THE EUROPEAN FOOD RISK ASSESSMENT FELLOWSHIP PROGRAMME

SERIES 1

2017-2018

EU-FORA

efsa
European Food Safety Authority

Luxembourg: Publications Office of the European Union, 2018

Print	ISBN 978-92-9499-057-0	ISSN 2599-7335	doi:10.2805/925804	TM-BC-18-001-EN-C
PDF	ISBN 978-92-9499-056-3	ISSN 2599-7343	doi:10.2805/307848	TM-BC-18-001-EN-N

Table of contents

Please note that each report has a separate numbering system beginning on p.1 in line with the policy of the EFSA Journal

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e160815

Dear reader,

I am delighted to share these reports from our first cohort of EFSA fellows.

The European Food Risk Assessment Fellowship (EU-FORA) programme was born in 2016 as part of EFSA's efforts to nurture and develop the next generation of Europe's food risk assessors. One of our strategic priorities – indeed, one of our duties – is to build and strengthen Europe's risk assessment capacity, and to promote harmonisation of methodologies across organisations and countries.

EU-FORA was designed specifically to support us in this essential ongoing endeavour.

The idea was simple: EFSA acts as a 'broker', matching early to mid-career scientists working in the field of food safety with food risk assessment authorities in EU Member States, Iceland or Norway.

The scientists gain invaluable hands-on experience and knowledge working alongside risk assessors in a host organisation outside their home country. They return home with new or enhanced skills in areas such as selecting and applying risk assessment methodologies, collecting and analysing relevant data, using computer models in risk assessment and providing effective risk communication.

In return, the host organisation enjoys the support of an enthusiastic scientist who brings a fresh eye and new insights to their host's work. He or she becomes an integral part of the workforce for one year, exchanging views, expertise and work practices with his or her temporary colleagues.

Reading these reports, it became clear to me that the experiences of our first group of fellows have matched the high hopes we had for the scheme. In this special issue of the *EFSA Journal*, the 15 scientists who came to Parma for initial training in late 2017 describe both the benefits and the challenges that they subsequently encountered during their placements with risk assessment bodies across Europe.

Our second round of fellows begin their placements in late 2018. I hope their experiences are as mutually enriching as those of their predecessors clearly were.

EU-FORA is already making a difference. Let's continue to work together and ensure that the future of risk assessment is in safe hands.

Finally, EFSA would like to thank everyone who contributed to the success of the first cycle of EU-FORA, in particular the fellows and their supervisors in the hosting organisations, members of the EU-FORA programme committee and members of the training consortium.

Bernhard Url,

Executive Director,

EFSA

ISSN: 2599-7335 (Print), 2599-7343 (PDF)

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e160816

Foreword

The first cycle of EFSA's Fellowship Programme (EU-FORA) is approaching its completion and, as foreseen at the beginning, it is time for the 15 fellows to present the key activities they have undertaken at the hosting sites during this past year. That is the purpose of this special issue of the *EFSA Journal*.

We were happy to see that the different work programmes across the various hosting organisations were quite diverse in nature and cover a broad swathe of issues related to risk assessment as it pertains to food safety.

The most impressive aspect for those of us involved in the programme at the European Food Safety Authority (EFSA) was that the fellows not only increased their knowledge of food risk assessment significantly (the main goal of the programme) but that many of them also worked on innovative topics, thereby creating new scientific knowledge. Many of these new developments will be the subject of scientific publications in the coming months and years.

A key added value of the EU-FORA programme, which unfortunately cannot be fully reflected in the present reports, was the strong networking that developed during the programme: between the various hosting organisations and the organisations of origin and of course among the fellows.

We hope that this special issue of the *EFSA Journal* will serve as inspiration for more Article 36 organisations to apply as hosting sites for the EU-FORA programme and that it will increase the interest of young- to middle-career scientists in the area of food safety risk assessment and encourage them to apply as candidate fellows.

For the fellows whose reports are included here, we would like to congratulate them and thank them for the professionalism, dedication, passion and resilience they have exhibited during the programme. We are sure that our paths will often meet again in different food safety risk assessment settings.

On behalf of the EU-FORA programme

Stylianos KOULOURIS
Engagement and Scientific Cooperation Officer
EU-FORA Programme Manager
ENCO/COMCO

ISSN: 2599-7335 (Print), 2599-7343 (PDF)

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

EU-FORA SERIES 1

EFSA Journal

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16081

Risk assessment of white willow (*Salix alba*) in food

German Federal Institute for Risk Assessment (BfR),
Unit of Food Toxicology, Germany
Ewa Matyjaszczyk and Regina Schumann

Abstract

This Technical Report contains a description of the activities within the work programme of the EU-FORA Fellowship on the risk assessment of white willow in food. The bark of different varieties of willow has had a long history of medical use as a means to reduce fever and as a painkiller. Willow bark is also used in weight loss and sports performance food supplements. The labelling of these products usually does not mention any restrictions to the length of use. The recommended doses for foods differ, sometimes exceeding doses recommended for pharmaceuticals. A systematic literature review on adverse effects potentially resulting from oral exposure to white willow (*Salix alba*) was performed. The aim of the study was to assess the risk for humans when consuming white willow bark in food. The preliminary results show that despite the long history of use only very limited data on toxicity of white willow bark are available. However, anaphylactic reactions in people with a history of allergy to salicylates may occur. Some other adverse effects of salicylates are considered to be of low relevance for the long-time consumption of white willow bark, mainly due to relatively low concentrations of salicin and the presence of compounds with gastroprotective action. However, it seems that the content of heavy metals, mainly cadmium, should be further addressed in risk assessment of white willow bark in food.

Keywords: *Salix alba*, cortex, White willow, bark, risk assessment, food, food supplements

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: German Federal Institute for Risk Assessment (BfR), Unit of Food Toxicology, Germany, Matyjaszczyk E and Schumann R, 2018. Risk assessment of white willow (*Salix alba*) in food. EFSA Journal 2018;16(8):e16081, 9 pp. https://doi.org/10.2903/j.efsa.2018.e16081

ISSN: 2599-7335 (Print), 2599-7343 (PDF)

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

This Technical Report focuses on the steps of work performed within the European Food Safety Authority (EFSA) founded European Food Risk Assessment Fellowship Programme (EU-FORA) Fellowship. It contains the description of activities that composed the programme of work in the German Federal Institute for Risk Assessment (BfR). The work programme entitled 'Risk assessment of plant and plant preparations in food' had foreseen the risk assessment of 1 of the 11 proposed plants: *Hypericum perforatum*, *Salix alba*, *Rubus suavissimus*, *Phyllanthus emblica*, *Panax ginseng*, *Uncaria tomentosa*, *Muira puama*, *Echinacea purpurea*, *Harpagophytum procumbens*, *Boswellia serrata* and *Eleutherococcus senticosus*.

EU-FORA is a practical ('training by doing') programme that aims to increase the expertise and capacity available to risk assessment bodies at both European and national levels. It is aimed specifically at early- to mid-career scientists working in food safety organisations across Europe. (Bronzwaer et al., 2016).

2. Description of work programme

2.1. Aims

It was the aim of the project to provide a detailed insight into a broad range of prepared methods to assess the safety of plants/plant preparations using a science-based approach. By stepwise walking through the entire process of modern risk assessment, the EFSA Grant fellow had the chance to extend the knowledge on how to draft a risk assessment of 'botanicals' at all stages of the approach including hazard identification, hazard characterisation, exposure assessment and risk characterisation according to international guidelines and standards. The assessment of putative health risks also comprised deviation and discussion of risk-preventing options based on the available data. Finally, there was the chance to get insight into modern risk communication measures as practised in BfR.

2.2. Choice of plant

The organisational issues needed some time and effort from the fellow, the supervisor and the international team that was very helpful in supporting the fellow's settlement in BfR. However, apart from organisational matters, the first task was to choose the plant to work on. There are quite a number of plants that are used in food and it would be a challenge to choose from among all of them. However, BfR Unit of Food Toxicology had presettled a list of plants from which to choose. The following plants were proposed for risk assessment: *H. perforatum* (known as St John's-wort), *S. alba* (white willow), *R. suavissimus* (Chinese blackberry), *P. emblica* (Indian gooseberry), *P. ginseng* (Asian ginseng), *U. tomentosa* (cat's claw), *M. puama* (potency wood), *E. purpurea* (purple coneflower), *H. procumbens* (devil's claw), *B. serrata* (Indian olibanum) and *E. senticosus* (Siberian ginseng).

The topic of risk assessment in plants used in food is very wide (Schumann et al., 2015; Bakhiya et al., 2017; Dusemund et al., 2017). Which plant to choose was a serious decision to make and the fellow used not only the supervisors' advice but also engaged an experienced colleague from the Unit of Food Toxicology in the process. The fellow was a food specialist by education, but for some time had been working in the area of plant protection. Therefore, a topic somehow connected to her regular job as a scientist in the Plant Protection Institute – National Research Institute in Poland was chosen. *S. alba* (white willow) was chosen for risk assessment. Apart from its use in medicine (Shara and Stohs, 2015) and as a food supplement, *Salix* bark is used in plant protection and is registered in the European Union (EU) as a basic substance (Marchand, 2015). *Salix* bark uses as a fungicide, having an eliciting action on the crop's self-defence mechanisms, are approved (Matyjaszczyk, 2018).

2.3. Identity and nature of the source material

A further step was the identification of the source material. It means that very precise defining the plant and the part of plant to work on was necessary.

The genus *Salix* is formed by around 400 species (Wikipedia). Most of them prefer moist soils and cold and temperate regions. Numerous species can be used and are used in medicine. 'Willow bark' is sometimes defined simply as the 'bark of *Salix* tree species' (Natural Medicines Comprehensive Database 2007). Willow bark constituents include flavonoids, tannins and salicylates. The active constituent of willow bark is thought to be salicin. In some publications, the type of willow the bark comes from is not even mentioned.

For risk assessment, it is not relevant if the source material is *S. alba* or some other species of big *Salix* family. It has been shown by numerous studies that the salicin, flavonoids and tannins content as well as that of other components in the *Salix* plant material depend on numerous factors (Sugier et al., 2013; Gawlik-Dziki et al., 2014). One of the factors is the species used (Mleczek et al., 2009; Krauze-Baranowska et al., 2013). Therefore, during the study, it was necessary to make sure that the data analysed concerned *S. alba* and not some other *Salix* plant species. The botanical identity of the plant was therefore as follows:

Family: Salicaceae
Genus: *Salix* L.
Species: *Salix alba*

Even this approach may be not sufficiently detailed, as the literature mentions a number of subspecies of *S. alba* and sometimes different terms are used to name the same plant. Moreover, sometimes systematics is not quite clear. Therefore, a list of different terms was prepared to consider during the study of *S. alba*.

The literature most often mentions the following subspecies:

Salix alba L.; *Salix alba* var. *caerulea* (Sm.) Sm.; *Salix alba* subsp. *micans* (Andersson) Rech. f.; *Salix alba* var. *vitellina* (L.) Stokes.

However, other names are also used, although these are often mentioned as synonyms:

Salix alba var. *alba*; *Salix alba* var. *australia* Poljakov; *Salix alba* f. *caerulea* (Sm.) Wimm.; *Salix alba* f. *ovalis* Wimm.; *Salix alba* f. *sericea* Wimm.; *Salix alba* var. *subintegra* N. Chao; *Salix alba* subsp. *vitelina* (L.) Arcangeli; *Salix alba* f. *vitelina* (L.) Wimm.

Another point of identification of the source material was to name the plant part used for risk assessment. For *Salix* (and other trees as well), the part of the plant strongly influences the content of numerous components, and it is therefore crucial for risk assessment to be specific (Unterbrunner et al., 2007; Zarubova et al., 2015). The source material 'white willow bark' is not as simple as it sounds, as this describes more than just the bark. The definition in the Martindale reference very precisely describes the plant part used as a source material for medical products as 'the whole or fragmented dried bark of young branches or whole dried pieces of current year twigs' (Brayfield, 2017).

Some analysis performed for willow could not be considered for studies because their data gave values of certain compounds in wood, leaves or roots.

2.4. Manufacturing process

Manufacturing processes can obviously influence the content of components in the final product. However, for willow bark, the manufacturing process is not very sophisticated as very often simply powdered or comminuted herbal substance is used. Therefore, for white willow bark, the manufacturing process does not seem to influence the composition of the source material.

It is also possible to use dried hydroalcoholic or aqueous extracts, tinctures or fluid extracts (Schilcher and Kammer, 2003); however, the inclusion of powdered or comminuted herbal substances is the worst-case scenario for risk assessment.

2.5. Chemical composition

In the next step of the research project, a list of secondary plant ingredients was to be established, as well as predictions of their genotoxic/carcinogenic potency based on the literature review. Based on the results obtained, substances were to be selected for subsequent *in vitro* studies with long/short time exposure to the selected secondary plant ingredients (induction of steatosis, microarray analysis, reporter gene assays: promoter analysis CYP7A1, interaction and nuclear receptors).

It was therefore necessary to use the literature as well as literature databases to look for the required information. However, regarding the description of the composition, old-fashioned printed compendia proved to be far more useful than internet sources.

In some cases, the most recent and most reliable data on the composition of products are to be found online. However, for plant components (at least white willow components), the printed compendia were easy to find and the information in these was compact and comprehensive. Mining the internet for the same data would be much more time consuming and would require much more

criticism and double-checking of the sources used. Of course the fellow was very conveniently placed for such studies. BfR is a governmental body dealing, among others, with food safety, therefore, the fellow had access to the whole library, as well as the small collection library of the Unit of Food Toxicology at her disposal. In addition, she was able to obtain advice from supervisors as to which were the most reliable and comprehensive manuals to use. However, in the current digitalised world, maybe it is worth to point out, that at least in some cases using books is still a much easier way to find relevant data, than looking for data on the Web.

2.6. Toxicological data

After identifying the secondary plant ingredients, the next task was to predict their genotoxic and carcinogenic potency. Here, it was necessary to make use of internet literature databases. However, the databases most often used by the fellow when searching for literature on plant protection were not always the best for human toxicology data. Regarding the toxicological data, PubMed and EMBASE databases were the most useful to work with. BfR as a governmental institution has a large number of databases available, so again the fellow was in a convenient position and could use these interchangeably. However, for people working in less equipped institutions, it may be a good tip that sometimes, especially while approaching certain topics from different angles, to try other databases from the library of another university or governmental institution, which may have access to databases that better suit the topic.

The use of papers containing toxicological data was the most challenging part of the fellow's task so far. She had no toxicological background and the technical vocabulary proved to be difficult for her to understand. In spite of the supervisor's assistance, looking for toxicological data on *S. alba* was a lengthy process because it was very difficult to find relevant studies. The most helpful publication was from the European Medicines Agency (EMA, 2017). It in fact did not contain or quoted any sources that contained data on toxicity of *S. alba*, but at least it stated information that confirmed findings or rather the lack of findings: namely that such data for the main components of *S. alba* are basically non-existent.

It remained, however, one more path to follow regarding the risk assessment of *S. alba*. From the fellow's agricultural knowledge, she was aware that different species of *Salix* are used for phytoremediation and phytostabilisation or are used to clean up soil contaminated with hazardous compounds, particularly heavy metals (Kacalkova et al., 2015; Mayerova et al., 2017). Therefore, as *Salix* species are known for their 'remarkable capacity to concentrate toxic heavy metals' (Chen et al., 2013), it was interesting to check if there is a risk that bark consumed by humans may contain excessive toxic heavy metals. Indeed, this idea proved to be the case. Heavy metals are present in willow bark in significant amounts.

2.7. Exposure

It is pretty logical that exposure occurs by oral consumption. In assessing exposure to *S. alba* in food, there should be consideration of how much white willow bark humans consume.

Tree bark is not typical component of meals in Europe and, when discussing the topic, people were generally surprised that it is consumed in food at all. However, it is consumed as an ingredient of weight-loss supplements (Sharpe et al., 2006) as well as sports performance products (Shara and Stohs, 2015). Some food supplements contain white willow bark as one of numerous components, plus willow bark is sold for human consumption as a powder.

When performing the exposure study for *S. alba* in food, a useful hint was given by an experienced colleague from the Unit of Food Toxicology to assume that the highest consumption level was that recommended for medical products.

However, the fellow carried out her own market research by checking different online shops with food supplements and analysed the labels of the offered products. In some cases, it was challenging to get information from the labels due to poor quality of pictures and sometimes the labels were not very informative. However, spending some time on this task it appeared that for food supplements containing multiple components, the recommended dose of *S. alba* was indeed usually lower than for medical products. Recommendations for medical products could therefore be considered as the worst case scenario. However, in food supplements containing white willow bark as the only or as the main component, there were cases in which maximum recommended dose of willow bark in food was higher than the maximum dose recommended for medical products.

2.8. Safety assessment based on available knowledge

It was not possible to consider the toxicity of secondary plant ingredients, as full data are not available. However, regarding the content of the salicylic compounds, there seems to be consensus in the literature that willow bark has a broader mechanism of action and is devoid of serious adverse events in comparison with aspirin (Vlachojannis et al., 2014; Dragos et al., 2017).

To calculate safety based on available knowledge on heavy metals, the EFSA published statements on tolerable weekly intake were considered (EFSA CONTAM Panel, 2010, 2011) as well as the literature data on content of heavy metals in willow bark.

It was debatable what should be considered as a worst-case scenario of heavy metal content in willow bark. In many European countries, willows grow in semi-wild conditions in different places. They can be often spotted in rural areas or alongside roads and streams, and among fields or meadows. Sometimes, however, they grow in proximity to abandoned buildings and uninhabited places. It is therefore neither unusual nor surprising for a passer-by to spot willows in the proximity of an abandoned mine. However, grounds surrounding some old mines and smelting places may contain uncommonly high levels of heavy metals. Moreover, different species of willows are often purposefully planted in places contaminated with heavy metals for their phytoremediation properties. Bark coming from such willows may be heavily contaminated with heavy metals. Conversely, it may be assumed that willow bark that serves as a raw material for food or medical industry would be acquired from safe places. Therefore, for the worst-case scenario calculation, the fellow decided to use a scenario in which willow bark comes from areas that are known to be polluted with heavy metals, but that are used in agriculture.

2.9. EU-FORA Fellowship supporting programme

In addition to her work in Unit of Food Toxicology, participation in weekly seminars and consultations with the supervisor as well as with some other colleagues, during the period of EU-FORA Fellowship Programme the fellow benefited from other activities. The four training modules in Parma, Vienna, Berlin and Athens were very interesting and were common for all EU-FORA fellows as described in the 'EU-FORA Fellowship Programme year 2018–2019'.

However, the hosting institution, BfR, provided additional training curriculum, as well as enabling the fellow to participate in some other activities that developed her general knowledge as well as her knowledge on risk assessment. Table 1 presents the supporting activities organised or facilitated for the fellow by BfR during the EU-FORA Fellowship.

Table 1: Supporting activities organised or facilitated by the hosting institution, the German Federal Institute for Risk Assessment, during the EU-FORA Fellowship

	Title	Date
Training on risk assessment	Workshop 'Risk assessment of plasticisers'	22.11.2017
	Workshop 'What does the future hold for harmonised human health risk assessment of plant protection products?'	23–24.11.2017
	GMO Risk Assessment Workshop	22–23.5.2018
Other activities	Participation once a week in department seminars with presentations of food safety-related (*in vitro* or *in vivo* experimental) research activities	Every week
	Training 'Library Introduction – Databases and Organisation of Information in BfR'	29.9.2017
	Conference 'Efficacy and risks of biorational products in integrated pest management (IPM) strategies - acceptable?'	13–14.12.2017
	Training 'Effective Presentations'	20–21.2.2018
	Participation in International Events organised by International Affairs team with an aim to provide an opportunity for social and professional networking	Every quarter
	Visit in Department of Pesticides Safety	TBC
	Visit in Department of Safety in the Food Chain	TBC

3. Conclusions

The programme of the fellowship reached two aims:

1) Enabling the fellow to gain first-hand experience on risk assessment as well as extend the knowledge on how to elaborate risk assessment of 'botanicals' at all stages of the approach including hazard identification, hazard characterisation, exposure assessment and risk characterisation according to international guidelines and standards.
2) Performing risk assessment of white willow (*S. alba*) in food. Very limited data on toxicity of white willow bark are available. However, regarding the available data, the content of heavy metals, mainly cadmium, may be of concern regarding white willow bark in food.

References

Bakhiya N, Ziegenhagen R, Hirsch-Ernst KI, Dusemund B, Richter K, Schultrich K, Pevny S, Schaefer B and Lampen A, 2017. Phytochemical compounds in sport nutrition: synephrine and hydroxycitric acid (HCA) as examples for evaluation of possible health risks. Molecular Nutrition and Food Research, 61, 1601020. https://doi.org/10.1002/mnfr.201601020

Brayfield A (ed.), 2017. Martindale: The Complete Drug Reference. Pharmaceutical Press, London, United Kingdom. 4142 pp. Available online: https://medicinescomplete.com

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

Chen G, Liu Y, Wang R, Zhang J and Owens G, 2013. Cadmium adsorption by willow root: the role of cell walls and their subfractions. Environmental Science and Pollution Research International, 20, 5665–5672. https://doi.org/10.1007/s11356-013-1506-3

Dragos D, Gilca M, Gaman L, Vlad A, Iosif L, Stoian I and Lupescu O, 2017. Phytomedicine in joint disorders. Nutrients, 9, 70.

Dusemund B, Rietjens I, Cartus A, Schaefer B and Lampen A, 2017. Plant-derived contaminants in food. Occurrence, effects and risk assessment. Bundesgesundheitsblatt-Gesundheitsforschung-Gesundheitsschutz, 60, 728–736. https://doi.org/10.1007/s00103–017–2561–6

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), 2011. Statement on tolerable weekly intake for cadmium. EFSA Journal 2011;9(2):1975, 19 pp. https://doi.org/10.2903/j.efsa.2011.1975

EMA (European Medicines Agency), 2017. Assessment report on *Salix* [various species including *S. purpurea* L., *S. daphnoides* Vill, *S. fragilis* L.], cortex. Available online: http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_HMPC_assessment_report/2017/07/WC500230918.pdf

Gawlik-Dziki U, Sugier D, Dziki D and Sugier P, 2014. Bioaccessibility *in vitro* of nutraceuticals from bark of selected *Salix* species. The Scientific World Journal, 2014, 782763. https://doi.org/10.1155/2014/782763

Kacalkova L, Tlustos P and Szakova J, 2015. Phytoextraction of risk elements by willow and poplar trees. International Journal of Phytoremediation, 17, 414–421. https://doi.org/10.1080/15226514.2014.910171

Krauze-Baranowska M, Poblocka-Olech L, Glod D, Wiwart M, Zielinski J and Migas P, 2013. HPLC of flavanones and chalcones in different species and clones of *Salix*. Acta Poloniae Pharmaceutica, 70, 27–34.

Marchand PA, 2015. Basic substances: an opportunity for approval of low-concern substances under EU pesticide regulation. Pest Management Science, 71, 1197–1200. https://doi.org/10.1002/ps.3997

Matyjaszczyk E, 2018. Plant protection means used in organic farming throughout the European Union. Pest Management Science, 74, 505–510. https://doi.org/10.1002/ps.4789

Mayerova M, Petrova S, Madaras M, Lipavsky J, Simon T and Vanek T, 2017. Non-enhanced phytoextraction of cadmium, zinc, and lead by high-yielding crops. Environmental Science and Pollution Research International, 24, 14706–14716. https://doi.org/10.1007/s11356-017-9051-0

Mleczek M, Rissmann I, Rutkowski P, Kaczmarek Z and Golinski P, 2009. Accumulation of selected heavy metals by different genotypes of *Salix*. Environmental and Experimental Botany, 66, 289–296. https://doi.org/org/10.1016/j.envexpbot.2009.02.010

Schilcher H and Kammer S, 2003. Weidenrinde (*Salix cortex*). In: von Schilcher H and Kammerer S (eds.). *Leitfaden Phytotherapie*. 2nd Edition. Urban and Fischer, Muenchen, Jena. pp. 257–259.

Schumann R, Klenow S, Dusemund B, Poeting A and Lampen A, 2015. Risk assessment of plants and plant preparations. Annals of Nutrition and Metabolism, 67, 503–504.

Shara M and Stohs SJ, 2015. Efficacy and safety of white willow bark (*Salix alba*) extracts. Phytotherapy Research, 29, 1112–1116. https://doi.org/10.100w2/ptr.5377

Sharpe PA, Granner ML, Conway JM, Ainsworth BE and Dobre M, 2006. Availability of weight-loss supplements: results of an audit of retail outlets in a southeastern city. Journal of the American Dietetic Association, 106, 2045–2051. https://doi.org/10.1016/j.jada.2006.09.014

Sugier DSP, Banas A and Szewczuk C, 2013. The content of phenolic glycosides and macroelements (K, Ca, Mg) in the bark of herbal willows. Acta Scientiarum Polonorum, Hortorum Cultus, 14, 31–41.

Unterbrunner R, Puschenreiter M, Sommer P, Wieshammer G, Tlustos P, Zupan M and Wenzel WW, 2007. Heavy metal accumulation in trees growing on contaminated sites in Central Europe. Environmental Pollution, 148, 107–114. https://doi.org/10.1016/j.envpol.2006.10.035

Vlachojannis C, Magora F and Chrubasik-Hausmann S, 2014. Pro and contra duration restriction of treatment with willow bark extract. Phytotherapy Research, 28, 148–149. https://doi.org/10.1002/ptr.5008

Willow bark, 2007. In: Jellin JM (ed.). *Natural Medicines Comprehensive Database*, 9th Ednition. Therapeutic Research Faculty Stockton, CA, USA. pp. 1358–1360.

Zarubova P, Hejcman M, Vondrackova S, Mrnka L, Szakova J and Tlustos P, 2015. Distribution of P, K, Ca, Mg, Cd, Cu, Fe, Mn, Pb and Zn in wood and bark age classes of willows and poplars used for phytoextraction on soils contaminated by risk elements. Environmental Science and Pollution Research International, 22, 18801–18813. https://doi.org/10.1007/s11356-015-5043-0

Abbreviations

BfR	German Federal Institute for Risk Assessment
EMA	European Medicines Agency
EU-FORA	The European Food Risk Assessment Fellowship Programme
GMO	genetically modified organism
IPM	integrated pest management

EU-FORA SERIES 1

ADOPTED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16082

Novel foods: a risk profile for the house cricket (*Acheta domesticus*)

SLU, Swedish University of Agricultural Sciences,
Department of Biomedical Sciences and Veterinary Public Health, Sweden,
X Fernandez-Cassi, A Supenu, A Jansson, S Boqvist and I Vagsholm

Abstract

Novel foods could represent a sustainable alternative to traditional farming and conventional foodstuffs. Starting in 2018, Regulation (EU) 2283/2015 entered into force, laying down provisions for the approval of novel foods in Europe, including insects. This Approved Regulation establishes the requirements that enable Food Business Operators to bring new foods into the EU market, while ensuring high levels of food safety for European consumers. The present risk profile tackles the hazards for one of the most promising novel food insects, the house cricket (*Acheta domesticus*). The risk profile envisages a closed *A. domesticus* crickets rearing system, under Hazard Analysis and Critical Control Points (HACCP) and good farming practices (GFP), in contrast with open cricket farms. The methodology used involves screening the literature and identifying possible hazards, followed by adding relevant inclusion criteria for the evidence obtained. These criteria include animal health and food safety aspects, for the entire lifespan of crickets, based on the farm to fork One Health principle. When data were scarce, comparative evidence from close relatives of the *Orthoptera* genus was used (e.g. grasshoppers, locusts and other cricket species). Nevertheless, significant data gaps in animal health and food safety are present. Even if HACCP-type systems are implemented, the risk profile identifies the following considerable concerns: (1) high total aerobic bacterial counts; (2) survival of spore-forming bacteria following thermal processing; (3) allergenicity of insects and insect-derived products; and (4) the bioaccumulation of heavy metals (e.g. cadmium). Other hazards like parasites, fungi, viruses, prions, antimicrobial resistance and toxins are ranked as low risk. For some hazards, a need for additional evidence is highlighted.

Keywords: house cricket, entomophagy, novel foods, food safety, risk profile

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: SLU, Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health, Sweden, Fernandez-Cassi X, Supenu A, Jansson A, Boqvist S and Vagsholm I, 2018. Novel foods: a risk profile for the house cricket (*Acheta domesticus*). EFSA Journal 2018;16(8):e16082, 15 pp. https://doi.org/10.2903/j.efsa.2018.e16082

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

Insects represent a significant part of the diet for many communities and are consumed in several countries in Africa, South America, Asia and Oceania. However, in western markets, the consumption of insects (entomophagy) is yet to be culturally and socially accepted (House, 2016). Insect biodiversity is huge, with estimations ranging from 2.6 to 7.8 million species (Stork et al., 2015). This biodiversity implies a huge diversity of metabolic pathways and microbiomes among the different species. Currently, more than 2,111 documented arthropods are being eaten worldwide (Jongema, 2017). Most consumed arthropods, ordered according to their consumption, belong to these eight groups: Coleoptera (beetles, often the larvae) (31%), Lepidoptera (caterpillars) (17%), Hymenoptera (wasps, bees and ants), Orthoptera (crickets, grasshoppers and locusts) (14%), Hemiptera (true bugs) (11%), Isoptera (termites) (3%), Odonata (dragonflies), Diptera (flies) and others (9%) (van Huis, 2018). However, only limited numbers of insect species are reared on a large scale as food and feed. According to some market studies, Europe is becoming the fastest growing market for edible insects, forecasting revenues of US$1.07 billion in 2022 (Persistence Market Research, 2018). The same sources highlight that the order Orthoptera is expected to advance even faster, due to the high demand for cricket-based products (e.g. protein powder, granola bars, crackers or cookies) (TECA, 2013).

From a nutritional point of view, insects have an interesting nutritional profile, offering important sources of vitamins, minerals and animal-derived proteins (Wang et al., 2004). They also require less feed for each kg of food produced, and have higher relative growth and lower emission of greenhouse gases (GHG) compared with pigs and cattle (Oonincx et al., 2010; Oonincx and de Boer, 2012). *Acheta domesticus* requires 1.7 kg of dried feed to produce 1 kg of food, compared with 2, 3.8 and 7 kg for poultry, pigs and cattle, respectively (Paoletti, 2005). According to the Food and Agricultural Organization (FAO) predictions, an increase of 70% of the global agricultural production will be needed to fulfil the expected demand. Considering their efficiency, edible insects could play an important role to meet this increasing demand, in particular as an important source of animal protein (FAO – High Level Expert Forum, 2009).

In some countries, insect consumption stands as common practice with a well stabilised industry. As an example, Thailand has released the first Good Agricultural Practices (GAPs) for cricket rearing (ACFS, 2017). According to European regulations and guidelines, insects should be considered as livestock. Hence, good farming practices (GFP) already enforced for other animal husbandry, such as swine, cattle or poultry should be applied. Despite this scenario, and due the particularities of insect rearing, those farming practices should be revised and adapted.

With Novel Foods Regulation (EU) 2283/2015 that entered into force in January 2018, insects and insect-derived products are considered to be novel foods and are subject to the novel foods approval procedure. General health risks associated with consumption of insects have already been tackled in several published risk profiles and scientific opinions (FAO, 2013; EFSA Scientific Committee, 2015; Finke et al., 2015; Schafer et al., 2016). However, due the huge diversity within the insects' world, there is a need to target insect species relevant for European consumers specifically. Therefore, a specific risk profile for the consumption of *A. domesticus* reared in controlled conditions has been developed (Fernandez-Cassi et al., submitted).

2. Description of the work programme

2.1. Aims

The aim of this work is to present a specific risk profile for the house cricket (*A. domesticus*) intended for human consumption. The risk profile will present the current knowledge available on house cricket as food. Also, during the development of the work, several data gaps have been identified. Risks will be ranked as low, medium or high according to information available in the scientific literature considering, in a qualitative manner, the probability that a genuine hazard exists and the consequences of exposure. A hazard is ranked as 'low' when measures can be applied during processing and before consumption, to decrease or inactivate/destroy the hazard. In a similar manner, a hazard is ranked 'medium' when measures applied are insufficient to guarantee the complete removal of the hazard or important data gaps about the likelihood or the consequences of being exposed to it exist. Finally, a hazard is ranked 'high' when its exposure can have serious consequences or it is very likely to happen despite measures applied during processing (EFSA BIOHAZ Panel, 2012).

2.2. Methodology

The literature was scanned and retrieved through searches using the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed), Google Scholar (https://scholar.google.com), Scopus® (https://www.scopus.com) and Web of Science (https://apps.webofknowledge.com). Selection of the included papers was carried out stepwise. Initially, the article titles from the literature were assessed for inclusion prior to reading the abstracts. So, if the abstracts were deemed relevant, the full paper was retrieved and read. Websites belonging to relevant organisations and authorities (e.g. World Health Organization, European Food Safety Authority) were also used to retrieve information. The literature search was carried out between October 2017 and April 2018. The search focused on available scientific evidence for insects as food. Field studies using cricket species as animal models were also included as scientific evidence. Due to the reduced volume of published articles on *A. domesticus* as food intended for human consumption, efforts have been made to identify similarities with crickets used as pet food, different cricket species and including other insects in the order Orthoptera.

3. Biological hazards

3.1. Bacteria and antimicrobial resistance genes

Currently, no specific microbial criteria are available in the European legislation for whole insects or insect-based products intended for human consumption. Some authors (e.g. Caparros Megido et al., 2017) have suggested using total aerobic counts (TAC) of minced meat as food safety and final product hygiene guidelines values (according to European Commission (EC) Regulation No. 2073/2005). However, the numbers provided for minced meat (5×10^5 CFU/g) are difficult to match in non-treated crickets according to published microbial loads (Table 1). The fact that whole animals are eaten, including their guts, which can contain around 10^6–10^{12} bacteria per mL might explain the high reported values (Cazemier et al., 1997). To decrease the high microbial counts, some farmers apply 24–48 h fasting prior to the killing step. However, the efficiency of this procedure to decrease microbial load is uncertain. Literature TAC values range from 10^4 CFU/g to 10^8 CFU/g. Different protocols used or the application of processing treatments such as heat-treated crickets might explain the different numbers reported.

Despite high microbial loads, food-borne bacteria such as *Listeria monocytogenes* have never been reported. Other important species, such as *Salmonella* spp. or *Escherichia coli*, have been rarely reported by plating (Caparros Megido et al., 2017; Grabowski and Klein, 2017a; Osimani et al., 2017; Vandeweyer et al., 2017a). Despite not being natural reservoirs for well known food-borne bacteria, crickets might be contaminated during processing (i.e. during farming, packaging, cooking or serving). *Yersinia* spp., *Citrobacter* spp., *Fusobacterium* spp. and *Bacteroides* spp. have been documented in previous studies in crickets (Ulrich et al., 1981).

