, USA). The fluorescence signal intensity was measured on a Jasco FP-8200 (JASCO, Tokyo, Japan) spectrofluorometer.

2.2.3. EST2-S35C as a bioreceptor in FRET-based methods

Fluorescence Resonance Energy Transfer (FRET) method (Medintz and Hildebrandt 2013) was applied, taking advantage of the intrinsic fluorescence of the protein tryptophans (donors) and its transference to the extrinsic IAEDANS (acceptor) (Figure 1). The stability of the labelled EST2-S35C was evaluated under different pH values. Food samples, such as fruit juices, constitute complex matrices rich in various organic molecules that can interfere in the fluorescence measurements by emitting intrinsic fluorescence or interacting with the enzyme's catalytic site. To test the possible interference of such organic molecules, three representative ones were selected (glucose, ascorbic acid, selected yeast extract) and tested in solution with EST2-S35C using FRET. The specificity of EST2-S25C towards phosphoryl OPs was also evaluated using single and complex mixtures of paraoxon and thio-OPs (parathion and diazinon).

2.2.4. EST2-S35C immobilisation and use in a 3D printed system

The fellow accomplished the immobilisation of purified EST2-S35C to a high-quality transfer membrane for biomolecules (Figure 2A,B), a PVDF polyvinylidene difluoride (PVDF) hydrophobic fluoropolymer membrane (pore size 0.20 μm, PORABLOT - MACHEREY-NAGEL GmbH & Co. KG, Germany), as described in detail in Rodrigues et al (Rodrigues et al., 2021). The immobilisation of the enzymatic bioreceptor was optimised. For that, different amounts of protein were spotted and tested. The conditions for adding paraoxon to the membrane (incubation time, spotting *vs* immersion) and the direct fluorescence quenching were evaluated. The vital components required for validating biosensor assays, such as precision, accuracy, linearity, stability of bioreceptor, specificity and sensitivity, were addressed.

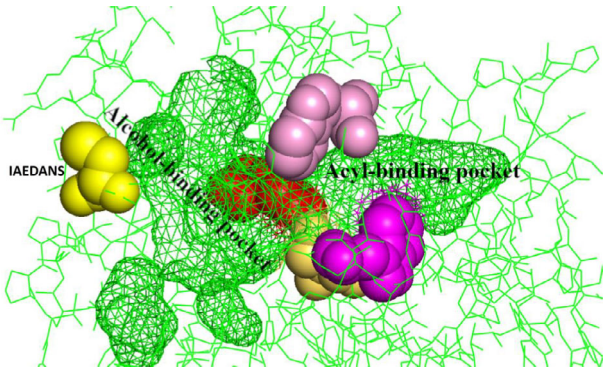

Figure 1: Mesh representation of the cavities inside the EST2-S35C shaping the catalytic site. The acyl- and alcohol-binding pockets and the residues of Cys35 (yellow), Ser155 (orange-yellow), His282 (red), Trp85 (magenta) and Trp 213 (pink) are indicated

2.2.5. Design and optimisation of 3D printed holder for fluorescence measurements

The need to stabilise and uniformise the use of the bioreceptor immobilised in the membrane during the spectrofluorimetric procedures led the fellow to design and develop a 3D printed membrane support (8 × 3 × 36 mm, Figure 2C) and an adapter for solid sample measurement (12 × 12 × 45 mm, Figure 2D), using the software SketchUp Make 2017 (Trimble Inc., USA). Slicing was done using the software Ultimate Cura v4.9.1.1 (Ultimaker B.V., Netherlands), and printing parameters were established on Labslicer 3D Slicing Software for Windows (Labists, Hongyu Zhineng Technology Co., Ltd., China). The bioplastic black polylactic acid (PLA) 1.75-mm filament was used to avoid light refraction. The components were printed in the High Precision Mini 3D printer, X1 entry-level 3D printer DIY kit, from Labists, as detailed in Rodrigues et al (Rodrigues et al., 2021). Different models for the adapter and membrane holder were printed, testing for the optimum angle of light incidence and reflection. The digital design files are available under a creative commons licence, free of charge (https://www.thingiverse.com/febbraio-research-group/designs).

The accuracy and versatility of the 3D adapter were evaluated in different equipment, in collaboration with a group from the Department of Chemistry, University of Naples. Furthermore, the critical factors required for validating biosensor assays, including precision, accuracy, linearity and sensitivity, were again addressed.

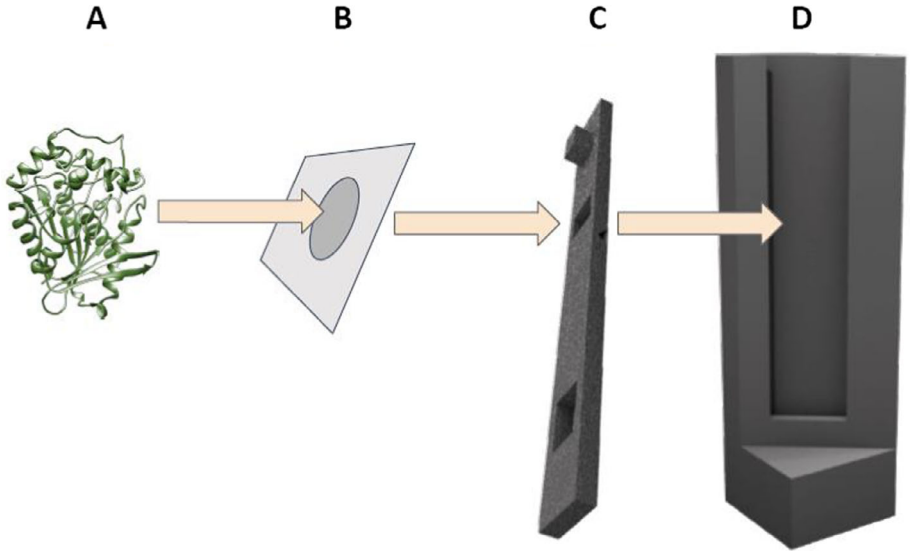

Figure 2: Schematic representation of the assemblage of the immobilised EST2-S35C (A) on the membrane (B) and the 3D printed membrane support (C) and, later, into the 3D printed adapter (D). The icons are not to scale

Part 6. Validation of the bioreceptor using fruit skin

The validation of the bioreceptor in the operative conditions for the detection of OPs concentration in fruit skin was performed. Different conditions were explored, the bioreceptor response on pretreated samples with increasing concentration of OP was evaluated, defining the range of linearity. Both developed methods were evaluated regarding the specificity and reduction of other organic molecules interference in the measurements of fluorescence quenching and consequent OPs detection.

3. Conclusions

3.1. EST2-S35C as a bioreceptor in FRET-based methods

The use of the FRET approach improved the efficiency of paraoxon detection in solution, with a limit of detection (LOD) of 0.09 μM obtained for paraoxon. Furthermore, it was observed that the addition of the fluorescent probes, such as IAEDANS, near the alcohol binding site, does not affect the enzyme's binding and function in the acyl binding site.

Several factors need to be considered when developing a biosensor for field application since the complexity of real samples increases. Therefore, the stability of labelled EST2-S35C at different pH values was tested in the range from 7.0 to 8.5, covering necessary liquid samples, such as drinking water or juice fruits (pH 7.0). The complexity of real samples is also illustrated by complex mixtures of different molecules, such as fruit juices that can contain sugars, vitamins and other proteins. Good stability of the bioreceptor using FRET measurements was also obtained when glucose, ascorbic acid or selected yeast extract were added to the solution.

In summary, two main goals were reached:

i) eliminate the interference in the fluorescence measurements of other organic molecules present in complex solutions;
ii) increase the protein specificity, as the use of FRET measurements allowed to observe changes affecting only the acyl-binding pocket, strongly reducing possible obstructions from nonspecific interactions at the alcohol-binding site.

3.2. EST2-S35C immobilisation and use in a 3D printed system

Similarly to the previous method, a linear relationship between the amount of EST2-S35C and the intensity of the measured fluorescence signal was obtained. A linear increase of fluorescence quenching would be expected until a 1:1 stoichiometric ratio of inhibitor/enzyme. In accordance, the 30 μM EST2-charged membranes presented a dynamic linear range up to 30 μM of paraoxon. A plateau was observed at 60 μM of paraoxon for the 90 μM EST2-charged membranes. So, the amount of bioreceptor should be higher than the expected OPs concentrations to be possible to determine the pesticide concentration in the sample by plotting the fluorescence quenching results, thus, avoiding some altered behaviour due to undesired fluorescence interference from adsorbed paraoxon at very high concentrations.

Regarding the versatility of the 3D printed system, the measurements performed at the different spectrofluorometers with the respective adapted support for the membrane gave comparable results, at similar wavelength (461–462 nm) and fluorescence intensity decrease with increasing paraoxon amount. Thus, these low-cost and straightforward designed 3D printed adapter and membrane supports can be used for fluorescence measurements and applied to detect chemicals in biosensing devices. Furthermore, the adapter's performance was very good, reaching similar results for sensitivity and replicability as a robotic workstation (Cetrangolo et al., 2019, 2020).

In conclusion, the designed 3D adapter has the fluorescence-cuvette dimension and the right angle to be used with minor light scattering. In addition, the developed membrane supports can be easily switched to match the specific heights of the instruments from different manufacturers, being quickly adjusted in different spectrofluorometers. Thus, this work provided an easy and accessible strategy to use low-cost tools for using ad hoc laboratory materials, overcoming the cost of commercial accessories. In fact, 3D printing would be an excellent ally to produce new accessories in different scientific fields, given the opportunity to improve existing methodologies or test new designs and methodologies.

In conclusion, the great advantage of using a 3D adapter for biosensing devices lies in the possibility of using immobilised enzymes on solid membranes, or direct measurement of optically active thin layers. Furthermore, the 3D support with the immobilised enzyme allowed the washing of the

membrane to remove the unreacted substances, including other organic compounds such as pigments, or amino acids, improving the fluorescence measurements and decreasing the background noise.

3.3. Additional scientific activities

In order to maximise knowledge transfer, during the fellowship programme, the fellow took part in internal data clubs, weekly as a participant and twice as a speaker. In addition, the fellow took part in the working meetings with groups at University Federico II (Naples, Italy) and Zewail City for Science and Technology (Cairo, Egypt) to plan collaborations and perform lab work.