Crickets are a suitable environment for bacterial regrowth according to reported pH and water activity (a_w) values (Vandeweyer et al., 2017a, 2018). Therefore, light heat treatments, such as blanching (i.e. for 1 min), might reduce microbial counts, but does not prevent rapid spoilage when insect products are stored at room temperature, as the environmental conditions favour bacterial regrowth taking advantage of the high-water content, favourable pH and nutrient-rich environment (Klunder et al., 2012). Intensive blanching treatments (4 min) combined with a rapid cooling procedure appear to ensure compliance with TAC levels for minced meat (Klunder et al., 2012). Despite the obtained numbers, the authors recommend boiling for 10 min to ensure acceptable microbial loads. Grabowski and Klein (2017c) assessed microbial loads in differently thermally processed *A. domesticus* products. Intense heat-treated products (deep-fried, dried and extruded) were compliant with the thresholds for TAC and Enterobacteriaceae in minced meat suggested by some competent authorities. However, powdered and dried insect products would require additional thermal processing before consumption to comply with the same TAC values.

Temperature/time combinations provided up to now might be insufficient to destroy sporulated bacteria (ANSES, 2015). In crickets, sporulated bacteria are described in the range of 10^2–10^5 CFU/g, depending on the study and the product analysed (Osimani et al., 2017; Vandeweyer et al., 2017a). Similarly, other sporulated bacteria, such as *Bacillus cereus*, were detected in grasshoppers in 88% of samples tested (15 out of 17), in counts lower than 10^2 CFU/g (NVWA, 2014). *B. cereus* has also been identified in *A. domesticus* extruded products (Grabowski and Klein, 2017c). *Clostridium perfringens* and other sulfite-reducing clostridia have rarely been detected, or were found in low concentrations (10^2 CFU/g) (Osimani et al., 2017). The removal of indigenous microbiota, for example by short

blanching treatment, could render the food vulnerable to spore-forming bacteria, leaving them free to grow without competition. Some of the mentioned species such as *Clostridium* spp. and *Bacillus* spp. could produce thermally stable toxins. On the storage of crickets' products, Vandeweyer et al. (2018) observed that microbial loads remained stable in different processed *Gryllodes sigillatus* products on a 6-month survey.

The advent of DNA sequencing technologies, such as high-throughput sequencing (HTS), have allowed the study of microbial communities of reared insects. For example, Garofalo et al. (2017) identified a low abundance of reads, taxonomically assigned to *Clostridium* spp., *Staphylococcus* spp., *Listeria* spp. and *Bacillus* spp., previously not detected by culturable methods. High-throughput methodologies rely on the DNA present in a given sample. Therefore, non-viable or viable non-culturable bacteria (VBNC) can still be detected. The analysis of the 16S subunit of ribosomal RNA is an useful tool for taxonomic assignment up to the genus level, but lacks sensitivity to reach the species level (Poretsky et al., 2014). Vandeweyer et al. (2017b) used a similar approach by studying reared crickets from three different establishments, while assessing microbial loads by culturable methods. Interestingly, the different batches reared in the same facilities presented different bacterial loads. In general, it seems that crickets present a high microbial diversity with a high relative abundance of minority operational taxonomic units (OTUs) (Vandeweyer et al., 2017b, 2018). The different rearing companies presented different OTUs profiles, suggesting that microbial communities are dependent on rearing conditions and are highly influenced by dietary and environmental factors (e.g. manipulation by breeders, food and water microbiota).

Insects can act as vectors for antimicrobial resistance genes (AMR). Milanović et al. (2016) studied the presence of AMR genes by using polymerase chain reaction (PCR) or nested-polymerase chain reaction (n-PCR) in edible insects. Tetracycline resistance genes (*tet* (K), *tet* (M) and *tet* (O)) were detected in cricket samples. The study showed different principal coordinates analyses (PCA) for AMR profiles of the insects reared in Europe compared with ones reared in Thailand. These results might reflect the different selective pressure caused by sanitisers used on different rearing companies on microorganisms carried by edible insects. Finally, these results suggest the possibility of using insects as sentinels for AMR in the environment.

3.2. Fungi, mycotoxins, yeasts and moulds

Insects are affected by most species of fungi and their presence is subject to several influencing factors (Boomsma et al., 2014). Visible fungi have been documented by breeders in insect-farming facilities (FAO, 2013). The presence of visible fungi has been also reported in breeding experiments at the Swedish University of Agricultural Sciences (SLU) without involving any major mortality or incidence.

Caparros Megido et al. (2017) found that yeast and mould counts for crickets were above the Good Manufacturing Practice (GMP) limits for raw meat. However, the addition of a heat treatment such as blanching reduced the yeast and mould counts to acceptable GMP levels. Comparatively, reared *G. sigillatus* crickets presented fungi isolates from the genera *Aspergillus*, *Candida*, *Kodamaea*, *Lichtheimia*, *Tetrapisispora*, *Trichoderma* and *Trichosporon* (Vandeweyer et al., 2018). The use of the denaturing gel gradient electrophoresis (DGGE) technique for cricket powder and small crickets allowed the detection of several fungi from the genera *Aspergillus*, *Tetrapisispora*, *Eurotium* and *Wallemia*. Yeasts from the genus *Debaryomyces* were detected in the same study. Most of the reported fungi genera are commonly found in soil and water (Guarro, 2012), but some are also involved in sporadic invasive or superficial infections (Roussel et al., 2004; Hubka et al., 2012).

Some fungi, such as *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp., can produce mycotoxins that have serious consequences for human health (Bennett and Klich, 2003). Vandeweyer et al. (2018) isolated mycotoxin-forming fungi from *Aspergillus* spp. and *Penicillium* spp. from the feed, substrate and/or within *G. sigillatus*. Noteworthy, once mycotoxins are present they are difficult to remove as some of these are thermally stable (Magan and Olsen, 2004). Other fungi species of the genus *Eurotium*, reported by Grabowski and Klein, produce echinulin and neoechinulin, which have been suggested to be toxic for animals (Ali et al., 1989; Pitt and Hocking, 2009). The potential toxicity of mycotoxins for insects is uncertain. Surprisingly, some aphid species have been documented to transform and detoxify mycotoxins produced by *Fusarium* (i.e. trichothecene deoxynivalenol (DON); De Zutter et al., 2016). In a similar way, other insect species might have biochemical pathways to detoxify mycotoxins (Camenzuli et al., 2018). More research is needed to assess the presence of mycotoxin-producing fungi in edible insects and their possible detoxification by insects including *A. domesticus*.

3.3. Parasites

Considering the present knowledge, no human parasites found in reared crickets have been described in the literature. Recently, some scientists have hypothesised about the possibility that *Abbreviata antarctica*, a lizard parasite that might have crickets as intermediate host, could infect human causing under-reported cases due to lack of knowledge (King and Jones, 2016).

Insects might transfer parasitic cysts from faeces to foods, so acting as vectors. However, all infective stages of parasites are destroyed by suitable heat treatments (Doyle, 2003). For example, food control of *Toxoplasma gondii*, a well-known zoonotic parasite, involves cooking meat at a temperature of at least +66°C (Dubey et al., 1990), or freezing it at −12°C (Dubey, 1996). Although an unexplored field that would merit further research, it seems reasonable to classify parasites as a low risk hazard.

3.4. Viruses

Insects are susceptible to be infected by a huge diversity of virus species. However, limited data are available about insects' virome. Shi et al. (2016) explored the transcriptome of more than 220 invertebrates, including crickets. Based on the analysis of the RNA-dependent RNA polymerase (RdRp), they discovered 1,445 different RNA viruses, some of these were sufficiently divergent to be considered as new families. Some virus families that infect insects are shared with humans and are well known human pathogens (*Poxviridae*, *Parvoviridae*, *Picornaviridae*, *Orthomyxoviridae* and *Reoviridae*) (EFSA, 2015).

Virus infections are a main concern for insect farmers, as they might induce high mortality rates, leading to economic losses. The cricket paralysis virus (CrPV) of the *Dicistroviridae* family, and the cricket densovirus (AdDV) from the *Parvoviridae* family are considered two of the most important virus pathogens for crickets (Maciel-Vergara and Ros, 2017). These virus families contain human pathogens, raising the concern of their pathogenicity for humans if the viruses cross the species barrier. The scientific evidence for the inability of insect viruses to infect vertebrate cell lines in combination with the high evolutionary distance between host taxa suggest an unlikely threat for human health (El-Far et al., 2004). No major human food-borne viruses, such as noroviruses and hepatitis A and E viruses, have been reported in insects. Considering the long phylogenetic distance between humans and crickets, their replication in crickets seems unlikely. The lack of hygienic measures during insect rearing (e.g. soil, water or feed contaminated with faeces) could represent an entrance point of human virus particles into the food chain. Also, the possibility that insect-based products could be contaminated during processing or handling could not be discarded. The survival of food-borne viruses via the gut of the crickets represents an important data gap and should be addressed by future studies. Although out of the scope of the present risk profile, we cannot exclude the scenario of crickets acting as mechanical vectors, when exposed to contaminated environment or feed. Finally, no single arbovirus that could constitute a human health threat has been detected in crickets.

3.5. Prions

Prions have been one of the main concerns in animal health and food safety over the last decades. Prion codifying genes or gene orthologues have not been detected in insects, making crickets naturally prion free (Thackray et al., 2012). This situation implies that the amplification/replication of prion proteins is impossible within crickets. However, their role as mechanical vectors should not be discarded (Post et al., 1999). Prions are highly stable in the environment, persisting as infective for long periods in water and soil (Maluquer de Motes et al., 2008; Smith et al., 2011). This high stability suggests the possibility of remaining infective for humans if previously ingested by insects. Hence, it is important to control the quality of feed used for cricket rearing, as well as complying with the feed provisions laid down in Commission Regulation (EU) 1148/2014, amending Regulation (EU) 999/2001, to avoid the entrance of prions into the cricket food chain. Recently, the legislation has been amended, relaxing the feed bans on the use of insect processed animal proteins (PAPs) for aquaculture animals, via Regulation (EU) 893/2017. Taking the available data into consideration, we can conclude that prions do not represent an important cause of concern for the envisaged food system.

4. Chemical hazards

4.1. Heavy metals

Crickets, as other food products, may contain cadmium, arsenic, lead and tin, but few studies have evaluated their presence. The concentration of heavy metals in crickets is dependent on their presence in animal feed or soil pollutants. Heavy metals can be bioaccumulated or bioconjugated. According to Bednarska et al. (2015), crickets are more efficient in regulating their dietary exposure to zinc than to cadmium, suggesting that crickets tend to accumulate cadmium. This hypothesis is supported by other authors, using data from other species from the *Orthoptera* genus (Devkota and Schmidt, 2000; Vijver et al., 2003; Zhang et al., 2009). Studies analysing the concentration of mercury and its organic forms in insects or crickets intended for food consumption are rare. Insects have, however, been proposed as sentinels to monitor the level of contaminants in the environment (Ortiz et al., 2015). Using these studies, it has been suggested that mercury concentration in crickets is influenced by their dietary/environmental exposure (Zhang et al., 2009; Rimmer et al., 2010). According to reported data, it appears that, under a controlled rearing process, low risk exists for mercury bioaccumulation. For other metals, such as lead, a low bioaccumulation for grasshoppers was reported in comparison with mercury or cadmium (Devkota and Schmidt, 2000). Moreover, this study also suggested that cadmium was more easily absorbed due to its higher chemical activity compared to lead.

The concentrations of heavy metals in edible insects or insect-derived products have been explored by Poma et al. (2017), including cricket-derived products. Concentrations of all tested heavy metals (cadmium, arsenic, chromium, lead and tin) were within the acceptable levels for human consumption.

Data from heavy-metal bioaccumulation in other insect species are available. However, the extrapolation of these data to crickets could be inaccurate, as important different metabolic and physiological differences exist between insect species. Seasonal variations in metal concentrations, as well as differences due to developmental stages, might play a role in the bioaccumulation phenomenon (Janssen et al., 1993). The presence of heavy metals, such as arsenic, aluminium, cadmium, chromium and mercury, in edible insects used for human consumption merits further research. Based on the few available studies, the levels detected in insect foodstuffs are compliant with Regulation (EU) 1881/2006 for contaminants.

4.2. Toxins and antinutrients

Insects can contain naturally toxigenic compounds or antinutrients for humans. Toxigenic compounds can be synthesised as defensive mechanisms or accumulated during the rearing processes. No internal toxins for humans are described in crickets (EFSA, 2015). Koc et al. (2014) performed a genotoxicity study using the water-soluble extracts of commercially available mole crickets, *Gryllotalpa* spp. were cultured human blood cells, and used for the micronucleus test to monitor DNA and chromosomal damage. The study concluded that cricket extracts have no genotoxic effects at tested concentrations (0–2,000 ppm). Similarly, crickets do not have specific organs to produce toxic compounds (phanerotoxic), nor can they bioaccumulate toxins (cryptotoxic) (EFSA, 2015; van der Spiegel et al., 2013). No case of acute intoxication due to toxins in crickets has been reported (FASFC, 2014). Rat-based animal studies using cricket powder determined a no-observed-adverse-effect-level (NOAEL) dose over 5,000 mg/kg without any adverse effect in a 13-week oral toxicity study (Ryu et al., 2016). The results point out that crickets could be suitable as food from a toxicological point of view. Currently, there is no single antinutritive compound identified in crickets. It is possible that fractioned products of crickets could be enriched in antinutrients or toxicological compounds that might have been unnoticed and represent a health problem in the future. Data on the toxicity of edible crickets and insects are scarce, representing a data gap that should be explored.

4.3. Dioxins, organochloride compounds, flame retaining compounds, polycyclic aromatic hydrocarbons and other chemical compounds

The presence of dioxins (polychlorinated biphenyls (PCBs)) and dioxin-like (DL-PCBs) in insects is an unexplored field. Paine et al. (1993) studied the concentration of PCBs in reared crickets without direct contact with soil in a naturally PCB polluted environment. Results suggest that PCBs are quickly absorbed by crickets but not accumulated. Other studies suggest that the *Orthoptera* genus is less efficient in bioaccumulation of PCBs compared with *Coleoptera* (Blankenship et al., 2005). Poma et al.

(2017) tested insects and insect-derived products placed on the market for 12 different PCBs compounds. The concentration detected in cricket-derived products showed that they were within the safe margins of PCBs levels according to EU legislations. In the same study, the insecticide organophosphorus pirimiphos-methyl was detected. Its presence could be attributed to the composition of cricket-derived products tested that had a high vegetable content. Finally, the possibility that other chemical compounds [e.g. heterocyclic aromatic amines (HAAs), polycyclic aromatic hydrocarbons (PAHs), chloropropanols, furans or acrylamide] could be generated due to chemical reactions between insect compounds and other ingredients during processing should not be discarded. This possibility will merit further studies and represents a gap in the scientific data (van der Spiegel et al., 2013).

4.4. Allergies

According to the World Health Organisation and the International Union of Immunological Societies (www.allergen.org, last accessed 19/01/2018), there is no single allergen reported under the order *Orthoptera* (crickets). Specific food-borne allergies derived from cricket consumption have not been notified in Europe. Likewise, allergic reactions linked to *A. domesticus* are rarely reported in regions where cricket consumption is more common. Cross-reactivity allergic reactions among crickets with other arthropods has been suggested (Panzani and Ariano, 2001). Cross-reactivity is based on the existence of commonly conserved (glycol) proteins present in different species (pan-allergens). With an increasing consumption of insects, an increase in allergic reactions against arthropods is predicted (i.e. shrimp, crab) as crickets share high protein homologies with arthropods. For example, tropomyosin, which is a well-known allergen in crustacean, is also present in crickets. So, people who are allergic to crustaceans can be sensitive to crickets and, with repeated exposures, prone to develop an allergic reaction. In those sensitised individuals, the consumption of crickets could therefore trigger allergic reactions as if they were exposed to the original allergen animal (e.g. shrimps). There is documented cross-reactivity with other arthropods (such as shellfish), with world-wide estimated prevalence as high as 10% (Moonesinghe et al., 2016). Therefore, cricket and cricket-derived products should be labelled to ensure safety of consumers allergic to crustaceans or molluscs (FASFC, 2014). Similarly, other important pan-allergens such as arginine-kinase (AK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are present in crustaceans (i.e. shrimp) and insects (Chuang et al., 2010; Khanaruksombat et al., 2014). Hexamerin B1 has been identified as an specific allergen of *Gryllus bimaculatus* (field cricket) (Srinroch et al., 2015).

The presence of fungi from the genera *Aspergillus* and *Penicillium* in insects could trigger secondary allergic reactions (Schlüter et al., 2017). The presence of allergens in insects and insect-based food can be modulated by applied food processing treatments. As an example, heat treatments can alter the protein structure, so triggering or shutting down the allergenicity of specific compounds. This effect has been documented by Phiriyangkul et al. (2015) who described a change in the allergenic profile of a locust species, *Patanga succincta*, when consumed raw or processed (fried). Similarly, the allergenic profile of crickets could be substantially different depending on the food processing technique used.

5. Conclusions

According to available scientific data, viruses, prions, fungi and parasites should be considered as low risk hazards. High microbial load, spore-forming bacteria and its regrowth after heat treatment, heavy metals bioaccumulation (in special cadmium) and allergenicity of crickets are considered the medium hazards. More research is needed to evaluate the safety of crickets as food intended for human consumption to cover identified data gaps (e.g. mycotoxins or chemical compounds such as heavy metals or dioxins on edible crickets placed on the market).

Crickets as food show higher microbial loads compared with other food products. Therefore, specific hygiene and safety criteria values for insects, including crickets, should be developed.

Commonly detected food-borne pathogenic bacteria, such as *L. monocytogenes or Salmonella* spp., have never been or are rarely reported in crickets intended for human consumption. Still, the use of HTS technologies has allowed the description of the cricket's microbiota and detected sequences taxonomically classified to the genera *Clostridium* spp., *Listeria* spp. and *Bacillus* spp., which contain relevant food-borne pathogens.

Thermal treatments, such as blanching, boiling or frying, can decrease microbial loads in edible insects. A mandatory thermal process for crickets or cricket-derived products should be implemented before product placement on the market. Furthermore, boiling before consumption could be advisable to ensure microbial loads that comply with both hygiene and food safety standards. However, such treatments may not be enough to kill spores from *Bacillus* spp. and *Clostridium* spp.

Heavy metals have been identified as putative chemical hazards when crickets are exposed to these during the rearing stage. Among heavy metals, cadmium bioaccumulation has been identified as a major concern. Available information on other heavy metals, such as aluminium, chromium and arsenic, is scarce and more data are required.

Crickets can trigger allergic reactions in sensitive consumers (e.g. prawns, crabs, lobsters). Homologue proteins shared between different species can trigger pan-allergic reactions. Tropomyosin, AK or GAPDH have been identified as a highly allergenic. Hexamerin B1, whose allergenic potential requires more research, has been described as a specific cricket allergen. For safety reasons, crickets and cricket-derived food products should be labelled to raise awareness in susceptible consumers (Table 2).

Table 1: Microbial loads reported for crickets reared as food, feed and cricket powder. Results are expressed in CFU/g. NT not tested; NEG: negative or under the limit of detection for the technique and microorganism

Product	Total aerobic counts	Enterobacteriaceae	Aerobic bacterial endospores	Moulds	Yeast	Reference
Whole crickets	3.16 × 108	1.6 × 107	5.01 × 103	4.0 × 105	4.0 × 105	Vandeweyer et al. (2018)[(a)]
Whole crickets	2.1 × 104	NEG	NT	NEG	7.9 × 104	Garofalo et al. (2017)[(c)]
Cricket powder	3.6 × 104	NEG	NT	1.1 × 103	NEG	
Whole crickets	1.59 × 104	NEG	3.98 × 103	NEG	NEG	Osimani et al. (2017)
Cricket powder	1.00 × 105	1.26 × 103	1.26 × 105	1 × 102	2.00 × 103	
Whole crickets	2.1 × 108	5.5 × 107	6.6 × 103	2.6 × 106	2.6 × 106	Vandeweyer et al. (2017a)[(a)]
Whole crickets	3.16 × 107	1 × 107	3.16 × 103	NEG	NEG	Grabowski and Klein (2017a,b,c)[(a),(b)]
Dead whole crickets	5.01 × 107	5.01 × 106	NEG	2.51 × 105	2.51 × 105	
Whole crickets	8.91 × 107	NT	NT	6.31 × 104	6.31 × 104	Caparros Megido et al. (2017)[(a)]
Cricket powder	8.2 × 104	NT	NT	NT	NT	Milanović et al. (2016)
Whole crickets	1.4 × 104	NT	NT	NT	NT	
Whole crickets	1.59 × 107	1.59 × 104	3.98 × 103	NT	NT	Klunder et al. (2012)

CFU: colony forming unit.
(a): Moulds and yeasts are cultured by using the same assay.
(b): Crickets intended for pet consumption. Crickets were already dead in the rearing facilities.
(c): Insects were crushed but not blended. Insects were boiled, dried and then sold.

Table 2: Microbial loads reported for thermally treated crickets reared as food or feed). Results are expressed in CFU/g. NT not tested; NEG: negative or under the limit of detection for the technique and microorganism

Thermal treatment	Total aerobic counts	*Enterobacteriaceae*	Aerobic bacterial endospores	Moulds	Yeast	Reference
Boiled	3.98×10^2	3.1×10^1	2.51×10^2	NEG	NEG	Vandeweyer et al. (2018)[(a)]
Frozen	2.51×10^2	NEG	1.0×10^2	NEG	NEG	
Oven dried	1.99×10^4	NEG	2.51×10^2	NEG	NEG	
Smoked and dried	7.94×10^7	NEG	2.51×10^3	NEG	NEG	
Blanching (4 min)	2.46×10^4	NT	NT	NEG	NEG	Caparros Megido et al. (2017)
Sterilised (16 min – 120°C)	5.50×10^3	NT	NT	NEG	NEG	
Freeze dried	1.12×10^4	NT	NT	NEG	NEG	
Boiled (5 min)	5.01×10^1	NEG	3.16×10^1	NT	NT	Klunder et al. (2012)
Stir fried (5 min)	5.01×10^2	NEG	3.16×10^1	NT	NT	

CFU: colony forming unit.
(a): Moulds and yeasts are cultured by using the same assay.

References

ACFS (Thailand's National Bureau of Agricultural Commodity and Food Standards), 2017. Good Agricultural Farming Practices, guidance on the application of Thai agricultural standard TAS 8202(g)-2017. Available online: http://www.acfs.go.th/standard/download/GUIDANCE_GAP_CRICKET_FARM.pdf

Ali M, Mohammed N, Alnaqeeb MA, Hassan RAH and Ahmad HSA, 1989. Toxicity of echinulin from *Aspergillus chevalieri* in rabbits. Toxicology Letters, 48, 235–241.

ANSES (French Agency for Food, Environmental and Occupational Health and Safety), 2015. Opinion on the use of insects as food and feed and the review of scientific knowledge on the health risks related to the consumption of insects. Available online: https://www.anses.fr/en/documents/BIORISK2014sa0153EN.pdf

Bednarska AJ, Opyd M, Żurawicz E and Laskowski R, 2015. Regulation of body metal concentrations: Toxicokinetics of cadmium and zinc in crickets. Ecotoxicology and Environmental Safety, 119, 9–14.

Bennett JW and Klich M, 2003. Mycotoxins. Clinical Microbiology Reviews, 16, 497–516.

Blankenship AL, Zwiernik MJ, Coady KK, Kay DP, Newsted JL, Strause K, Park C, Bradley PW, Neigh AM, Millsap SD, Jones PD and Giesy JP, 2005. Differential accumulation of polychlorinated biphenyl congeners in the terrestrial food web of the Kalamazoo River Superfund site, Michigan. Environmental Science and Technology, 39, 5954–5963.

Boomsma JJ, Jensen AB, Meyling NV and Eilenberg J, 2014. Evolutionary interaction networks of insect pathogenic fungi. Annual Review of Entomology, 59, 467–485.

Camenzuli L, Van Dam R, de Rijk T, Andriessen R, Van Schelt J and Van der Fels-Klerx H, 2018. Tolerance and excretion of the mycotoxins aflatoxin B1, zearalenone, deoxynivalenol, and ochratoxin A by *Alphitobius diaperinus* and *Hermetia illucens* from contaminated substrates. Toxins, 10, 91.

Caparros Megido R, Desmedt S, Blecker C, Béra F, Haubruge É, Alabi T and Francis F, 2017. Microbiological load of edible insects found in Belgium. Insects, 8, 12.

Cazemier AE, Hackstein JHP, den Camp HJMO, Rosenberg J and van der Drift C, 1997. Bacteria in the intestinal tract of different species of arthropods. Microbial Ecology, 33, 189–197.

Chuang JG, Su SN, Chiang BL, Lee HJ and Chow LP, 2010. Proteome mining for novel IgE-binding proteins from the German cockroach (*Blattella germanica*) and allergen profiling of patients. Proteomics, 10, 3854–3867.

De Zutter N, Audenaert K, Arroyo-Manzanares N, De Boevre M, Van Poucke C, De Saeger S, Haesaert G and Smagghe G, 2016. Aphids transform and detoxify the mycotoxin deoxynivalenol via a type II biotransformation mechanism yet unknown in animals. Scientific Reports, 6, 38640.

Devkota B and Schmidt GH, 2000. Accumulation of heavy metals in food plants and grasshoppers from the Taigetos Mountains, Greece. Agriculture, Ecosystems and Environment, 78, 85–91.

Doyle ME, 2003. Foodborne Parasites A Review of the Scientific Literature Review. Wisconsin.

Dubey JP, 1996. Strategies to reduce transmission of *Toxoplasma gondii* to animals and humans. Veterinary Parasitology, 64, 65–70.

Dubey JP, Kotula AW, Sharar A, Andrews CD and Lindsay DS, 1990. Effect of high temperature on infectivity of Toxoplasma gondii tissue cysts in pork. Journal of Parasitology, 76, 201–204.

EFSA Biohaz Panel (EFSA Panel on Biological Hazards), 2012. Scientific opinion on the development of a risk ranking framework on biological hazards. EFSA Journal, 2012;10(6):2724, 88 pp. https://doi.org/10.2903/j.efsa.2012.2724

EFSA Scientific Committee, 2015. Risk profile related to production and consumption of insects as food and feed. EFSA Journal, 2015;13(10):4257, 60 pp. https://doi.org/10.2903/j.efsa.2015.4257

El-Far M, Li Y, Fédière G, Abol-Ela S and Tijssen P, 2004. Lack of infection of vertebrate cells by the densovirus from the maize worm *Mythimna loreyi* (MlDNV). Virus Research, 99, 17–24.

FAO (Food and Agriculture Organization of the United Nations) – High Level Expert Forum, 2009. How to feed the world 2050 – global agriculture towards 2050. High Level Expert Forum – How to feed the world 2050, pp. 1–4.

FAO (Food and Agriculture Organization of the United Nations), 2013. Edible insects. Future prospects for food and feed security. Vol. 171.

FASFC (Belgian Scientific Committee of the Federal Agency for the Safety of the Food Chain), 2014. Food safety aspects of insects intended for human consumption. Common advice of the Belgian Scientific Committee of the Federal Agency for the Safety of the Food Chain (FASFC) and of the Superior Health Council (SHC). Available online: http://www.favv-afsca.fgov.be/scientificcommittee/advices/_documents/ADVICE14–2014_ENG_DOSSIER2014–04.pdf

Fernandez-Cassi X, Supenu Alexandru S, Vaga M, Jansson A, Boqvist S and Vagsholm I. Risk profiling the house cricket (*Acheta domesticus*) as a novel food. International Journal of Insects as Food and Feed. Submitted.

Finke MD, Rojo S, Roos N, van Huis A and Yen AL, 2015. The European Food Safety Authority scientific opinion on a risk profile related to production and consumption of insects as food and feed. Journal of Insects as Food and Feed, 1, 245–247.

Garofalo C, Osimani A, Milanović V, Taccari M, Cardinali F, Aquilanti L, Riolo P, Ruschioni S, Isidoro N and Clementi F, 2017. The microbiota of marketed processed edible insects as revealed by high-throughput sequencing. Food Microbiology, 62, 15–22.

Grabowski NT and Klein G, 2017a. Microbiology of cooked and dried edible Mediterranean field crickets (*Gryllus bimaculatus*) and superworms (*Zophobas atratus*) submitted to four different heating treatments. Food Science and Technology International, 23, 17–23.
Grabowski NT and Klein G, 2017b. Microbiology of processed edible insect products – results of a preliminary survey. International Journal of Food Microbiology, 243, 103–107.
Grabowski NT and Klein G, 2017c. Microbiological analysis of raw edible insects. Journal of Insects as Food and Feed, 3, 7–14.
Guarro J, 2012. Atlas of soil ascomycetes. CBS-KNAW Fungal Biodiversity Centre.
House J, 2016. Consumer acceptance of insect-based foods in the Netherlands: Academic and commercial implications. Appetite, 107, 47–58.
Hubka V, Kubatova A, Mallatova N, Sedlacek P, Melichar J, Skorepova M, Mencl K, Lyskova P, Sramkova B, Chudickova M, Hamal P and Kolarik M, 2012. Rare and new etiological agents revealed among 178 clinical *Aspergillus* strains obtained from Czech patients and characterized by molecular sequencing. Medical Mycology, 50, 601–610.
van Huis A, 2018. Insects as human food. Ethnozoology. Elsevier, pp. 195–213.
Janssen MPM, Ma WC and Van Straalen NM, 1993. Biomagnification of metals in terrestrial ecosystems. Science of The Total Environment, 134, 511–524.
Jongema Y, 2017. List of edible insects of the world, 2017. Laboratory of Entomology, Wageningen University, Wageningen, the Netherlands. Available online: https://www.wur.nl/upload_mm/8/a/6/0fdfc700–3929–4a74–8b69-f02fd35a1696_Worldwide%20list%20of%20edible%20insects%202017.pdf. [Accessed 1 April 2018.]
Khanaruksombat S, Srisomsap C, Chokchaichamnankit D, Punyarit P and Phiriyangkul P, 2014. Identification of a novel allergen from muscle and various organs in banana shrimp (*Fenneropenaeus merguiensis*). Annals of Allergy, Asthma & Immunology, 113, 301–306.
King C and Jones HI, 2016. The life cycle of the reptile-inhabiting nematode *Abbreviata hastaspicula* (Spirurida: Physalopteridae: Physalopterinae) in Australia. International Journal for Parasitology: Parasites and Wildlife, 5, 258–262.
Klunder HC, Wolkers-Rooijackers J, Korpela JM and Nout MJR, 2012. Microbiological aspects of processing and storage of edible insects. Food Control, 26, 628–631.
Koc K, Incekara U and Turkez H, 2014. Biomonitoring of the genotoxic effects and oxidative potentials of commercial edible dung beetles (*Onitis* sp.), grasshopper (*Caelifera* sp.) and mole crickets (*Gryllotalpa* sp.) *in vitro*. Toxicology and Industrial Health, 30, 683–689.
Maciel-Vergara G and Ros VID, 2017. Viruses of insects reared for food and feed. Journal of Invertebrate Pathology, 147, 60–75.
Magan N and Olsen M, 2004. Mycotoxins in food : detection and control. CRC Press.
Maluquer de Motes C, Cano MJ, Torres JM, Pumarola M and Girones R, 2008. Detection and survival of prion agents in aquatic environments. Water Research, 42, 2465–2472.
Milanović V, Osimani A, Pasquini M, Aquilanti L, Garofalo C, Taccari M, Cardinali F, Riolo P and Clementi F, 2016. Getting insight into the prevalence of antibiotic resistance genes in specimens of marketed edible insects. International Journal of Food Microbiology, 227, 22–28.
Moonesinghe H, Mackenzie H, Venter C, Kilburn S, Turner P, Weir K and Dean T, 2016. Prevalence of fish and shellfish allergy. Annals of Allergy, Asthma & Immunology, 117, 264–272.e4
NVWA (Netherlands Food and Consumer Product Safety Authority), 2014. Advisory report on the risks associated with the consumption of mass-reared insects. Available online: http://www.nvwa.nl/actueel/risicobeoordelingen/bestand/2207475/consumptie-gekweekte-insecten-advies-buro
Oonincx DGAB and de Boer IJM, 2012. environmental impact of the production of mealworms as a protein source for humans – a life cycle assessment. PLoS ONE, 7, e51145.
Oonincx DGAB, van Itterbeeck J, Heetkamp MJW, van den Brand H, van Loon JJA and van Huis A, 2010. An exploration on greenhouse gas and ammonia production by insect species suitable for animal or human consumption. PLoS ONE, 5, e14445.
Ortiz C, Weiss-Penzias PS, Fork S and Flegal AR, 2015. Total and monomethyl mercury in terrestrial arthropods from the central California coast. Bulletin of Environmental Contamination and Toxicology, 94, 425–430.
Osimani A, Garofalo C, Milanović V, Taccari M, Cardinali F, Aquilanti L, Pasquini M, Mozzon M, Raffaelli N, Ruschioni S, Riolo P, Isidoro N and Clementi F, 2017. Insight into the proximate composition and microbial diversity of edible insects marketed in the European Union. European Food Research and Technology, 243, 1157–1171.
Paine JM, McKee MJ and Ryan ME, 1993. Toxicity and bioaccumulation of soil PCBs in crickets: Comparison of laboratory and field studies. Environmental Toxicology and Chemistry, 12, 2097–2103.
Panzani RC and Ariano R, 2001. Arthropods and invertebrates allergy (with the exclusion of mites): the concept of panallergy. Allergy, 56(Suppl 6), 1–22.
Paoletti MG, 2005. Ecological implications of minilivestock : potential of insects, rodents, frogs, and snails. Science Publishers.
Persistence Market Research, 2018. Edible insects market: global analysis, size, share, value, demand, market growth By 2024. Available online: https://www.persistencemarketresearch.com/market-research/edible-insects-market.asp (Accessed: 1 March 2018).