The fellow participated in the EUROTOX 2021 meeting as presenting author of the poster presentation 'A FRET approach to detect organophosphate pesticides using a fluorescent biosensor' and as co-author of the poster presentation 'Direct detection of organophosphate pesticides in water by a fluorescence-based biosensor', held on a virtual platform from 27 September to 1 October 2021 (https://www.eurotox2021.com/abstracts/). Two abstracts, one as presenting author and the second as co-author ('Detection of neurotoxic compounds at environmentally relevant concentrations by using a fluorescence-based biosensing device' and 'Application of a fluorescence-based biosensing device for the detection of organophosphate pesticides in water samples') have been accepted as a poster presentation at ONE – Health, Environment, Society – Conference, 21–24 June 2022.

The data resulting from activities 2.2.4 and 2.2.5 are accepted for publication (Rodrigues et al., 2021). In addition, the data resulting from activity 2.2.3 are submitted to another peer-reviewed journal (Sensors), and other publications are planned.

References

Bhattu M, Verma M and Kathuria D, 2021. Recent advancements in the detection of organophosphate pesticides: a review. Analytical Methods, 13, 4390–4428. https://doi.org/10.1039/D1AY01186C

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bronzwaer S, Le Gourierec N and Koulouris S, 2016. Editorial: The European Food Risk Assessment Fellowship Programme (EU-FORA). EFSA Journal, 14, e14111. https://doi.org/10.2903/j.efsa.2016.e14111

Carullo P, Cetrangolo GP, Mandrich L, Manco G and Febbraio F, 2015. Fluorescence spectroscopy approaches for the development of a real-time organophosphate detection system using an enzymatic sensor. Sensors, 15, 3932–3951. https://doi.org/10.3390/s150203932

Carullo P, Chino M, Cetrangolo GP, Terreri S, Lombardi A, Manco G, Cimmino A and Febbraio F, 2018. Direct detection of organophosphate compounds in water by a fluorescence-based biosensing device. Sensors and Actuators B: Chemical, 255, 3257–3266. https://doi.org/10.1016/j.snb.2017.09.152

Cetrangolo GP, Gori C, Rusko J, Terreri S, Manco G, Cimmino A and Febbraio F, 2019. Determination of picomolar concentrations of paraoxon in human urine by fluorescence-based enzymatic assay. Sensors (Basel, Switzerland), 19, 4852. https://doi.org/10.3390/s19224852

Cetrangolo GP, Rusko J, Gori C, Carullo P, Manco G, Chino M and Febbraio F, 2020. Highly sensitive detection of chemically modified thio-organophosphates by an enzymatic biosensing device: an automated robotic approach. Sensors, 20, 1365. https://doi.org/10.3390/s20051365

De Simone G, Galdiero S, Manco G, Lang D, Rossi M and Pedone C, 2000. A snapshot of a transition state analogue of a novel thermophilic esterase belonging to the subfamily of mammalian hormone-sensitive lipase. Journal of Molecular Biology, 303, 761–771. https://doi.org/10.1006/jmbi.2000.4195

EFSA (European Food Safety Authority), Carrasco Cabrera L and Medina Pastor P, 2021. The 2019 European Union report on pesticide residues in food. EFSA Journal 2021;19(4):e06491, 45 pp. https://doi.org/10.2903/j.efsa.2021.6491

Febbraio F, Merone L, Cetrangolo GP, Rossi M, Nucci R and Manco G, 2011. Thermostable esterase 2 from Alicyclobacillus acidocaldarius as biosensor for the detection of Organophosphate Pesticides. Analytical Chemistry, 83, 1530–1536. https://doi.org/10.1021/ac102025z

Rodrigues ACM, Barbieri MV, Chino M, Manco G and Febbraio F, 2021. A 3D printable adapter for solid-state fluorescence measurements: the case of an immobilized enzymatic bioreceptor for organophosphate pesticides detection. Analytical and Bioanalytical Chemistry, 415, 1999–2008. https://doi.org/10.1007/s00216-021-03835-1

Umapathi R, Park B, Sonwal S, Rani GM, Cho Y and Huh YS, 2021. Advances in optical-sensing strategies for the on-site detection of pesticides in agricultural foods. Trends in Food Science & Technology, 119, 69–89. https://doi.org/10.1016/j.tifs.2021.11.018

Abbreviations

EST2	esterase 2 from *Alicyclobacillus acidocaldarius*
EST2-S35C	EST2 where the serine 35 has been replaced by a cysteine residue
EU-FORA	European Food Risk Assessment Fellowship Programme
EUROTOX	Congress of the European Societies of Toxicology
FRET	fluorescence resonance energy transfer
LOD	limit of detection
MRL	maximum residue level

EU-FORA SERIES 4

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Benefit and risk assessment of replacing of sodium chloride by other salt/substances in industrial seafood products

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[2]Poznan University of Economics and Business, Poznan, Poland

Abstract

Sodium (Na) is primarily consumed as salt (sodium chloride, NaCl), which is a critical food ingredient that contributes to improve preservation, shelf-life and sensory attributes (e.g. texture and taste). On the other hand, the excessive Na intake is a risk factor for cardiovascular diseases including stroke and heart diseases. The actual NaCl intake in most countries is far above recommended level of 5 g NaCl/day. Therefore, the reduction of NaCl is among top priorities for health authorities around the globe and the World Health Organization (WHO) adopted a strategy to reduce NaCl intake by 30% until 2025. Integral part of the reduction strategy is to limit NaCl intake from seafood, which is especially relevant for regions with a significant fish and shellfish consumption. The purpose of the project was to (i) review the current situation of relevant strategies to reducing NaCl content in seafood (literature review), (ii) assess benefit/risk of NaCl replacement with other substances/ingredients in seafood and (iii) disseminate results obtained. In the first phase of the project, the literature review was performed and the review paper was prepared. The second part of the project was focused on the experimental studies on smoked trout which commercially available products can deliver up to 4 g NaCl in 100 g. The aim of this study was to optimise the development process of smoked trout with reduced NaCl content without compromising quality and safety attributes. Another part of the project was related to the dissemination of results which resulted in the preparation of three conference abstracts and two experimental papers.

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Keywords: salt reduction, sodium chloride (NaCl), potassium chloride (KCl), seafood, fish, trout

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Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

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1. Introduction

Reducing **salt (sodium chloride, NaCl)** intake has been identified as one of the most cost-effective measures that can be taken to improve the health of worldwide population (WHO, 2020). The excessive NaCl intake is linked with cardiovascular diseases such as stroke or heart disease (Strazzullo et al., 2009). It is estimated that reaching a global target of a 30% reduction in salt intake by 2025 (compared to 2010 levels) would save about 40 million lives over 30 years (WHO, 2012). The recommended daily intake is 5 g NaCl but the actual intake in Europe is two to three times higher (EFSA, 2019) (Figure 1).

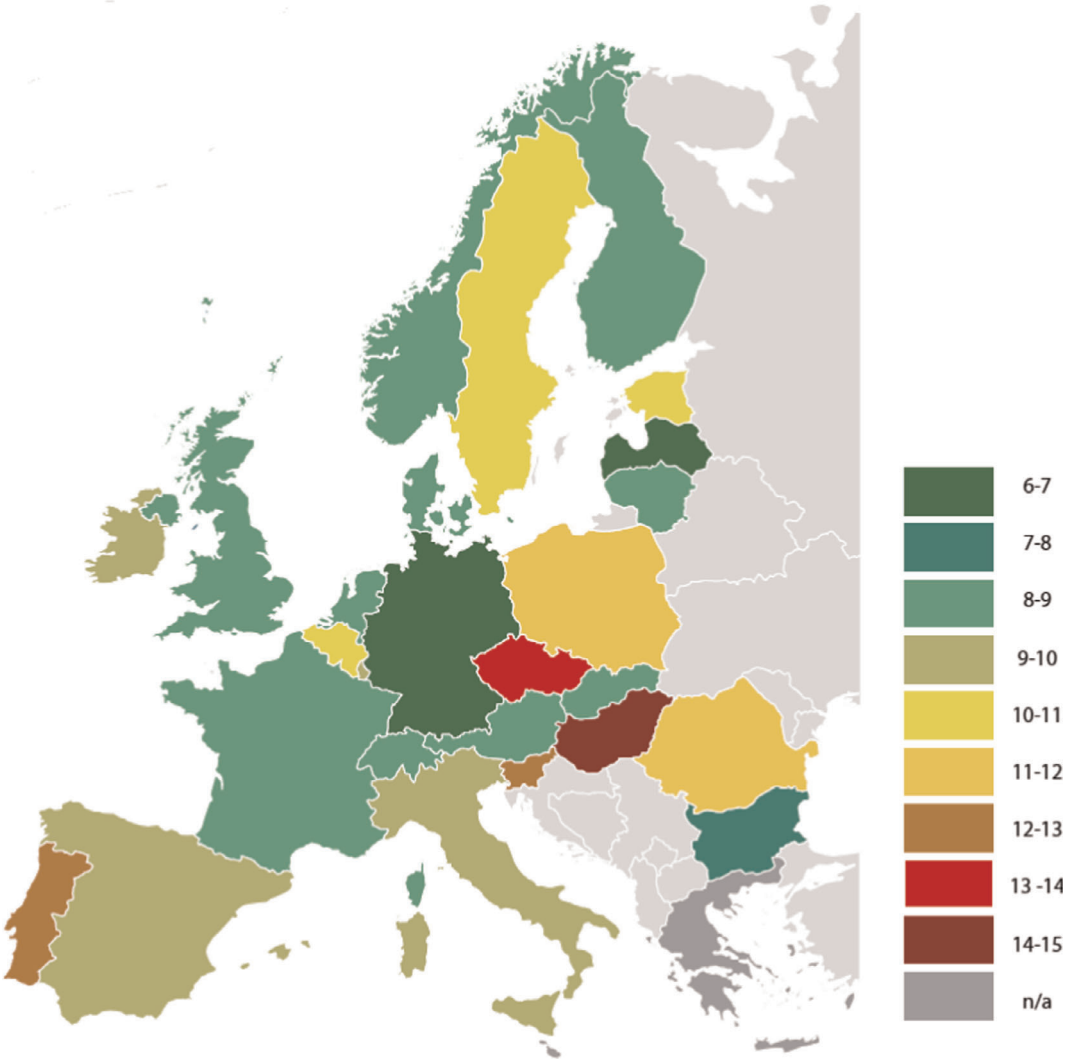

Figure 1: Daily intake of salt (presented in g of NaCl per person per day) in Europe [European Commission, 2012]

Different strategies for salt reduction include governmental polices, co-operation with food industry to reformulate products, social campaigns and monitoring the population salt intake (European Commission, 2012; WHO, 2020). Most initiatives apply to NaCl reduction in ready-to-eat products and meals served in schools or nurseries. In terms of particular food category, they primarily apply to bakery products (bread), snacks and cheese. The **food reformulation** is mostly based on lowering NaCl content during processing, the replacement of NaCl by substitutes such as KCl, $MgCl_2$, $CaCl_2$, amino acids and the addition of flavour enhancers or their combinations. KCl. The most often used a replacer is KCl which has similar properties to NaCl and its consumption can promote a protective effect against high blood pressure, stroke or osteoporosis (Hall, 2003). However, a very limited number of studies was conducted for salt reduction in seafood products so far.