Phiriyangkul P, Srinroch C, Srisomsap C, Chokchaichamnankit D and Punyarit P, 2015. Effect of food thermal processing on allergenicity proteins in Bombay locust (*Patanga succincta*). ETP International Journal of Food Engineering, 1, 23–28.
Pitt JI and Hocking AD, 2009. Fungi and food spoilage. Springer-Verlag.
Poma G, Cuykx M, Amato E, Calaprice C, Focant JF and Covaci A, 2017. Evaluation of hazardous chemicals in edible insects and insect-based food intended for human consumption. Food and Chemical Toxicology, 100, 70–79.
Poretsky R, Rodriguez-R LM, Luo C, Tsementzi D and Konstantinidis KT, 2014. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS ONE, 9, e93827.
Post K, Riesner D, Walldorf V and Mehlhorn H, 1999. Fly larvae and pupae as vectors for scrapie. The Lancet, 354, 1969–1970.
Rimmer CC, Miller EK, McFarland KP, Taylor RJ and Faccio SD, 2010. Mercury bioaccumulation and trophic transfer in the terrestrial food web of a montane forest. Ecotoxicology, 19, 697–709.
Roussel S, Reboux G, Dalphin JC, Bardonnet K, Millon L and Piarroux R, 2004. Microbiological evolution of hay and relapse in patients with farmer's lung. Occupational and Environmental Medicine, 61, e3.
Ryu HY, Lee S, Ahn KS, Kim HJ, Lee SS, Ko HJ, Lee JK, Cho MH, Ahn MY, Kim EM, Lim JH and Song KS, 2016. Oral toxicity study and skin sensitization test of a cricket. Toxicological Research, 32, 159–173.
Schafer B, Bandick N, Epp A, Hirsch-Ernst KI, Pucher J, Schumann R, Spolders M, Wagner B and Lampen A, 2016. BfR Symposium 'Insects as food or feed: Food for the future?' Report on the Symposium on 24 May 2016. BfR Symposium 'Insekten als Lebens- oder Futtermittel: Nahrung der Zukunft?' Bericht zum Symposium am 24 Mai 2016.
Schlüter O, Rumpold B, Holzhauser T, Roth A, Vogel RF, Quasigroch W, Vogel S, Heinz V, Jäger H, Bandick N, Kulling S, Knorr D, Steinberg P and Engel KH, 2017. Safety aspects of the production of foods and food ingredients from insects. Molecular Nutrition & Food Research, 61, 1600520.
Shi M, Lin XD, Tian JH, Chen LJ, Chen X, Li CX, Qin XC, Li J, Cao JP, Eden JS, Buchmann J, Wang W, Xu J, Holmes EC and Zhang YZ, 2016. Redefining the invertebrate RNA virosphere. Nature, 540, 539–543.
Smith CB, Booth CJ and Pedersen JA, 2011. Fate of prions in soil: a review. Journal of Environmental Quality, 40, 449–461.
van der Spiegel M, Noordam MY and van der Fels-Klerx HJ, 2013. Safety of Novel Protein Sources (Insects, Microalgae, Seaweed, Duckweed, and Rapeseed) and legislative aspects for their application in food and feed production. Comprehensive Reviews in Food Science and Food Safety, 12, 662–678.
Srinroch C, Srisomsap C, Chokchaichamnankit D, Punyarit P and Phiriyangkul P, 2015. Identification of novel allergen in edible insect, *Gryllus bimaculatus* and its cross-reactivity with *Macrobrachium* spp. allergens. Food Chemistry, 184, 160–166.
Stork NE, McBroom J, Gely C and Hamilton AJ, 2015. New approaches narrow global species estimates for beetles, insects, and terrestrial arthropods. Proceedings of the National Academy of Sciences of the United States of America, 112, 7519–7523.
TECA, 2013. Cricket Farming for Human Consumption. Available online: http://teca.fao.org/read/7927
Thackray AM, Muhammad F, Zhang C, Denyer M, Spiropoulos J, Crowther DC and Bujdoso R, 2012. Prion-induced toxicity in PrP transgenic *Drosophila*. Experimental and Molecular Pathology, 92, 194–201.
Ulrich RG, Buthala DA and Klug MJ, 1981. Microbiota associated with the gastrointestinal tract of the common house cricket, *Acheta domestica*. Applied and Environmental Microbiology, 41, 246–254.
Vandeweyer D, Crauwels S, Lievens B and Van Campenhout L, 2017a. Microbial counts of mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different rearing companies and different production batches. International Journal of Food Microbiology, 242, 13–18.
Vandeweyer D, Crauwels S, Lievens B and Van Campenhout L, 2017b. Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies. International Journal of Food Microbiology, 261, 11–18.
Vandeweyer D, Wynants E, Crauwels S, Verreth C, Viaene N, Claes J, Lievens B and Van Campenhout L, 2018. Microbial dynamics during industrial rearing, processing, and storage of the tropical house cricket (*Gryllodes sigillatus*) for human consumption. Applied and Environmental Microbiology, 84, AEM.00255–18. https://doi.org/10.1128/aem.00255-18
Vijver M, Jäger T, Posthuma L and Peijnenburg W, 2003. Metal uptake from soils and soil–sediment mixtures by larvae of *Tenebrio molitor* (L.) (Coleoptera). Ecotoxicology and Environmental Safety, 54, 277–289.
Wang D, Bai Y-Y, Li J-H and Zhang C-X, 2004. Nutritional value of the field cricket (*Gryllus testaceus* Walker). Insect Science, 11, 275–283.
Zhang ZS, Lu XG, Wang QC and Zheng DM, 2009. Mercury, cadmium and lead biogeochemistry in the soil–plant–insect system in Huludao city. Bulletin of Environmental Contamination and Toxicology, 83, 255–259.

Abbreviations

AdDV	cricket densovirus
AK	arginine-kinase
AMR	antimicrobial resistance genes

ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (France)
a_w	water activity
cfu	colony forming unit
CrPV	Cricket paralysis virus
DGGE	denaturing gel gradient electrophoresis
DL-PCBs	dioxin-like polychlorinated biphenyls
DON	deoxynivalenol
FASFC	Federal Agency for the Safety of the Food Chain (Belgium)
FAO	Food and Agricultural Organization
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	good farming practices
GHG	greenhouse gases
GMP	good manufacturing practice
HAAs	heterocyclic aromatic amines
HACCP	Hazard Analysis and Critical Control Points
HTS	high-throughput Sequencing
NOAEL	no-observed-adverse-effect-level
n-PCR	nested polymerase chain reaction
NVWA	Nederlandse Voedsel- en Warenautoriteit (Netherlands)
OTUs	operational taxonomic units
PAH	polycyclic aromatic hydrocarbons
PAPs	processed animal proteins
PCA	principal coordinates analysis
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
SLU	Swedish University of Agricultural Sciences
TAC	total aerobic counts
VBNC	non-viable or viable non-culturable bacteria

EU-FORA SERIES 1

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16083

Risk assessment of substances used in food supplements: the example of the botanical *Gymnema sylvestre*

German Federal Institute for Risk Assessment (BfR), Germany
G Marakis, R Ziegenhagen, A Lampen and KI Hirsch-Ernst

Abstract

Botanicals and preparations derived from these are among the substances frequently added to foods and food supplements, yet the safety of many botanicals has not been systematically assessed. In the context of the EU-FORA fellowship programme, the fellow performed an assessment on the safety of the botanical *Gymnema sylvestre,* in accordance with EFSA's guidance on the assessment of safety of botanicals. Although preparations of *G. sylvestre* are marketed as food supplements, they may appeal to people who are suffering from metabolic syndrome and/or diabetes mellitus. A scientific literature search was carried out using PubMed/MEDLINE and EMBASE electronic databases. Experience was gained by the fellow in systematic data extraction from scientific publications, structuring of the data and evaluating toxicological key parameters, outcomes of clinical significance, pharmacokinetic and pharmacodynamic interactions, uncertainties and methodological shortcomings of studies. Limited evidence from toxicological *in vivo* studies and human clinical studies suggested lack of relevant adverse effects of this botanical. However, human studies provided some indications that certain *Gymnema* extracts may enhance the glucose-lowering effects of certain antidiabetic drugs. Considering the uncertainties for the composition of different *Gymnema* preparations, potential herb–drug interactions and the indications of glucose lowering or hypoglycaemic effects, the use of *Gymnema*-based food supplements in combination with authorised antidiabetic drugs may be associated with risks. The procedures learned for the safety evaluation of *Gymnema* may be similarly applied by the fellow for the risk assessment of other substances with nutritional or physiological effect added to foods and food supplements. Furthermore, apart from learning by conducting exercises in risk assessment, the fellow was able to develop other skills (e.g. communication skills), diversify his competencies and expand his network of scientific connections for future collaborations in the field of nutritional risk assessment.

Keywords: *Gymnema sylvestre*, botanical, food supplement, risk assessment

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: German Federal Institute for Risk Assessment (BfR), Germany, Marakis G, Ziegenhagen R, Lampen A and Hirsch-Ernst KI, 2018. Risk assessment of substances used in food supplements: the example of the botanical *Gymnema sylvestre*. EFSA Journal 2018;16(8):e16083, 10 pp. https://doi.org/10.2903/j.efsa.2018.e16083

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

Directive 2002/46/EC and Regulation (EC) 1925/2006 regulate the addition to food supplements or foods of (a) vitamins and minerals; and (b) 'other substances with a nutritional or physiological effect' (Directive 2002/46/EC) or 'other substances' (Regulation (EC) 1925/2006), respectively. Currently, the European Union (EU) legislation only lays down which vitamins and minerals may be added to food supplements or foods and which vitamin/mineral substances may be used; daily maximum amounts for vitamins and minerals have not been established. For 'other substances with a nutritional or physiological effect' or 'other substances', there are currently no specific provisions as to the substances (with the exception of two substances) which may be used in food supplements or foods. In addition, there are no provisions for daily maximum amounts in single products (food supplements or fortified foods) for individual substances.

Substances with nutritional or physiological effects that are frequently added to foods and food supplements include, among others, amino acids, essential fatty acids or certain botanicals or preparations derived from these. Food supplements and fortified foods containing botanicals have gained a substantial and constantly growing market share across Europe (Restani et al., 2018). The reasons for their increasing availability and use by consumers are complex, but the assumption that 'natural' can be equated with 'safe' is deemed to be a major factor. Nevertheless, herbs and/or herbal extracts may contain active ingredients that might be associated with harmful health effects. Examples of botanicals that have been shown or suspected to pose risks to human health include '*Ephedra* herb and its preparations originating from *Ephedra* species', which are now prohibited in foods according to Commission Regulation (EU) 2015/403 (Part A, Annex III of the Regulation (EC) No 1925/2006) and 'Yohimbe bark and its preparations originating from Yohimbe (*Pausinystalia yohimbe* (K. Schum) Pierre ex Beille)', which has been placed under European Union scrutiny, pending a decision on whether or not to allow the use of the substance in foods (Part C, Annex III of the Regulation (EC) No 1925/2006). In many cases, safety aspects of botanicals used in food supplements for human health have been insufficiently evaluated and have not been covered adequately.

The plant *Gymnema sylvestre* and botanical preparations from these have had a long tradition of use, mainly in the Ayurvedic system of medicine, for a range of health ailments. While *Gymnema* has been primarily used with the intention of lowering raised blood glucose levels and ameliorating other co-morbid metabolic disorders such as dyslipidaemia, it has also been claimed to exhibit a wide range of other therapeutic effects, most of which lack sufficient scientific evidence (among others, antiarthritic, immunostimulatory, antimicrobial, hepatoprotective or anticancer effects) (Tiwari et al., 2014). *Gymnema* preparations have not been approved as drugs in Europe but are marketed as ingredients of certain food supplements, either alone or in combination with other herbs and/or micronutrients. On account of its use in the Ayurvedic system of medicine as an anticipated remedy for diabetes, food supplements containing *Gymnema* preparations may appeal to people who display one or more symptoms of metabolic syndrome. As a food supplement, it is used without medical supervision but it is conceivable that *Gymnema*-based food supplements may also be consumed instead of or in combination with antidiabetic drugs.

The risk assessment of *G. sylvestre* and preparations from these was performed in the context of the EFSA EU-FORA fellowship programme. This programme offers motivated professionals from EU national risk assessment authorities or other Article 36 organisations the opportunity to increase their knowledge and experience in food safety risk assessment (Bronzwaer et al., 2016). The aim of this programme is to contribute towards expanding the EU's community of scientists working in the field of risk assessment and at the same time enhance cooperation among Europe's food safety agencies as well as between them and EFSA. The fellow, whose home institution is the Hellenic Food Authority (EFET), Nutrition Policy and Research Directorate, was hosted by the German Federal Institute for Risk Assessment (BfR), Department of Food Safety, Unit of Nutritional Risks, Novel Foods and Allergies. The task assigned to the EU-FORA fellow was the preparation of a monograph for the risk assessment for *G. sylvestre* and preparations from these, under the guidance of unit members.

2. Description of work programme

2.1. Aims

The primary aim of the work programme was to become acquainted with the general aspects of risk assessment and risk communication as well as to gain experience specifically in the risk

assessment of botanicals (i.e. *G. sylvestre*) and other substances used in food supplements and fortified foods. The general methodology applied for the risk assessment of the chosen botanical should be suitable for application by the fellow also for the risk assessment of other substances with nutritional or physiological effect added to foods and food supplements. A further aim of the programme was to build professional connections with other colleagues in nutritional risk assessment (Bronzwaer et al., 2016), which can be expected to provide a supportive resource long after the completion of the fellowship, through exchange of views or common projects between the fellow's hosting site and the fellow's home institute.

2.2. Activities/methods

2.2.1. Preparation of a monograph for the risk assessment of *Gymnema sylvestre*

The selection of the botanical *G. sylvestre* was discussed with members of the BfR, also considering suggestions for botanicals that are currently being marketed as food supplements and for which a detailed safety assessment was deemed to be of importance. To avoid duplicating existing work, a prerequisite for the selection of this botanical was that its safety had not been previously evaluated by scientific bodies, international organisations (such as EFSA or the European Medicines Agency) or national authorities (such as BfR or the EFET). In the EFSA Compendium of Botanicals (EFSA, 2012), *G. sylvestre* (leaves) was listed in Annex A ('insufficient information' list), which includes botanicals appearing on a negative list or subject to restricted use in at least one European Member State but for which not enough information on possible substances of concern or adverse effects could be found, or for which the information present could not be verified. It is worth mentioning that this compendium does not address possible interactions between botanical substances or other products (e.g. allopathic medication) that would need to be taken into account when assessing safety, as described in the EFSA Guidance for the safety assessment of botanicals and botanical preparations (EFSA, 2012). Therefore, *Gymnema* preparations were selected as an example for risk assessment of substances used in food supplements. If *Gymnema* preparations are to be considered as a novel food or may be classified as pharmaceuticals was not addressed. (Such questions lie within the remit of the relevant authorities.)

The methodology that was followed for the preparation of the monograph of the *Gymnema/ Gymnema* preparations was in accordance with the EFSA *Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements* (EFSA Scientific Committee, 2009). As specified in the EFSA Guidance, the scope of the present risk assessment does not address hazards linked to the presence of contaminants and food-borne pathogens in the botanical and the preparations from these.

Electronic literature searches were conducted to identify relevant scientific articles on *G. sylvestre*. Two of the most important scientific databases of references and abstracts on life sciences and biomedical topics, PubMed/MEDLINE and EMBASE, were systematically searched. Additional information from relevant webpages from Health Agencies/Bodies was sought as well as references from identified review articles. No restrictions on language or time of publication were imposed. Special attention was paid to identify and include in the assessment relevant scientific articles with common indigenous names as well as synonyms of the botanical. When common indigenous names (e.g. gurmar) were used in the search engine, special attention was paid that the correct species was identified.

A tiered approach was used for the identification of relevant scientific articles. An initial screening was conducted of the article titles identified via the two electronic scientific databases. Following this, the abstracts of potentially relevant articles were scrutinised. The complete manuscripts of the articles that appeared useful and necessary for the risk assessment of the botanical were retrieved for further detailed and thorough evaluation. The studies that were identified as relevant included: (a) those on chemical composition of the botanical/botanical preparations, the nature of extracts and bioavailability of their active constituents; (b) human intervention studies in healthy subjects as well as in people with disease; (c) acute, subchronic and chronic toxicological animal studies as well as animal studies investigating specific health effects of oral *Gymnema* administration; and (d) studies on potential pharmacokinetic and pharmacodynamic interactions between the botanical and allopathic drugs (especially antidiabetic drugs). Limited *in vitro* studies were also retrieved to understand better possible mechanisms related to health or safety aspects. Systematic reviews were also used to identify other scientific articles that were not included in the electronic databases used.

All identified human intervention studies on *G. sylvestre* were retrieved and evaluated. For safety aspects, human studies can be categorised in one of the three types: (1) studies in which no information is given for the occurrence or absence of adverse effects; (2) studies that report only the absence of adverse effects, without providing any further details; and (3) studies containing more detailed information on the occurrence of adverse effects in verum and control groups. For risk assessment purposes, studies that do not report on the occurrence or absence of adverse effects cannot be taken as proof that no adverse events occurred.

To facilitate systematic data extraction from scientific publications and the structuring of scientific data, tables were drawn for the selected human and animal studies. The tables contained information for the population groups (number of enrolled subjects, health status and concomitant use of antidiabetic medication), the type of *Gymnema* preparation (parts of the herb used, nature of extract and any other information for the standardisation of the extract), daily dosage, duration of the intervention, as well as safety laboratory parameters, clinical outcomes and information on absence/occurrence of adverse effects.

The focus was laid primarily on studies examining the effects of preparations of *G. sylvestre* alone, in healthy humans, as food supplements are intended mainly to complement one's diet and not to treat or cure diseases. However, *G. sylvestre* is marketed as a supplement that is purported to favourably affect blood glucose levels. As such, human intervention studies in which *Gymnema* preparations were administered to those suffering from glucose intolerance and diabetes mellitus (both insulin dependent and non-insulin dependent), with or without concomitant administration of antidiabetic drugs, were also evaluated for the safety of the preparations (e.g. Baskaran et al., 1990; Shanmugasundaram et al., 1990b; Al-Romaiyan et al., 2010; Zuniga et al., 2017). Furthermore, studies on the effects of formulas containing *G. sylvestre* in combination with other herbs and/or micronutrients were also studied for potential toxicity (e.g. Kurian et al., 2014; Mahajan et al., 2015). Information on sensitive population groups such as children, pregnant/lactating women, etc., was sought separately.

Randomised, double-blinded, placebo-controlled human intervention studies employing a sufficient number of participants that investigate not only the efficacy of the preparation but also potential toxic effects are usually the first line of choice. Due to the lack of these types of studies in the scientific literature for *G. sylvestre*, all human intervention studies were considered carefully for relevant information that might be used for the risk assessment of this botanical and preparations from these, including non-blinded studies and/or studies without control groups. Case reports of adverse effects were also considered. However, caution is to be exercised when interpreting such case studies for any conclusion on potential causality. For example, a published case report of liver toxicity related to the consumption of a *Gymnema* tea (Shiyovich et al., 2010) could point to potential adulteration or contamination of the tea with hepatotoxic substances or to a hepatotoxic potential of certain *G. sylvestre* constituents. However, in the light of preliminary evidence from animal studies, rather potential hepatoprotective effects of *Gymnema* preparations were observed (Srividya et al., 2010).

For the animal studies, emphasis was placed primarily on 'classical' toxicological studies (single dose or repeated dose toxicity studies) (Ogawa et al., 2004). However, animal studies that were carried out to assess the efficacy of the herb on various clinical, biochemical and laboratory parameters were also examined to obtain any useful information for potential safety issues (e.g. Shanmugasundaram et al., 1990a; Chattopadhyay, 1999; Shigematsu et al., 2001; Yadav et al., 2010).

Based on indications from both animal and human studies for potential interactions of *G. sylvestre* preparations with allopathic drugs (especially antidiabetic drugs), specific attention was paid to *in vitro* and *in vivo* studies that investigated primarily pharmacodynamic and/or pharmacokinetic interactions. For pharmacodynamic interactions, concomitant administration of the test substance has effects on targets such as receptors, enzymes, transcription factors, etc., leading to synergistic, additive or antagonistic effects with respect to the therapeutic effects, without altering the drug's concentration in the body. In pharmacokinetic interactions, co-administered test substance enhances or interferes with the absorption, distribution, metabolism or excretion of the conventional drug(s), resulting in changes in drug concentration in the body (e.g. Kamble et al., 2016).

It is known that pharmacokinetic interactions often involve inhibition or induction of the cytochrome P450 (CYP450) family of xenobiotic-metabolising enzymes. Enzyme inhibitors decrease enzyme activity, leading to increased concentrations of substrates (i.e. drugs being metabolised by enzyme system) and so, predisposing to drug toxicity. Enzyme inducers, conversely, increase the number of enzymes, leading to decreased concentrations of substrates, and have the potential to decrease the effectiveness of the drug.

In conclusion, the focus of the present project was to assess the possible risks and critical health aspects for the use of *G. sylvestre* in dietary supplements. The intention of this exercise was also to explore whether the derivation of health-based values that might form the basis of recommendations on *Gymnema* consumption would be possible and, in addition, to evaluate the uncertainties for this risk assessment.

2.2.2. Other activities during the EU-FORA fellowship

At the hosting site (BfR), the fellow participated in:

- a one-day seminar on literature search given by a staff member of the BfR. During the seminar, the fellow became acquainted with the electronic scientific databases that the BfR has access to and how to retrieve scientific articles.
- a two-day seminar on improving presentation skills 'Effective presentations'. The seminar focused on techniques for structuring a presentation and presenting facts and figures, ways to establish contact with the audience and finally ways to conclude a presentation.
- a seminar on risk assessment of foods containing genetically modified organisms.
- regular meetings of the Unit of Nutritional Risks, Allergies and Novel Foods of the BfR (which were held in English). These meetings offered the opportunity to the fellow to have fruitful and interesting discussions on the activities of other members of the Unit as well as on current nutrition-related risk assessment issues, e.g. for micronutrient supplementation or food fortification.
- short seminars (20 min each) organised regularly by the Department of Food Safety of BfR on the current scientific work carried out at different units of the BfR.

Additional activities:

- Presentation of the EU-FORA fellowship programme during a pre-Christmas one-day event at the BfR.
- Participation in international events organised by the International Affairs team of the BfR. These events provided the possibility for networking with other colleagues not only from Germany but also from around the world.
- Presentation of the project on *G. sylvestre* at the seminar of the Department of Food Safety of the BfR.
- Preparation of a poster for the EFSA conference on 'Science, Food, Society' in Parma on the 18–21st September 2018.

3. Conclusions

3.1. Conclusions for the assessment of the botanical

The safety assessment of *G. sylvestre* was complicated by a number of aspects.

In particular, the lack of standardisation in and comparability of *G. sylvestre* preparations posed a problem. The plant *G. sylvestre* may have different chemical composition, depending on geographical area (Pandey and Yadav, 2010) and growing conditions. For *Gymnema* preparations, different parts of the plant (leaves, stem and flowers) may be used which have different concentrations of active constituents. Furthermore, different modes of producing the preparation, including procedures involving different methods of extraction, may be used, resulting in very different composition and content of phytochemicals (Yadav et al., 2010). Certain *Gymnema* preparations that have been used in studies or in products found on the market have been reported to be standardised based on varying percentages of 'gymnemic acid', which is in itself a mixture of different compounds (Zarrelli et al., 2014).

Animal and human studies published in scientific journals have used a wide range of different *Gymnema* preparations. Therefore, extrapolating from one preparation to another with different chemical composition would not be possible, particularly as gymnemic acid (which is purported to be the active 'substance') is actually a mixture of compounds, some of which have not yet been characterised. Based on the resulting difficulties in performing a quantitative risk assessment, the available data were not regarded as being sufficient for the derivation of health-based guidance values.

The assessment of the botanical *G. sylvestre* was also challenging also to the insufficient number of studies containing relevant data for the safety assessment, the poor methodological approaches or poor study design, the lack of systematic data on dose and effect relationship and poor reporting or presentation of the outcomes in the scientific publications. Along these lines, it appears possible that unwanted or adverse effects resulting from the use of certain botanical preparations may be underreported (Bakhiya et al., 2017).

There are limited toxicological data from animal studies most of which were not adequately described or meeting the requirements of existing guidelines (e.g. the Organisation for Economic Co-operation and Development (OECD) test guidelines). Only with one type of *Gymnema* preparation (i.e. aqueous extract) a 'classical' subchronic or chronic toxicological study could be identified (Ogawa et al., 2004) (article only available in Japanese language). In this study, 0, 100, 1,000 or 10,000 mg of a *Gymnema* extract/kg feed were administered and no relevant adverse effects were observed. The authors identified a no-observed-effect level (NOAEL) 10,000 mg/kg feed per day (highest dose investigated), corresponding to 504 mg/kg body weight (bw) per day for male and 563 mg/kg bw per day for female rats. A slight but statistically significant increase in the relative weight of ovaries in all dose groups of female rats treated with the *Gymnema* extract deserves further clarification. It should also be pointed out that long-term repeated dose toxicity studies have not been performed with other types of extract such as ethanol extracts (used in some human clinical studies) or with higher doses.

A few other available experimental animal studies assessed the efficacy of different *Gymnema* preparations on plasma glucose and lipid parameters. In these studies, no adverse effects on liver, kidney or other organs were reported, when safety laboratory parameters were investigated.

No animal or human study could be identified for the effects of *G. sylvestre* alone on fertility and gestation.

The available data from a small number of human studies in healthy individuals do not point to any serious adverse effects following consumption of *G sylvestre*. While a significant blood glucose lowering effect was observed after 10 days with administration of 2 g of dry leaf powder per day (Shanmugasundaram et al., 1981) and 6 g/day of an aqueous decoction of shade-dried powdered leaves (Khare et al., 1983) to a small number of individuals, no hypoglycaemic episodes were reported.

However, studies that investigated the efficacy of a water-soluble ethanol extract of *G. sylvestre* (GS4) at a dose of 400 mg/day in insulin-dependent diabetic patients (Shanmugasundaram et al., 1990b) and in non-insulin-dependent diabetic patients on antidiabetic drugs (Baskaran et al., 1990), reported the occurrence of hypoglycaemic episodes and adjustment of the drug regime. For example in the latter study by Baskaran et al. (1990), several weeks after *Gymnema* supplementation, virtually all patients developed secondary hypoglycaemic symptoms and the dose of drugs was reduced or discontinued (23% discontinued their conventional drug therapy). This serves as an indication of the possibility of herb–drug interactions. In addition, animal studies have suggested the risk of hypoglycaemia when *G. sylvestre* is taken concomitantly with antidiabetic drugs, pointing to potential pharmacodynamic interactions (Kamble et al., 2016) or tissue regeneration mechanisms (as presumed by Shanmugasundaram et al., 1990a for the specific animal model that was employed). Animal studies have provided some indications for potential pharmacokinetic interactions of *Gymnema* preparations with allopathic drugs (e.g. Vaghela et al., 2017), so suggesting possible interactions with CYP450 enzymes.

In conclusion, there are presently considerable knowledge gaps for the risk assessment of *G. sylvestre* preparations and open questions for whether results obtained with one preparation can be extrapolated to another *Gymnema* preparation. Also based on the lack of systematic data on dose and effect relationships, the available information was regarded as not being sufficient for the derivation of health-based guidance values for *Gymnema* or *Gymnema* preparations. Considering the uncertainties for the composition of different *Gymnema* preparations, potential herb–drug interactions and the concerns about glucose lowering or hypoglycaemic effects, the use of *Gymnema*-based food supplements in combination with (or as a substitute for) authorised antidiabetic drugs may be associated with risks when used without medical supervision.

3.2. Conclusions for the benefits gained from the fellowship

The fellow gained experience in systematic data extraction in a time-efficient manner from scientific publications and in the evaluation of toxicological and toxicokinetic studies. The general procedure for the safety evaluation of *Gymnema* can be followed by the fellow for the risk assessment of other

substances with nutritional or physiological effect added to foods and food supplements. No exposure assessment was performed due to the lack of available dietary intake data in the scientific literature.

The fellow realised the importance of not relying solely and automatically on the information presented in the abstract and/or the discussion/conclusions made by the authors but of the need to carefully analyse the data presented in the results section (Tables and Figures). A deeper insight of the presented data can certainly help any assessor decide whether the data are according to the authors' conclusion or to which extent an individual study might contribute to the overall weight of evidence. Various exercises were given to the fellow to establish an understanding of the importance of evaluating the presented data (ranging from biochemical and toxicological parameters to clinical outcomes) from individual articles that helped him to realise that, on some occasions, a different understanding of the presented data may explain the different conclusions made by different scientists for the risk assessment of the same substance.

The EU-FORA programme has provided a unique opportunity for the fellow to interact with experts in the field of nutritional risk assessment, obtain valuable experience and improve skills in performing nutritional risk assessment. The experience gained at the BfR may also lead to international collaboration opportunities well beyond the fellowship time.

While many nutritionists tend to focus primarily on diet/nutrient efficacy aspects, safety issues and potential adverse effects of nutrients and substances in foods and food supplements are often not of primary interest and hence, not adequately considered. To develop food-based dietary guidelines, one ought to be skilled in assessing not only the benefits but also the potential risks associated with consumption/over-consumption of certain foods, dietary supplements or individual substances added to supplements or fortified foods, in a systematic and time-efficient way. Therefore, early-to-middle career nutritionists (particularly those in the area of public health) and nutrition toxicologists who are interested in visiting an institution abroad for experience in nutrition risk assessment are greatly encouraged to look into and apply to the EU-FORA programme. Such programmes can greatly stimulate the fellow to think and work in a different framework and with a different mind-set, allow the fellow to diversify his/her competence and at the same time provide considerable opportunities for networking.

References

Al-Romaiyan A, Liu B, Asare-Anane H, Maity CR, Chatterjee SK, Koley N, Biswas T, Chatterji AK, Huang G-C, Amiel SA, Persaud SJ and Jones PM, 2010. A novel *Gymnema sylvestre* extract stimulates insulin secretion from human islets *in vivo* and *in vitro*. Phytotherapy Research, 24, 1370–1376.

Bakhiya N, Ziegenhagen R, Hirsch-Ernst KI, Dusemund B, Richter K, Schultrich K, Pevny S, Schäfer B and Lampen A, 2017. Phytochemical compounds in sport nutrition: synephrine and hydroxycitric acid (HCA) as examples for evaluation of possible health risks. Molecular Nutrition and Food Research, 61. https://doi.org/10.1002/mnfr.201601020

Baskaran K, Kizar Ahamath B, Radha Shanmugasundaram K and Shanmugasundaram ERB, 1990. Antidiabetic effect of a leaf extract from *Gymnema sylvestre* in non-insulin-dependent diabetes mellitus patients. Journal of Ethnopharmacology, 30, 295–305.

Bronzwaer S, Le Gourierec N and Koulouris S, 2016. 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

Chattopadhyay RR, 1999. A comparative evaluation of some blood sugar lowering agents of plant origin. Journal of Ethnopharmacology, 67, 367–372.

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, 60 pp. https://doi.org/10.2903/j.efsa.2012.2663

EFSA Scientific Committee, 2009. Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements, on request of EFSA. EFSA Journal 2009;7(9):1249, 19 pp. https://doi.org/10.2093/j.efsa.2009.1249

Kamble B, Gupta A, Moothedath I, Khatal L, Janrao S, Jadhav A and Duraiswamy B, 2016. Effects of *Gymnema sylvestre* extract on the pharmacokinetics and pharmacodynamics of glimepiride in streptozotocin induced diabetic rats. Chemico-Biological Interactions, 245, 30–38.

Khare AK, Tondon RN and Tewari JP, 1983. Hypoglycaemic activity of an indigenous drug (*Gymnema sylvestre*, 'gurmar') in normal and diabetic persons. Indian Journal of Physiology and Pharmacology, 27, 257–258.

Kurian GA, Manjusha V, Nair SS, Varghese T and Padikkala J, 2014. Short-term effect of G-400, polyherbal formulation in the management of hyperglycemia and hyperlipidemia conditions in patients with type 2 diabetes mellitus. Nutrition, 30, 1158–1164.

Mahajan S, Chauhan P, Subramani SK, Anand A, Borole D, Goswamy H and Prasad GBKS, 2015. Evaluation of 'GSPF *kwath*': a *Gymnema sylvestre*-containing polyherbal formulation for the treatment of human type 2 diabetes mellitus. European Journal of Integrative Medicine, 7, 303–311.

Ogawa Y, Sekita K, Umemura T, Saito M, Ono A, Kawasaki Y, Uchida O, Matsushima Y, Inoue T and Kanno J, 2004. *Gymnema sylvestre* leaf extract: a 52-week dietary toxicity study in Wistar rats. Shokuhin Eiseigaku Zasshi, 45, 8–18.

Pandey AK and Yadav S, 2010. Variation in gymnemic acid content and non-destructive harvesting of *Gymnema sylvestre* (Gudmar). Pharmacognosy Research, 2, 309–312.

Restani P, Di Lorenzo C, Garcia-Alvarez A, Frigerio G, Colombo F, Maggi FM, Milà-Villarroel R and Serra-Majem L, 2018. The PlantLIBRA consumer survey: findings on the use of plant food supplements in Italy. PLoS ONE, 13, e0190915.

Shanmugasundaram KR, Panneerselvam C, Samudram P and Shanmugasundaram ER, 1981. The insulinotropic activity of *Gymnema sylvestre*, R. Br. An Indian medical herb used in controlling diabetes mellitus. Pharmacological Research Communications, 13, 475–486.

Shanmugasundaram ERB, Leela Gopinath K, Radha Shanmugasundaram K and Rajendran VM, 1990a. Possible regeneration of the islets of Langerhans in streptozotocin-diabetic rats given *Gymnema sylvestre* leaf extracts. Journal of Ethnopharmacology, 30, 265–279.

Shanmugasundaram ERB, Rajeswari G, Baskaran K, Rajesh Kumar BR, Radha Shanmugasundaram K and Kizar Ahamath B, 1990b. Use of *Gymnema sylvestre* leaf extract in the control of blood glucose in insulin-dependent diabetes mellitus. Journal of Ethnopharmacology, 30, 281–294.

Shigematsu N, Asano R, Shimosaka M and Okazaki M, 2001. Effect of long term-administration with *Gymnema sylvestre* R. BR on plasma and liver lipid in rats. Biological and Pharmaceutical Bulletin, 24, 643–649.

Shiyovich A, Sztarkier I and Nesher L, 2010. Toxic hepatitis induced by *Gymnema sylvestre*, a natural remedy for type 2 diabetes mellitus. The American Journal of the Medical Sciences, 340, 514–517.

Srividya AR, Varma SK, Dhanapal SP, Vadivelan R and Vijayan P, 2010. *In vitro* and *in vivo* evaluation of hepatoprotective activity of *Gymnema sylvestre*. International Journal of Pharmaceutical Sciences and Nanotechnology, 2, 768–773.

Tiwari P, Mishra BN and Sangwan NS, 2014. Phytochemical and pharmacological properties of *Gymnema sylvestre*: an important medicinal plant. BioMed Research International, 2014, 830285. https://doi.org/10.1155/2014/830285

Vaghela M, Sahu N, Kharkar P and Pandita N, 2017. *In vivo* pharmacokinetic interaction by ethanolic extract of *Gymnema sylvestre* with CYP2C9 (tolbutamide), CYP3A4 (amlodipine) and CYP1A2 (phenacetin) in rats. Chemico-Biological Interactions, 278, 141–151.

Yadav M, Lavania A, Tomar R, Prasad GBKS, Jain S and Yadav H, 2010. Complementary and comparative study on hypoglycemic and antihyperglycemic activity of various extracts of *Eugenia jambolana* seed, *Momordica charantia* fruits, *Gymnema sylvestre*, and *Trigonella foenum graecum* seeds in rats. Applied Biochemistry and Biotechnology, 160, 2388–2400.

Zarrelli A, Romanucci V, Gravante R, Di Marino C and Di Fabio G, 2014. History of gymnemic acid, a molecule that does not exist. Natural Product Communication, 9, 1429–1432.