The reduction of salt intake from fish and shellfish products is especially important for regions with high seafood consumption such as such as Iceland, Maldives, Micronesia and Hong Kong (≥ 70 kg per capita/year; global average = 20 kg per capita/year) (Statista, 2021). On the other, smoked seafood are eagerly chosen by many consumers due their specific taste, aroma and colour. Smoked fish and shellfish should be given a special attention as processing of raw fish significantly increases NaCl content in the product. The portion of 100 g of commercially available **smoked fish** often deliver up to 100% of recommended daily intake of NaCl. Therefore, the project was focused on the benefit and risk assessment of NaCl replacement with other salts/substances in seafood.

2. Description of work programme

2.1. Aim and activities/methods

The project was aimed at three **tasks**:

1) preparation and submission of guidelines for the application of relevant strategies to reduce NaCl content in seafood (literature review);
2) assessing benefit/risk of NaCl replacement with other substances/ingredients in seafood;
3) elaborating and disseminating results obtained.

3. Results, discussion and conclusions

The time schedule of the project is presented in Table 1.

Table 1: The schedule of the project

Year	2021											
Month number	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
Month	Jan	Febr	March	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
EFSA induction training												
Task 1. Guidelines for salt reduction in seafood												
1.1. Data extraction												
1.2. Manuscript preparation												
1.3. Manuscript submission												
Task 2. Experimental studies												
2.1. Experimental I												
2.2. Experimental II												
Task 3. Dissemination												
3.1. Research papers												
3.2. Conferences												

3.1. Task 1: Guidelines for salt reduction in seafood

Taking into account COVID-19 restrictions in Portugal at the beginning of 2021, the project was conducted in a remote version from February 1 until March 31. It was agreed that project will initially include the preparation of a review paper on substitution of NaCl by other ingredients in seafood products. The aim was to: (i) discuss the Na content in seafood, (ii) indicate the positive and negative roles of NaCl in seafood processing, (iii) identify the main strategies to reduce NaCl in seafood products, (iv) categorise the ingredients used as substitutes of NaCl, (v) specify how the current substitutes of NaCl affect its technological function in seafood production and (vi) discuss the nutritional (dietary) impact of NaCl substitution, (vii) debate the influence of NaCl replacement on physicochemical properties of seafood products, (viii) discuss the influence of NaCl replacement on sensory attributes of seafood products and (ix) discuss the influence of NaCl replacement on microbiological safety of seafood products. The preparation of the paper included three subtasks presented in Table 1: data extraction, manuscript preparation and manuscript submission. Subtask 1 was performed within close collaboration between the fellow and supervisor and included the following steps (Figure 2).

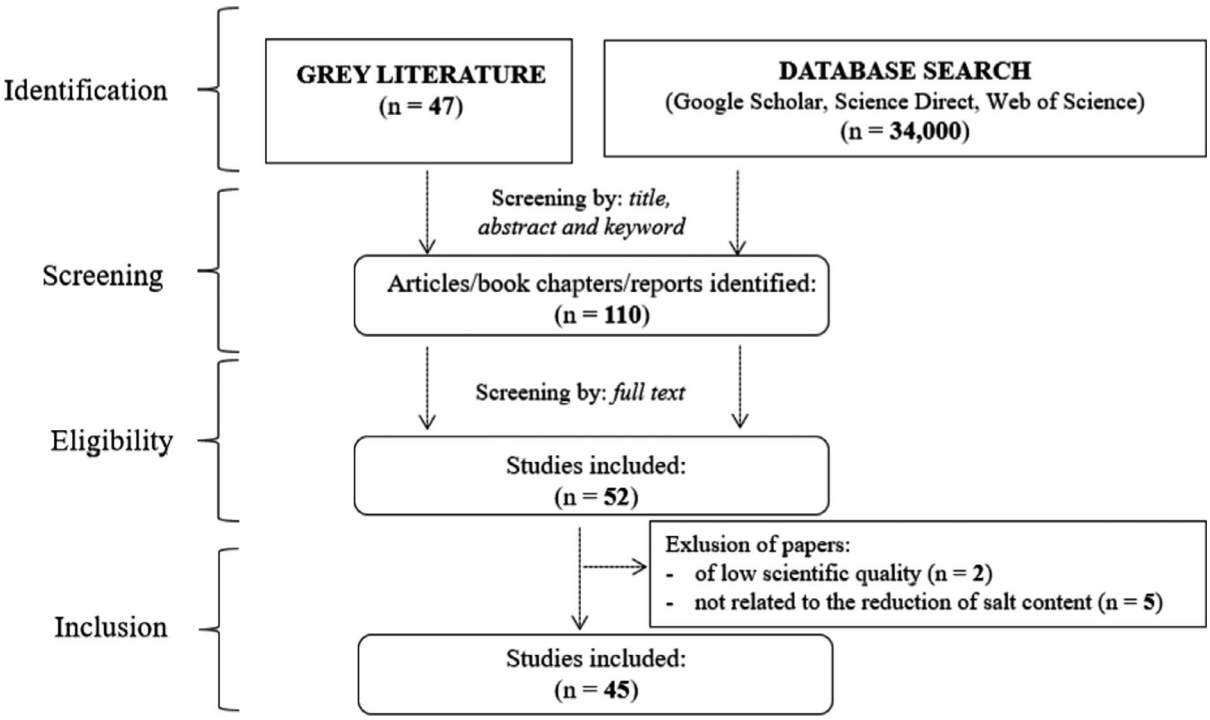

Figure 2: Studies comprised in the review paper [adapted from draft of the paper]

Once the data were collected, the manuscript was prepared by the fellow first and then revised by the team from the hosting site. From April 1, the preparation of the review paper was continued in the hosting site and was finalised in August 23 by submitting it to the scientific journal. As of November 9, the manuscript status is 'under revision' in Food Control (Elsevier).

3.2. Task 2: Experimental studies

Task 2 applied to experimental studies on the replacement of NaCl with other substances/ ingredients in smoked trout. The aim of these studies was to assess the benefit and risk of this substitution in terms of multiply chemical, physical, microbiological and sensory attributes.

Task 2.1. Experiment I

The study was conducted in April–June 2021 using hosting site facilities. It covered all steps of experimental study namely: conceptualisation, investigation and formal analysis. The fellow was personally involved in all steps.

AIM: To develop safe, healthy and attractive smoked trout with reduced content of NaCl
MATERIAL: Hot smoked rainbow trout (*Oncorhynchus mykiss*) developed using eight formulations with different concentrations of NaCl, KCl, sugar and bitterness masking agent

Collecting the fish **Filleting** **Salting** **Smoking**

Figure 3: Experiment I: the development of smoked trout with reduced content of NaCl [own data]

METHODS: Determinations in raw material and smoked products included:

- microbiological analysis,
- instrumental texture/colour,
- sensory analysis

RESULTS: Satisfactory results were obtained for microbiological, texture and colour factors, however, the results obtained for sensory analysis (very high/high scores for salty taste) precludes the application of conditions applied in the experiment.

Task 2.2. Experiment II

The study was conducted in July–November 2021 and included conceptualisation, investigation and formal analysis. The fellow was personally involved in all steps. The results from Experiment I were carefully taken into consideration and appropriate corrections were included in the Experiment II.

AIM: To develop safe, healthy and attractive smoked trout with reduced content of NaCl

MATERIAL: Hot smoked rainbow trout (*Oncorhynchus mykiss*) developed using eight formulations with different concentrations of NaCl, KCl, sugar, microencapsulated plants + spices (ME) and bitterness masking agent

METHODS: Determinations in raw material and smoked products included the following analyses:

- microbiological,
- moisture, fat, protein,
- chlorides, Na, K,
- pH, water activity (a_w),
- water holding capacity (WHC),
- instrumental texture/colour,
- sensory

RESULTS: The last results for Experiment II were obtained in mid-November. Results allowed for identification of formulations with most desirable attributes especially in relation to NaCl reduction/sensory attributes ratio. The results indicate that the replacement of NaCl with KCl and ME, with or without masking agent, is a potential solution towards more sustainable and healthy diets (with lower Na and higher K content).

As expected, there was a significant Na reduction in smoked trout developed either with KCl, or with microencapsulated plants + spies (with or without bitterness masking agent). Smoked trout with reduced NaCl levels delivered significantly less NaCl which can limit its intake to the recommended level of 5 g of per day (EFSA NDA Panel, 2019). An increase in the K level was also observed for formulations developed with KCl addition and is expected to bring other health benefits. Smoked trout produced with KCl can be labelled as a 'source of K' according to the definition of this claim (Regulation (EC) No 1924/2006) (European Commission, 2006), which is essential for supporting blood pressure, cardiovascular health, bone and muscle strength (EFSA NDA Panel, 2016). Moreover, the Na: K ratio, which is also positively associated with blood pressure and is a predictor of cardiovascular risk,

decreased in products prepared with NaCl replacement compared to traditional smoked products. These ratios observed in samples prepared with reduced Na levels are closer or in line with the WHO that recommended Na:K ratio ≤ 1 (O'Halloran et al., 2016).