Zuniga LY, Gonzalez-Ortiz M and Martinez-Abundis E, 2017. Effect of *Gymnema sylvestre* on metabolic syndrome, insulin sensitivity, and insulin secretion. Journal of Medicinal Food, 20, 750–754.

Abbreviations

BfR	Bundesinstitut für Risikobewertung
bw	body weight
CYP450	cytochrome P450
EFET	Hellenic Food Authority
EU-FORA	The European Food Risk Assessment Fellowship Programme
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development

EU-FORA SERIES 1

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16084

Development of an automated multienzymatic biosensor for risk assessment of pesticide contamination in water and food

Institute of Protein Biochemistry – National Research Council, Naples, Italy
Janis Rusko and Ferdinando Febbraio

Abstract

The goal of this research is to better address the problems related to the widespread presence of pesticides in the environment. Despite the unquestionable utility of the pesticides against various pests in the agricultural field, most pesticides and the corresponding pesticide residues are toxic to the environment and hazardous to human health. The recent literature on organophosphate compounds emphasises a clear correlation between their use and the occurrence of disorders in the nervous system, especially in children. The conventional systems for the detection and analysis of these compounds are expensive, time-consuming and require highly specialised operators; moreover, no online automated screening systems are yet available, that would allow the identification and quantification of the presence of these chemicals in samples from industrial sectors such as the food industry. Esterase-based biosensors represent a viable alternative to this problem. In this fellowship programme, we aim to develop a robust and sensitive methodology that enables the screening of toxic compounds using a streamlined process, using an automated robotic system to achieve a continuous monitoring for risk assessment of pesticides.

Keywords: organophosphate pesticides, biosensing device, thermophilic esterase, environmental monitoring

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: Institute of Protein Biochemistry – National Research Council, Naples, Italy, Rusko J and Febbraio F, 2018. Development of an automated multienzymatic biosensor for risk assessment of pesticide contamination in water and food. EFSA Journal 2018;16(8):e16084, 10 pp. https://doi.org/10.2903/j.efsa.2018.e16084

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

The presence of pesticide residues and the corresponding metabolites has been one of the major issues in food safety research. Although pesticides have improved agricultural productivity, they are associated with many health effects. Quantitative analysis of pesticides by chromatographic and spectroscopic technologies is limited by the time required to analyse a high number of samples, as the workloads and workforce involved in pesticide laboratories are immense. However, to perform food safety risk assessment much data are needed that is derived from large quantities of samples and a methodology that enables rapid quantitative analysis. To propose a solution for the issue, we are in the process of developing specific enzymes for the detection of select organophosphate pesticides (OPPs) and implementing the designed biosensing devices in a robotic system in combination with fluorescence and mass spectrometry (MS) detection.

Previous research has indicated several biosensors for OPPs detection, based on enzymatic or biological bioreceptors (Whangsuk et al., 2016; Hassani et al., 2017). Most widely used are biosensors based on the inhibition of acetylcholinesterase (AChE) (Guler et al., 2016). However, there are some limitations – the low stability of corresponding proteins and the susceptibility to react with inhibitors such as metals (Frasco et al., 2005; Amine et al., 2006), carbamates (Colovic et al., 2013) and even tetrahydrocannabinol (THC) (Eubanks et al., 2006), present in cannabis.

Esterase 2 from *Alicyclobacillus acidocaldarius* (EST2) is highly sensitive to the action of paraoxon, and appears to be endowed with a high affinity toward this OPP (Febbraio et al., 2008). EST2 is a carboxylesterase belonging to the hormone-sensitive lipase (HSL) family that includes several AChEs. Previous research has demonstrated the characteristics of EST2 – stability over time, prime resistance and activity at different pH values and temperatures (Mandrich et al., 2004, 2005; Foglia et al., 2007), as well as satisfactory stability in the presence of low concentrations of organic solvents and detergents (Del Vecchio et al., 2002; Mandrich et al., 2006), so supporting the potential further use in biosensing devices selective for a variety of inhibitors such as OPPs. In previous research, our group has already described the use of EST2 as a biological part of a colorimetric biosensor for paraoxon detection (Febbraio et al., 2011).

Furthermore, the EST2 3D structure has been solved at 2.6 Å resolution (De Simone et al., 1999, 2000), allowing modelling of the structure *in silico* and permitting molecular docking predictions. EST2's peculiarity for stability, in addition to the terms of irreversible inhibition, the sensitivity and selectivity toward phosphoryl OPPs, such as paraoxon and methyl paraoxon (Foglia et al., 2007; Febbraio et al., 2011), makes this enzyme a prime candidate for use as a bioreceptor in biosensors for qualitative and quantitative OPP detection.

Using our proposed bioassay methodology, analysis time for multiple samples by fluorescence measurement takes about 1 min, which is at least 10 times faster than common quantification methods. If any positives for OPPs are found, analysis is continued, provided by the extra selectivity of tandem MS (fragmentation patterns) or high-resolution MS (accurate mass and fragmentation). Generally, the system is optimised to minimise the means of sample preparation and analyse the native samples. Our methodology enables a rapid preliminary testing approach and works as a filter before chromatographic analysis, avoiding the testing of most negative samples.

The goal of the research involved in the EU-FORA fellowship programme (Bronzwaer et al., 2016), granted by the European Food Safety Authority (EFSA), is to develop a method capable of providing rapid quality control and achieving continuous monitoring of the OPPs in the environmental (water and liquid food) samples.

2. Description of work programme

2.1. Aims

The work programme activities or aims were summarised in three principal parts. The first part was the participation of the selection and preparation of the suitable enzymes that would further be utilised in the second part of the work programme as bioreceptors for multienzymatic biosensors, developed using a robotic workstation. Third and final part of the work programme was dedicated to the collection and analysis of real world samples using the developed bioenzymatic method and to compare the achieved results with the use of conventional methods to further ensure confidence in the developed method. After data collection, the data were processed and analysed to provide the risk assessment of pesticide content in fruit juice and wine samples.

2.2. Methods

2.2.1. Part 1

The fellow accomplished the expression in mesophilic hosts, such as *Escherichia coli* strain BL21 (DE3), of several mutants of a thermostable esterase (Manco et al., 1998), sensitive to OPP inhibition (Febbraio et al., 2011), already available as transformed cells in the laboratory. Locally available equipment such as fermentation facilities, centrifuges, incubators and sonicators were used to accomplish this part. Proteins were further purified via thermoprecipitation steps, and size-exclusion chromatography (gel permeation chromatography (GPC)). Proteins were quantified using Bradford protein assays. Biochemical characterisation of purified proteins was performed by enzymatic activity assays using fluorescent substrates. The biochemical constants of inhibition in the presence of different concentration of OPPs for each enzyme were determined to catalogue them, depending on individual affinities for these compounds.

2.2.2. Part 2

In the second part of the work programme, the fellow was trained to use a robotic workstation Hamilton MICROLAB® STAR equipped with a robotic arm connected to a Multilabel Plate Reader PerkinElmer Victor X3, to develop methods that were used for application of real world sample analysis and further method development and investigation stages. In particular, residual activities of each selected enzyme for the respective OPPs were determined. Assay analysis times were optimised to obtain prime reproducibility of data and to better investigate the effects of unique residual activities. To support the bioenzymatic method and confirm the robustness, a conventional mass spectroscopy method was developed and verified using an AB Sciex QTrap 4,500 triple quadrupole MS system coupled to a Shimadzu Nextera x2 liquid chromatography system.

2.2.3. Part 3

In the last part of the work programme, various kinds of fruit juice, such as apple, apricot, peach and other commercial fruit juices were collected, to be used for method development and validation. The samples were collected at commercial sources in Naples, Italy. In total, there were more than 60 unique samples of both wine and juice. No information was available on possible previous treatment of these fruit juices or wines, except for products labelled with the 'BIO' label. The samples were shaken until homogeneous, two aliquots of 50 mL were transferred to screw-cap polypropylene sample tubes and stored at −20°C. Only one tube was thawed at one time to avoid unnecessary matrix product degradation.

Initially, the samples were analysed and the presence (or absence) of the OPPs was determined by use of the developed confirmatory MS method over a short period (48 h) of time after the initial acquisition of the samples. The method specificity and the occurrence of OPPs were studied. In particular, the bioenzymatic assays were optimised for the detection of OPPs in complex matrix solutions by measuring the efficiency of the assays in the presence of various matrix constituents. The obtained concentration data were used for the risk assessment of OPPs in liquid foods.

3. Conclusions

3.1. Method development

3.1.1. Part 1

Enzymes (EST2 mutants, displayed in Figure 1) were overexpressed in the mesophilic host *E. coli* strain BL21 (DE3) and purified. Protocols described by Manco et al. (1998) and Pezzullo et al. (2012) were used to obtain reference enzymes. As the purification method was excessive and not viable for large-scale preparation, final purification steps, including fast protein liquid chromatography (FPLC) and high-performance liquid chromatography (HPLC), were excluded. Two crude enzyme extracts, the first after thermoprecipitation and the second after thermoprecipitation followed by GPC were tested for the enzyme stability and activity against the reference purified enzymes. Results indicated that unpurified enzymes were more stable and active with respect to the purified ones. Even though the proteins obtained using the previous protocols were more pure, it was not viable to include the

redundant and time-consuming flash chromatography and FPLC steps, during which significant loss pf activity occurred. The experiment was continued using the final extracts obtained after a basic thermoprecipitation step, which allowed us to obtain all six of the mutant enzymes in a short period of time and in large quantities.

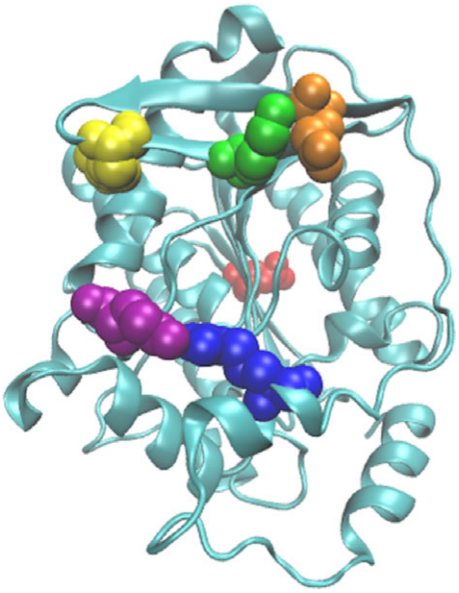

Figure 1: Representation of an EST2 3D structure in cyan, mutated groups are indicated using the van der Waals (VDW) structure, each colour referring to a different mutant

3.1.2. Parts 2 and 3

We proposed a modernised method that would allow a clean analysis process. The method development process started off with sample collection and preparation. During April 2018, we collected 60 combined commercial samples of fruit juice and wine. However, in the last months of the fellowship programme, we increased the variety and amount of the sample matrices.

The sample preparation was necessary to be robust and as simple as it could be. There were two opposite directions from which we had to decide on one. First direction was to improve the sensitivity and selectivity of the analysis using multiple sample preparation steps such as SPE (dSPE, μSPE), LLE or, the most common one, QuEChERS. Selective procedure helps to remove the excess matrix constituents such as sugars and antioxidants and enables the possibility of enrichment factor. Multiple sample preparation steps do come with a bigger price tag and generally take more time. The other direction was to be as easy as possible and enable high throughput analysis using simple sample preparation steps such as dilution, filtration and centrifugation. The issue with such basic sample preparation is that the matrix constituents are prone to diminish the detection efficiency and introduce bias in the analysis results. A problem was that we had to perform the confirmatory analysis using MS, which is very easily influenced by the matrix constituents providing signal suppression or enhancement effects, which can vary for individual samples.

We underwent preliminary method development steps using sample centrifugation, filtering with 0.22-μm polytetrafluoroethylene (PTFE) filters and dilution in water with final pH correction to 7.0 using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Standard addition was performed to samples before the sample preparation procedure. Then 60 individual samples were tested using MS to determine that the matrix influence was minor and contributed to no more than 20% chromatographic signal peak area deviation from the standard addition (spiked) samples without the matrix (blank solvent). The results indicated that the best options were to choose convenience, use the previously mentioned rapid sample preparation methods and omit the extensive sample preparation practices. Then, we continued the streamlined method development, using the robotic workstation to prepare the samples automatically.

Investigation on the bioenzymatic assays in the presence of matrix influence was continued. To increase the threshold of OPP detection using the EST2 enzyme, we included the use of a fluorogenic substrate – 4-methylumbelliferyl butyrate (4-MUBu) to measure the residual enzymatic activity. Note

that all the matrix samples were diluted with HEPES buffer. The preliminary condition was to determine the effect of the matrix on fluorescence detection – whether there was signal suppression or enhancement. The hydrolysed substrate (4-MU) was used as the fluorescence agent. Matrix blank (containing no OPPs or inhibitors) samples were used for multiple concentration levels of 4-MU standard addition. Another condition was to determine whether 4-MUBu was hydrolysed within the matrix blank samples by standard addition of a single concentration level of the compound and by measuring the fluorescence emission change over time (kinetic measurements).

Furthermore, we continued to examine how different concentration levels of HEPES buffer and concentration of matrix in the final sample extracts affected the suppression or enhancement effects. Note that the amount of matrix used in the final sample affects the sensitivity of the method.

We continued to determine the specific kinetic constants for six of our expressed EST2 mutant enzymes under standard assay conditions in the presence of 60 unique matrices and a control blank solvent. 4-MUBu was used as a substrate in a range of concentrations. The kinetic constant values (K*m* and k_{cat}) were calculated by plotting the reciprocals of EST2 hydrolysis rates versus the substrate concentrations.

Next, we continued to perform the inhibition assays for each of the enzymes of the following 12 OPPs – ethyl-paraoxon, methyl-paraoxon, coumaphos, fensulfothion, methyl-parathion, ethyl-parathion, cyanophose, methyl-pirimiphos, diazinon, phosmet, chlorpyrifos and tolclofos-methyl. The inhibition assays were carried out under standard assay conditions in the presence of 60 unique matrices and blank solvent. The matrix added to final sample aliquots was constant and the amount of inhibitors added to the assays was in a continuous concentration parts per thousand (ppt) scale. Aliquots were tested for the residual activity of EST2 measured in the presence of 1 mM 4-MUBu.

Furthermore, we tested the inhibition assay performance in the presence of an oxidising agent *N*-bromosuccinimide (NBS), which transforms the sulfur-containing pesticides to the corresponding oxidated forms. Prior to the bioassay analysis, the NBS oxidation efficiency was tested using MS and deemed efficient. As for the bioassay, NBS was added to the samples (both blank and matrix) containing only the inhibitors and, after a brief period of incubation in room temperature, the inhibition assay protocol was continued and the residual activity of EST2 was determined.

3.2. Streamlined method

To propose a solution to planned issues such as lack of data, slow analysis methods and extensive resource use, our goal was to develop and apply a streamlined method for OPP analysis in various target matrices.

The developed method was designed to be equivalent to and possibly improve on the common pesticide analysis methods using MS methodologies, such as QuEChERS. A brief, illustrative comparison can be seen in Figure 2.

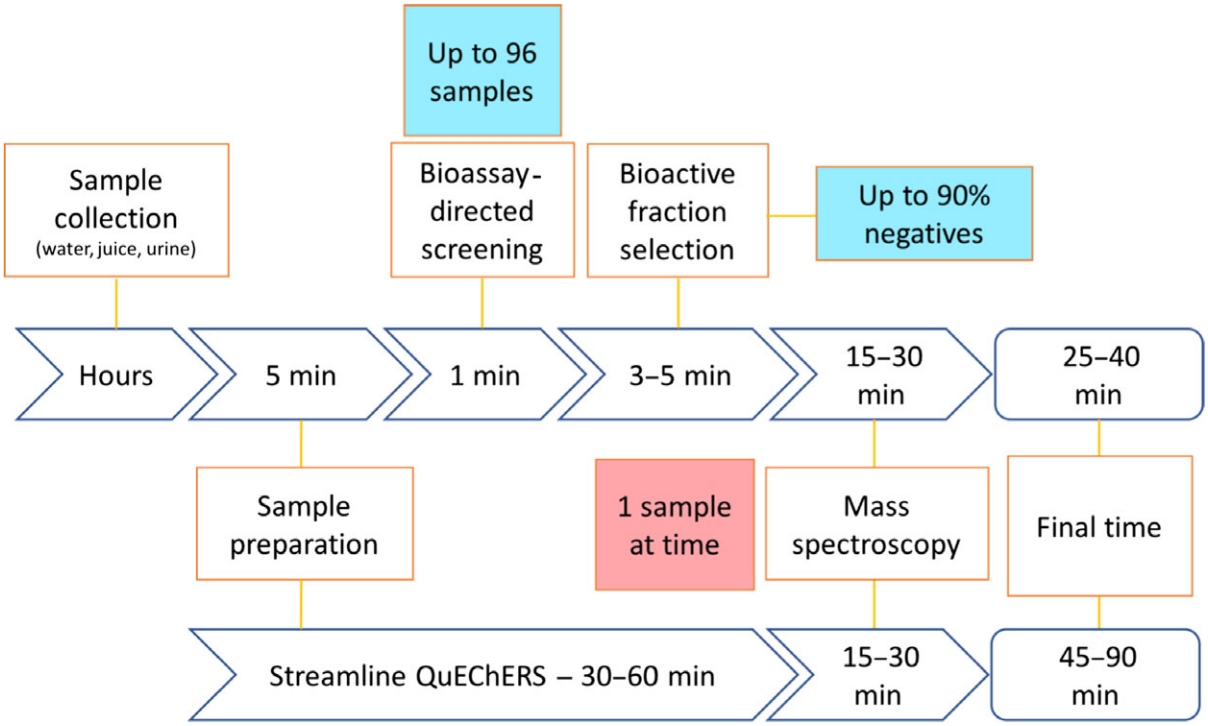

Figure 2: Analysis method process, in comparison with a conventional pesticide analysis method (QuEChERs + mass spectroscopy)

The figure illustrates the processes involved in the streamlining of both our developed method and the common MS analysis method for pesticides. For a single sample, the preparation takes significantly less time than using QuEChERS. The latter is then directly followed by MS analysis. In our developed methodology, sample preparation is followed by rapid bioassay analysis and the preliminary result evaluation. Positive samples are confirmed by MS.

The major difference is that our methodology provides a preliminary bioassay mass-screening of up to 96 (or even 384) samples at a time, compared with the conventional pesticide analysis method, which allows the processing of a single sample at time using MS detection. This approach is slightly more convenient as the results provided by the bioassays allowed us to discard up to 90% of the samples that had negative results. Therefore, the large-scale sample analysis workload and time of use of the MS systems is decreased.

3.3. Future goals

Direct influence on further method development by allocating more time would allow the incorporation of technology also in solid samples, which typically require extra treatment (SPE, QuEChERS, etc.) before pesticide data procurement. Continuous research would allow the application of genetically modified sensors to bind new, more complex target molecules and enable the application of analysis to new environmental and food matrices. Also, there is a possible line of research in binding the designed enzymes to a stationary phase, developing a SPE approach, so opening the possibilities of more sensitive and selective methods.

In future, it is possible to develop systems for people to use even in household applications, by performing the analysis using a simple set up of mobile phone and a sample cell.

The ultimate goal is to develop an accessible methodology in which only the biosensor would be used. However, we are limited by the extensive resources needed to develop such a method, as we would further require professionals with a background in bioinformatics and software development that would help in the development of software packages for data analysis and profiling. Such a system would allow accurate fingerprinting of the OPPs. Data processing and elaboration for fluorescence measurements and fingerprint determination would be continued by employing artificial neural networks (ANNs). ANN databases are collected through the determination of patterns and relationships in data and so are trained via experiences from sample dataset.

The possibility of further research would enable not only the ability for fast routine screening in environmental or quality control (QC) laboratories, but also for on-site operation in places significantly remote for the immediate identification of hazard, risk assessment and action. This approach would allow immediate operation, so preventing the placement of positive products on the market locally.

3.4. Extracurricular activities

The fellow participated in scientific seminars of interest and also presented the current research idea pitch in a mutual and poster presentation at EFSA organised Risk Assessment Research Assembly (RARA).

3.5. Disclaimer

The results obtained from the method development, sample analysis and risk assessment are not included in this report to avoid certain copyright claims, as the research is still ongoing over the last months of the fellowship programme and these results will be subsequently published in other scientific journals.

References

Amine A, Mohammadi H, Bourais I and Palleschi G, 2006. Enzyme inhibition-based biosensors for food safety and environmental monitoring. Biosensors and Bioelectronics, 21, 1405–1423.

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

Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM and Vasic VM, 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. Current Neuropharmacology, 11, 315–335.

De Simone G, Manco G, Galdiero S, Lombardi A, Rossi M and Pavone V, 1999. Crystallisation and preliminary X-ray diffraction studies of the carboxylesterase EST2 from *Alicyclobacillus acidocaldarius*. Acta Crystallographica Section D: Biological Crystallography, 55, 1348–1349.

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 lipase1. Journal of Molecular Biology, 303, 761–771.

Del Vecchio P, Graziano G, Granata V, Barone G, Mandrich L, Rossi M and Manco G, 2002. Denaturing action of urea and guanidine hydrochloride toward two thermophilic esterases. Biochemical Journal, 367, 857–863.

Eubanks LM, Rogers CJ, Beuscher AE IV, Koob GF, Olson AJ, Dickerson TJ and Janda KD, 2006. A molecular link between the active component of marijuana and Alzheimer's disease pathology. Molecular Pharmaceutics, 3, 773–777.

Febbraio F, D'Andrea SE, Mandrich L, Merone L, Rossi M, Nucci R and Manco G, 2008. Irreversible inhibition of the thermophilic esterase EST2 from *Alicyclobacillus acidocaldarius*. Extremophiles, 12, 719–728.

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.

Foglia F, Mandrich L, Pezzullo M, Graziano G, Barone G, Rossi M, Manco G and Del Vecchio P, 2007. Role of the N-terminal region for the conformational stability of esterase 2 from *Alicyclobacillus acidocaldarius*. Biophysical Chemistry, 127, 113–122.

Frasco MF, Fournier D, Carvalho F and Guilhermino L, 2005. Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers, 10, 360–375.

Guler M, Turkoglu V and Kivrak A, 2016. Electrochemical detection of malathion pesticide using acetylcholinesterase biosensor based on glassy carbon electrode modified with conducting polymer film. Environmental Science and Pollution Research, 23, 12343–12351.

Hassani S, Momtaz S, Vakhshiteh F, Maghsoudi AS, Ganjali MR, Norouzi P and Abdollahi M, 2017. Biosensors and their applications in detection of organophosphorus pesticides in the environment. Archives of Toxicology, 91, 109–130.

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, 203–212.

Mandrich L, Pezzullo M, Del Vecchio P, Barone G, Rossi M and Manco G, 2004. Analysis of thermal adaptation in the HSL enzyme family. Journal of Molecular Biology, 335, 357–369.

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.

Mandrich L, Manco G, Rossi M, Floris E, Jansen-van den Bosch T, Smit G and Wouters JA, 2006. *Alicyclobacillus acidocaldarius* thermophilic esterase EST2's activity in milk and cheese models. Applied and Environmental Microbiology, 72, 3191–3197.

Pezzullo M, Del Vecchio P, Mandrich L, Nucci R, Rossi M and Manco G, 2012. Comprehensive analysis of surface charged residues involved in thermal stability in *Alicyclobacillus acidocaldarius* esterase 2. Protein Engineering, Design and Selection, 26, 47–58.

Whangsuk W, Thiengmag S, Dubbs J, Mongkolsuk S and Loprasert S, 2016. Specific detection of the pesticide chlorpyrifos by a sensitive genetic-based whole cell biosensor. Analytical Biochemistry, 493, 11–13.

Glossary and Abbreviations

AChE	acetylcholinesterase
ANN	artificial neural network
dSPE	dispersive solid-phase extraction
EST2	esterase 2 from *Alicyclobacillus acidocaldarius*
EU-FORA	The European Food Risk Assessment Fellowship Programme
FPLC	fast protein liquid chromatography, technique used to analyse purified mixtures of proteins
GPC	gel permeation chromatography, technique of size-exclusion chromatography used to separate analytes on the basis of their molecular size
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-pressure liquid chromatography, technique used to separate, identify and quantify each component in a mixture
HSL	hormone-sensitive lipase
LLE	liquid–liquid extraction
4-MUBu	4-methylumbelliferyl butyrate
4-MUBu	4-methylumbelliferyl butyrate
MS	mass spectrometry
NBS	*N*-bromosuccinimide
OPPs	organophosphorus pesticides
PTFE	polytetrafluoroethylene
QC	quality control
QuEChERS	'Quick Easy Cheap Effective Rugged Safe', dispersive solid phase extraction method for detection of pesticide residues in food
RARA	Risk Assessment Research Assembly
μSPE	micro solid-phase extraction
THC	tetrahydrocannabinol
Thermoprecipitation	Purification of sample using multiple high and low temperature steps to separate the impurities from target compound

EU-FORA SERIES 1

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16085

Identification and evaluation of potentially mutagenic and carcinogenic food contaminants

German Federal Institute for Risk Assessment (BfR),
Department Food Safety, Berlin, Germany
Josef D Rasinger, Falko Frenzel, Albert Braeuning and Alfonso Lampen

Abstract

Heat processing of food gives rise to a plethora of chemical compounds whose toxicological effects are largely unknown. Due to a general lack of experimental toxicological data, assessing the risks associated with the consumption of these substances remains a challenge. Computer models that allow for an *in silico* prediction of physicochemical and toxicological characteristics, may be able to fill current data gaps and facilitate the risk assessment of toxicologically uncharacterised chemicals, their transformation products and their biological metabolites. The overall aims of the present project were for the fellow: (i) to get acquainted with the application of computational toxicological analyses tools in risk assessment based on results and experiences from previous research performed at the German Federal Institute for Risk Assessment (BfR); and (ii) to apply the newly gained skills on historic and novel data using updated and additional *in silico* tools. The project contributed to the continuous further education of the fellow in the use of computational toxicology tools, corroborated findings related to the safety of heat-induced contaminants and laid the foundations for future collaborations between the fellow's home institution, the Institute of Marine Research (IMR) in Norway, and the BfR in Germany.

Keywords: *in silico* toxicology, QSAR, data mining, R

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: German Federal Institute for Risk Assessment (BfR), Department Food Safety, Berlin, Germany, Rasinger JD, Frenzel F, Braeuning A and Lampen A, 2018. Identification and evaluation of potentially mutagenic and carcinogenic food contaminants. EFSA Journal 2018;16(8):e16085, 9 pp. https://doi.org/10.2903/j.efsa.2018.e16085

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

Heat processing gives rise to a plethora of substances whose toxicological effects are largely unknown. Particularly, the presence of chemical compounds that are considered possibly or probably carcinogenic to humans has attracted the attention of the public and initiated a debate on the healthiness of heated foods and beverages (Wenzl et al., 2007). Many of these undesired compounds are products of the Maillard reaction or lipid oxidation reactions and include, among others, legacy contaminants such as acrylamide, furan, acrolein, 5-hydroxymethyl furan. Contaminants of recent concern include 3-chloro-1,2,-propanediol (3-MCPD), 2-chloro-1,3,-propanediol (2-MCPD), glycidol and their fatty acid esters. Risk assessment has been performed for some of these substances but for many of the hundreds of heat-induced substances known to date, data on their toxicological properties are still lacking (Frenzel et al., 2017).

Conventionally, for single compounds, *in vivo* experiments are the method of choice for identifying the toxic effects of xenobiotics (Pradeep et al., 2016). However, time, costs and ethical constraints render these assays ineffective when the high throughput hazard assessment of large numbers of novel contaminants is required. In particular, the envisaged reduction in the use of animals in research, as outlined in the European Union (EU) Directive 2010/63/EU (Directive 2010/63/EU 2010), calls for a further development and wider application of alternative testing methods that are able to predict or estimate inherent toxic properties of chemical substances without the need for animal testing. In this context, computer-based (*in silico*) predictions such as quantitative structure–activity relationship models (QSAR) are gaining increased importance, especially as screening tools for prioritisation purposes as they provide faster, non-animal-based alternatives for the prediction of complex toxicological endpoints (Maunz et al., 2013; Pradeep et al., 2016).

In the risk assessment of chemicals, *in silico* tools are typically used in combination with other non-testing methods such as read-across in the context of Integrated Testing Strategies (ITS) and Weight-of-Evidence (WoE) approaches (EFSA 2014). Several QSAR models have been used and validated by US regulatory agencies and a set of internationally agreed upon validation principles for regulatory acceptance were laid out by the Organisation for Economic Co-operation and Development (OECD) (Pradeep et al., 2016). Also in the EU, QSAR are gaining acceptance in the prediction of toxicity reference values and the classification of thresholds for human and environmental risk assessments (Benfenati et al., 2017).

However, despite the recent advances in computational toxicology, challenges do remain that still hamper the use of QSAR in safety assessment decisions and reports. For example, different *in silico* tools using different mathematical algorithms and different training data sets were found to provide conflicting predictions (Gleeson et al., 2012). Therefore, regulators may compile predictions from different QSAR tools to come to a decision. Research is currently ongoing to investigate how to best combine the outputs of these tools to gain improved predictive performances for various toxic endpoints (Pradeep et al., 2016; Frenzel et al., 2017).

2. Description of work programme

At the Department of Food Safety of the German Federal Institute for Risk Assessment (BfR), recently a combine and conquer strategy for QSAR analyses was developed and applied to ~ 800 heat-induced food contaminants (Frenzel et al., 2017) as well as to ~ 600 secondary plant compounds (Glück et al., 2018). In the course of the EU-FORA programme (Bronzwaer et al., 2016), the fellow, Dr Josef D Rasinger from the Institute of Marine Research (IMR)[1] in Norway, was placed at the BfR, to become familiar with the *in silico* tools and approaches described in Frenzel et al. (2017) and to corroborate findings related to the safety of heat-induced contaminants through re-analyses of the already published data using updated and additional *in silico* tools and approaches.

Dr Rasinger is a nutritional toxicologist whose research at the IMR is focused on the development and implementation of molecular ('omics), biostatistical and bioinformatics tools for use in food and feed safety assessments (Rasinger et al., 2014, 2017; Reffatto et al., 2018; Nøstbakken et al., accepted). During his placement at the BfR, Dr Rasinger was introduced to modern *in silico* methodologies for hazard assessment, namely QSAR and further developed his knowledge and experience in risk assessment.

[1] In January 2018, the IMR was merged with The National Institute of Nutrition and Seafood Research (NIFES). The new institute will be a leading supplier of knowledge on the sustainable management of resources in marine ecosystems and the whole food chain from the sea to the fork: http://www.imr.no/en

The project work at the BfR took place in close collaboration with senior scientists of the Unit 51, 'Effect-based Analytics and Toxicogenomics Unit', who as part of the BfR Department of Food Safety (Department 5), examine effects of food ingredients and contaminants. This involves the analysis of the intake, distribution, metabolism and excretion of these substances (toxicokinetics) using *in vitro* and molecular biological methods including *in silico* tools, 'omics techniques, as well as classic chemical analytical methods. The fellow was supervised by Dr Albert Braeuning, the head of Unit 51, and Dr Falko Frenzel, a post-doctoral researcher in Unit 51 and principal investigator in the project around which the fellow's work is centred.

2.1. Aims

The aims of the present project were for the fellow: (i) to become familiar with the application of computational *in silico* toxicological analysis tools in risk assessment based on results and experiences from previous research performed at the BfR; (ii) to obtain transferable skills increasing the scientific capacity of the fellow's home institute; and (iii) to assess the possibility of and set up further networking and bilateral cooperation between the fellow's home and host institutions; the IMR in Norway and the BfR in Germany.

2.2. Activities/Methods

2.2.1. Application of computational *in silico* toxicological analyses tools in risk assessment

For a successful completion of this project, the fellow became familiar with commonly used open source QSAR tools and software tools for data management and data mining. The computer models employed included VEGA, T.E.S.T. and lazar. According to Frenzel et al. (2017), the fellow used five different prediction tools for mutagenicity and three prediction tools for carcinogenicity and two relevant toxicological endpoints for heat-induced food contaminants.

The VEGA platform[2] (v1.1.4) provides several different software tools to predict physicochemical, ecotoxicological and toxicological properties for compounds of interest. In addition, VEGA includes tools for read-across (ToxRead) and prioritisation (JANUS), and allows the integration of results using a weight-of-evidence approach (ToxWeight). Within VEGA, four models are available to predict carcinogenicity and mutagenicity and one mutagenicity consensus model is provided. The Toxicity Estimation Software Tool (T.E.S.T.[3]; v4.2.1) provided by the United States Environmental Protection Agency (US EPA) predicts mutagenicity using three different QSAR methodologies based on either hierarchical clustering, a so-called FDA approach as applied by the US Food and Drug Administration (FDA), a nearest-neighbour approach, and a consensus model. lazar[4] (lazy, structure–activity relationship) (Maunz et al., 2013) comprises two models for mutagenicity and six models for carcinogenicity. In addition to VEGA, T.E.S.T. and lazar, the OECD QSAR Toolbox[5] (v4.1) was used to retrieve information on experimental data on mutagenicity and carcinogenicity of the test compounds.

As described in Frenzel et al. (2017), a list of 814 substances comprising approximately 600 products of the Maillard reaction and 200 lipid oxidation products prepared within the HEATOX project[6] and 24 compounds of current interest in food safety risk assessment was subjected to VEGA, T.E.S.T. and lazar. All settings and parameters within each software tool were set as previously described (Frenzel et al., 2017).

For both handling of the input files and the combination of the output files, the fellow was introduced to R[7] (The R Project for Statistical Computing) and Python[8] (Python Software Foundation, version 2.7) based in-house scripts and KNIME[9] workflows. These tools allowed for the generation of Simplified Molecular Input Line Entry System (SMILES), International Chemical Identifier Keys (InChIKeys), MDL Mol files and structured data files (SDF); common file formats, needed for the translation of a test compound's chemical structure into a computer readable format. In addition,

[2] https://www.vegahub.eu/
[3] https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test
[4] http://lazar.in-silico.de/predict
[5] http://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm
[6] https://cordis.europa.eu/publication/rcn/12731_en.html
[7] https://www.r-project.org/
[8] http://www.python.org
[9] https://www.knime.com/

scripts and snippets adapted from Frenzel et al. (2017) were deployed that allowed for the harmonisation of the heterogeneous output styles of the different QSAR software tools used.

During the re-evaluation of the data published in Frenzel et al. (2017), it became apparent that no major improvements could be achieved applying updated versions of the QSAR tools listed above. In the present EU-FORA project, the data set and QSAR workflow published in Frenzel et al. (2017) were therefore solely used as the training material to teach the fellow best practice approaches for QSAR-based data analysis, data preparation approaches and the proper use and output interpretation of the freely available *in silico* software tools. The dataset also was used to highlight and discuss the benefits and limitations of *in silico* approaches in risk assessment.