3.3. Task 3: Dissemination

This task included dissemination of results by the preparation of research article and conferences participation. First dissemination activity applied to the description of salt reduction strategies utilised in the European food industry. The 1st International Conference on Quality and Management Sciences 2021 (**conference 1**) was organised by Poznan University of Economics and Business in September 13–15, 2021, in Poznan (Poland). The abstract entitled 'European strategies for salt reduction in food' was authored by Iga Rybicka, António Marques, Amparo Gonçalves, Helena Oliveira and Maria Leonor Nunes and presented by fellow during remote participation in the conference.

The results from experimental studies (Experiments I and II) are being analysed and the **manuscript 1** is under preparation. It is projected to finish the draft of the paper by the end of December 2021. After its revision by the research team, the manuscript will be submitted to the journal (expected date: 1st Q of 2022). Working title of the manuscript is 'Development and quality assessment of smoked rainbow trout with reduced sodium content' by Iga Rybicka, Marlene Silva, Amparo Gonçalves, Helena Oliveira, António Marques, Maria João Fraqueza and Maria Leonor Nunes. Moreover, results from Experiments I and II were summarised and described in abstract for ONE – Health, Environment, Society – Conference 2022 which is organised by, e.g. EFSA in June 21–24, 2022, in Brussels (Belgium) (**conference 2**). The submission (entitled 'Development of safe, nutritious and attractive smoked trout with reduced salt content', which was authored by Iga Rybicka, Marlene Silva, Amparo Gonçalves, Helena Oliveira, Maria João Fraqueza, António Marques and Maria Leonor Nunes) was already accepted by organising committee. Depending on the organisational and financial capabilities of the fellow her participation will be in physical or remote attendance (organisers allow both possibilities).

Dissemination activities were expanded by the elaboration of results obtained for smoked mackerels with reduced content of NaCl which were developed and analysed in CIIMAR at the beginning of 2021. The fellow did not participate in person in the experiment, but the results from this experiment were analysed and the draft of the **manuscript 2** was prepared by Iga Rybicka. The draft of the manuscript is now revised by the research team and its submission is planned for the end of November 2021 (title 'Development and quality of smoked mackerel (*Scomber japonicus*) with reduced sodium content' by Iga Rybicka, Marlene Silva, Amparo Gonçalves, Helena Oliveira, António Marques, Maria José Fernandes, Maria Helena Fernandes, Maria João Fraqueza and Maria Leonor Nunes). Moreover, the results from this experiment were already presented in the 35th EFFoST International Conference: Healthy Individuals, Resilient Communities, and Global Food Security (**conference 3**) which was held November 1–4, 2021, in Lausanne (Switzerland) among fellow's physical attendance in the event (funded by EU-FORA programme). The title of the poster was 'Salt reduction strategies in processed seafood'.

Additionally, to the work programme, the fellow participated in the experimental study aimed at optimising smoking conditions for the development of industrial seafood. In October 2021, the fellow was involved in the preparation of raw rainbow trout for further processing of the fish which are planned for the 1st quarter of 2022. The fellow and supervisor will be co-authors of the research results published in **manuscript 3** by the end of 2022.

All dissemination outputs (papers and conference submissions) are and will be acknowledged by EFSA EU-FORA programme. Posters for conference 1 and conference 2 are attached as annexes to the technical report.

References

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), Turck D, Bresson J-L, Burlingame B, Dean T, Fairweather-Tait S, Heinonen M, Hirsch-Ernst KI, Mangelsdorf I, McArdle H, Neuhäuser-Berthold M, Nowicka G, Pentieva K, Sanz Y, Siani A, Sjödin A, Stern M, Tomé D, Van Loveren H, Vinceti M, Willatts P, Aggett P, Martin A, Przyrembel H, Brönstrup A, Ciok J, Gómez Ruiz JÁ, de Sesmaisons-Lecarré A and Naska A, 2016. Dietary reference values for potassium. EFSA Journal 2016;14(10):4592, 55 pp. https://doi.org/10.2903/j.efsa.2016.4592

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), Turck D, Castenmiller J, de Henauw S, Hirsch-Ernst K-I, Kearney J, Knutsen HK, Maciuk A, Mangelsdorf I, McArdle HJ, Pelaez C, Pentieva K, Siani A, Thies F, Tsabouri S, Vinceti M, Aggett P, Fairweather-Tait S, Martin A, Przyrembel H, Ciccolallo L, de Sesmaisons-Lecarre A, Martinez SV, Martino L and Naska A, 2019. Scientific Opinion on the dietary reference values for sodium. EFSA Journal 2019;17(9):5778, 191 pp. https://doi.org/10.2903/j.efsa.2019.5778

European Commission, 2006. Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. In: Official Journal, L 404, 30.12.2006, p. 9–25.

European Commission, 2012. EU Salt Reduction Framework. European Commission.

Hall JE, 2003. The kidney, hypertension, and obesity. Hypertension, 41, 625–633. https://doi.org/10.1161/01.HYP.0000052314.95497.78

O'Halloran SA, Grimes CA, Lacy KE, Campbell KJ and Nowson CA, 2016. Dietary intake and sources of potassium and the relationship to dietary sodium in a sample of Australian pre-school children. Nutrients, 8, 496.

Pedro S and Nunes ML, 2019. Reducing salt levels in seafood. In: Groves K and Titoria PM (Eds.), Beeren, C, Reducing Salt in Foods, Woodhead Publishing Series in Food Science, Technology and Nutrition, 2nd Edition. pp. 185–212.

Statista, 2021. Seafood industry. Available online: https://www-1statista-1com-1s8fui2em0efe.han3.ue.poznan.pl/study/22230/seafood-industry-statista-dossier/ [Accessed: 8 November 2021].

Strazzullo P, D'Elia L, Kandala NB and Cappuccio FP, 2009. Salt intake, stroke, and cardiovascular disease: meta-analysis of prospective studies. BMJ (Online), 339, 1296. https://doi.org/10.1136/bmj.b4567

WHO (World Health Organization), 2012. Available online: https://www.who.int/elena/global-targets/en/ [Accessed: 18 November 2021].

WHO (World Health Organization), 2020. Accelerating salt reduction in Europe: a country support package to reduce population salt intake in the WHO European Region (2020). Available online: https://www.euro.who.int/en/health-topics/disease-prevention/nutrition/publications/2020/accelerating-salt-reduction-in-europe-a-country-support-package-to-reduce-population-salt-intake-in-the-who-european-region-2020 [Accessed: 18 November 2021].

Abbreviations

a_w	water activity
ME	microencapsulated herbs and spices
WHC	water holding capacity
WHO	world health organization

Glossary

Salt	Applies to sodium chloride (NaCl) as it is a major salt used in food industry
Salt reduction	Applies to the reduction of NaCl as ~ 95% of Na intake originates from NaCl

Annex A – The poster for conference 1

EUROPEAN STRATEGIES FOR SALT REDUCTION IN FOOD

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OBJECTIVE

The aim of the study was to **review** the current status of (1) salt (NaCl, sodium chloride) intake and (2) activities related to salt reduction in Europe

METHODS & RESULTS

RECOMMENDATIONS

- The World Health Organization (WHO) recommends reducing salt intake to ≤ 5 g per day [1]
- 5 g of NaCl (~1 tablespoon) corresponds to 2 g of sodium (Na)

SOURCES OF NaCl

- EUROPE: 75-95% processed food
- WORLD: America – mainly from processed food; Asia – majority from salt added during cooking at home (incl. soya sauce)[2]]

Fig. 1. Salt (NaCl) content in food products [g NaCl/100 g of product] (adopted from [3])

(1) SALT INTAKE

CURRENT STATUS IN EUROPE

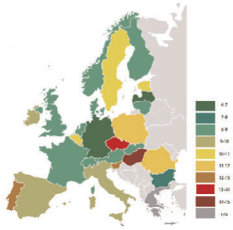


Fig. 2. Salt intake [g/person/day] in some European countries [4]

- NaCl intake is above the WHO recommended levels in approximately 99% of the world's adult population (in 181 of 187 countries),
- [WORLD: The highest intake in Tianjin (China); the lowest in Yanomamo Indians (Brazil) [2]

CONSEQUENCES

HEALTH

- NaCl is a main source of Na. Na intake around the world is well in excess of physiological need and a diet high in Na is ranked as the second most important dietary risk factor to health globally [5]
- Excessive Na intake is strongly linked with raised blood pressure and increased risks of cardiovascular disease (CVD), as well as with kidney disease, gastric cancer, and osteoporosis [5]
- Raised blood pressure is responsible for 62% of stroke and 49% of coronary heart disease [3]

SOCIAL

- CVD is one of the leading causes of mortality and morbidity from non-communicable disease globally. In 2017, high Na intake led to approximately 3 million deaths and the loss of 70 million disability-adjusted life-years (with four out of five of these deaths occurring in low- and middle-income countries) [6]

ECONOMICAL

- The total global cost of CVD was approximately $863 billion in 2010 and it is predicted to raise up to $1,044 billion by 2030. The costs per episode of hypertension/generic CVD ranges between $500 and $1500 [7]

APPROACHES

- ✓ Twenty-nine WHO member states have committed to reduce the salt intake by 30% until 2025 [1]
- ✓ Efforts to reduce Na have been increasing at national, regional, and global levels: 1) the number of countries that have national Na reduction strategies doubled from 2010 to 2014; 2) since 2016–2017, eighty-nine countries have national policies that support Na reduction [6]

Tab. 1. Existing salt/sodium reduction strategies [6]

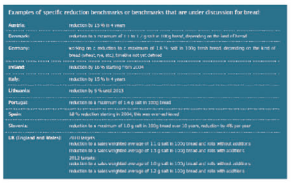
Salt/sodium from packaged foods
Labelling: front-of-pack labelling regulations
Labelling: mandatory nutrient declaration on labels
Labelling: nutrition/health claims on food packaging
Reformulation targets for packaged food (voluntary or mandatory)
Regulation of foods marketing of foods and beverages to children
Fiscal policies: taxation on high salt/sodium foods
Supermarket interventions using products placements, price, or promotion strategies (4Ps)
Salt/sodium from food prepared outside home
Standards for salt/sodium as part of food procurement policies for public institutions
Restaurants: menu labelling of high or low salt/sodium items (primarily chain restaurants)
Restaurant: removal of salt shakers and high salt/sodium condiments from tables
Restaurants: chef training to reduce salt/sodium in food
Restaurants: providing the provision of low salt/sodium or no salt/no sodium added items on menus
Restaurants: food reformulation targets for restaurants (voluntary or mandatory; primarily chain restaurants)
Salt/sodium added at home
Mass media campaigns
Community education (e.g., through schools, community venues, workplaces, etc.)
Individual education and counselling (usually through primary health care)
Increase the variety of low salt/low sodium (preparation, distribution, availability)