2.2.2. Acquiring transferable skills increasing the scientific capacity of the home institution

At the fellow's home institution currently efforts are ongoing to assess the safety of ethoxyquin (EQ; 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline). EQ is a synthetic antioxidant that is used as a technological additive to protect against lipid peroxidation in feed for pets, livestock and farmed fish. The use of EQ may result in residues of EQ and its transformation products (TP) being detected in edible tissues of animal origin, so humans can be exposed to these compounds through their diet and safety limits are to be set up.

Concerns on the safety of EQ and its TP and an overall lack of data led to a suspension of the authorisation of EQ as a feed additive for all animal species and categories (Commission Implementing Regulation EU 2017/962). A re-consideration of this assessment by the European Commission (EC) is possible if supplementary data on the safety of use and the efficacy of this additive are brought forward and current data gaps in the assessment of the exposure and the safety of EQ and its TP for animals, consumers and the environment are filled.

Based on novel data, which recently became available at the fellow's home institution, the fellow applied the newly gained computational *in silico* toxicology skills to predict toxicities and develop a prioritisation strategy for the risk assessment of the transformation TP of EQ. At the IMR, using a travelling-wave ion mobility spectrometry (TWIMS) coupled to quadrupole time-of-flight mass spectrometry (QTOFMS), 27 EQ TP were identified in oxidation experiments (Negreira et al., 2017). In subsequent experiments, 25 of those TP were detected in fish feed and 24 in fish from EQ exposure experiments (unpublished data). Using the *in silico* toxicology workflow outlined above, the fellow translated the list of EQ TP into machine readable chemical structure files and set out to predict *in silico* the toxicity of EQ TPs in both fish and mammals.

Work on this data set is currently ongoing. Similar to the prioritisation of the heat-induced contaminants performed in Frenzel et al. (2017), the output of this *in silico* analysis will allow a prioritisation of EQ TP according to their theoretical toxicities and highlight the compounds of most concern for consumers and farmed fish that need to be analysed further using *in vitro* or *in vivo* models of toxicity. The results of this work will be presented at the EFSA 2018 conference in Parma[10] and it is envisaged that a manuscript will be submitted for publication in a peer-reviewed journal before the end of the fellowship.

In addition to computational *in silico* toxicology competencies, the fellow also took part in the following activities that further developed his knowledge and experience in risk assessment:

- Three weeks of induction training in chemical and microbiological risk assessment at the EFSA premises in Parma (September 2017), and three 1-week modules focusing on different aspects of risk assessment and risk communications at the Austrian Agency for Health and Food Safety (AGES) in Vienna (December 2017), the BfR in Berlin (March 2018) and the Hellenic Food Authority (EFET) in Greece (June 2018).
- A 1-day seminar at the BfR on systematic literature search and review by a member of staff from the BfR library (November 2017).
- A 1-day risk assessment workshop at the BfR on food contamination by plasticisers (November 2017).
- A 1-day workshop at the BfR on mathematical modelling of metabolism and contaminant transfer in farm animals (December 2017).
- A 1-day introductory course to the KNIME-based BfR Food Chain-Lab software tools (PMM) laboratory at the BfR (March 2018).

[10] https://conference.efsa.europa.eu/

- The 24th EFSA colloquium: "Omics in risk assessment: state-of-the-art and next steps' held in Berlin (April 2018).
- A 2-day workshop at the BfR on risk assessment and risk management of genetically modified organisms (GMO; May 2018).

Throughout the year, the fellow also was given the opportunity to gain insight into other food-related research of the 'Department of Food Safety' (Department 5) by attending weekly seminars and through individual introductions to current projects related to risk assessment, food toxicology, novel foods and GMO. These meetings provided the opportunity to obtain an overview of the scientific work performed at Department 5, to discuss current issues in food safety risk assessment and to extend the fellow's network at the BfR. The fellow was also given the opportunity to present and discuss work accomplished under the auspices of the EU-FORA project at a departmental seminar in June 2018.

2.2.3. Establishment of further networking and bilateral cooperation between the fellow's home and host institutions

The placement of the fellow at the BfR in the course of the EU-FORA fellowship programme provided a unique opportunity to lay the foundations for future collaborations in the assessment of the applicability of modern methodologies in risk assessment research.

The 'Effect-based Analytics and Toxicogenomics Unit' at the BfR hosts the 'National Reference Laboratory for Animal protein in Feed (NRL-AP)'. The NRL-AP performs research on the prevention of food and feed fraud, allergen detection and risk assessment with the overall aim to provide farmed animals and food consumers trustful, healthy and low risk feed and food along the whole chain from farm to fork. Safe feed and food are also areas of research that are central to the fellow's home institution, the IMR.[1]

Currently, researchers at both institutes are working independently to develop molecular tools for food and feed safety risk assessment. At the IMR, in Norwegian Research Council (NRC) funded projects (NRC: 227387[11] and NRC: 268344[12]), in collaboration with the European Reference Laboratory for Animal Proteins in Feedstuffs (CRA-W), the University of Namur (UN) and the Leiden University Medical Centre (LUMC), global spectral library-based mass spectrometry methods are being developed for species- and tissue-specific differentiation of processed animal proteins (PAP) (Rasinger et al., 2016). In the course of this work, databases were created that are suitable for large-scale data mining for tissue- and species-specific peptide markers and the *in silico* prediction of potentially bioactive peptides such as allergens. Research at the BfR in projects funded by the German Federal Ministry of Food and Agriculture currently also focuses on mass spectrometry methods for detection of terrestrial animal species in highly processed feed. Unlike the global spectral library-based methods developed at the IMR, researchers at the BfR and its collaboration partner, the Natural and Medical Sciences Institute at the University of Tübingen (NMI), focus on targeted mass spectrometry analysis (Steinhilber et al., 2018). This approach allows a more accurate estimation of the abundance of selected PAP animal species in unknown samples, but strongly relies on the discovery of suitable marker peptides, which the IMR database can provide. In other words, combining these two complementary approaches will allow a more comprehensive proteomics-based screening and characterisation of proteic material.

Based on the common interest and complementary experience in the application of 'omics tools for PAP detection, the fellow and his home institute were named key collaborators in the Unit 51's EU-FORA hosting site application for the second cycle starting in autumn 2018. The application was successful and the Unit's next EU-FORA fellow will be working on a project entitled 'The use of novel DNA- and mass spectrometry-based detection methods for the identification of potential allergenic species and food authentication'; the mass spectrometry-based work will be conducted in close cooperation with the former fellow from the IMR.

In addition to the EU-FORA hosting site application, the fellow and his supervisor also drafted a joint application for funding under the NRC INTPART (International Partnerships for Excellent Education, Research and Innovation) programme.[13] The objective of the INTPART programme is to develop world-class research and education in Norway through long-term international cooperation and will provide funding for the establishment and further development of the institutional cooperation on

[11] https://www.forskningsradet.no/prosjektbanken/#/project/NFR/227387/Sprak=en
[12] https://www.forskningsradet.no/prosjektbanken/#/project/NFR/268344/Sprak=en
[13] https://www.forskningsradet.no/en/Funding/INTPART/1254007331831

research and higher education. The project proposal was entitled 'Food and feed risk assessment in a circular economy' and aims to set up a long-term research and education partnership between the IMR, a globally recognised institute for aquaculture and fisheries research, and the BfR, a leading European centre for risk assessment. At the time of writing, the project proposal was under review by the NRC.

3. Conclusions

In the course of the EU-FORA fellowship programme, the fellow was introduced to and gained experience in the use of modern *in silico* tools for chemical hazard assessment. In this process, the fellow learned best practice approaches for QSAR-based data analysis principles and strengthened personal skills related to the refinement of computational tools for data management and data mining. The EU-FORA project at the BfR contributed to the continuous further education of the fellow in the use of *in silico* toxicology tools, corroborated findings related to the safety of food contaminants and laid the foundations for future collaboration between the fellow's home institution, the IMR in Norway, and the BfR in Germany.

In addition to the practical 'learning by doing' education, the EU-FORA programme also provided for a rich social experience both during the training modules and the placement at the BfR. During the theoretical models close ties and valuable personal and professional networks were formed with other fellows, course tutors and course organisers. At the BfR, under the lead of Dr Gollnick, the very competent and helpful International Affairs team organised an introductory workshop to the BfR and various international events throughout the year. These fora proved a very valuable way to quickly become familiar with the institute and the city of Berlin and to establish close links and friendships with international guest scientists and resident researchers and staff at the BfR.

References

Benfenati E, Como F, Manzo Marco, Gadaleta D, Toropov A and Toropova A, 2017. Developing innovative *in silico* models with EFSA's OpenFoodTox database. EFSA supporting publication 2017:EN-1206, 19 pp. Available online: http://www.efsa.europa.eu/en/supporting/pub/1206e

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

EFSA, 2014. Modern methodologies and tools for human hazard assessment of chemicals. EFSA Journal, 12, 3638.

EU, 2010. 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 (text with EEA relevance). Official Journal of the European Union, L 276, 33–79: OJ L 276, 20.10.2010.

EU, 2017. Commission Implementing Regulation (EU) 2017/962 of 7 June 2017 suspending the authorisation of ethoxyquin as a feed additive for all animal species and categories (text with EEA relevance). Official Journal of the European Union, L 145, 13–17: OJ L 145, 8.6.2017.

Frenzel F, Buhrke T, Wenzel I, Andrack J, Hielscher J and Lampen A, 2017. Use of *in silico* models for prioritization of heat-induced food contaminants in mutagenicity and carcinogenicity testing. Archives of Toxicology, 91, 3157–3174. https://doi.org/10.1007/s00204-016-1924-3

Gleeson MP, Modi S, Bender A, Robinson RLM, Kirchmair J, Promkatkaew M, Hannongbua S and Glen RC, 2012. The challenges involved in modelling toxicity data *in silico*: a review. Current Pharmaceutical Design, 18, 1266–1291.

Glück J, Buhrke T, Frenzel F, Braeuning A and Lampen A, 2018. *In silico* genotoxicity and carcinogenicity prediction for food-relevant secondary plant metabolites. Food and Chemical Toxicology, 116, 298–306.

Maunz A, Gütlein M, Rautenberg M, Vorgrimmler D, Gebele D and Helma C, 2013. lazar: a modular predictive toxicology framework. Frontiers in Pharmacology, 4, 38.

Negreira N, Regueiro J, Valdersnes S, Berntssen MHG and Ørnsrud R, 2017. Comprehensive characterization of ethoxyquin transformation products in fish feed by traveling-wave ion mobility spectrometry coupled to quadrupole time-of-flight mass spectrometry. Analytica Chimica Acta, 965, 72–82.

Nøstbakken OJ, Duinker A, Rasinger JD, Nilsen B, Sanden M, Frantzen S, Hove HT, Lundebye A-K, Berntssen MHG, Hannisdal R, Madsen L and Maage A. Factors influencing risk assessments of brominated flame-retardants; evidence based on seafood from the North East Atlantic Ocean. Environment International (accepted).

Pradeep P, Povinelli RJ, White S and Merrill SJ, 2016. An ensemble model of QSAR tools for regulatory risk assessment. Journal of Cheminformatics, 8, 48.

Rasinger JD, Carroll TS, Lundebye AK and Hogstrand C, 2014. Cross-omics gene and protein expression profiling in juvenile female mice highlights disruption of calcium and zinc signalling in the brain following dietary exposure to CB-153, BDE-47, HBCD or TCDD. Toxicology, 321, 1–12.

Rasinger JD, Marbaix H, Dieu M, Fumière O, Mauro S, Palmblad M, Raes M and Berntssen MHG, 2016. Species and tissues specific differentiation of processed animal proteins in aquafeeds using proteomics tools. Journal of Proteomics, 147, 125–131.

Rasinger JD, Lundebye A-K, Penglase SJ, Ellingsen S and Amlund H, 2017. methylmercury induced neurotoxicity and the influence of selenium in the brains of adult zebrafish (*Danio rerio*). International Journal of Molecular Science, 18, 725.

Reffatto V, Rasinger JD, Carroll TS, Ganay T, Lundebye A-K, Sekler I, Hershfinkel M and Hogstrand C, 2018. Parallel *in vivo* and *in vitro* transcriptomics analysis reveals calcium and zinc signalling in the brain as sensitive targets of HBCD neurotoxicity. Archives of Toxicology, 92, 1189–1203.

Steinhilber AE, Schmidt FF, Naboulsi W, Planatscher H, Niedzwiecka A, Zagon J, Braeuning A, Lampen A, Joos TO and Poetz O, 2018. Mass spectrometry-based immunoassay for the quantification of banned ruminant processed animal proteins in vegetal feeds. Analytical Chemistry, 90, 4135–4143.

Wenzl T, Lachenmeier DW and Gökmen V, 2007. Analysis of heat-induced contaminants (acrylamide, chloropropanols and furan) in carbohydrate-rich food. Analytical and Bioanalytical Chemistry, 389, 119–137.

Abbreviations

2-MCPD	2-chloro-1,3,-propanediol
3-MCPD	3-chloro-1,2,-propanediol
AGES	Austrian Agency for Health and Food Safety
BfR	German Federal Institute for Risk Assessment
CRA-W	European Reference Laboratory for Animal Proteins in Feedstuffs
EFET	Hellenic Food Authority
EPA	Environmental Protection Agency
EQ	6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline
EU-FORA	The European Food Risk Assessment Fellowship Programme
FDA	Food and Drug Administration
GMO	genetically modified organisms
IMR	Institute of Marine Research
InChIKeys	International Chemical Identifier Keys
INTPART	International Partnerships for Excellent Education, Research and Innovation
ITS	Integrated Testing Strategies
lazar	lazy structure–activity relationships
LUMC	Leiden University Medical Centre
NMI	Natural and Medical Sciences Institute at the University of Tübingen
NRC	Norwegian Research Council
NRL-AP	National Reference Laboratory for Animal protein in Feed
OECD	Organisation for Economic Co-operation and Development
PAP	processed animal proteins
QSAR	quantitative structure–activity relationship
QTOFMS	quadrupole time-of-flight mass spectrometry
SDF	structured data files
SMILES	simplified molecular input line entry system
T.E.S.T	Toxicity Estimation Software Tool
TP	transformation products
TWIMS	travelling-wave ion mobility spectrometry
UN	University of Namur
WoE	Weight-of-Evidence

EU-FORA SERIES 1

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16086

Use of next-generation sequencing in microbial risk assessment

UCD Centre for Food Safety, Dublin, Ireland,
K Van Hoorde and F Butler

Abstract

Despite the ever increase in rigorous control and monitoring measures to assure safe food along the entire farm-to-fork chain, the past decade has also witnessed an increase in microbial food alerts. Hence, research on food safety and quality remain of utmost importance. Complementary, and at least as important, is the necessity to be able to assess the potential microbial risks along the food chain. Risk assessment relies on sound scientific data. Unfortunately, often, quality data are limited if not lacking. High-throughput tools such as next-generation sequencing (NGS) could fill this gap. NGS approaches can be used to generate ample qualitative and quantitative data to be used in the risk assessment process. NGS applications are not new in food microbiology with applications ranging from pathogen detection along the food chain, food epidemiology studies, whole genome analysis of food-associated microorganisms up to describing complete food microbiomes. Yet, its application in the area of microbial risk assessment is still at an early stage and faces important challenges. The possibilities of NGS for risk assessment are ample, but so are the questions on the subject. One of the major strengths of NGS lies in its capacity to generate a lot of data, but to what extend can this wealth be of use in hazard identification, hazard characterisation and exposure assessment to perform a sound risk characterisation, which in turn will make it possible to take substantiated risk management decisions.

Keywords: food safety, next-generation sequencing, whole genome sequencing, microbial risk assessment, Illumina, Oxford Nanopore Technologies, MiSeq, MinION

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: UCD Centre for Food Safety, Dublin, Ireland, Van Hoorde K and Butler F, 2018. Use of next-generation sequencing in microbial risk assessment. EFSA Journal 2018;16(8): e16086, 13 pp. https://doi.org/10.2903/j.efsa.2018.e16086

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

The pathogenic properties of food-borne microorganisms are strain dependent. In addition, the epidemic potential of a food-borne strain within its population can vary in function of its genetic make-up and ecological items (EFSA, 2013). Hence, for microbial pathogens in a context of food safety, whether it is for the detection and/or investigation of food-borne outbreaks (e.g. route- and source-tracking, cross-contamination events), attribution studies, assessment of possible virulence properties or epidemic potential and integrating all these data into risk assessment evaluations, even up to intervention and control strategy studies, it is of utmost importance that the pathogen under investigation is unambiguously identified and characterised. To this end, a wide variety of microbial typing methods is at our disposal.

Microbial typing refers to the process of identifying or discriminating between different types of microorganisms within the same microbial species. Although classical phenotyping methods, i.e. methods relying on phenotypic properties (e.g. serotyping, phage-typing, the use of antimicrobial profiling, chemotaxonomic profiling) are still being used, in the last decades due to advances in molecular techniques, we have experienced an important and inevitable shift towards genotyping methods relying on the information present in DNA or RNA molecules. The typing technique of choice largely depends on the hazard, the purpose and required level of resolution and the appropriate state-of the art. Sometimes, a combination of methods is advised (Figure 1).

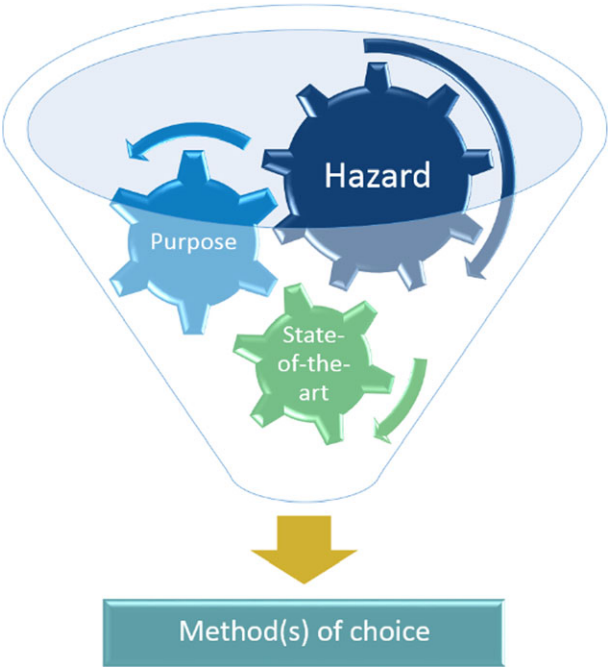

Figure 1: Factors determining the choice of typing method

Which typing method is chosen depends on the hazard, the purpose of the analysis and the most appropriate technique(s) at hand for the purpose.

Fingerprinting techniques, such as pulsed-field gel electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (MLVA), and the DNA sequence-based approach, multilocus sequence typing (MLST), have proven to be valuable tools for the surveillance and detection of food-borne disease outbreaks (Swaminathan et al., 2001; Joseph and Forsythe, 2012; EFSA, 2013; Lindstedt et al., 2013). However, a shortcoming of PFGE and MLVA is that they often fail to provide appropriate discriminatory power for specific subtypes within a given pathogen species to discriminate between outbreak-related and sporadic cases (Allard et al., 2012; Franz et al., 2016). Also, MLST has problems when confronted with pathogens that are highly conserved and have a high level of clonal population structure. For such low levels of diversity, the resolution of MLST might not always suffice (Ranieri et al., 2013).

More recently, next-generation sequencing (NGS) technologies have entered the field, offering opportunities to characterise food-borne pathogens in much more great detail and giving access to the

genetic information of pathogens at the highest resolution (Croucher and Didelot, 2015; Franz et al., 2016). With respect to the broad context of microbial hazards and food safety, within NGS applications, two tendencies can largely be discerned: whole genome sequencing (WGS) and metagenomics. In the same way, if interest lies in functionality, the methodology can be extrapolated at the RNA level with whole transcriptome sequencing and metatranscriptomic approaches, respectively. All of which, be it at the DNA or RNA level, or in combination, can deliver ample information that potentially could be used to contribute to the processes of hazard identification and characterisation, or even to be integrated in exposure assessment studies.

2. Description of work programme

2.1. Aims

The objective of this EFSA EU-FORA project is to use state-of-the-art NGS techniques to characterise and track microbial pathogens in a food-processing facility to identify contamination sources and transmission routes of pathogens through the process facility. These molecular data will be used to underpin the development of the next generation of microbial risk assessments of pathogen transmission in food processing facilities and advance existing EFSA risk assessment methodologies in this area. The project will focus on dairy powder products that present ongoing food safety challenges.

The first objective will be an intensive induction and training period during which the fellow will be trained in advanced laboratory and bioinformatics techniques so that the fellow can successfully sequence a microbial isolate on the Illumina Platform MiSeq sequencer facility at UCD as well as apply the appropriate bioinformatics pipelines to characterise and genetically compare isolates sourced from a dairy production facility. In addition, the fellow will be trained in the use of Nanopore sequencing at the Center for Food Safety and Applied Nutrition (CFSAM) facilities of the Food and Drug Administration (FDA), Maryland, USA. Through this approach by learning and doing, the fellow will get a better understanding of different NGS solutions, their strengths and weaknesses, and how the generated data can be used in the context of food safety and ultimately risk assessment.

The second objective of the fellowship, through collaboration with Teagasc and Irish dairy manufacturing facilities, is coupled to studies using WGS to characterise the spatial and temporal distribution and genetic diversity of selected groups of bacteria (e.g. *Cronobacter* spp. and spore-forming bacteria such as *Bacillus* spp.) at key stages in the production process and in the final product.

In a last phase, which will run to the end of the fellowship period, it is the objective to undertake the bioinformatics associated with all of the sequence data assembled in the course of the project as well as the large amount of relevant sequence data already collected by the UCD Centre for Food Safety through ongoing process facility surveillance projects. The main goal will be to determine the phylogenetic relationship of isolates based on for example single nucleotide polymorphism (SNP) analysis and through core genome data set analysis (i.e. genes shared by $\geq$ 95% of isolates). These techniques allow a comparison of the molecular similarity or diversity of bacterial isolates recovered from a production facility and gives enormous power to identify contamination sources and transmission routes of pathogens through a process facility. In addition to this end, for a selection of isolates, data generated through Illumina and Nanopore sequencing will be compared and complemented.

2.2. Activities/methods

2.2.1. Overview of activities

The performed work in this EFSA EU-FORA project constituted out of three separate parts:

- In a first part, a collection of isolates recovered from a dairy processing environment was identified using a WGS approach. The intent of the work performed in this first part was threefold: (i) learning the theoretical and practical aspects of WGS using Illumina technology on a MiSeq; (ii) getting familiar with the type of data generated and on bioinformatics tools for the analysis of the sequence data; and (iii) achieve an unambiguous identification of the isolates.
- The second part consisted of applying the acquired skills of Illumina sequencing for the identification of a selection of 24 *Bacillus* spp. isolates using WGS.

- The third part involved a comparative but also complementary study using Illumina and Nanopore technology for WGS of a set of *Cronobacter sakazakii* isolates representing four different MLST sequence types. Aims of this part consisted of:

  i) learning the theoretical and practical items of whole genome sequencing using Nanopore technology on a MinION instrument;
  ii) gain insights on different NGS methodologies for WGS;
  iii) compare and combine the complementary data sets for the assembly of the whole genomes of the sequenced isolates;
  iv) transferring acquired knowledge on Nanopore sequencing to the UCD host site.

2.2.2. Description of applied next-generation sequencing methodologies

In the fellowship, two different NGS technologies were used: sequencing-by-synthesis (SBS) using the reversible terminator technology by Illumina, and single molecule sequencing using Nanopore technology by Oxford Nanopore Technologies (https://nanoporetech.com/about-us/for-the-media) (Figure 2).

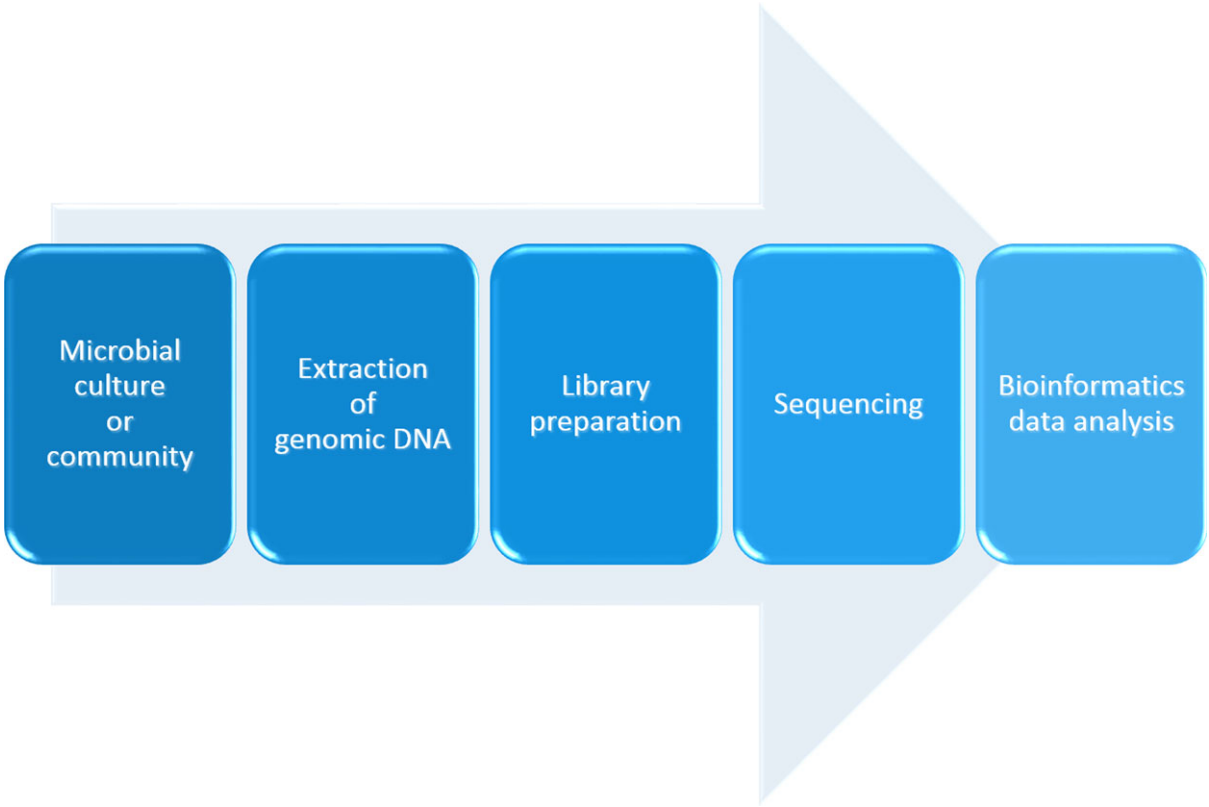

Figure 2: Next-generation sequencing workflow

A NGS approach in general starts with the extraction of total DNA from a pure culture, for whole genome sequencing, or from the community, in a metagenomics study. Following a library preparation step, which can involve polymerase chain reaction (PCR), and sequencing of the generated library pool, bioinformatics tools are used for data analysis.

2.2.2.1. Illumina technology (Figures 3 and 4) (Courtesy of Illumina, Inc.)

The reversible terminator technology employed by Illumina uses a sequencing concept that is similar to the chain termination procedure used in Sanger sequencing, in that, the strand elongation is stopped after the incorporation of a fluorescently labelled base that prevents further strand elongation and the label of the incorporated base is read out to reveal sequence information (Figure 3). However, contrary to Sanger sequencing with an irreversible termination of strand elongation and with sequence information retrieved from strands that differ in length by one base (i.e. the fluorescently labelled

base), in the reversible terminator technology, termination is reversible and the sequence is determined in real time at the moment of incorporation of the fluorescently labelled bases.

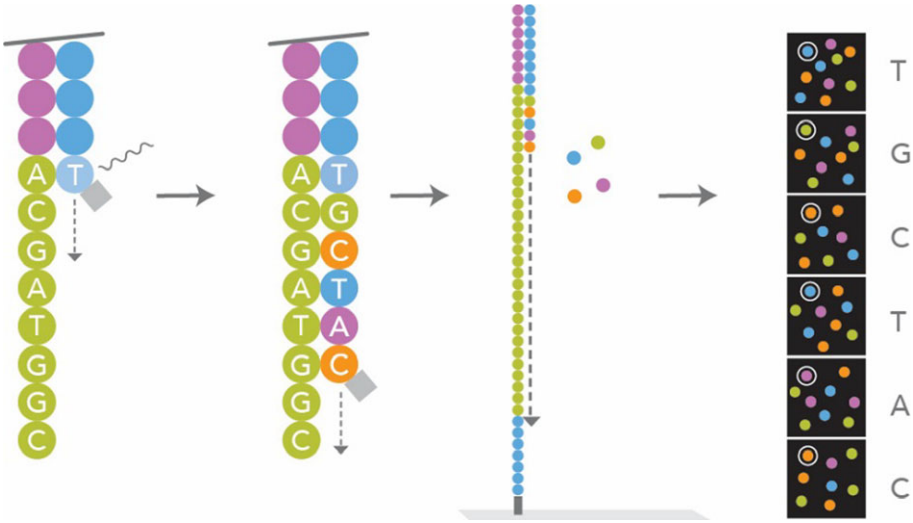

Figure 3: Reversible terminator technology during sequencing-by-synthesis

During each sequencing cycle, all four nucleotides are presented to the growing strand as fluorescently labelled (colour of the nucleotide) and blocked (grey square) bases. Only the complementary base will be incorporated and its complementary fluorescent signal is detected (i.e. base calling). No further elongation of the strand occurs as the incorporated base is blocked at its 3′OH. Unincorporated bases are washed away. Before a new cycle, the fluorescent label is removed and the nucleotide is unblocked to allow the incorporation of the next base (Courtesy of Illumina, Inc.).

Following DNA extraction, similar to many other NGS approaches, first a sequencing library needs to be constructed to amplify and immobilise the templates for sequencing. Resulting library fragments consist of the fragment to be sequenced flanked by two different adapters (Figure 4A). This double-stranded library is denatured to obtain single-stranded DNAs that are applied on a flow cell. This flow cell has on its surface two populations of immobilised oligonucleotides complementary to the two different single-stranded adapter ends of the sequencing library. Denatured oligonucleotides anneal to the complementary single-stranded library oligonucleotides on the flow cell surface (pink and blue). Via reverse strand synthesis starting from the 3′ end of the surface bound oligo (the double-stranded part), a new strand is created. Upon denaturation, the original strand is removed and the newly synthesised copy remains covalently bound to the flow cell. If this new strand bends over and attaches to the other oligonucleotide type complementary to the second adapter sequence that is present on the free 3′ end of the strand, it can be used to synthesise a second covalently bound reverse strand. The process of bending and reverse strand synthesis is called bridge amplification. Bridge amplification is repeated several times and creates clusters of several 1,000 copies of the original sequence in a very close proximity to each other on the flow cell (Figure 4B). At the end, each cluster on the flow cell consists of a single-stranded, identically oriented copies of the same sequence. These can be sequenced by hybridising the sequencing primer onto the adapter sequences and starting the reversible terminator chemistry (Figure 4C).

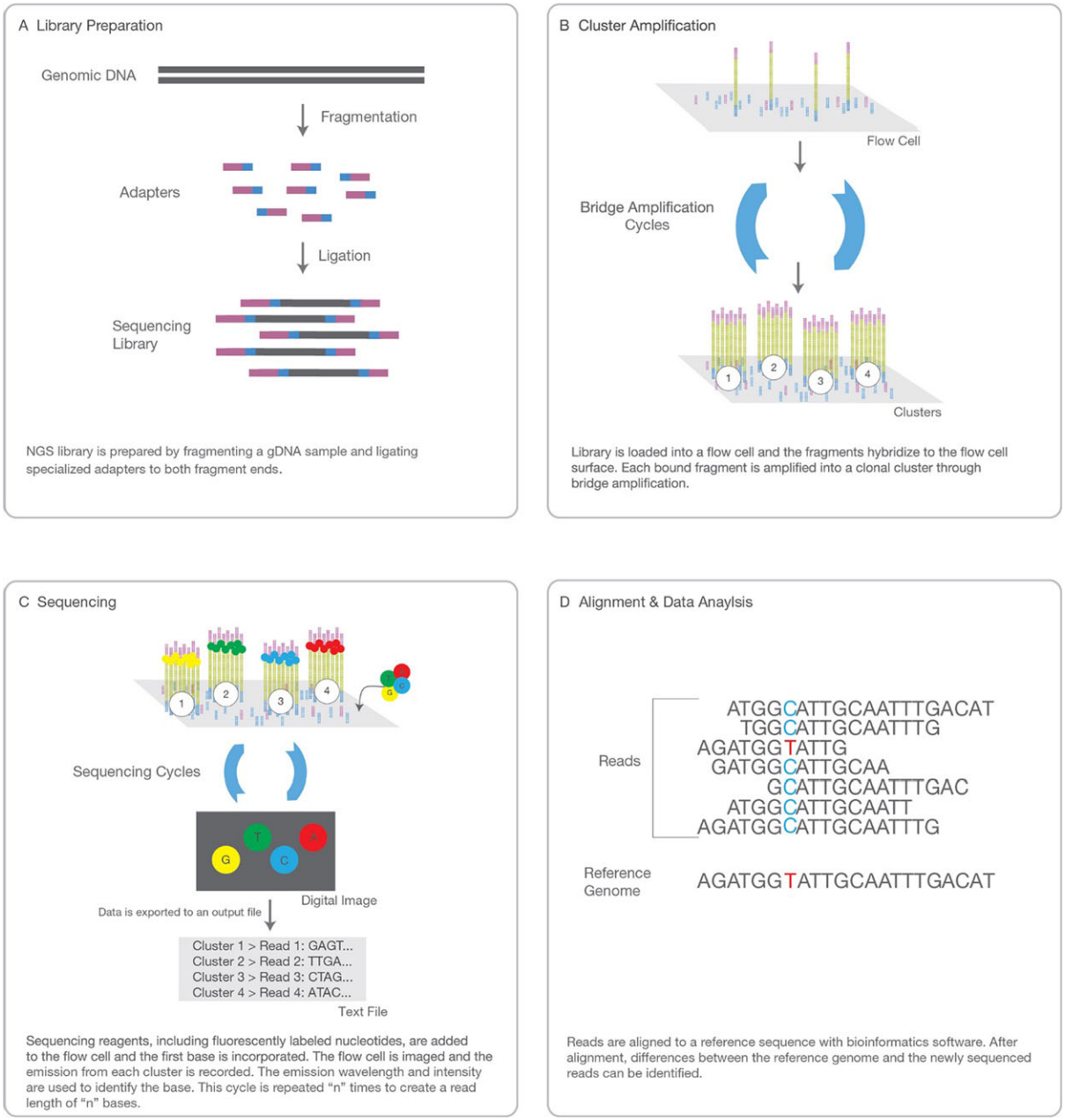

Figure 4: Illumina sequencing workflow

Illumina sequencing workflow consisting of library preparation (A), cluster amplification (B), sequencing (C) and bioinformatics data analysis (D) (Courtesy of Illumina, Inc.).

2.2.2.2. Nanopore technology (Figures 5 and 6) (Courtesy of Oxford Nanopore Technologies)

The Nanopore technology by Oxford Nanopore Technologies can be considered as a true 'third-generation' sequencing (TGS) technology, i.e. a single molecule sequencing (SMS) technology were reads represent the sequencing of a single molecule without the need of a replication enzymatic system (Schadt et al., 2010). In addition, the technology allows for real-time sequencing and of long reads up to 1 Mb and longer (Loose, 2018a,b), which offers advantages during genome assembly.

After a DNA extraction to obtain high-molecular weight genomic DNA, a library is constructed without the need for PCR amplification steps. During the library preparation, adapters are ligated to the DNA fragments and a processive enzyme is coupled. Upon interaction of the sequence–enzyme complex with the Nanopore, sequencing commences (Figure 5). The sequencing technology relies on nanopores that function as a channel between two chambers of an electrophoretic system (Figure 6).

When a small voltage (~ 100 mV) is applied across the Nanopore, the resulting current can be measured. Molecules going through the Nanopore are responsible for the disruption in the ionic current. Measuring of this disruption allows the identification of the molecule. In the context of DNA, each base gives a subtly different reading as it passes through the pore, allowing direct reading of the sequence. Raw output used for base calling is an electronic trace of current changes generated not by individual bases but by 5- or 6-nucleotide 'words' known as k-mers. Sequence data are streamed as DNA fragments translocate through the pore, permitting real-time analysis (Loman and Watson, 2015; Lu et al., 2016).

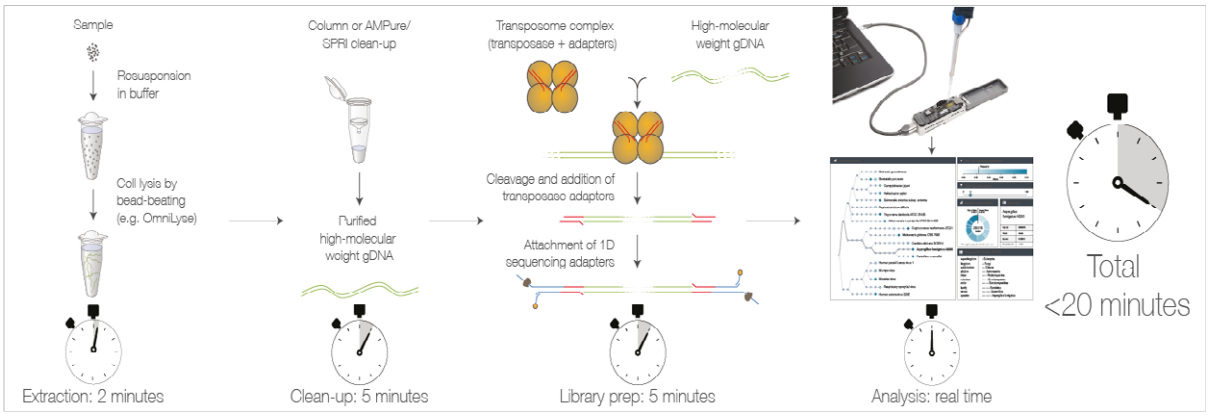

Figure 5: Oxford Nanopore Technology sequencing workflow on the MinION instrument

Schematic overview of sequencing workflow on Oxford Nanopore Technologies' MinION instrument. Following a DNA extraction to obtain high-molecular weight genomic DNA (gDNA), a library is constructed prior to the real-time sequencing analysis (courtesy of Oxford Nanopore Technologies: https://nanoporetech.com/resource-centre/posters/dna-extraction-and-library-preparation-rapid-genus-and-species-level).

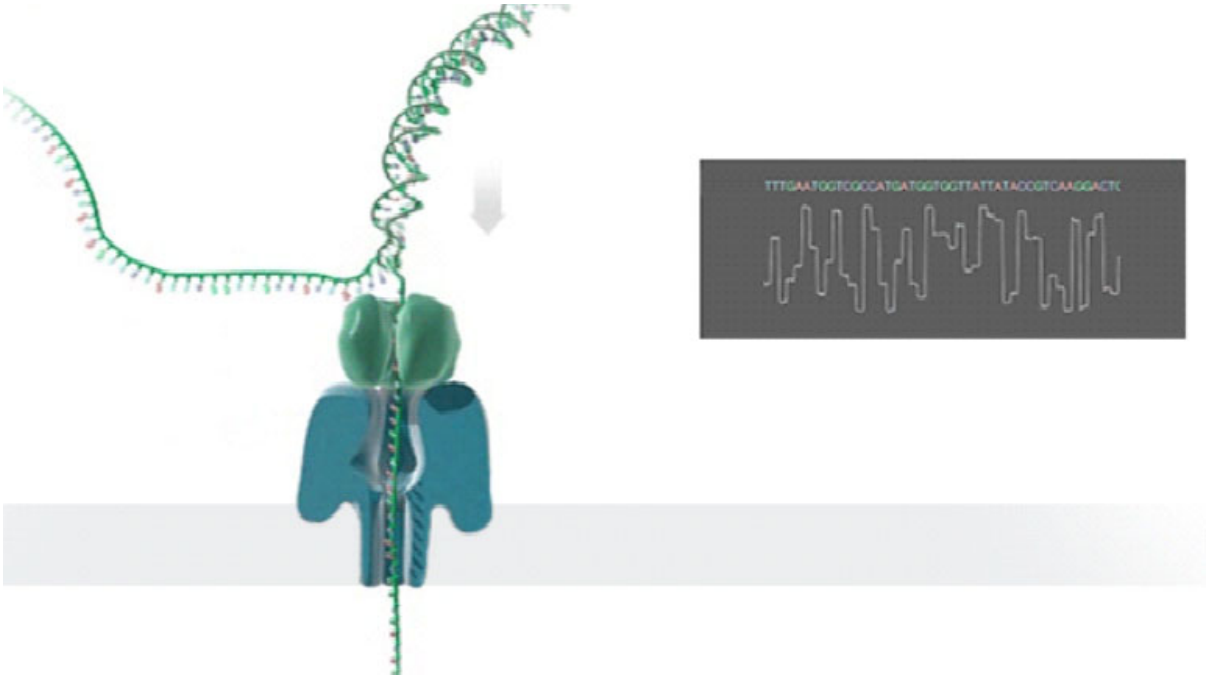

Figure 6: Oxford Nanopore sequencing

The DNA is coupled to a processive enzyme (green). The DNA–enzyme complex interacts with the Nanopore (blue). Single-stranded DNA is pulled through the Nanopore aperture one base at a time. As the DNA moves through the pore, the combination of nucleotides in the strand being processed creates a characteristic disruption in the electrical current. This signal can then be used to determine

the order of bases on that particular DNA strand (current trace diagram on the right) (courtesy of Oxford Nanopore Technologies).

2.2.3. Whole genome sequencing of dairy processing environment isolates

To become familiar with the technology and bring theoretical knowledge into practice, a set of 18 isolates from a dairy processing environment was sequenced with Illumina technology using a MiSeq instrument. For library preparation, the NEBNext® Ultra™ II FS DNA library prep kit (NEB) was used. For sequencing the MiSeq reagent kit V3 (Illumina) 600 cycles and a paired-end run was used.

The isolates were considered to belong to the genus *Bacillus*. However, preliminary identification using partial 16S rRNA sequencing and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) did not allow the correct identification, either due to the lack of resolution of 16S rRNA sequencing and/or due to conflicting results between 16S and MALDI-TOF MS. As WGS-based approaches are considered to be superior compared with traditional 16S rRNA sequence analysis due to their much higher resolution because they are based on a much larger part of the genome, it was decided, in the project, to sequence the whole genome of these isolates to get a correct identification. Obtained sequences were analysed using bioinformatics tools available at https://cge.cbs.dtu.dk/services/.

2.2.4. Whole genome sequencing of *Bacillus* spp. isolates

The second part of work was part of a larger study identifying key or core spores and spore-forming bacteria in skimmed milk powders. Twenty-one samples of medium-heat skim milk powders from eight different facilities were analysed for their spore-forming community using 16 different microbiological tests. A preliminary identification of a selection of 285 isolates was performed using partial 16S rRNA (a 570-bp part of the V3–V5 region) sequencing. Blast analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and pairwise and multiple alignment using the BioNumerics Software version 7.6.3. (Applied Maths, bioMérieux) revealed the presence of eight different genera divided over 21 sequence clusters (Figure 7).

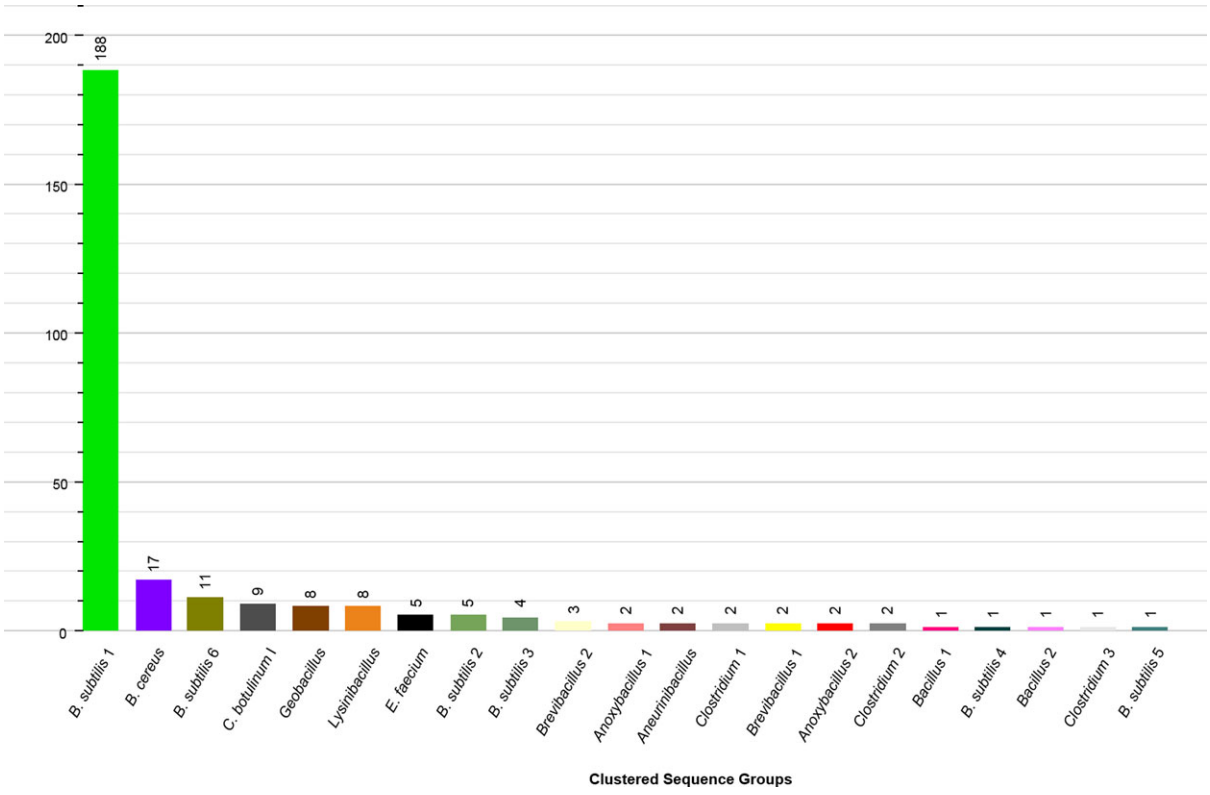

Figure 7: Observed 16S rRNA based diversity

Preliminary identification results, of 285 isolates, based on a 570-bp fragment of the 16S rRNA gene.

Although 16S rRNA sequencing has long been the gold standard for species delineation, it has become evident that the resolution is not sufficient for closely generated species. Increasing the cut-off from 97% to 98.7% 16S rRNA sequence similarity to be considered belonging to the same species (Stackebrandt and Ebers, 2006) still resulted in conflicting results within many genera. This is also the case for the genus *Bacillus*, the 16S rRNA gene encountering problems in resolving species within this genus (Caamaño-Antelo et al., 2015). For example, the species within the *Bacillus subtilis* group exhibited 16S sequence similarity varying between 98.1% up to 99.8%. A selection of 24 isolates, identified as belonging to the *B. subtilis* group based on the partial 16S sequence, was picked for whole genome sequencing using Illumina technology with a MiSeq machine to obtain a more correct species identification and more information on the genetic diversity within the species. For library preparation, Illumina's Nextera® XT (Illumina) DNA library preparation method was used and for paired-end run sequencing, the MiSeq reagent kit V3 for 600 cycles was selected. A pipeline of several bioinformatics tools will be used for the quality control, trimming and assembly of the sequences. Subsequently assembled WGS contigs will be used for identification and characterisation purposes.

2.2.5. Oxford Nanopore sequencing of selected *Cronobacter sakazakii* isolates

One isolate of each of the four MLST sequence types (1, 4, 41 and 42) of *Cronobacter sakazakii,* retrieved from a dairy powder processing environment and previously characterised via a whole genome MLST (wgMLST) approach, was selected for WGS using Oxford Nanopore technology. Despite the higher error rate compared, the much longer fragment reads that can be sequenced aided the assembly of a reduced number of bigger contigs. The already available MiSeq accurate short-read sequences will complement any errors to increase the accuracy of the assembly.

2.2.6. Other secondary activities performed in the EU-FORA fellowship

i) Cooperation with the Agriculture and Food Development Authority of Ireland (Teagasc).
ii) Attended a food safety regulation training (a module that is part of the MSc Food Safety and Risk Analysis, University College Dublin) organised by the Food Safety Authority of Ireland (FSAI).
iii) Attended the EFSA Scientific Colloquium No. 24, 'OMICS in risk assessment: state-of-the-art and next steps' (24–25 April 2018, Berlin, Germany). As a member of the discussion group 'Genomics for the identification and characterisation of microbial strains used in food and feed products', discussed the potential of WGS analysis for improving hazard identification of microbial strains that enter the food chain or used as production strains.
iv) Panel member 'building a global monitoring system for food-borne illness and AMR' during Asset 2018, 28–31 May 2018 Belfast, Northern Ireland.
v) Visiting the Center for Food Safety and Applied Nutrition (CFSAM) facilities of the FDA, Maryland, USA that has a close collaboration with the UCD, Centre for Food Safety hosting site.
vi) A short outreach moment on the EU-FORA programme at the FSAI premises in the presence of FSAI staff and other Article 36 organisations. This trip will be coupled by a 1- to 2-day visit to the FSAI to get a better insight into the activities of the Irish food safety authority.

3. Conclusions

With the introduction of high-throughput sequencing platforms, NGS applications have become widely applied. It is without any doubt that the application of NGS, be it through whole genome sequencing or more elaborate metagenomics approaches, offers a plethora of opportunities in the areas of food safety and microbial risk assessment. The most important difference between both approaches lies in the difference in target used, i.e. for WGS, the nucleic acids obtained from a pure culture isolate, and in metagenomics, total nucleic acids retrieved directly from the microbiota present in the environment under investigation. This can be extrapolated at the RNA level with whole transcriptome sequencing and metatranscriptomics, respectively.

For WGS that relies on a culture-based approach, microbial growth is required using selective culture medium and conditions to recover the target organism. The output of WGS is the complete DNA content of a microorganism, inferring information on genes that are under diversifying selection

(e.g. antigen genes), genes that are under stabilising selection (e.g. housekeeping genes) and genes that might be of relevance from a food safety perspective (e.g. virulence genes, AMR genes, toxin production), but also of non-coding regions and possible episomal DNA present. Hence, WGS has the potential to describe a bacterial strain at the highest genetic detail allowing the full characterisation of a strain. WGS is also universal applicable, in contrast with for example MLST or PFGE, the choice of loci or restriction enzymes, respectively, depending on the taxonomic group or pathogen under investigation. However, this will require standardisation in the way the data are generated and analysed and at an interlaboratory level preferably on a global scale. Initiatives to promote this harmonisation exist, such as www.globalmicrobialidentifier.com and efforts have been undertaken to standardise for example some quality parameters and on guidelines how WGS data can be used to its best (Chun et al., 2018). Moreover, once fully characterised, the information can be used for detection and/or investigation of food-borne outbreaks (e.g. route- and source-tracking, cross-contamination events), attribution studies, assessment of possible virulence properties or epidemic potential, and integrating all these data into risk assessment evaluations, even up to intervention and control strategy studies. Taking these applications into consideration, it is important to be aware of some of issues inherent to culturing approaches: they cannot be considered quantitative as they might under- or overestimate the true diversity present. In addition, currently, the genetic content of a microorganism cannot be seen detached from its phenotypic traits (e.g. its pathogenicity) in relation to host and environmental factors.

Metagenomics approaches target the nucleic acids of the microorganisms in the environment or food matrix and have the advantage that no prior cultivation steps are necessary to investigate the full community, it is a so-called culture-independent technique. The complexity of such kind of analyses makes it even a more daunting task to use the generated data in a context of food safety and risk assessment. A solution lies in the identification of biomarkers that are linked and might predict microbial behaviour (Brul et al., 2012). Also here, it is important that a connection is made between host and environmental conditions and expression level of the biomarker on the one hand and phenotypic behaviour on the other hand. The continued increase in other 'omics data from proteomics and metabolomics studies will certainly contribute in this process.

Finally, similar to many other currently applied identification and typing methods in which the method of choice depends on the pathogen and the question that needs to be addressed, for NGS, the type of approach and the type and extent of data used will depend on the purpose (Franz et al., 2016).

The programme itself aimed at familiarising the fellow with NGS in the context of food safety and risk assessment and this via a principle of 'learning by doing'. First, in addition to the necessary theoretical insights, wet-laboratory experience was gained for two NGS techniques: high-throughput sequencing of short reads using Illumina technology with a MiSeq instrument and long-read sequencing using a MinION based on Nanopore technology. In addition, the fellow acquired bioinformatics skills using Unix-based and other tools necessary for the analysis (quality, trimming, assembly) of generated sequence data. Given the acquired theoretical and practical insights, additional discussion will aid the interpretation on how these techniques and data can be implemented in a microbial risk assessment framework.

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, 32.

Brul S, Bassett J, Cook P, Kathariou S, McClure P, Jasti PR and Betts R, 2012. 'Omics' technologies in quantitative microbial risk assessment. Trends in Food Science and Technology, 27, 12–24.

Caamaño-Antelo S, Fernández-No IC, Böhme K, Ezzat-Alnakip M, Quintela-Baluja M, Barros-Velázquez J and Calo-Mata P, 2015. Genetic discrimination of foodborne pathogenic and spoilage *Bacillus* spp. based on three housekeeping genes. Food Microbiology, 46, 288–298.

Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, Rooney AP, Yi H, Xu X-W, De Meyer S and Trujillo ME, 2018. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. International Journal of Systematic and Evolutionary Microbiology, 68, 461–466.

Croucher NJ and Didelot X, 2015. The application of genomics to tracing bacterial pathogen transmission. Current Opinion in Microbiology, 23, 62–67.

EFSA (European Food Safety Authority), 2013. Scientific opinion on the evaluation of molecular typing methods for major food-borne microbiological hazards and their use for attribution modelling, outbreak investigation and scanning surveillance: part 1 (evaluation of methods and applications). EFSA Journal 2013;11(12):3502, 84 pp. https://doi.org/10.2903/j.efsa.2013.3502

Franz E, Gras LM and Dallman T, 2016. Significance of whole genome sequencing for surveillance, source attribution and microbial risk assessment of foodborne pathogens. Current Opinion in Food Science, 8, 74–79.

Joseph S and Forsythe SJ, 2012. Insights into the emergent bacterial pathogen *Cronobacter* spp., generated by Multilocus Sequence Typing and Analysis. Frontiers in Microbiology, 3, 397.

Lindstedt BA, Torpdahl M, Vergnaud G, Le Hello S, Weill FX, Tietze E, Malorny B, Prendergast DM, Ní Ghallchóir E, Lista RF, Schouls LM, Söderlund R, Börjesson S and Åkerström S, 2013. Use of multilocus variable-number tandem repeat analysis (MLVA) in eight European countries, 2012. Eurosurveillance, 18, 20385.

Loman NJ and Watson M, 2015. Successful test launch for nanopore sequencing. Nature Methods, 12, 303–304.

Loose M, 2018a. Available online: https://twitter.com/mattloose/status/954147458778587136 [Accessed: 27 May 2018]

Loose M, 2018b. Available online: https://twitter.com/mattloose/status/992117714289217536 [Accessed: 27 May 2018]

Lu H, Giordano F and Ning Z, 2016. Oxford Nanopore MinION sequencing and genome assembly. Genomics, Proteomics and Bioinformatics, 14, 265–279.

Ranieri ML, Shi C, Moreno Switt AI, den Bakker HC and Wiedmann M, 2013. Comparison of typing methods with a new procedure based on sequence characterization for *Salmonella* serovar prediction. Journal of Clinical Microbiology, 51, 1786–1797.

Schadt EE, Turner S and Kasarskis A, 2010. A window into third-generation sequencing. Human Molecular Genetics, 19, R227–R240.

Stackebrandt E and Ebers J, 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiology Today, 33, 152–155.

Swaminathan B, Barrett TJ, Hunter SB and Tauxe RV, 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerging Infectious Disease Journal, 7, 382–389.

Abbreviations

AMR	antimicrobial resistance
bp	base pairs
CFSAM	Center for Food Safety and Applied Nutrition
EU-FORA	The European Food Risk Assessment Fellowship Programme
FDA	Food and Drug Administration
FSAI	Food Safety Authority Ireland
gDNA	genomic DNA
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
MLST	multilocus sequence typing
MLVA	multiple-locus variable number tandem repeat analysis
NGS	next-generation sequencing
PFGE	pulsed-field gel electrophoresis
rRNA	ribosomal RNA
SBS	sequencing-by-synthesis
SNP	single nucleotide polymorphism
TGS	'third-generation' sequencing
UCD	University College Dublin
WGS	whole genome sequencing
wgMLST	whole genome multilocus sequence typing

EU-FORA SERIES 1

National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport

EFSA Journal

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16087

Assessment of occupational and dietary exposure to pesticide residues

National Institute for Public Health and the Environment (RIVM)

The Netherlands

Chemical Food Safety Department, Centre for Nutrition, Prevention and Health Services

M Markantonis, T van der Velde-Koerts, C Graven, JD te Biesebeek, M Zeilmaker, AG Rietveld and BC Ossendorp

Abstract

Plant protection products (PPPs) are pesticides containing at least one active substance that drives specific actions against pests (diseases). PPPs are regulated in the EU and cannot be placed on the market or used without prior authorisation. EFSA assesses the possible risks of the use of active substances to humans and environment. Member States decide whether or not to approve their use at EU level. Furthermore, Member States decide at national level on the authorisation of PPPs containing approved substances. In agriculture, exposure to PPPs and their residues during occupational tasks is estimated prior to product authorisation, using models fed with study-specific (e.g. absorption, dissipation) and default values. Exposure of workers to pesticide residues reduces with the pesticide's dissipation time during crop-related tasks. However, the current risk assessment gap is that no methodology is available to calculate the re-entry interval (REI) for workers, which specifies how long they should wear personal protective clothing during their first entry into pesticide-sprayed crops. Protective clothing (such as gloves) can reduce pesticide residue exposure to an acceptable level of worker safety. Within the European Food Risk Assessment Fellowship Programme (EU-FORA) assignment, a methodology was developed to calculate agricultural-use-specific and pesticide-specific REIs for which period workers should wear gloves. This was an assignment of the Dutch Ministry of Social Affairs and Employment. Another important aspect of risk assessment to ensure consumer safety is dietary risk assessment. A critical evaluation of residue studies and metabolism of the pesticide in question in crops results in a residue definition for dietary risk assessment and for enforcement and monitoring to define maximum residue limits allowed legally on or in raw agricultural commodities when applying pesticides according to good agricultural practices. This work was assigned by the Dutch Ministry of Health, Welfare and Sport and contributes to the work of the Joint FAO/WHO Meeting on Pesticide Residues.

Keywords: plant protection products, residue exposure, re-entry interval, dermal absorption, dietary risk assessment, maximum residue level

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: National Institute for Public Health and the Environment (RIVM) The Netherlands, Chemical Food Safety Department, Centre for Nutrition, Prevention and Health Services, Markantonis M, van der Velde-Koerts T, Graven C, te Biesebeek JD, Zeilmaker M, Rietveld AG and Ossendorp BC, 2018. Assessment of occupational and dietary exposure to pesticide residues. EFSA Journal 2018;16(8):e16087, 8 pp. https://doi.org/10.2903/j.efsa.2018.e16087

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

The principal focus of the European Food Risk Assessment Fellowship Programme (EU-FORA) is to provide hands-on skills in chemical and microbiological risk assessment for food safety. This individual work programme focused on two aspects of exposure assessment for plant protection product (PPP) residues: non-dietary and dietary exposure.

The work was performed at the Dutch National Institute for Public Health and the Environment (RIVM) in the Chemical Food Safety Department. The department works on the safety of food by contributing nationally, at the European Union (EU) level and globally to the development and harmonisation of risk assessment methodology, carrying out risk assessments and advising policy-makers and scrutinising decision-making. Chemical substances in food include pesticides, biocides, and veterinary drug residues, food and feed additives, contaminants, substances in food contact materials and botanicals. The department contributes to the evaluation of substances in biocides and PPPs prior to their registration based on manufacturers' applications and focuses on method and model development for use in exposure and risk assessment.

The following paragraphs give background information on the non-dietary and dietary exposure assessment. To start with, PPPs and their current regulation in the EU are briefly described. Second, a short overview of the risk assessment of PPPs in an occupational setting is given, focusing on exposure assessment of residues for crop workers. A gap in the current risk assessment methodology and in the regulation of PPPs is identified. At present, there is no methodology available to calculate product-specific re-entry intervals (REI) for workers entering pesticide-sprayed crops, which specify the period of wearing personal protective clothing. The final paragraph briefly presents the purpose of setting maximum residue limits (MRLs) for PPPs in or on raw agricultural commodities, as a result of the extensive evaluation of residue studies as a basis for the dietary exposure assessment and the relevance of the work of the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) for the EU authorisation system.

1.1. Regulation of plant protection products

PPPs are mainly used to keep crops healthy and prevent them from being destroyed by disease and infestation. They include herbicides, fungicides, insecticides, acaricides, plant growth regulators and repellents. PPPs contain at least one active substance (chemicals or microorganisms) that enables the product to perform its action. Before an active substance can be used in a PPP in the EU, it must be approved by the European Commission. Active substances undergo an intensive evaluation process before a decision can be made as to approval. PPPs are regulated by Regulation EC 1107/2009 concerning the placing of PPPs on the market including basic substances[1] (art. 23).

A large body of EU legislation regulates the marketing and use of PPPs and their residues in food. PPPs cannot be placed on the market or used without prior authorisation. A system was established whereby: (1) Member State competent authorities peer review the application, and after discussion between the competent authorities, the European Food Safety Authority (EFSA) comes to a conclusion; (2) Member State risk managers decide in Brussels, together with the European Commission, on the approval of the active substances; and (3) Member States decide at national level on the approval of products.

Regulation (EC) No 396/2005[2] covers compliance with legal limits for pesticide residues in food and feed, including provisions on official controls of pesticide residues in food (plant or animal origin).

Meeting current and future food safety demands, an optimal food safety assessment (risk assessment) requires scientific understanding and measurement of chemical hazards, estimating or modelling exposures, and ultimately concluding on risks associated with them.

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

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

1.2. Risk assessment of occupational exposure of agricultural workers to PPP residues

During occupational tasks, operators and workers may be exposed to pesticides either directly through contact with spray drift (via dermal or inhalation routes) or indirectly through contact with drift deposits (dermal or ingestion) or vapour drift arising from volatilisation of deposits. Exposure is expected to decline over time from the initial value at, or close to, the time of application.

Therefore, the total exposure from the application of PPPs results from different exposure routes (dermal, inhalation, ingestion). The Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products (EFSA, 2014) provides a model for assessing the major exposure pathways. Nevertheless, the EFSA working group on pesticides recommends that further research must be carried out to perform a more representative exposure assessment. However, for non-dietary exposure pathways, other than dermal or inhalation, in most cases few data are available to provide quantification of their impact on the overall exposure assessment.

Agricultural workers are potentially exposed to pesticide residues when they enter pesticide-treated fields to perform a variety of hand-labour tasks, such as pruning, thinning, scouting and harvesting of agricultural crops. These exposures can occur in different crops throughout the growing season and can be of similar magnitude to the exposure of operators, who mix, load and apply pesticides. For authorisation of PPPs, risk assessments must be carried out for all scenarios of exposure of operators, workers, residents and bystanders that can be expected to occur as a consequence of the proposed uses of a PPP. The risk assessment methodology focuses on scenarios for workers, and specifically assesses the period for safe re-entry of workers into a treated crop area after application of a PPP (e.g. risk-based restricted entry interval).

The practical options for managing exposures through the use of personal protective equipment, or other measures such as technical solutions, are considerably more limited for re-entry workers performing crop-related tasks, than for operators during mixing, loading and application.

Establishment of a period for safe re-entry, the REI, is a risk mitigation option although currently not used in the EU for active substance approval and PPP authorisation. The REI is intended to provide sufficient time for pesticide residues to degrade to a safe level, after which workers can safely enter a field.

Currently, risks for workers are mitigated by prescribing gloves for re-entry tasks on the PPP label. However, at present, there is no harmonised methodology available to estimate the pesticide-specific and agricultural-use-related interval for which these gloves should be worn for each intended use. A methodology has been developed to determine the REI (in this report framed as the period during which workers must wear protective clothing, e.g. gloves).

Additionally, current exposure assessment methodology takes into account principal parameters such as the dissipation time of the active substance in the PPP (DT_{50}), the transfer coefficient (from crop to worker) and the dermal absorption (DA) factor. However, current approaches take into account the DA of the spray dilution of the PPP, but not of its dried residues to which a worker is exposed. At present, there is no harmonised methodology to determine the DA factor for dried residues.

1.3. Assessment of dietary exposure to PPP residues

If PPPs are used in accordance with the regulations, residues are expected to remain in or on the harvested commodities, especially when applications are close to harvest or post-harvest. PPP residues are defined as residues of active substances and their (toxicologically) relevant degradation products that remain on or in food or feed. MRLs are stipulated for residues of PPPs in food and feed.

The Codex Alimentarius, or 'Food Code', is a collection of standards, guidelines and codes of practice adopted by the Codex Alimentarius Commission. The Commission is the central part of the Joint FAO/WHO Food Standards Programme and was established by the FAO and WHO to protect consumer health and promote fair practices in food trade. The 188 Codex members have negotiated science-based recommendations in all areas related to food safety and quality, including in the area of pesticide residues (FAO, online-a). The JMPR is an expert ad hoc body governed jointly by the FAO and WHO that proposes MRLs that can be used as Codex maximum residue limits. Furthermore, the JMPR produces guidance on the applicable risk assessment methodology. It consists of experts who act in a personal capacity and not as Member State representatives, and has performed annual

evaluations of pesticide residues in food since 1963, advising on the acceptable levels of pesticide residues, namely MRLs in food (FAO, online-b).

The current JMPR comprises the WHO Core Assessment Group and the FAO Panel of Experts on Pesticide Residues in Food and the Environment. The WHO Core Assessment Group is responsible for reviewing pesticide toxicological data and estimating acceptable daily intakes, acute reference doses and characterises other toxicological criteria. The FAO Panel reviews pesticide residue data for estimated MRLs, supervised trials median residue values and highest residues in food and feed (FAO, online-b; WHO, online). Combining the efforts of both panels, a dietary risk assessment is performed to evaluate the toxicological acceptability of the proposed MRLs. The MRLs are recommended to the Codex Committee on Pesticide Residues (CCPR) for consideration for adoption by the Codex Alimentarius Commission as Codex MRLs. Harmonised MRLs benefit trade.

2. Description of work programme

2.1. Aims

The objective of the work programme was to work on two aspects of exposure to PPP residues: (i) exposure of workers upon entry into pesticide-treated crops; and (ii) dietary exposure to residues. The first part of the project involved the use of exposure models for pesticides and identifies the information required to develop a methodology to calculate a safe REI for crop workers. The second part of the project aimed to gain a thorough understanding of critical evaluations of residue field trials, metabolism studies, and other residue studies for PPPs that were submitted by the product applicant, in order to provide an MRL for the respective food commodities and to perform a dietary risk assessment for these food commodities in the context of the JMPR.

Overall, the objective was to be involved in all aspects of daily routine work and ad hoc projects at the Department of Chemical Food Safety at the RIVM.

2.2. Activities/methods

2.2.1. Exposure of workers upon entry into pesticide-treated crops

A report was prepared that provides a methodology to determine a safe REI, during which workers must wear protective clothing (such as gloves) during entry into previously pesticide-treated crops as an exposure mitigation option for authorised PPPs. Moreover, the report contains a discussion of the parameters that have an impact on the exposure to residues, such as DA and product properties, like acute toxicity, skin irritation or sensibilisation of spray dilutions and foliar dry residues.

The drafting of the report provided an in-depth understanding of the process of legal authorisation of PPPs and the assessment requirements of the exposure risk of workers during crop entry after treatment. Moreover, information was collected through a literature search (e.g. scientific publications and EU guidance documents (Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products (EFSA, 2014) and Guidance on Dermal Absorption (EFSA, 2017)), EU Regulation (EC) No 1272/2008[3] (Regulation on Classification and Labelling) and up-to-date modelling techniques (e.g. EFSA OPEX) were applied and a new methodology developed to calculate the REI. A visit was made to the Dutch competent authority for authorisation of PPPs and biocides, namely the Dutch Board for the Authorisation of Plant Protection Products and Biocides (College voor de toelating van gewasbeschermingsmiddelen en biociden). This offered the opportunity to discuss which mathematical models were previously and currently used for the preregistration exposure risk assessment (for operators, workers, residents and bystanders) for the respective agrochemicals to be authorised in the Netherlands. On another occasion, a representative of STIGAS (the certified occupational health and safety service in the Netherlands) was invited by RIVM to give a presentation on agricultural practices in the Netherlands in terms of occupational safety for farmers, operators and farm workers. Moreover, STIGAS presented their mode of communication with farmers, including training for the workers, and provided leaflets that were handed out to farmers and workers advising on the 'dos and don'ts' for workers performing hand-labour tasks in crops previously treated with agrochemicals (in fields and greenhouses).

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

The output of this work programme is foreseen to be made publically available as a report on the RIVM website in 2018.

2.2.2. Dietary exposure to pesticide residues

The evaluation of pesticide residue studies for the purpose of setting MRLs in plant and animal commodities for a new PPP (mandestrobin) and its dietary risk assessment was performed in the period January–August 2018. This part of the work programme gave an insight into the practices of the JMPR and the CCPR. As the European Commission and EFSA represent the EU Member States at the CCPR, the work programme facilitates worldwide trade in agricultural commodities.