(2) SALT REDUCTION

BY FOOD CATEGORY

- In Europe, bread is frequently identified as the main source of NaCl (in average: a 20% contribution to salt intake; up to 28% in France)
- 2nd: meat and meat products (from 8% in Switzerland to 26% in Spain)
- 3rd: cheese and other dairy products (approximately 10% in most of European countries)
- Other important categories: ready-to-eat products/meals and soups [4]
- Generally, labels for processed food can be grouped into:
  - Low-salt (< 0.3 g NaCl/100 g of product)
  - Medium-salt (0.3 – 1.5 g NaCl/100 g)
  - High-salt (>1.5 g NaCl/100 g)

Tab. 2. Examples of specific reduction benchmarks for bread [4]



BENEFITS

- Salt reduction is considered as a simple, low-cost and effective way to protect public health
- The reduction of salt intake to the recommended level is predicted to save an estimated 1.65 million lives each year
- It is assumed that $1 spent on salt reduction efforts can save up to $19 as a result of reduced disability and mortality in high-income countries [7]
- **United Kingdom (UK) example:** In 2003, the UK was one of the first countries introducing a salt reduction program to reduce the incidence of cardiovascular disease (CVD). Between 2000 and 2018, the NaCl intake was limited to 1 g (from 9.4 to 8.4 g/day) which was calculated as: 194K CVD cases averted + 542K quality-adjusted life-years added + £1,640m saved [8]

CONSLUSIONS

- ✓ Reducing NaCl/Na intake is among the main priority interventions for WHO members and contributes toward achieving UN Sustainable Development Goals, especially Goal 3 (ensuring healthy lives and promoting well-being for all at all ages) and Goal 12 (ensuring sustainable consumption and production patterns)
- ✓ Strategies to reduce NaCl intake are focused on governmental policies, including taxation, social campaigns aimed at increasing consumer awareness, support producers to manufacture healthier foods and regular monitoring such intake. They mainly apply to bread and meat products which are main contributors to the daily intake of salt in all European countries
- ✓ Public health efforts should be maximized to achieve the ambitious 30% reduction target, as no country has yet achieved recommended level of salt intake [7]
- ✓ Future areas of work are likely to include work on modelling the economic costs of salt intakes and the positive impact of salt reductions [1]

LITERATURE

[1] WHO, 2021, https://www.who.int/activities/reducing-population-sodium-salt-intakes (accessed August 17, 2021]
[2] Ian J Brown, Ioanna Tzoulaki, Vanessa Candeias, Paul Elliott, 2009, Salt intakes around the world: implications for public health, International Journal of Epidemiology, 38, 3, 791–813, https://doi.org/10.1093/ije/dyp139
[3] Polish National center for nutritional education (in Polish: Narodowe Centrum Edukacji Żywieniowej), 2016, https://ncez.pzh.gov.pl/choroba-a-dieta/nadcisnienie-mniej-soli/ [accessed August 18, 2021]
[4] WHO, 2014, https://www.google.com/search?client=firefox-b-d&q=Survey+on+Members+States%E2%80%99+Implementation+of+the+EU+Salt+Reduction+Framework [accessed August 18, 2021]
[5] Nhung Nghiem, Tony Blakely, Linda J., Chritine L. Cleghorn, Nick Wilson, 2016, The health gains and cost savings of dietary salt reduction interventions, with equity and age distributional aspects. BMC Public Health, 16, 423, https://doi.org/10.1186/s12889-016-3102-1
[6] Nicole Ide, Adefunke Ajenikoko, Lindsay Steele, Jennifer Cohn, Christine J. Curtis, Thomas R. Frieden, Laura K. Cobb, 2020, Priority actions to advance population sodium reduction, Nutrients 12, 9, 2543, https://doi.org/10.3390/nu12092543
[7] Adrian, Gheorghe, Ulla Griffiths, Adrianna Murphy, Helen Legido-Quigley, Peter Lamptey, Pablo Perel, 2018, The economic burden of cardiovascular disease and hypertension in low- and middle-income countries: a systematic review, BMC Public Health, 18, 975, https://doi.org/10.1186/s12889-018-5806-x
[8] Sergi Alonso, Monique Tan, Changqiong Wang, Seamus Kent, Linda Cobiac, Graham A. MacGregor, Feng J. He, Borislava Mihaylova, 2021, Impact of the 2003 to 2018 population salt intake reduction program England, Hypertension., 77,1086–1094, https://doi.org/10.1161/HYPERTENSIONAHA.120.16649

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Annex B – The poster for conference 3

SALT REDUCTION STRATEGIES IN PROCESSED SEAFOOD – SMOKED MACKEREL CASE

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INTRODUCTION

High NaCl intake [1]:

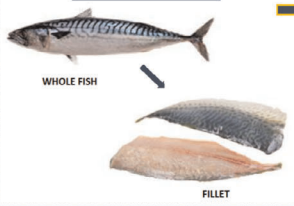
- ↑ risk of cardiovascular diseases
- 3 M deaths and 70 M disability-adjusted life-years each year
- global cost: $850 B in 2010
- global cost: $1,044 B by 2030

- World Health Organization recommendations [1]: NaCl ≤ 5 g per day
- Actual daily NaCl intake is 9-12 g in Europe and 15 g in Asia [2]
- NaCl in commercial smoked mackarel – up to 4 g/100 g

AIM: Developing safe, nutritious & attractive smoked mackerel with reduced NaCl/Na content

MATERIAL & METHODS

PREPARATION



SALTING

- **BRINE 5%** (1 h, 7 °C):
  - 5A – 100% NaCl
  - 5B – 75% NaCl + 25% KCl
  - 5C – 50% NaCl + 50% KCl
  - 5D – 25% NaCl + 75% KCl
- **BRINE 10%** (1 h, 7 °C):
  - 10A – 100% NaCl
  - 10B – 75% NaCl + 25% KCl
  - 10C – 50% NaCl + 50% KCl
  - 10D – 25% NaCl + 75% KCl

HOT SMOKING

- Conditions: drying (40-55 °C, 90-95% relative humidity, 2 h); smoking (65 °C, 85% relative humidity, 40 min) in a semi-industrial unit

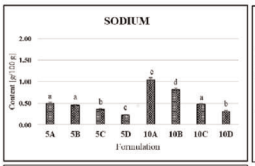
ANALYSIS

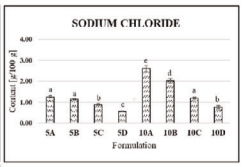
- Chemical: moisture, protein, fat, sodium, potassium
- Physical: pH, water activity (a_w), water holding capacity (WHC), colour, texture
- Microbiology: Total viable counts (TVC), Enterobacteriaceae, *Listeria monocytogenes*
- Sensory evaluation
- Statistical analysis was performed using factorial analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$)

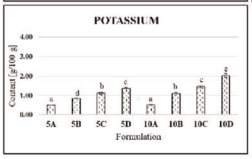
RESULTS

CHEMICAL COMPOSITION AND PHYSICAL PARAMETERS

- **No significant differences** for:
  - Moisture (49-56%)
  - Protein (28-30%)
  - Fat (9.2-10.5%)
  - pH (6.1-6.2)
  - a_w (0.94-0.96)
  - WHC (58-65%)
  - Yield (78-81%)
- **Significant differences** for:
  - Sodium
  - Sodium chloride (NaCl = Na x 2.5)
  - Potassium





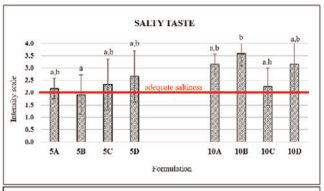


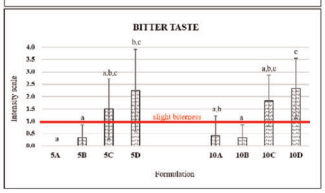
- Results are given as mean values ± standard deviations;
- Different small letters indicate significant differences between formulations

TEXTURE

- **No significant differences** for:
  - Hardness (33-40 N)
  - Springiness (~0.5 N)
  - Cohesiveness (~0.4 N)
  - Chewinesss (7.2-.86 N)

SENSORY





- **No significant differences** for:
  - Smoking taste/aroma (1.5-2.8)
  - Firmness (1.5-3.0)
- **Significant differences** for:
  - Saltiness
  - Bitterness
- Formulation with the best attributes (negligible bitterness and moderate saltiness): **5B**
- Limited saltiness and bitterness perception were also noticed in Na-reduced smoked salmon (replaced by KCl) [3]

- Results are given as mean values ± standard deviations
- Different small letters indicate significant differences between formulations
- Intensity scale: 0 – absent, 1 – slight, 2 – moderate (adequate for salty), 3 – strong, 4 – extreme

COLOUR

- **No significant differences** for:
  - L^* (49-56)
  - a^* (2.5-3.5)
  - b^* (18-21)

MICROBIOLOGY

- All products were considered **safe**
- **No significant differences** for:
  - TVC (1.3 – 2.1 log CFU/g)
  - Enterobacteriaceae (<LOQ)
  - *L. monocytogenes* (<LOQ)

CONCLUSIONS

- ✓ Chemical composition, physical parameters and safety of smoked mackerel were not affected by the different formulations used to reduce NaCl content
- ✓ The formulation **5B (5% brine** and **75% NaCl + 25% KCl)** provided the best relation sodium reduction: sensory properties
- ✓ 5B formulation allowed for a **70% reduction of Na/NaCl compared to conventional commercial products**
- ✓ Higher sodium chloride substitution may be possible through the addition of bitterness masking ingredients, such as aromatic herbs

REFERENCES

[1] WHO, 2021, https://www.who.int/activities/reducing-population-sodium-salt-intakes (accessed: August 31, 2021)

[2] WHO, 2018, https://www.euro.who.int/en/health-topics/disease-prevention/pages/news/news/2018/4/whoeurope-develops-new-tool-to-support-countries-in-achieving-salt-reduction-targets (accessed: August 30, 2021)

[3] Muñoz, I., Guàrdia, M. D., Arnau, J., Dalgaard, P. & et al. 2020. https://doi.org/10.1016/j.fct.2020.111554

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Association between dietary exposure to bisphenols and body mass index in Spanish schoolchildren

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Abstract

The increase in children obesity worldwide has been of particular concern in recent decades. Environmental factors have been proposed as contributors to obesity, and there is a growing concern over obesogens, environmental chemicals with potential obesity-related endocrine-disrupting properties. In this regard, bisphenol A (BPA) and its analogues are suspected to have obesogenic properties. Current document report on the activities of the fellow, undertaken during the fourth, 2020–2021 cycle of the EU-FORA programme at the University of Granada, Institute of Nutrition and Food Science, in Spain. The work programme offered by the hosting site was related to the extrapolation of bisphenols exposure following the determination of these compounds in food frequently consumed by children and in their biological samples. The fellow has participated in the recruitment of the study population in the health centres. In addition, she has participated in the collection of the children biological samples, anthropometric measurements and dietary surveys and in the optimisation of the laboratory methodology for the extraction of bisphenols in biological samples. All these activities also provided the fellow an opportunity to develop her data science related skills, which will benefit her professional development. In addition, the fellow gained an overview of various topics related to food safety risk assessment by attending the EU-FORA dedicated training modules.