The work consisted of drafting a summary of evaluations based on residue studies conducted by the manufacturer of the pesticide in question. Numerous studies were evaluated including the physical chemical properties of the pesticide, its metabolism in plant and animal commodities, its degradation in soil and water, the validity of analytical methods, the magnitude of pesticide residues in raw agricultural commodities following application according to good agricultural practices, the stability of pesticide residues during freezer storage, the degradation of pesticide residues during food processing and the magnitude of pesticide residues in animal commodities following livestock feeding. The rationale for methodologies for long- and short-term dietary risk assessments are described in detail in the FAO manual on the submission and evaluation of pesticide residue data for the estimation of MRLs in food and feed (FAO, 2012).

Participation at the international Fresenius conference (Mainz, April 2018) on cumulative risk assessment and dietary risk assessment (MRL setting) offered the opportunity to have a dialogue with EU and national food safety authorities and representatives of the industrial sector to discuss new advances in the EU and worldwide (including the US Environmental Protection Agency).

This work package will provide two written outputs to which the EU-FORA work programme contributed. The detailed evaluation report and the appraisal document, proposing MRLs for the respective food plant commodities, will be finalised for submission to the FAO secretariat (due date August 2018). These reports will be discussed (and adapted) at the JMPR meeting in September 2018 and will then be published in the JMPR 2018 report and JMPR 2018 evaluation on the FAO website (due dates December 2018 and January 2019).

3. Conclusions

The work programme at the RIVM in the Chemical Food Safety Department provided the opportunity to get a thorough insight into the EU Member States' responsibility in risk assessment for the authorisation of PPPs. The preparation of a report on exposure assessment of pesticide residues focused on risk mitigation measures for workers during re-entry into sprayed crops, with consideration of the EU guidance (Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products (EFSA, 2014) and Guidance on dermal absorption (EFSA, 2017)), EU Regulation (EC) No 1272/2008 and exposure assessment models (EFSA OPEX). The development of a new methodology to calculate a safe REI indicates the period during which workers should wear personal protective clothing to reduce the risk of residue exposure to a safe level. The initial report may serve as a basis for developing a guidance document on the estimation of safe REIs for workers during first crop entry post application.

The preparation of the critical in-depth evaluation of the manufacturer-submitted residue studies of the pesticide in question was based on numerous studies (See Section 2.2.2). The final summary document will contain the proposed MRLs for different food commodities and a dietary risk assessment. The document will serve as the starting point for the discussion by the FAO and WHO panels in the JMPR, resulting in agreed MRLs for the pesticide in question and the respective food commodities. The MRLs are evaluated for food safety and help to accommodate the world trade of goods. However, MRLs are not health-based safety levels.

The EU-FORA training on chemical and microbiological risk assessment gave a broad overview of the European and global risk assessment practices. The projects performed in the Chemical Food Safety Department at the RIVM perfectly complemented the previously gained knowledge in the theory-based training programmes at EFSA. Newly acquired knowledge was immediately applied in the exposure evaluations of PPPs in an occupational and dietary setting.

In a pleasant working atmosphere in the department, the daily exchange with experts of different chemical food safety aspects through flexible working places, group meetings and the opportunity to

participate in international congresses enabled the fellow to learn new methodologies, gain more expertise and start building a network in the European food safety community.

References

EFSA (European Food Safety Authority), 2014. Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products. EFSA Journal 2014;12(10):3874, 55 pp. https://doi.org/10.2903/j.efsa.2014.3874

EFSA (European Food Safety Authority), Buist H, Craig P, Dewhurst I, Hougaard Bennekou S, Kneuer C, Machera K, Pieper C, Court Marques D, Guillot G, Ruffo F and Chiusolo A, 2017. Guidance on dermal absorption. EFSA Journal 2017;15(6):4873, 60 pp. https://doi.org/10.2903/j.efsa.2017.4873

FAO (Food and Agriculture Organization), 2012. Submission and evaluation of pesticide residues data for the estimation of maximum residue levels in food and feed. (FAO plant production and protection paper 225). FAO, Rome, 298 pp.

FAO (Food and Agriculture Organization), online-a. Codex Alimentarius. Available online: http://www.fao.org/fao-who-codexalimentarius/en/ [Accessed: 30 May 2018]

FAO (Food and Agriculture Organization), online-b. AGP - JMPR Guidance and related documents. Available online: http://www.fao.org/agriculture/crops/thematic-sitemap/theme/pests/jmpr/jmpr-docs/en/ [Accessed: 30 May 2018]

WHO (World Health Organization), online. Food safety. Joint FAO/WHO Meeting on Pesticide Residues (JMPR). Available online: http://www.who.int/foodsafety/areas_work/chemical-risks/jmpr/en/ [Accessed: 30 May 2018]

Abbreviations

CCPR	Codex Committee on Pesticide Residues
DA	dermal absorption
DT_{50}	50% dissipation time of the active substance in the PPP
EU-FORA	The European Food Risk Assessment Fellowship Programme
FAO	Food and Agriculture Organization of the United Nations
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
MRL	maximum residue level
PPP	plant protection product
REI	re-entry interval
RIVM	National Institute for Public Health and the Environment (the Netherlands)
WHO	World Health Organization

EU-FORA SERIES 1

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16088

Application of data science in risk assessment and early warning

Bundesinstitut für Risikobewertung, Berlin, Germany,
Individual authors: Michał Jan Czyż, Matthias Filter and Anja Buschulte

Abstract

The currently applied approaches, procedures and tools used for the identification of emerging risks vary greatly among Member States of the EU. EFSA established a structured approach for emerging risk identification that mainly consists of systematically searching, collecting, collating and analysing information and data. In addition, EFSA concluded that new methodologies and tools are needed to facilitate efficient and transparent sharing of data, knowledge and methods in the field of emerging risk identification between Member States. As the result of an open call issued by EFSA, the 'Determination and metrics of emerging risks' (DEMETER) project was established in spring 2017 to support current and future procedures for identification of emerging risks. As the Bundesinstitut für Risikobewertung (BfR) hosting site is involved in the DEMETER project, as well as in several other software development activities in the area of quantitative microbiological risk assessment, the fellow had the opportunity to play an active role in the project work and development of the running DEMETER project. The training and close integration in the project team enabled the fellow to make significant contributions, e.g. with the creation of new open source data processing workflows and by contributing to the Emerging Risk Knowledge Exchange Platform (ERKEP) Framework Concept Note. Besides DEMETER, the fellow participated in other activities of the Unit for Food Technologies, Supply Chains and Food Defence, including testing and applying several BfR open source software tools which had been developed in previous projects and that are used in microbiological risk assessment (e.g. Predictive Microbial Modelling Lab (PMM-Lab)) or as automatic data retrieval systems (e.g. SiLeBAT NewsRadar) – see https://foodrisklabs.bfr.bund.de.

Keywords: data science, emerging risks, early warning

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: Bundesinstitut für Risikobewertung, Berlin, Germany, Czyż MJ, Filter M and Buschulte A, 2018. Application of data science in risk assessment and early warning. EFSA Journal 2018;16(8):e16088, 7 pp. https://doi.org/10.2903/j.efsa.2018.e16088

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

The European Food Risk Assessment Fellowship Programme (EU-FORA) began in September 2017 (Bronzwaer et al., 2016). It was developed by members of the European Food Safety Authority (EFSA) (Fellowship Programme Committee) with additional support from representatives of Member State authorities. The main goal of the programme is to train a new generation of risk assessors. In the first edition, 15 early to mid-career scientists working in national authorities were placed for 1 year in the competent authority of another Member State. During this stay, each fellow was integrated into the work of the hosting site.

In addition to the work programme, all fellows were trained in microbiological and chemical risk assessment during a three-week introductory training at EFSA. Furthermore, three-one-week training courses covering different risk assessment subjects and risk communication were an integral part of the fellowship programme.

1.1. Bundesinstitut für Risikobewertung Unit 41

The project took place in the Department for Biological Safety (Dep. 4) of the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung (BfR)) and was supervised by the Department's Unit for Food Technologies, Supply Chains and Food Defence (Unit 41). The Department for Biological Safety deals with health risks to humans that may arise from microorganisms, the toxins formed by them and other microbial metabolites. This includes not only bacteria but also viruses, parasites and transmissible spongiform encephalopathy pathogens. The department is involved in establishing the cause of outbreaks of food-borne diseases and zoonoses. It has a number of national reference laboratories for the diagnosis and fine typing of pathogens, antibiotic resistance and the microbiological contamination of foods (a task anchored in food legislation). In this scope, Unit 41 deals with the identification and evaluation of hazards that may be present in food. Performing vulnerability assessments concerning these hazards and developing risk mitigation strategy concepts are related tasks of the unit. Unit 41 is furthermore involved in microbiological risk assessments and provides Germany's national expert for the EFSA Scientific Network on Microbiological Risk Assessment. Other key focuses are national and international research projects that aim to develop new data-driven infrastructure and knowledge supporting the efficient generation of risk assessments. In this context, several software tools have been developed to facilitate risk assessment. Examples are 'Predictive Microbial Modelling Lab' (PMM-Lab; aims to ease and standardise the statistical analysis of experimental microbial data and the development of predictive microbial models), 'FoodProcess-Lab' (for the application of predictive microbial models on food process chains) or 'Food Safety Model Repository' (openFSMR; a community-driven search engine for predictive microbial models). Other core activities are national and international research projects. Scientists from this unit were or are involved in many other projects, for instance: SiLeBAT, AniBioThreat, ZooGloW, FoodAuthent, AGINFRA+, DEMETER.

The fellowship programme was supervised by two senior research scientists from Unit 41: Dr Anja Buschulte, Veterinarian, Senior Research Scientist and Matthias Filter, Biochemist, Senior Research Scientist. Further support was given by other research scientists in the unit with many years of professional background in the field of either performing microbiological risk assessments or data deployment. Cross-unit activities provided the opportunity to get a closer insight into other risk assessment related issues.

Furthermore, the fellowship activities were integrated with the ongoing research project 'Determination and metrics of emerging risks (DEMETER)' that has received a grant from EFSA. This enabled the fellow to also learn from the other leading research institutes that are participating in this project, like Wageningen University, the Netherlands, the University of Newcastle upon Tyne, United Kingdom, and the National Food Chain Safety Office, Hungary.

1.2. DEMETER project

The general food law implemented in Regulation (EC) No 178/2002[1] obliges EFSA to establish monitoring procedures for the identification of emerging risks through searching, collecting, collating

[1] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

and analysing information and data. With regard to these requirements, EFSA has developed and implemented an approach to identify emerging risks. EFSA also recognised that the improvement of the coordination of resources, expertise and data across Europe and internationally, is of crucial importance. It concluded that new methodologies and tools were needed in order to facilitate efficient and transparent sharing of data, knowledge and methods in the field of emerging risk identification. As a result, an open call was issued by EFSA in which the DEMETER consortium was selected. DEMETER aims to support the current procedure for emerging risk identification by developing a collaborative platform to allow EU Member States and EFSA to share data, knowledge and methods for the identification of emerging food-related risks. One of the aims of this project was to develop a prototypic collaborative Emerging Risk Knowledge Exchange Platform (ERKEP).

The ERKEP was defined by the DEMETER project as 'a technical resource based on KNIME Server infrastructure hosted at BfR supporting the exchange of data analytics workflows for emerging risk identification'. The platform will be a technical solution to support the exchange of data analytics knowledge relevant for emerging issues and risk identification.

Besides this technical solution, EFSA asked the DEMETER project to develop a broader vision of what an 'ideal' knowledge exchange framework on emerging issues and risks should look like (ERKEP Framework). This vision includes specific concerns, e.g.:

- Which functionalities are desired?
- What should be shared?
- What side factors should be considered?

Several needs and functionalities were identified to be taken into account for the ERKEP Framework. The vision paper articulates the shared understanding about the values, mission, and approach that could guide EFSA's and DEMETER's efforts for the future development of resources for efficient knowledge exchange in regard to the emerging risk identification area.

2. Description of work programme

The fellow was associated with Unit 41 of BfR and had the chance to be involved in the DEMETER research project which started in spring 2017. The objectives and research proposed in this project were specifically designed to support current (and future) EFSA procedures for emerging risk identification by providing a set of integrated, open source solutions that would allow EFSA and EU Member State authorities to share data, knowledge and methods in a rapid and effective manner. As part of this work, the fellow had the opportunity to generate his own research results that could be published as a research paper or as an oral presentation or poster at scientific conferences.

2.1. Aims

The main goals of the fellowship project were:

- Learning best practice on data analysis principles (including transparency, validation and documentation).
- Getting insight into tasks of the department concerning microbiological risk assessment with emphasis on the application of scientific data for risk assessment.
- Getting familiar with software tools for data mining (KNIME) and predictive microbial modelling (e.g. PMM-Lab) and quantitative microbiological risk assessment (QMRA) (e.g. FSK-Lab).
- Analysing EFSA's emerging risk identification framework – current approaches and tools (e.g. MediSys, SiLeBAT NewsRadar).
- Generating automated data retrieval and monitoring pipelines using KNIME.
- Contributing to the design of DEMETER ERKEP and the ERKEP Framework vision.

2.2. Activities/methods

The fellow got close insights into the area of QMRA. He participated in BfR internal activities in this area and contributed to the ongoing development of software tools for the related community. Furthermore, the fellow was closely involved in the planning and conducting of activities in the DEMETER and other national and international research projects of the unit, e.g. AGINFRA+ and the national food project FoodAuthent. Among these activities were: collaboration in writing the DEMETER concept note, attendance at AGINFRA+ and DEMETER project meetings and contribution and

development of open source data analysis workflows and services for virtual research environments (VRE).

In addition to the individual training opportunities provided by Unit 41, the fellow had the opportunity to participate in the accompanying training programme by BfR for EU-FORA fellows. The training sessions and seminars were:

- Reviewing scientific literature systematically – an introduction.
- Risk assessment and risk management of genetically modified organisms.
- Presenting in English.
- Introduction to the BfR FoodRisk-Lab software tools.

As an extension to the main goals and primary agreements, a script book with introduction to the application of R scripting language in the area of risk modelling was written by the fellow. This tutorial will be made available as an open resource in an online open source repository and as an online course inside a VRE.

3. Conclusions

During AGINFRA+ and DEMETER project meetings in Wageningen (November 2017), the fellow presented sample workflows in the KNIME platform to automatically obtain, arrange and analyse data from social media and the European and Mediterranean Plant Protection Organization (EPPO) data services (EPPO, online).

In cooperation with other partners from the DEMETER project, an ERKEP framework vision paper was developed. Important parts of this document are the definition of a common vocabulary, the participants, and the current and future technical possibilities of both the ERKEP platform and framework.

The development of new workflows for data processing, as well as upgrading old workflows in collaboration with other Unit 41 members, resulted in providing open or better tools for emerging risk identification and assessment. Further tools are currently under development and will be deployed via the VRE or the BfR KNIME server infrastructure which serves as a basis for the DEMETER ERKEP prototype. Moreover, other developments on VREs are ongoing, including the creation of Wiki pages and improvements in the general VRE user experience.

Last, but not least, an introductory script book for using R language in risk assessment was developed. After review by members of Unit 41 and EU-FORA fellows it is available for the general public via the GitHub repository (Czyż, 2018). This open source project allows the broad community of R developers to share their expertise and knowledge in order to create a modern and comprehensive script book. As a result, risk assessors as well as others interested in modelling will have an opportunity to become familiar with R language which is one of the most appreciated tools in data analysis nowadays (Cass, 2017).

References

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

Cass S, 2017. The 2017 Top Programming Languages. IEEE Spectrum, 18 July. Available online: https://spectrum.ieee.org/computing/software/the-2017-top-programming-languages

Czyż MJ, 2018. A Beginners Guide to R's Galaxy. Available online: https://github.com/mczyzj/BeginnersGuideToGalaxy

EPPO (European and Mediterranean Plant Protection Organization), online. EPPO Data Services. Available online: https://data.eppo.int/

Abbreviations

AGINFRA+	Accelerating user-driven e-infrastructure innovation in food and agriculture
BfR	Bundesinstitut für Risikobewertung
DEMETER	Determination and Metrics of Emerging Risks
EPPO	European and Mediterranean Plant Protection Organization
ERKEP	Emerging Risk Knowledge Exchange Platform
EU-FORA	European Food Risk Assessment Fellowship Programme
FoodAuthent	Development of a system for collection, analysis, and utilisation of product data for authenticity in the food sector
FSK-Lab	Food Safety Knowledge Lab

openFSMR	Food Safety Model Repository
PMM-Lab	Predictive Microbial Modelling Lab
QMRA	quantitative microbiological risk assessment
SiLeBAT	Sicherstellung der Futter- und Lebensmittelwarenkette bei bio- und agro-terroristischen (BAT)-Schadenslagen
VRE	virtual research environment
ZooGloW	Zoonosen und Lebensmittelsicherheit entlang globaler Warenketten

EU-FORA SERIES 1

National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport

EFSA Journal

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e16089

Modelling of inactivation through heating for quantitative microbiological risk assessment (QMRA)

National Institute for Public Health and the Environment (RIVM), the Netherlands
M Pesciaroli, JE Chardon and EG Evers

Abstract

EFSA regards the household as a stage in the food chain that is important for the final number of food-borne infections. The fate of a pathogen in the private kitchen largely depends on consumer hygiene during preparation of food and on its proper cooking, especially in the case of meat. Unfortunately, detailed information on the microbiological survival in meat products after heating in the consumer kitchen is lacking. The aim of the study was to improve the estimation of the inactivating effect on pathogens by heating meat or a meat product by the consumer in the kitchen. On that account, artificially contaminated meat and meat products were cooked according to several degrees of doneness and simulating real world conditions, and bacterial survival was measured. Heat camera pictures and button temperature loggers inserted into the food matrix served to record time and the temperature of heating. Temperature, time and the microbial survival ratio observed served to inform a mathematical model able to explain the thermal inactivation of meat or a meat product in home settings. The results of the study would help to improve microbiological comparative exposure assessments of pathogens in food, as an attribution tool and as a supportive tool for risk-based sampling in monitoring and surveillance.

Keywords: QMRA, Exposure assessment, D/z model, home preparation, meat, heating, inactivation

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: National Institute for Public Health and the Environment (RIVM), the Netherlands, Pesciaroli M, Chardon JE and Evers EG, 2018. Modelling of inactivation through heating for quantitative microbiological risk assessment (QMRA). EFSA Journal 2018;16(8):e16089, 8 pp. https://doi.org/10.2903/j.efsa.2018.e16089

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

Quantitative microbiological risk assessment (QMRA) is a methodology that combines information, data and mathematical models to evaluate food-, direct contact- and environmental-related microbiological public health risks (Evers et al., 2008). In the case of food, QMRAs rely on mathematical models to describe the sequential steps of a pathogen along the food chain, embracing the food animals on the farm, transport, slaughter, processing, retail and preparation by the consumer in the households. The exposure of a population to a microbiological hazard is a key step of QMRA. The estimated exposure of consumers to a pathogen, coupled with a dose–response relationship, enables an approximation to be made of the number of human cases attributable to the pathogen occurring in the population of interest (Chardon and Evers, 2017). The assessment of exposure to microbiological agents requires, as a basic input, data such as the number of portions of products eaten by the consumers (food consumption data), the prevalence of contaminated portions and the concentration of the microorganism in the portion. Nevertheless, the fate of a pathogen in food and, in turn, the level of human exposure, is associated with consumer behaviour in the kitchen. The European Food Safety Authority (EFSA) estimates that the largest proportion of food-borne outbreaks that occur in the European Union originate in the household, with meat and meat products the type of food most frequently implicated (EFSA, 2016). Notably, information on the extent of microbiological survival in meat products after heating in the consumer's kitchen is lacking. Many studies have simulated the heat inactivation of bacterial cultures grown in liquid media (De Jesús and Whiting, 2003; Gabriel, 2012; Smelt and Brul, 2014; Adhikari et al., 2016; Haberbeck et al., 2017). The results of these studies have been used to predict the thermal inactivation of the bacteria present in meat. Correct estimation of this inactivating effect in a real-world situation (a consumer preparing food in the kitchen) would serve to decrease the uncertainty in exposure assessment calculations. The hypothesis behind our study is that the bacterial survival in meat after heating is higher than in microbiological culture media. Therefore, we performed experiments to quantify the degree of thermal inactivation of bacteria in meat and meat preparations. By recording the time and the temperature to which food was exposed, we wanted to ascertain whether the resulting bacterial inactivation could be explained by a D/z model (Smelt and Brul, 2014) with, only when necessary, the inclusion of additional explanatory variables.

2. Description of work programme

The work programme was carried out at the Centre for Zoonoses and Environmental Microbiology (Z&O) at RIVM, the Netherlands, which has extensive and long experience of performing QMRAs for pathogens in food, water and the environment. The work programme was integrated into a wider project on microbiological comparative exposure assessment of pathogens in food, as an attribution tool and as a supportive tool for risk-based sampling in monitoring and surveillance. The activities proposed were aimed at improving the estimation of the inactivating effect on pathogens by the heating of meat or a meat product by the consumer in the kitchen.

2.1. Aims

The activities of the work programme were aimed at:

i) obtaining survival ratios for the domestic preparation of meat or meat products to then be used in a QMRA;
ii) developing and characterising a thermal inactivation model explaining bacterial heat survival in meat prepared at home.

2.2. Activities/methods

The work programme used the results of some realistic experiments using meat and meat preparations artificially contaminated with *Escherichia coli*, which served as model bacterium, while monitoring the temperature with a heat camera and button data loggers inserted in the food matrix. Beefsteaks, plain beef and mixed (50% beef and 50% pork) meat preparations (meatball, hamburger and meat crumble) were spiked with a known amount of *E. coli* and then fried simulating home conditions until meeting various levels of readiness preferred by consumers. The temperature of the food during frying was measured and recorded by taking pictures with a heat camera (Figure 1) and, in the case of hamburger and meatballs, also by button loggers embedded in the food matrix (Figure 2). The extent of survival together with the recorded temperature and duration of frying

served to build a D/z model explaining the survival observed in the different food types based of the frying style applied.

The first activity of the programme was a thorough statistical analysis of the inactivation resulting from the heating. We used a Bayesian approach to interpret the results of plate counts or of a combination of plate counts and presence/absence after enrichment.

In the second step of the programme, we took advantage of the inactivation observed in the experiments and the matrix of time–temperature–location (on or in the meat) data to estimate the parameter values of the heating (D/z) model. The result of this step was the generation of a set of D_{ref} and Z values compatible with the survival ratio observed in the experiments. This activity is still ongoing (at the time of writing) and is expected to be finished by the end of May 2018.

The final step of the work programme foresees an in-depth study of the current available scientific articles on the bacterial survival after heating in laboratory liquid media or in or on meat. The ultimate aim is to develop a theory and possibly an explicit mathematical model which can explain and describe the inactivation results obtained by these studies. Again, this task is expected to be accomplished in June 2018.

The main of output of the work programme will be the preparation of a manuscript describing a model explaining the thermal inactivation of bacteria occurring during meat preparation at home. In order to reach the widest range of possible readers and give the results of the work programme the greatest visibility, the manuscript will be submitted to a journal dealing with microbial food safety rather than one dealing with mathematical modelling and risk analysis.

3. Conclusions

The experiments performed, of which the analyses have yet to be finalised, revealed that the residual bacteria found after frying the beefsteak will almost all originate from the side. This result has its explanation in the higher bacterial contamination present on the side and the lower temperature values this part of the beefsteak experiences during heating when compared to the top and bottom surfaces. The number of microorganisms found in the hamburgers after frying declined with the length of the thermal treatment. The composition of the hamburger, either beef or a blend of beef and pork, seems to have no influence on the number of bacteria surviving the heating process. Similarly, in the case of meatballs, no difference in the number of microorganisms was found between meatballs made of plain beef or a blend of beef and pork when the same level of readiness was compared. Interestingly, variability of survival was observed, probably connected to the shape of the meat balls which results in an uneven temperature distribution during the frying process.

The work programme proposed made the fellow acquainted with the best-suited statistical methods to describe the uncertainty associated with microbiological data. This process was also coupled with learning how to use @RISK, one of the software packages often used as a risk analysis tool. In addition, the fellow learned how to combine temperature and time data to explain thermal inactivation of microorganisms by means of a D/z model. Overall, the proposed work programme was in line with the background and expertise of the fellow. Furthermore, he joined and actively participated in the meetings and seminars organised by the Z&O Centre throughout the year. No issue has hampered or slowed the implementation of the agreed work programme, given the constant and effective guidance offered by the supervisor and his collaborators. The programme enables the fellow to return to his home institution with the expertise to build QMRAs able to answer relevant microbiological risk questions. Both the fellow and the supervisor agree that the EU-FORA programme **provides** a valuable opportunity to exchange opinions and methodologies on relevant public health issues and to build a professional and personal network that will serve as the basis for future cooperation.

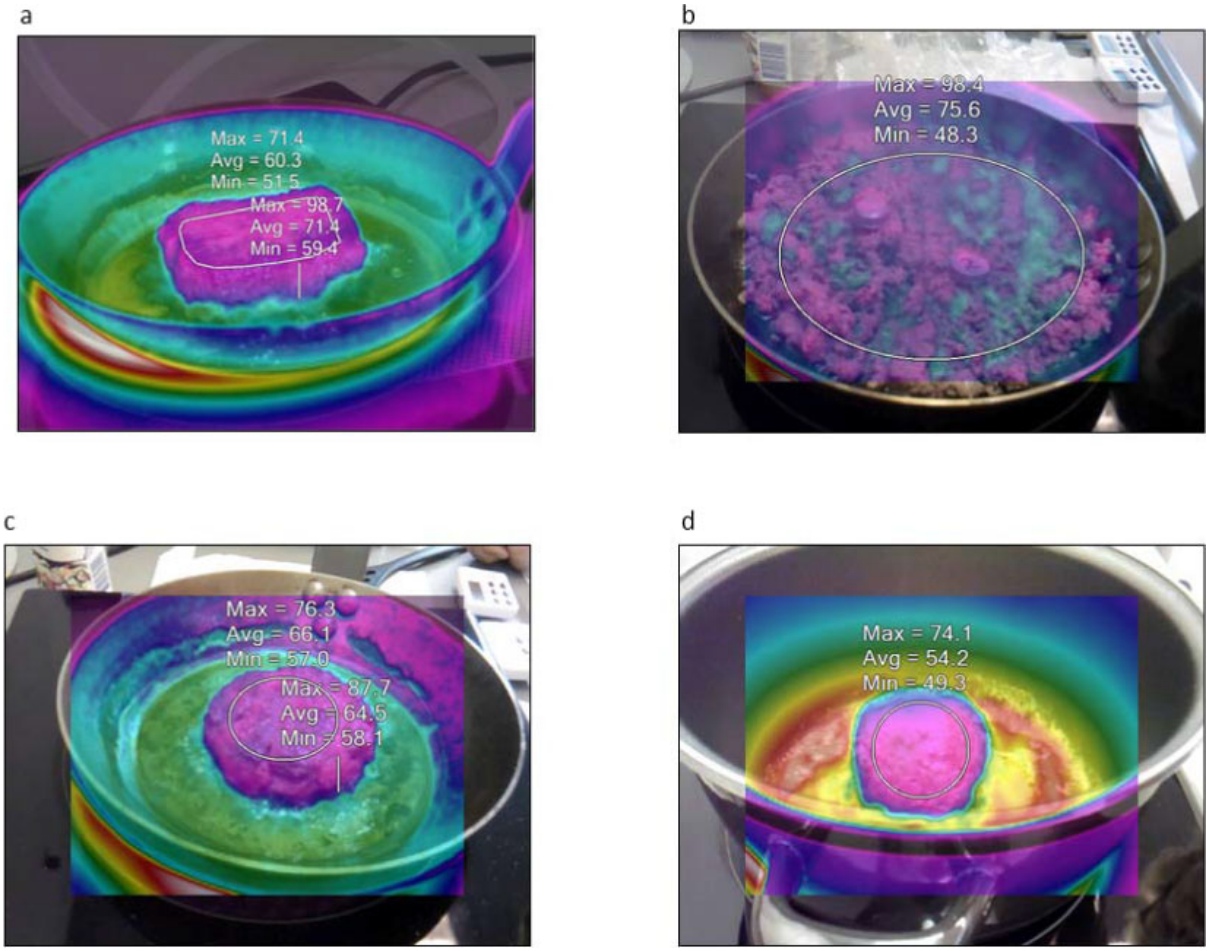

Figure 1: Infrared pictures with temperature measurements of the beefsteak, crumbs, hamburger and meatball (Figure 1a–d)

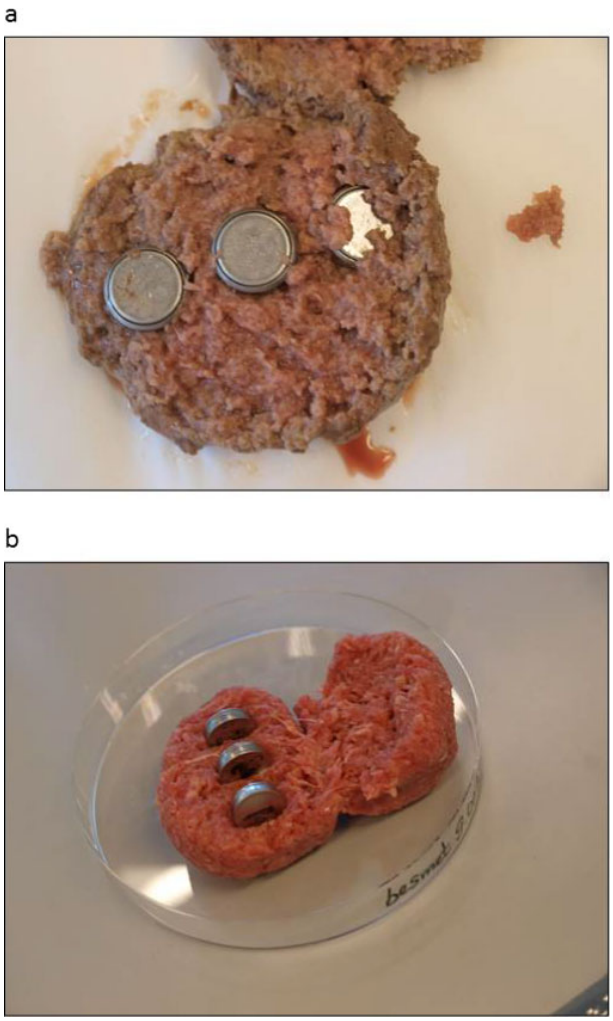

Figure 2: Temperature log buttons inserted in the medium fried pork–beef hamburger and meatball (Figure 2a and b)

References

Adhikari A, Bary A, Cogger C, James C, Ünlü G and Killinger K, 2016. Thermal and starvation stress response of *Escherichia coli* O157:H7 isolates selected from agricultural environments. Journal of Food Protection, 79, 1673–1679.

Chardon JE and Evers EG, 2017. Improved swift Quantitative Microbiological Risk Assessment (sQMRA) methodology. Food Control, 73, 1285–1297.

De Jesús AJ and Whiting RC, 2003. Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. Journal of Food Protection, 66, 1611–1617.

EFSA (European Food Safety Authority), 2016. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. EFSA Journal 2016;14(12):4634, 231 pp. https://doi.org/10.2903/j.efsa.2016.4634

Evers EG, Klerx HJVDF, Nauta MJ, Schijven JF, Havelaar AH, 2008. *Campylobacter* source attribution by exposure assessment. International Journal of Risk Assessment and Management, 8, 174.

Gabriel AA, 2012. Influences of heating temperature, pH, and soluble solids on the decimal reduction times of acid-adapted and non-adapted *Escherichia coli* O157:H7 (HCIPH 96055) in a defined liquid heating medium. International Journal of Food Microbiology, 160, 50–57.

Haberbeck LU, Wang X, Michiels C, Devlieghere F, Uyttendaele M and Geeraerd AH, 2017. Cross-protection between controlled acid-adaptation and thermal inactivation for 48 *Escherichia coli* strains. International Journal of Food Microbiology, 241, 206–214.

Smelt JPPM and Brul S, 2014. Thermal Inactivation of Microorganisms. Critical Reviews in Food Science and Nutrition, 54, 1371–1385.

Abbreviations

RIVM	Rijksinstituut voor Volksgezondheid en Milieu
QMRA	quantitative microbiological risk assessment
Z&O	Centre for Zoonoses and Environmental Microbiology

EU-FORA SERIES 1

National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport

EFSA Journal

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e160810

Preparation of Dutch food consumption data for risk assessment

National Institute for Public Health and the Environment (RIVM), the Netherlands
K Nelis and C van Rossum

Abstract

The availability of detailed and high-quality food consumption data collected at an individual level is essential for assessing the exposure to potential risks in the food chain. During the years 2012–2016, the Dutch National Food Consumption Survey was conducted in the Netherlands as part of the EU Menu survey, following the EFSA 2009 guidance on 'General principles for the collection of national food consumption data in the view of a pan-European dietary survey'. Complete results were obtained for 4,313 persons aged 1–79 years (response rate 65%). The work programme proposed to the European Food Risk Assessment (EU-FORA) Fellow included FoodEx2 mapping of the Dutch food consumption data and preparing the final scientific report for EFSA as well as analysing habitual intake of nutrients using the SPADE programme. Further activities were added, such as performing a literature search as to the validity and usability of mobile applications for collecting food consumption data and exploring methods for estimating added-sugar/free-sugar intake.

Keywords: EU-FORA, Fellowship, food consumption survey, habitual intake, SPADE

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: National Institute for Public Health and the Environment (RIVM), the Netherlands, Nelis K and van Rossum C, 2018. Preparation of Dutch food consumption data for risk assessment. EFSA Journal. EFSA Journal 2018;16(8):e160810, 7 pp. https://doi.org/10.2903/j.efsa.2018.e160810

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

The availability of detailed and high-quality food consumption data collected at an individual level is essential for assessing the exposure to potential risks in the food chain. The collection of accurate and harmonised food consumption data at a European level is therefore considered a primary long-term objective for the European Food Safety Authority (EFSA, 2014). In 2009, the EFSA guidance on 'General principles for the collection of national food consumption data in the view of a pan-European dietary survey' was published, and a pan-European food consumption survey, also known as the 'EU Menu', was launched (EFSA, 2014).

During the years 2012–2016, the Dutch National Food Consumption Survey (DNFCS) was conducted by the National Institute for Public Health and the Environment (RIVM) as part of the EU Menu survey. For it, a random sample was drawn from consumer panels stratified by age and gender, and representative of the population with regard to region, address density and educational level. Complete results were obtained for 4,313 persons (response rate 65%) including toddlers, children, adolescents, adults and elderly. Food consumption data were collected by means of two non-consecutive 24-h dietary recalls conducted by trained dieticians; for participants aged 1–8 and 71–79 years, this was done in combination with a food diary. The dietary recalls were conducted using the computer-directed interview program GloboDiet (formerly EPIC-soft©) provided by the International Agency for Research on Cancer (IARC, Lyon, France).