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Keywords: bisphenols, obesity, food consumption, schoolchildren daily intake, biomonitoring

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1. Introduction

Endocrine disrupter chemicals (EDCs) are a family of exogenous substances able to alter the hormonal equilibrium of the body, they can cause adverse effects in nervous, cardiovascular and reproductive system in both men and women (Kabir et al., 2015). In the last decade, some studies have demonstrated EDCs can also behave as obesogens altering the endocrine system and then leading to the onset of obesity (Vaamonde and Álvarez-Món, 2020). Obesity is worldwide increasing over the last decades in adults as well as in children. In 2016, more than 1.9 billion adults, 18 years and older, were overweight, of these over 650 million were obese. World Health Organization (WHO) estimates the prevalence of overweight and obesity among children and adolescents aged 5–19 has risen dramatically from just 4% in 1975 to just over 18% in 2016. Nowadays over 340 million children and adolescents aged 5–19 are overweight or obese (Vaamonde and Álvarez-Món, 2020). This is considered of particular concern as this population can develop diabetes mellitus type II and cardiovascular disease among others. Recently different scientific studies have started to consider additional factors playing a role in the onset of obesity including diet and lifestyle habits, genetics (Herbert, 2008; Heindel and Blumberg, 2019), living environment (Nappi et al., 2016) and exposure to obesogens. Obesogens can be found in food, dust, water, drugs and personal care products (PCPs), so they can enter the human body by oral and dermal contact or inhalation (Monneret, 2017).

One of the most studied EDCs is bisphenol A (BPA). Since 1930, BPA has been used in the manufacture of polycarbonate plastics and epoxy resins, and their use is widely extended as cans coating in processed and ultra-processed food, where BPA can migrate from food contact material to food, and enter the body by diet. The European Food Safety Authority (EFSA) established a limit of 4 μg kg weight^{-1} day^{-1} (EFSA, 2015); however, recently, a new limit of 0.04 ng kg weight^{-1} day^{-1} has been established (EFSA, 2021). Because of legal limits, industries have started to use BPA analogues (bisphenols, BPs) with similar chemical structures and behaviour. However, specific limit values of exposure have not been established yet for these compounds. BPs more commonly used today are bisphenol S (BPS), bisphenol F (BPF), bisphenol E (BPE), bisphenol AF (BPAF), bisphenol P (BPP) and bisphenol B (BPB). However, some studies have demonstrated that BPA analogues show endocrine disrupting activities similar to BPA, and could act as obesogens at low concentrations (Andújar et al., 2019; Martínez et al., 2020; Reina-Pérez et al., 2021). We have recently demonstrated that some of those BPs including BPA are present in foodstuffs (Gálvez-Ontiveros et al., 2021; García-Córcoles et al., 2018).

Moreover, evidences of toxicity related to other BPA analogues used as substitutes of BPA are still missing due to their recent appearance; they include bisphenol C (BPC), bisphenol Z (BPZ), bisphenol AP (BPAP), bisphenol M (BPM) or bisphenol FL (BPFL). All chemical structures of target bisphenols are shown in Appendix A.

The interest in finding an association between BPA and analogues exposure with obesity is currently growing in the scientific community. In a recently published study, we have demonstrated an association between dietary exposure and both total bisphenols and BPA in overweight/obese adolescent girls (Robles-Aguilera et al., 2021). Therefore, the main objective of the fellow work program was to learn how to measure bisphenols exposure in a children population to establish a correlation between exposure and overweight/obesity.

1.1. Ethical considerations

This proposal was developed according to the Helsinki Declaration and human rights and biomedical research. This proposal respect UNESCO Universal Declaration about human genome and human rights. Moreover, the proposal was carried out according to the protocol established by the Organic Law 15/1999, 13 December, which includes data about Personal Data Protection Law 41/2002.

2. Description of work programme

2.1. Aims

As part of the EU-FORA fellowship, the focus of this study was for the fellow to be involved in all the activities required to obtain data, tools and possible biomarkers to correlate the estimation of dietary exposure to BPA and analogues with endocrine-disrupting activity (BPS, BPP, BPF, BPB, BPE and BPAF) in schoolchildren population and its impact in obesity. Her work was included the EFSA Partnering Grants 'OBEMIRISK-Knowledge platform for assessing the risk of Bisphenols on gut

microbiota and its role in obesogenic phenotype: looking for biomarkers' (Grant Agreement Number – GP/EFSA/ENCO/2018/03 – GA04). Changes were proposed due to delays in field work in the collection of samples due to the COVID-19 pandemic. The objectives were focus mainly on the estimation of BPA and analogues in foods frequently consumed by the population under study, to acquire knowledge in measuring the levels of these endocrine disruptors in different biological samples and to collaborate in the know-how platform database.

In support of this objectives, the fellow also learn how to determine individual SNPs from hormonal receptor genes. However, she does not participate in the genetic laboratory analyses due to COVID restriction of the use of the laboratory.

2.2. Activities/methods

As part of the fellowship, the priority of the hosting site was to provide the fellow with the basic theoretical background required to develop the questionnaires and perform the biological samples analyses. The fellow joined a working team with proved expertise in the subjects.

- Population recruitment: sample collection (swabs, urine, saliva, hair and nails), FFQ (Food frequency questionnaire) and anthropometric analyses in health centres.
- Experimental design (procedure of bisphenols extraction from saliva and urine, analytical methods validations)
- Software tools specific for design of experiments, databases and data interpretation (Statgraphics plus v.5.0; SPSS v.23; MassLynx v4.1).
- Analysis of bisphenols in food and biological samples.
- In addition, the fellow benefited from the EU-FORA dedicated training modules.

2.3. Food analysis

A total of 100 food products were purchased from the main Spanish supermarket chains. The selected foods represent 95% of the daily intake of energy, macronutrients and micronutrients of the children selected in this study (Gálvez-Ontiveros et al., 2021). Most of the foods were packaged in plastic containers, cans and tetra bricks. The foods were divided into three categories based on the NOVA classification (Monteiro et al., 2018). The categories recognised were minimally processed or unprocessed, processed and ultra-processed.

The bisphenols determined in food were a total of 7 (BPA, BPAF, BPB, BPE, BPF, BPP and BPS).

The fellow learned the extraction and quantification protocol for bisphenols in food along with the analysis and interpretation of the results (Gálvez-Ontiveros et al., 2021) and carried out the entire procedure for better understanding and consolidation.

Regarding the foods analysed (Appendix B. Food list), the presence of bisphenols was detected in 51% of samples. BPA was detected in the majority of food samples analysed (28%) followed by BPS (26%) and BPE (4%). The rest of the studied bisphenols were not detected. The concentrations ranged from 1 ng g^{-1} (chocolate palm tree) to 409 ng g^{-1} (canned tuna in oil). The Figure 1 and Table 1 below show the described results.

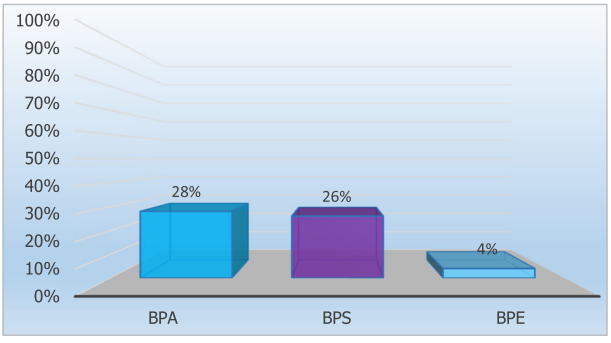


Figure 1: Percentage of detected bisphenols in analysed food samples

Table 1: Frequencies (%) and mean (ng g^{-1}) of bisphenols in analysed food samples (Gálvez-Ontiveros et al., 2021)

	Bisphenols in foods			
	BPS	**BPE**	**BPA**	**ΣBPs**
Unprocessed or minimally processed foods (n = 32)				
Frequency (%)	46.88	6.3	21.88	63
Mean (ng g^{-1})	17.27	< LOQ	6	18.35
Processed foods (n = 21)				
Frequency (%)	38.1	0	38.1	67
Mean (ng g^{-1})	39.49	0	86.3	35
Ultra-processed foods (n = 47)				
Frecuency (%)	6.38	4.26	27.66	36
Mean (ng g^{-1})	47.48	< LOQ	35.3	38.34
All (n = 100)				
Frecuency (%)	26	4	28	51
Mean (ng g^{-1})	28.99	< LOQ	43.28	30.4

ΣBPs: ΣBisphenols; LOQ: limit of quantification.

The obtained results agreed with other evidences obtained in previous scientific studies where it was observed that the highest concentrations of bisphenols have been found in canned foods (Gallart-Ayala et al., 2011; Alabi et al., 2014; Russo et al., 2019). This may be due to the food contact material covering the cans; it is usually epoxy resin obtained mainly from BPA (Abraham and Chakraborty, 2020) that can migrate from the container to the food. In addition, it was observed that the greater is the contact time of food with the container, the greater will be the migration towards food, with canned foods being the foods that spend the longest time stored in the pantries of our houses.

2.4. Recruitment of the study population

2.4.1. Study population

The fellow has participated in the recruitment of the studied population. The age of the children selected for this study was ranged between 6 and 12 years old. The recruitment of the population was carried out in different health centres where the fellow proceeded to collect the biological samples (hair, swab, nails, saliva and urine), to take anthropometric measurements (weight, height, circumference of waist and hip and bioimpedance) and to submit the survey containing questions related to dietary habits, physical activity and exposure to bisphenols (Appendix C). Moreover, the fellow learnt how to perform bisphenol exposure assessment using the data collected in the questionnaires.

Urine and saliva samples were stored at −80°C, while hair, nail and swab samples were stored at room temperature until laboratory analysis.

2.4.2. Anthropometry

The anthropometric measurements were collected taking into consideration the documents recommended by the WHO and the recommendations of the THAO program for the prevention of childhood obesity (Gomez et al., 2014). The measuring instruments applied included: Floor scale (model SECA 872), Tallimeter (model SECA 214 (20–207 cm)) measuring tape to measure the waist circumference (model SECA 201) and the Plicometer (model Harpenden Skinfold Caliper-0120) to measure skinfolds.

The fellow collaborated with the Dieticians-Nutritionists to learn how to make anthropometric measurements for the subsequent classification of the population.

2.4.3. Survey

A questionnaire has been designed and validated to assess the risk of schoolchildren exposure to BPA and its analogues through food consumption (Appendix C) (Robles-Aguilera et al., 2021). The fellow had the opportunity to improve the questionnaire and contribute to the final version.

2.5. Analysis of biological matrixes

Saliva, urine, nails and hair have been taken from children and new methodologies to determine target endocrine disrupters have been developed. Selected bisphenols to be determine in all samples were BPAF, BPF, BPE, BPA, BPC, BPB, BPZ, BPS, BPAP, BPM, BPP and BPFL (Appendix A).

2.5.1. Saliva

Procedures to analyse saliva were out-of-date, so first step was to develop a new original method to guarantee optimum extraction of all bisphenols from the samples. The fellow helped to design the experiments and to apply the extraction procedure. Final results about the optimisation and validation have been published recently (Moscoso-Ruiz et al., 2022). Briefly, 1 g of fresh saliva is subjected to a protein precipitation with ACN and acidic medium, followed by an ultrasound-assisted extraction with ethanol and re-extracted with acetone (30 min, 35% power). Finally, the dry residue was reconstituted with MeOH/H2O (30/70) (v/v) and injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) equipment. The fellow also learned how to optimise parameters in the equipment and the theory behind spectrometry technique. Once the method was validated for the sensitivity and sensibility, it was possible to use it to analyse samples of children saliva.

A total of 74 samples were analysed following this protocol. Results in Figure 2 show that 38% of samples contained BPA, being the most frequently detected bisphenol. However, several BPA analogues have been detected as BPP (28%), BPAP, BPAF, BPM (11%), BPB (8%), BPE, BPC (5.4%) and BPZ (4%).

These results cannot be compared with other scientific studies, indeed as far as we know this is the first study analysing BPA and analogues content in children saliva. However, Gomes et al. (2020) reported evidences about BPA presence in saliva after dental treatment, as the dental composites and resins contain this compound.

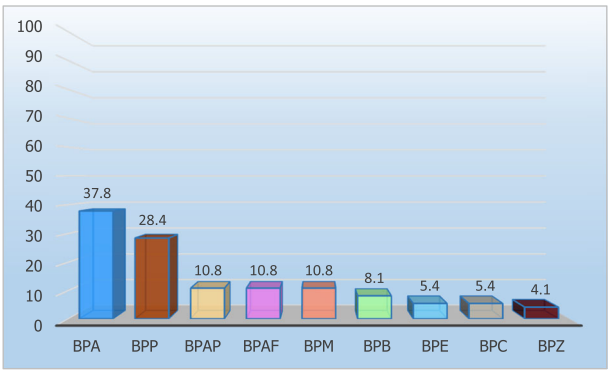


Figure 2: Results from children saliva

2.5.2. Urine

The procedure to analyse urine samples was taken from Vela-Soria et al. (2014) with some modifications. Analysis of urine was performed by duplicate, with and without enzyme (total and free BPs). Briefly, for free BPA, 4 mL of urine were taken and 4 mL of NaCl (aq) 10% (p/v) were added to provoke 'salting out' effect, plus 100 μL of HCl 6 N to adjust pH to 2. Extraction was made by injecting a solution of 400 μL of acetone and 600 μL of ethanol. The inferior portion of the mix was separated in a new tube and the extraction was repeated four times, mixing the inferior parts. After that, an evaporation of the mix was carried out, reconstituted in MeOH/H_2O (30/70) (v/v) and injected into the LC–MS/MS equipment. Total BPs was determined by adding to urine aliquot two different enzymes: 100 μL of β-glucuronidase from Helix Pomatia (solid) in acetate buffer and 25 μL of β-glucuronidase/ sulfatase (liquid). The same procedure was repeated to determine free BPs. After the method validation, the procedure was carried out for all samples of urine collected. Results are illustrated in Figure 3. They show that 40% of urine samples contained detectable quantity of BPA, but also we found BPAF (5%) and BPF (1.3%) in different samples. These results are in concordance with other studies concerning BPs in children urine; some of them detected BPA in remarkable concentrations and

even established an association between BPA level in urine and body mass index (Tschersich et al., 2021; Mahfouz et al., 2021).

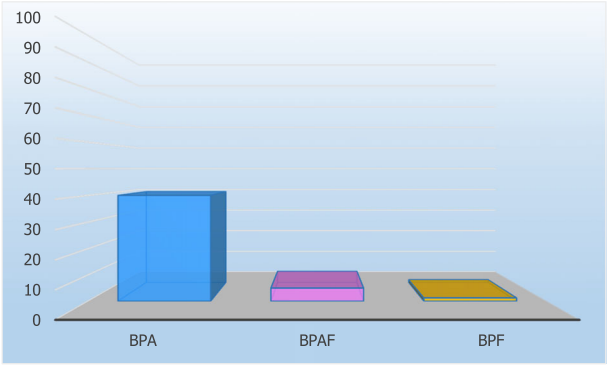


Figure 3: Results from children urines (free + conjugated)

3. Conclusions

3.1. Conclusions from the laboratory results

This project focused on the analysis and study of several matrices where BPA and its analogues can be accumulated and evaluated as potential non-invasive human samples. Furthermore, food analysis, in concordance with other studies, has demonstrated there are remarkable levels of BPs in foodstuffs. On the other hand, the presence of BPs in children biological samples confirmed that BPs bioaccumulate into the body, and further studies are needed to establish reliable biomarkers of exposure. The final objective of the project was to demonstrate whether exposure to bisphenols is associated to overweight and obesity in children. The statistical analysis of the results is still ongoing to evaluate the relationship between BPs dietary exposure, their presence in biological matrices and overweight and/or obesity in children.

3.2. Scientific activities during fellowship

During the fellowship, the fellow had the opportunity to participate to various scientific activities including participation in various conferences/seminars/webinars:

XXV Jornadas Internacionales Nutrición Práctica y el XIV Congreso Internacional de la SEDCA. She has contributed with the communication 'Consumo de los alimentos en niños en edad escolar para su uso en la estimación de la ingesta a disruptores endocrinos'. Held in Madrid, Spain, from April 20 to 22, 2021.

XXV Jornadas Internacionales Nutrición Práctica y el XIV Congreso Internacional de la SEDCA. She has contributed with the communication 'Concordancia entre la autopercepción del peso corporal y el índice de masa corporal en una muestra de adolescentes españoles'. Held in Madrid, Spain, from April 20 to 22, 2021.

3.3. Conclusions from the participation in the fellowship programme

The EU-FORA programme offered the fellow an opportunity to learn about BPs dietary exposure and its association with overweight/obesity in children. The fellow participated and learnt field work such as submitting questionnaires to children in health centres, collecting biological samples such as urine, saliva, hair and nails and performing chemical analyses to evaluate their presence in foods frequently consumed by the population under study and in the biological samples collected. The fellow learn about risk assessment of bisphenols reviewing literature related to the physico-chemical characteristics of the substances, their health effects and the regulatory framework they are framed, as well as practical hands-on exercises on risk assessment. This experience also provided the fellow an opportunity to develop her data science related skills, which will benefit her professional development as a data analyst.

In addition, the fellow gained an overview of various topics related to food safety risk assessment by attending the EU-FORA dedicated training modules.

References

Abraham A and Chakraborty P, 2020. A review on sources and health impacts of bisphenol A. Reviews on Environmental Health, 35, 201–210. https://doi.org/10.1515/reveh-2019-0034

Alabi A, Caballero-Casero N and Rubio S, 2014. Quick and simple sample treatment for multiresidue analysis of bisphenols, bisphenol diglycidyl ethers and their derivatives in canned food prior to liquid chromatography and fluorescence detection. Journal of Chromatography. A, 1336, 23–33. https://doi.org/10.1016/j.chroma.2014.02.008

Andújar N, Gálvez-Ontiveros Y, Zafra-Gómez A, Rodrigo L, Álvarez-Cubero MJ, Aguilera M, Monteagudo C and Rivas A, 2019. Bisphenol A analogues in food and their hormonal and obesogenic effects: a review. Nutrients, 11, 2136. https://doi.org/10.3390/nu11092136

EFSA (European Food Safety Authority), 2015. Scientific Opinion. Scientific Opinion on the risk to public health related to the presence of bisphenol A (BPA) in foodstuffs: Executive summary. EFSA panel on food contact materials, enzymes, flavourings and processing aids (CEF). EFSA Journal 2015;13(2):3978, 66 pp. https://doi.org/10.2903/j.efsa.2015.3978

EFSA (European Food Safety Authority), 2021. Bisphenol A: EFSA draft opinion proposes lowering the tolerable daily intake. EFSA panel on food contact materials, enzymes, flavourings and processing aids (CEF). EFSA Newsroom. https://www.efsa.europa.eu/en/news/bisphenol-efsa-draft-opinion-proposes-lowering-tolerable-daily-intake

Gallart-Ayala H, Moyano E and Galceran MT, 2011. Fast liquid chromatography-tandem mass spectrometry for the analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives in canned food and beverages. Journal of Chromatography. A, 1218, 1603–1610. https://doi.org/10.1016/j.chroma.2011.01.026

Gálvez-Ontiveros Y, Moscoso-Ruiz I, Rodrigo L, Aguilera M, Rivas A and Zafra-Gómez A, 2021. Presence of parabens and bisphenols in food commonly consumed in Spain. Foods, 10, 92. https://doi.org/10.3390/foods10010092

García-Córcoles MT, Cipa M, Rodríguez-Gómez R, Rivas A, Olea-Serrano F, Vílchez JL, and Zafra-Gómez A, 2018. Determination of bisphenols with estrogenic activity in plastic packaged baby food samples using solid-liquid extraction and clean-up with dispersive sorbents followed by gas chromatography tandem mass spectrometry analysis. Talanta, 178, 441–448. https://doi.org/10.1016/j.talanta.2017.09.067

Gomes JM, Almeida TFA, da Silva TA, de Lourdes CZ and Menezes HC, 2020. Saliva biomonitoring using LPME-GC/MS method to assess dentristry exposure to plasticizers. Analytical and Bioanalytical Chemistry, 412, 7799–7810. https://doi.org/10.1007/s00216-020-02908-x

Gomez SF, Casas R, Palomo VT, Martin Pujol A, Fíto M and Schröder H, 2014. Study protocol: effects of the THAO-child health intervention program on the prevention of childhood obesity - the POIBC study. BMC Pediatrics, 14, 215. https://doi.org/10.1186/1471-2431-14-215

Heindel JJ and Blumberg B, 2019. Environmental Obesogens: mechanisms and controversies. Annual Review of Pharmacology and Toxicology, 59, 89–106. https://doi.org/10.1146/annurev-pharmtox-010818-021304

Herbert A, 2008. The fat tail of obesity as told by the genome. Current Opinion in Clinical Nutrition and Metabolic Care, 11, 366–370. https://doi.org/10.1097/MCO.0b013e3283034990

Kabir ER, Rahman MS and Rahman I, 2015. A review on endocrine disruptors and their possible impacts on human health. Environmental Toxicology and Pharmacology, 40, 241–258. https://doi.org/10.1016/j.etap.2015.06.009

Mahfouz N, Salah E, Armaneous A, Youssef MM, Abu Shady MM, Sallam S, Anwar M, Morsy S and Hussein J, 2021. Association between bisphenol A urine level with low-grade albuminuria in egyptian children and adolescents. Open Access Macedonian Journal of Medical Sciences, 9, 1092–1097. https://doi.org/10.3889/oamjms.2021.6499

Martínez MÁ, Blanco J, Rovira J, Kumar V, Domingo JL and Schuhmacher M, 2020. Bisphenol A analogues (BPS and BPF) present a greater obesogenic capacity in 3T3-L1 cell line. Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association, 140, 111298. https://doi.org/10.1016/j.fct.2020.111298

Monneret C, 2017. What is an endocrine disruptor? Comptes Rendus Biologies, 340, 403–405. https://doi.org/10.1016/j.crvi.2017.07.004

Monteiro CA, Cannon G, Moubarac J, Levy RB, Louzada MLC and Jaime PC, 2018. The UN Decade of Nutrition, the NOVA Food Classification and the Trouble with Ultra-Processing. Public Health Nutrition, 21, 5–17. https://doi.org/10.1017/S1368980017000234

Moscoso-Ruiz I, Gálvez-Ontiveros Y, Cantarero-Malagón S, Rivas A and Zafra-Gómez A, 2022. Optimization of an ultrasound-assisted extraction method for the determination of parabens and bisphenol homologues in human saliva by liquid chromatography-tandem mass spectrometry. Microchemical Journal, 175, 107122.

Nappi F, Barrea L, Di Somma C, Savanelli MC, Muscogiuri G, Orio F and Savastano S, 2016. Endocrine aspects of environmental "obesogen" pollutants. International Journal of Environmental Research and Public Health, 13, 765. https://doi.org/10.3390/ijerph13080765

Reina-Pérez I, Olivas-Martínez A, Mustieles V, Ruiz-Ojeda FJ, Molina-Molina JM, Olea N and Fernández MF, 2021. Bisphenol F and bisphenol S promote lipid accumulation and adipogenesis in human adipose-derived stem cells. Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association, 152, 112216. https://doi.org/10.1016/j.fct.2021.112216

Robles-Aguilera V, Gálvez-Ontiveros Y, Rodrigo L, Salcedo-Bellido I, Aguilera M, Zafra-Gómez A, Monteagudo C and Rivas A, 2021. Factors associated with exposure to dietary bisphenols in adolescents. Nutrients, 13, 1553. https://doi.org/10.3390/nu13051553

Russo G, Barbato F, Mita DG and Grumetto L, 2019. Occurrence of Bisphenol A and its analogues in some foodstuff marketed in Europe. Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association, 131, 110575. https://doi.org/10.1016/j.fct.2019.110575

Tschersich C, Murawski A, Schwedler G, Rucic E, Moos RK, Kasper-Sonnenberg M, Koch HM, Brüning T and Kolossa-Gehring M, 2021. Bisphenol A and six other environmental phenols in urine of children and adolescents in Germany - human biomonitoring results of the German Environmental Survey 2014–2017 (GerES V). The Science of the Total Environment, 763, 144615. https://doi.org/10.1016/j.scitotenv.2020.144615

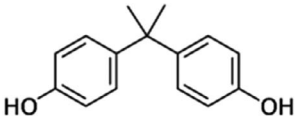
Vela-Soria F, Ballesteros O, Zafra-Gómez A, Ballesteros L and Navalón A, 2014. UHPLC-MS/MS method for the determzination of bisphenol A and its chlorinated derivatives, bisphenol S, parabens, and benzophenones in human urine samples. Analytical and Bioanalytical Chemistry, 406, 3773–3785. https://doi.org/10.1007/s00216-014-7785-9

Vaamonde J and Álvarez-Món MA, 2020. Obesidad y Sobrepeso. Enfermedades endocrinológicas y metabólicas (II). Obesidad Y Desnutrición, 13, 767–776. https://doi.org/10.1016/j.med.2020.07.010

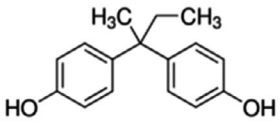
Abbreviations

AESAN	Spanish Agency for Food Safety and Nutrition
BFPL	bisphenol FL
BPA	bisphenol A
BPAF	bisphenol AF
BPAP	bisphenol AP
BPB	bisphenol B
BPC	bisphenol C
BPE	bisphenol E
BPF	bisphenol F
BPM	bisphenol M
BPP	bisphenol P
BPS	bisphenol S
BPZ	bisphenol Z
Bw	body weight
ECHA	European Chemical Agency
HCl	hydrochloric acid
LC–MS	liquid chromatography–mass spectrometry
MeOH	methanol
MRM	multiple-reaction-monitoring
NaCl	sodium chloride
TDI	tolerable dietary intake

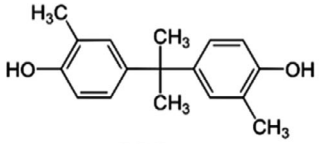
Appendix A – Structures of target bisphenols



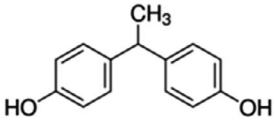
BPA



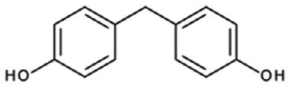
BPB



BPC



BPE



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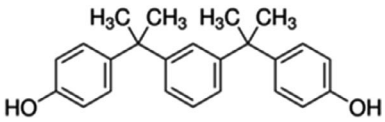

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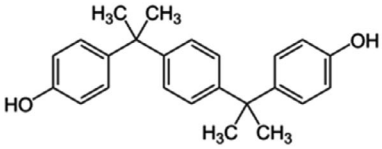

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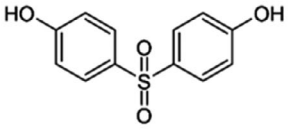

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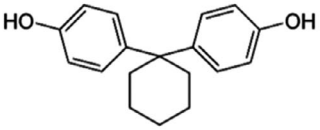

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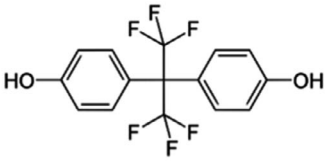

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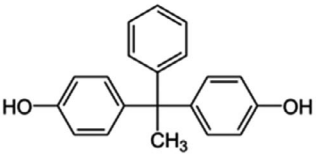

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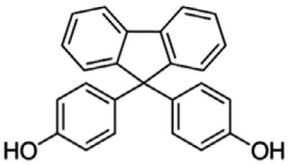

BPFL

Appendix B – Food List

FOOD LIST	
Chocolate doughnuts	Canned tuna in oil
Milk bread	Frozen hake
Croissants	Tuna dumplings (frozen precooked)
Chocolate palm (puff pastry with chocolate)	Battered hake sticks (frozen precooked)
Cacao-filled roll (Bollycao)	Bottle water
Chocolate rice pancakes	Pizza
Homemade cake	Ketchup
Muffins	Tomato sauce
Hamburger bun	Grape
Sandwich bread	Blueberries
Milk bread with chocolate (Weikis)	Pineapple
Cooked ham	Raspberries
Sausage (salchichon, chorizo, frankfurt sausages, turkey cold cut and mortadella)	Melon
Chicken burger	Apple
Chicken	Packaged apple
Serrano ham	Pear
Olive	Frozen red fruit mix
Anchovy stuffed olives	Frozen mango
Whole milk plastic bottle	Frozen chopped garlic
Tetra brick whole milk	Frozen chopped onion
Chocolate milkshake	Frozen chopped parsley
Semi-fermented milk	Frozen spinach
Yogurts (fruits and natural)	Tomato
Liquid yogurt	Packaged tomato
Cheese (slice, spread and melting)	striped carrot
Lentils	Packaged carrot
Pasta	packaged lettuce
Microwave rice cups	Packaged pumpkin
Candy jelly	packaged mushrooms
Corn snacks	Guacamole
Chips	Green pepper
Nachos	Packaged eggs
Canned sweet corn	

Appendix C – Exposure to Bisphenols Food Questionnaire

Link:

https://docs.google.com/document/d/1Ryq2WwbnJRZgGP5qaHsCuJ1UQo62LqDR/edit?usp=sharing&ouid=112625456176696250428&rtpof=true&sd=true

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