To fully utilise the collected data and make it available to EFSA as well as other researchers working on risk assessment, the data had to be cleaned and coded in a common classification and description system. Alongside the food consumption data, a scientific report on the methodology of the survey needed to be provided to EFSA. These tasks, together with analyses of habitual nutrient intake from foods and supplements, formed the core of the work programme proposed by the RIVM to the Fellow. Further activities carried out by the Fellow during the European Food Risk Assessment (EU-FORA) year included performing a literature search on validity and usability studies concerning mobile applications used for recording food intake and exploring methods for estimating added-sugar/free-sugar intake. Some activities are ongoing and will be finalised after submitting this report.

2. Description of work programme

2.1. Aims

The aim of the proposed work programme was to prepare the Dutch food consumption data for risk assessment. The Fellow was placed at the RIVM, in the team working on the DNFCS. The Fellow worked with members of the team on the following tasks:

- Mapping food codes recorded in the final version of the national food consumption database to the EFSA food classification system, FoodEx2.

FoodEx2 is a comprehensive food classification and description system aimed at covering the need to describe food in data collections across different food safety domains (EFSA, 2015). The system consists of a large number of individual food items aggregated into food groups and broader food categories in a hierarchical structure of parent–child relationships, which may be complemented with additional information through the use of facets and facet descriptors (EFSA, 2011). The ability to capture all the useful details of food groups in exposure assessments by EFSA is a crucial requirement for the process of risk assessment (EFSA, 2015).

- Preparing a final DNFCS scientific report for EFSA, including an evaluation of the adaptation of the methodology according to EFSA guidance for national dietary survey data collection.

In 2009, EFSA published guidance on the general principles for collection of national food consumption data in the context of a pan-European dietary survey (EFSA, 2009) that has provided the basis for the quality criteria for EFSA's food consumption data collection (EFSA, 2014). To further improve the harmonisation of food consumption data collected in Europe, EFSA initiated a project 'What's on the Menu in Europe? (EU Menu)' in 2009. To make it possible to evaluate the success of the harmonisation efforts, it is considered important that the reporting of the results is also harmonised (EFSA, 2014).

- Performing data analyses to estimate habitual intake from intake measured on specific days using the SPADE programme, and preparing reports and scientific article(s) from the DNFCS findings concerning food consumption and dietary intake.

SPADE is a program in R (R Development Core Team, 2011) developed at RIVM for estimating the habitual intake of dietary components (e.g. micronutrients, macronutrients, contaminants) or food (groups) from repeated short-term dietary intake data. In addition to the basic modelling of daily and episodic consumption, SPADE applies a first-shrink-then-add approach to combine the intake from different sources to overcome problems with multimodality and heterogeneous variances (Dekkers et al., 2014).

The wider objective of the work programme was to give the Fellow more insight into how the data from the DNFCS is used in different fields of food safety risk assessment at a national and international level and to provide the opportunity to work with the Dutch experts in these fields.

2.2. Activities

2.2.1. FoodEx2 mapping of the DNFCS data

To provide EFSA with the food consumption data collected during the DNFCS, a process of FoodEx2 mapping had to be carried out. The Fellow was involved in team meetings with the people working on mapping and data cleaning. She presented the FoodEx2 mapping procedures used in her home institute and advised the team on some of the more challenging issues. One of the issues included finding a more efficient way of matching the highly detailed and therefore long list of unique foods from the Dutch national food composition database with the corresponding FoodEx2 codes. The other challenge arose from discrepancies between the classification of foods in the national database and in the FoodEx2 system regarding some of the recipes/mixed food groups that had to be broken down into ingredients. While working on these issues, the Fellow had the opportunity to learn about the structure and distinct features of the output data from GloboDiet© (IARC, Lyon, France), the software used for collecting food consumption data in the Netherlands. The mapping and data cleaning process were finalised by the team on time and the data submitted to EFSA.

2.2.2. Preparing the scientific report for DNFCS

The food consumption data collected as part of the EU Menu survey must be complemented with a scientific report describing the methodology used and its compliance with the EFSA guidance published in 2009. The Fellow prepared the first version of the report, presented it during a team meeting, and was engaged in discussions concerning adaptations and additions needed. The Fellow was actively involved in the reviewing and refining process leading to the final version of the report. While preparing the final report on the DNFCS for EFSA, the Fellow was able to use her previous knowledge from the Estonian food consumption survey and also to gain a detailed insight into how these surveys are carried out in the Netherlands. The Fellow had the opportunity to evaluate the adaptations made to the methodology proposed by the EFSA guidance for data collection in national dietary surveys and to learn about the challenges faced in the Dutch survey as well as potential ways of coping with them in the future. For that purpose, the Fellow also provided a detailed comparison of EFSA guidance documents from 2009 and 2014 on topics relevant to the methodology used in Dutch food consumption surveys. The scientific report was provided to EFSA together with the food consumption data as scheduled in the initial time frame.

2.2.3. Calculating the habitual nutrient intake

To learn about the principles of habitual intake modelling for populations and the use of the SPADE model in R, the Fellow attended a 2.5-day course organised in Wageningen by the VLAG Graduate School. As input to the SPADE model, the Fellow prepared the necessary datasets including files with dietary reference values from EFSA as well as from the Dutch Health Council (Scientific Committee on Food and EFSA Scientific Panel on Dietetic Products, Nutrition and Allergies, 2006; Gezondheidsraad, 2014; EFSA, 2017). The Fellow also tested the new version of SPADE that allows the model to be run simultaneously for different age classes and various sets of dietary reference values and provides the habitual intake of multiple subgroups of the population. Using the new SPADE, the Fellow carried out statistical analyses to calculate the habitual intake of nutrients from foods based on the latest Dutch food consumption survey and estimate the proportion of the population below or above a certain cut-off value for each nutrient. After submitting this progress report, more analyses of habitual nutrient intake will be performed to estimate the intake of nutrients from different sources (foods and dietary supplements), followed by calculations of the confidence intervals for distributions with a parametric

bootstrap. These data are necessary for the interpretation of the results. The Fellow will also be included in activities related to disseminating the results of the survey including preparing the national reports and creating a website to present the results to the public.

2.2.4. Other activities

Given the availability of new technologies like apps and scanners to identify barcodes on foods, it is worthwhile to further investigate less labour-intensive alternatives for collecting food consumption data while continuing to maintain the level of detail and harmonisation across Europe. For this reason, the Fellow performed a literature search of validity and usability studies on mobile applications used for recording food intake. The aim was to identify the features supported by these applications, the methods used to validate them and the main outcomes of these validation and usability studies. The Fellow gave a presentation on her findings to colleagues working on developing a mobile application for future Dutch food consumption surveys.

A high sugar intake has become a subject of scientific debate because of the potential health implications, free sugar recommendations by the WHO and the upcoming assessment of dietary sugars by EFSA (WHO, 2015; EFSA, 2018). RIVM has been requested by the Dutch Ministry of Health, Welfare and Sport to calculate the habitual intake of added and free sugars based on the data from DNFCS. The Fellow made an overview of the different concepts used to describe intake of dietary sugars as well as methods applied to calculate the amount of both free and added sugars in foods. During the last 4 months of the programme, the Fellow is expected to link the DNFCS data with the estimated values of free and added sugar and use SPADE to calculate the habitual intake of the Dutch population. Based on these results, it is possible to evaluate the adherence to the guidelines set by WHO and other relevant authorities as well as to look for trends in free sugar consumption in the Netherlands based on the previous survey by Sluik et al. (2016).

3. Conclusions

The main focus of the work programme proposed by the RIVM to the EU-FORA Fellow was on the preparation of the Dutch food consumption data for risk assessment. The activities of the Fellow proceeded in accordance with the work programme and the expected time frame.

The Fellow contributed to the FoodEx2 mapping of the Dutch food consumption data and to write the final scientific report for EFSA as well as to the preparation of the national reports by analysing, for example, the habitual intake of nutrients using the SPADE programme and comparing these results with the dietary reference values. During this work, the Fellow had the opportunity to provide input on practices used in her home institute. Besides the activities described in the initial working programme, the Fellow was assigned additional tasks such as performing a literature search of validity and usability studies of mobile applications developed for collecting food consumption data and exploring methods for estimating added-sugar/free-sugar intake. After submitting this progress report, the analyses of habitual nutrient intake will continue with estimating the nutrient intake from supplements and calculating confidence intervals for distributions obtained by a parametric bootstrap. The Fellow will also be included in activities related to disseminating the results of the survey. During the last months of the EU-FORA year, the Fellow is expected to link the DNFCS data with the estimated values of free and added sugar and calculate the habitual intake of the Dutch population.

The proposed working programme was an excellent fit with the experience and expectations of the Fellow and provided the opportunity for her to get acquainted with the different facets of risk assessment work performed at the RIVM. Throughout the year, the Fellow received sufficient supervision and support from her supervisor and other team members responsible for specific tasks. She was invited to join all social and topic-specific activities of the team, experts, department and centre. Besides gaining valuable insight into topics directly related to the Fellow's work at her home institute, she was able to share her experiences and contribute to the workflow of the hosting site. As a result, the EU-FORA programme provided a great environment for mutual learning and created a strong basis for future scientific collaboration.

References

Dekkers ALM, Verkaik-Kloosterman J, van Rossum CTM and Ocké MC, 2014. SPADE, a new statistical program to estimate habitual dietary intake from multiple food sources and dietary supplements. The Journal of Nutrition, 144, 2083–2091. https://doi.org/10.3945/jn.114.191288

EFSA (European Food Safety Authority), 2009. General principles for the collection of national food consumption data in the view of a pan-European dietary survey. EFSA Journal 2009;7(12):1435, 51 pp. https://doi.org/10.2903/j.efsa.2009.1435
EFSA (European Food Safety Authority), 2011. The food classification and description system FoodEx 2 (draft-revision 1). EFSA supporting publications 2011:EN-215, 438pp. Available online: http://www.efsa.europa.eu/en/supporting/doc/215e.pdf
EFSA (European Food Safety Authority), 2014. Guidance on the EU Menu methodology. EFSA Journal 2014;12(12):3944, 77 pp. https://doi.org/10.2903/j.efsa.2014.3944
EFSA (European Food Safety Authority), 2015. The food classification and description system FoodEx2 (revision 2). EFSA supporting publication 2015:EN-804, 90pp. https://doi.org/10.2903/sp.efsa.2015.en-804
EFSA (European Food Safety Authority), 2017. Dietary reference values for nutrients: summary report. EFSA supporting publication 2017:e15121, 92 pp. https://doi.org/10.2903/sp.efsa.2017.e15121
EFSA (European Food Safety Authority), 2018. Draft protocol for the Scientific Opinion on free sugars from all dietary sources. Available online: https://www.efsa.europa.eu/sites/default/files/engage/180109.pdf
Scientific Committee on Food and EFSA Scientific Panel on Dietetic Products, Nutrition and Allergies, 2006. Tolerable upper intake levels for vitamins and minerals. Available online: http://www.efsa.europa.eu/sites/default/files/efsa_rep/blobserver_assets/ndatolerableuil.pdf
Gezondheidsraad, 2014. Tijdelijke voedingsnormen. Available online: http://www.gezondheidsraad.nl/nl/nieuws/tijdelijke-voedingsnormen
R Development Core Team, 2011. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online: https://www.gbif.org/tool/81287/r-a-language-and-environment-for-statistical-computing
Sluik D, van Lee L, Engelen AI and Feskens EJM, 2016. Total, free, and added sugar consumption and adherence to guidelines: The Dutch National Food Consumption Survey 2007–2010. Nutrients, 8, 70. https://doi.org/org/10.3390/nu8020070
WHO (World Health Organization), 2015. Guideline: sugars intake for adults and children. Available online: http://apps.who.int/iris/bitstream/handle/10665/149782/9789241549028_eng.pdf?sequence=1

Glossary and Abbreviations

DNFCS	Dutch National Food Consumption Survey
EPIC-SOFT	Dietary software created by International Agency for Research on Cancer
EU Menu	Fully harmonised pan-European Food Consumption Survey called 'What's on the Menu in Europe?'
EU-FORA	The EUropean FOod Risk Assessment Fellowship Programme
FoodEx2	Version 2 of the EFSA Food classification and description system for exposure assessment
GloboDiet	Computer-directed interview program GloboDiet© (former EPIC-soft©) provided by the International Agency for Research on Cancer (IARC, Lyon, France). With the GloboDiet program, the 24-hour recalls are standardised, and the answers can be entered directly into the computer.
IARC	International Agency for Research on Cancer
RIVM	Dutch Institute of Public Health and the Environment
SPADE	Statistical Program to Assess Dietary Exposure developed by RIVM
WHO	World Health Organization

EU-FORA SERIES 1

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e160811

Risk assessment of antimicrobial resistance along the food chain through culture-independent methodologies

Host institution: Department of Food Hygiene and Technology, Institute of Food Science and Technology, Universidad de León, León, Spain,
E Likotrafiti, EA Oniciuc, M Prieto, JA Santos, S López and A Alvarez-Ordóñez

Abstract

Antimicrobial resistance (AMR) represents a major challenge for Public Health and the scientific community, and requires immediate and drastic solutions. Acquired resistance to certain antimicrobials is already widespread to such an extent that their efficacy in the treatment of certain life-threatening infections is already compromised. To date, the emergence and spread of AMR has been attributed to the use, misuse or indiscriminate use of antibiotics as therapeutic drugs in human, animal and plant health, or as growth promoters in veterinary husbandry. In addition, there is growing concern over the possibility of AMR transmission via the food chain. Food processing environments could act as potential hotspots for AMR acquisition and spread. Indeed, biocide use and exposure to food-related stresses and food processing technologies could presumably act as selection pressures for increased microbial resistance against clinically relevant antibiotics. Global AMR surveillance is critical for providing the necessary information to form global strategies and to monitor the effectiveness of public health interventions as well as to detect new trends and emerging threats. Surveillance of AMR is currently based on the isolation of indicator microorganisms and the phenotypic characterisation of the strains isolated. However, this approach provides very limited information on the mechanisms driving AMR or on the presence and spread of AMR genes. Whole genome sequencing (WGS) of bacterial pathogens is a powerful tool that can be used for epidemiological surveillance, outbreak detection and infection control. In addition, whole metagenome sequencing (WMS) allows for the culture-independent analysis of complex microbial communities, providing useful information on the occurrence of AMR genes. Both approaches can be used to provide the information necessary for the implementation of quantitative risk assessment of AMR transmission routes along the food chain.

Keywords: Antimicrobial resistance, risk assessment, surveillance, food-borne pathogens, whole genome sequencing, metagenomics

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: Host institution: Department of Food Hygiene and Technology, Institute of Food Science and Technology, Universidad de León, León, Spain, Likotrafiti E, Oniciuc EA, Prieto M, Santos JA, López S and Alvarez-Ordóñez A, 2018. Risk assessment of antimicrobial resistance along the food chain through culture-independent methodologies. EFSA Journal 2018;16(8):e160811, 8 pp. https://doi.org/10.2903/j.efsa.2018.e160811

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

1.1. Antimicrobial resistance

Antimicrobials have been used for several decades to fight infections in humans, animals and plants caused by pathogenic microorganisms. In the last two decades, the emergence and transmission of multidrug-resistant pathogenic bacteria has created an urgent need to understand the underlying mechanisms involved. This is necessary to combat this global phenomenon, which is one of the main public health challenges of the 21st century (Smith et al., 2016; WHO, 2015). The ability of bacteria to resist the action of one or several antimicrobial agents through bacterial gene mutations or by acquiring exogenous resistance genes carried on mobile elements is defined as antimicrobial resistance (AMR) (ECDC, 2017). Acquired resistance to some antimicrobials is already so widespread as to compromise their value for the treatment of life-threatening infections (EFSA BIOHAZ Panel, 2008). Harmonised outcome indicators have been jointly established by the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) to assist Member States in assessing the progress made in reducing antimicrobial use and AMR both in humans and food-producing animals (ECDC, EFSA BIOHAZ Panel and CVMP, 2017). The possibility of AMR transmission via the food chain is currently under investigation (Verraes et al., 2013; Bengtsson-Palme, 2017), but the relative contribution of the food chain to the global burden of infections caused by antimicrobial-resistant microorganisms remains unknown. Controlling the emergence and spread of resistant bacteria and resistance genes in primary food production and food processing must therefore be a priority to reduce the occurrence of untreatable infections.

Risk analysis schemes provide a framework for the evaluation and communication of risks related to foods. For a risk assessment to be successful, adequate surveillance systems should be implemented. Surveillance of AMR is currently based on the isolation of indicator microorganisms and their phenotypic characterisation, but this culture-dependent approach does not provide complete information on the mechanisms driving AMR or on the presence or spread of AMR genes throughout the food chain. Metagenomics is a powerful tool that allows culture-independent analysis of complex microbial communities and thus has potential applications in AMR surveillance (Bonham et al., 2017; Flórez et al., 2017; Walsh et al., 2017). It can provide access to all the genetic resources in a given environmental niche, which is essential for obtaining the genomes of fastidious or non-culturable microorganisms. Metagenomics could therefore facilitate the tracking of AMR genes and mobile genetic elements, providing the essential information for quantitative risk assessments that will allow for the identification of hotspots and routes of transmission of AMR in the food chain.

2. Description of work programme

2.1. Aims

The aim of the work programme was to familiarise the Fellows with the plethora of risk assessment methods and to train them in the execution of qualitative and quantitative risk assessments in relation to AMR. The specific objectives were: (i) to perform a complete qualitative risk assessment to characterise the role of the food supply chain in the spread of AMR; (ii) to develop and validate a toolbox for the execution of next-generation-sequencing laboratory experiments and bioinformatics analyses for use in AMR surveillance programmes. The application of these methods in global surveys may provide insights into the mechanisms of selection and spread of antibiotic resistance in food-related settings and help identify hotspots of AMR spread in the food chain. Thus, knowledge-based interventions could be developed to reduce the spread and dispersal of multidrug-resistant bacteria throughout the food chain.

2.2. Activities/Methods

The objectives of the work programme were as follows:

Objective 1: The EU-FORA Fellows were trained in risk assessment methodologies routinely used by the supervisors and collaborators at the host institution. The Fellows received training in risk prioritisation, identification of risk factors through regression analyses and in-depth training in specific risk assessment software tools. The Fellows used this knowledge to compile a report calculating the percentage of transmissions that could be considered food-borne for *Cronobacter*, histamine and marine biotoxins. This opinion report along with additional data was used by Professor Jesús A. Santos,

who is a member of the Scientific Committee of the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN), as part of a risk assessment study carried out by this Institution.

Objective 2: The Fellows were trained on the execution of a qualitative risk assessment of the role of the food chain in the spread of AMR. A systematic approach mimicking that followed by EFSA panels in their Scientific Opinions was used for hazard identification and characterisation. A review article presenting the findings of this exercise was published in the international open-access peer-reviewed journal, *Genes* (impact factor 3.6), in the special issue *Genetics and Genomics of Foodborne Pathogens*, with the title 'The present and future of whole genome sequencing (WGS) and whole metagenome sequencing (WMS) for surveillance of antimicrobial resistant microorganisms and antimicrobial resistance genes across the food chain'. At the time of writing, a second review article, 'Food processing activities as a risk factor for AMR spread through the food chain', is in preparation for submission to the international peer-reviewed journal, *Current Opinion in Food Science*. In addition, the Fellows developed two survey questionnaires, one addressed to food companies in Spain concerning the use of biocides and the cleaning and disinfection protocols used in the food industry, and the other directed to the general public across the EU and concerning consumer awareness and risk perception of antimicrobial resistance.

Over a thousand answers were collected from the risk perception questionnaire on antimicrobial resistance, thanks to help from EFSA, which distributed the questionnaire link to all EFSA focal points and thence to all Article 36 organisations. The questionnaire was also distributed via web-based social network platforms such as Facebook and Twitter, and through emails, food- and health-related professional groups and associations as well as in a printed format. In brief, initial results indicate that almost 70% of the participants considered they were well- or very well-informed about antibiotics, which could be explained by the facts that 72% had received an education or specialisation related to the health or food safety sector and 67% had a profession related to those sectors. More than 90% of the participants had obtained antibiotics by medical prescription and adhered to the prescription guidelines. Almost all participants (98.4%) were aware of bacteria being resistant to antibiotics, and the majority believed that the most likely cause is the inappropriate use of antibiotics at farms and in household and clinical settings. About a quarter of the participants believed that antibiotics are used in the EU to stimulate the growth of farm animals, and another quarter did not know the answer to this question. Almost half of the participants either thought that antibiotics used on farm animals are different from those used on people or did not know the answer to this question. Approximately 80% of the participants had received information about antibiotic-resistant bacteria in humans compared with 56% of the participants who had received information about antibiotic-resistant bacteria in farm animals and 45% who had received information about antibiotic-resistant bacteria in foods. Participants felt more confident receiving information about antibiotic resistance in farm animals from scientists and national and European Food Safety Agencies and Institutions than national governments, consumer organisations, farmers, food manufacturers and family and friends. Almost 20% of the participants had changed their eating habits in the past 12 months because of antibiotic-resistant bacteria in farm animals, and 34%, when buying a meat product, considered whether the animal had been bred without the use of antibiotics, although 40% did not know where to check that information. Interestingly, 80% of the participants would be willing to pay more for a meat product that had been obtained from an animal produced without antibiotics, and more than half felt strongly or very strongly threatened by antibiotic-resistant bacteria. According to the participants, the highest priorities for reducing resistance to antibiotics should be the implementation of legislation further restricting the use of antibiotics, informing consumers of the risks of picking up resistant bacteria, helping farmers to shift to modes of production that require less or no antibiotic use, and more investment in research to replace ineffective antibiotics. Information from this survey will provide an overview of consumers' current awareness and perception of antimicrobial resistance. This survey could serve as a baseline for developing new strategies or adjusting existing ones to increase public awareness. To support future risk communication strategies at the European level, analysis of the results of the survey is in progress to identify how different sociodemographic factors influence consumers' perceptions.

The second survey questionnaire, relating to the use of biocides and the cleaning and disinfection protocols used in the food industry, is ongoing at the time of writing. This questionnaire is divided into three sections. Section one includes business details and questions relating to quality management, section two mostly focuses on manufacturing procedures, and section three on cleaning and disinfection protocols and procedures.

Objective 3: The Fellows contributed to the development and validation of harmonised methods and protocols suitable for monitoring AMR genes through next-generation sequencing. At the time of

writing, standard operating procedures (SOPs) are being built for the execution of culture-independent analyses of samples in routine AMR surveillance of foods and food processing samples. The aim is to build a toolbox of methods and protocols that can potentially be used for the surveillance of AMR. For this, the Fellows attended a training course organised by the Institute of Food Science and Technology of the University of León focused on acquiring knowledge in the use of genomics and metagenomics tools. To achieve this, the Fellows participated in taking food and environmental samples (swabs) from a Spanish meat industry site on several occasions and extracting genomic DNA from over 150 samples using established protocols for metagenomic analysis. In addition, the Fellows were involved in the extraction of bacterial fosmid DNA from a metagenomic library previously built from dairy and environmental samples. The fosmid DNA was then purified, subjected to enzymatic restriction analysis and sequenced to identify genes associated with phenotypically increased resistance to antibiotics including ciprofloxacin, gentamycin, tetracycline, cefotaxime and ampicillin. Laboratory research was performed within the framework of the research project 'Identification of routes and mechanisms of antibiotic resistance spread throughout the food chain through culture-dependent and culture-independent methodologies' funded by the Spanish Ministry of Economy and headed by the mentor.

Objective 4: The Fellows participated in dissemination and outreach activities. Two reports were published in international peer-reviewed high-impact-factor journals. Both Fellows presented their work and research interests at seminars in the Institute of Food Science and Technology (ICTAL), which belongs to the University of León. Results were disseminated by the Press Office of the University of León on several occasions, e.g. meeting with the Dean of Research of the University of León, meeting with the EFSA scientific coordinator of EU-FORA, etc. In addition, two poster presentations by the Fellows were accepted for the 2018 EFSA conference 'Science, Food, Society – contextualising risk assessment' to be held in Parma, Italy, 18–21 September 2018. The title of the first poster was 'A European survey questionnaire on consumers' awareness and risk perception of antimicrobial resistance', focused on the overall results of the survey questionnaire on consumers' awareness and risk perception of antimicrobial resistance at the EU level. The title of the second poster was 'Consumer awareness in Greece of issues concerning antibiotic use and the risk of antimicrobial resistance in bacteria', which focused on the results of the same survey, particularly in Greece, which was the country contributing the highest number of answers to the survey questionnaire.

The Fellows also participated in a three-day training course (20 h duration, 1 ECTS) entitled 'Practical workshop on genomics and metagenomics applications in the fields of food microbiology and veterinary microbiology' organised by the Institute of Food Science and Technology of the University of León. The objectives of this workshop were to acquire knowledge of the main applications of genomics and metagenomics techniques in food science as well as to become familiar with the main bioinformatics tools available for genomics and metagenomics studies. In brief, the Fellows independently learned to solve complex problems in the fields of genomics and metagenomics (hands-on exercises in sequence quality control, assembly and genome annotation, mobile genetic elements, antimicrobial resistance genes, virulence genes and others), designed experiments, understood the limitations of the experimental approach and were trained in the review of scientific information relating to genomics and metagenomics. The Fellows also attended a conference entitled 'University-enterprise conference: knowledge transfer in the agrifood sector', organised by the Asociación de la Industria Alimentaria de Castilla y León (Vitartis) and academics from the University of León.

In addition, Erasmus agreements for exchange of students and staff were signed between the Department of Food Hygiene and Technology of the University of León, Spain (hosting site) and the Department of Food Technology, Alexander Technological Educational Institute of Thessaloniki (ATEI-The), Greece (the Fellow's home organisation). One undergraduate student from the Department of Food Technology of ATEI-The is already undertaking her Erasmus placement in the Department of Food Hygiene and Technology of the University of León. Erasmus agreements were also signed between the home institutions of the two Fellows hosted in the University of León: The Faculty of Food Science and Engineering, Dunarea de Jos University of Galati, Romania; and the Department of Food Technology of ATEI-The, Greece. An additional Erasmus agreement was signed between the Department of Food Technology of ATEI-The, Greece and the Faculty of Agriculture and Life Sciences, University of Maribor, Slovenia (the home organisation of another participant in the EU-FORA Fellowship programme).

Two research grant proposals were also prepared jointly between the Laboratory of Food Microbiology, Department of Food Technology, ATEI-The, Greece (the Fellow's home organisation) and the Laboratory of Food Microbiology, Department of Food Hygiene and Technology and Institute of Food Science and Technology, University of León, Spain (hosting site) and submitted to national and European funding bodies. In addition, a postdoctoral fellowship proposal (Juan de la Cierva –

Formación Fellowship) was submitted to the Ministry of Economy of Spain by the Fellow from Dunarea de Jos University of Galati, Romania, in collaboration with the University of León in the area of Food Science and Technology.

Finally, the Fellows took Spanish lessons to get acquainted with both the language and cultural heritage of Spain, and also participated in outdoor activities organised by the University of León (talks/ seminars, skiing lessons, mountain walks, etc.).

3. Conclusions

Both Fellows have so far successfully fulfilled the objectives and tasks of the work programme proposal. Activities performed allowed the Fellows to communicate and disseminate the results. In this context, the Fellows undertook to prepare manuscripts for publication in peer-reviewed journals; one has been published in the journal *Genes*, which has an impact factor of 3.6, and another is currently being written. In addition, two survey questionnaires have been developed, one focused on addressing the use of biocides and the cleaning and disinfection protocols used in Spanish food industries, and another focused on current awareness and consumer perceptions of antimicrobial resistance. Both survey questionnaires can provide a basis for future risk management and communication strategies at the European level. Two poster presentations were submitted by the Fellows and accepted for the EFSA 2018 conference 'Science, Food, Society – contextualising risk assessment' to be held in Parma, Italy 18–21 September 2018. Both posters were linked to the results provided by the questionnaire of the survey on consumer awareness and risk perception regarding antimicrobial resistance. Moreover, Fellows were familiarised with a three-day training course entitled 'Practical workshop on genomics and metagenomics applications in the fields of food microbiology and veterinary microbiology' organised by the Institute of Food Science and Technology of the University of León.

Certain objectives were slightly amended during the placement and/or new objectives were introduced that still served the same purpose: training the Fellows in risk assessment methodology. Monthly laboratory meetings were arranged with the supervisors to monitor the status of the work objectives. The Fellows adjusted and integrated very easily at the University of León and worked in harmony with the rest of the Department staff (Figure 1) .

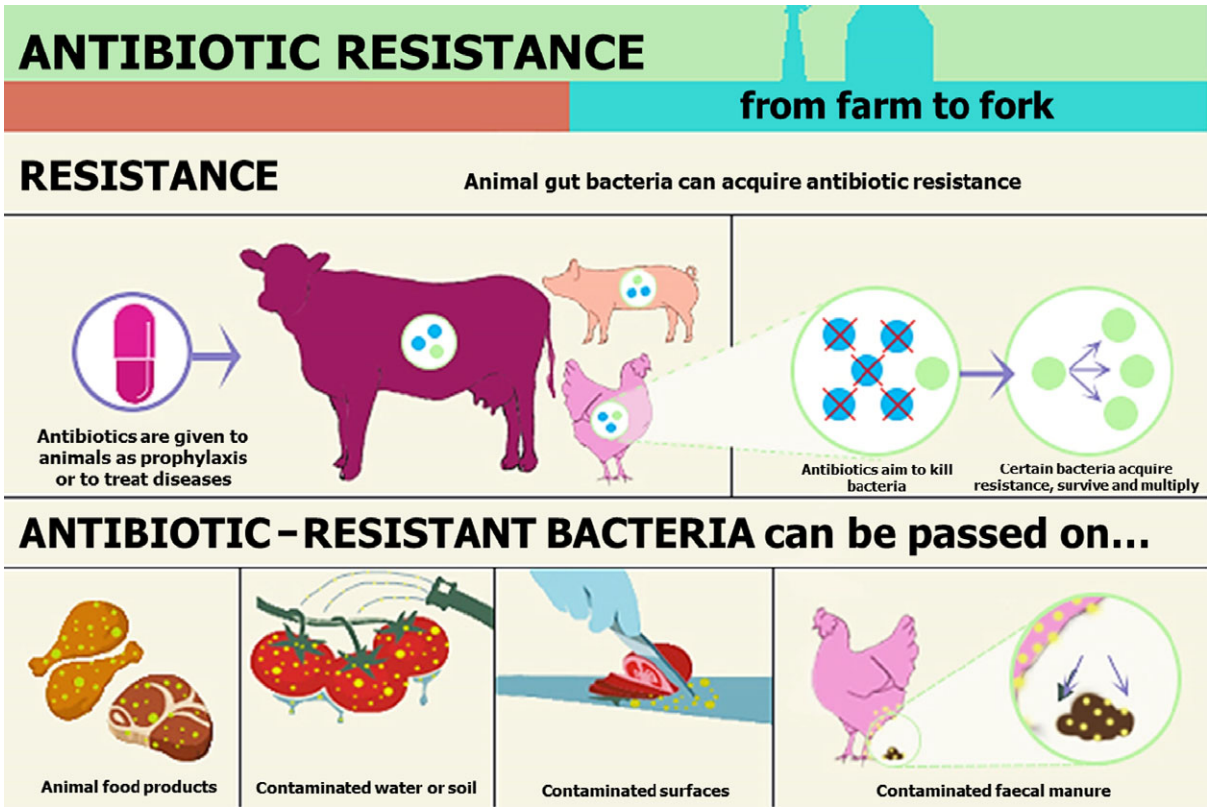

Figure 1: Schematic representation of antibiotic resistance spread through the food chain

References

Bengtsson-Palme J, 2017. Antibiotic resistance in the food supply chain: where can sequencing and metagenomics aid risk assessment? Current Opinion in Food Science, 14, 66–71. https://doi.org/10.1016/j.cofs.2017.01.010

Bonham KS, Wolfe BE and Dutton RJ, 2017. Extensive horizontal gene transfer in cheese-associated bacteria. ELife, 6, e22144. https://doi.org/10.7554/elife.22144

ECDC (European Centre for Disease Prevention and Control), 2017. Surveillance of antimicrobial resistance in Europe 2016. ECDC Stockholm, Sweden. 88 pp. Available online: https://ecdc.europa.eu/sites/portal/files/documents/AMR-surveillance-Europe-2016.pdf

ECDC, EFSA BIOHAZ Panel and CVMP (European Centre for Disease Prevention and Control, EFSA Panel on Biological Hazards and EMA Committee for Medicinal Products for Veterinary Use), 2017. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. EFSA Journal 2017;15(10):5017, 70 pp. https://doi.org/10.2903/j.efsa.2017.5017

EFSA (European Food Safety Authority), 2008. Factsheet on antimicrobial resistance. Available online: http://www.efsa.europa.eu/sites/default/files/corporate_publications/files/factsheetamr.pdf

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2008. Scientific Opinion on foodborne antimicrobial resistance as a biological hazard. EFSA Journal 2008;6(8):765, 87 pp. https://doi.org/10.2903/j.efsa.2008.765

Flórez AB, Vázquez L and Mayo BA, 2017. A functional metagenomic analysis of tetracycline resistance in cheese bacteria. Frontiers in Microbiology, 8, 1–12. https://doi.org/10.3389/fmicb.2017.00907

Smith E, Lichten CA, Taylor J, MacLure C, Lepetit L, Harte E, Martin A, Ghiga I, Pitchforth E, Sussex J, Dujso E and Littmann J, 2016. Evaluation of the EC action plan against the rising threats from antimicrobial resistance. European Commission, Brussels. 150 pp. Available online: https://ec.europa.eu/health/amr/sites/amr/files/amr_final-report_2016_rand.pdf

Verraes C, Van Boxstael S, Van Meervenne E, Van Coillie E, Butaye P, Catry B, de Schaetzen M-A, Van Huffel X, Imberechts H, Dierick K, Daube G, Saegerman C, De Block J, Dewulf J and Herman L, 2013. Antimicrobial resistance in the food chain: a review. International Journal of Environmental Research and Public Health, 10, 2643–2669. https://doi.org/10.3390/ijerph10072643

Walsh AM, Crispie F, Daari K, O'Sullivan O, Martin JC, Arthur CT, et al., 2017. Strain-level metagenomic analysis of the fermented dairy beverage nunu highlights potential food safety risks. Applied and Environmental Microbiology, 83, e01144–17. https://doi.org/10.1128/AEM.01144-17

WHO (World Health Organization), 2015. Global action plan on antimicrobial resistance. WHO, Geneva, Switzerland. 28 pp. Available online: http://www.wpro.who.int/entity/drug_resistance/resources/global_action_plan_eng.pdf

Abbreviations

AECOSAN	Spanish Agency for Consumer Affairs, Food Safety and Nutrition
AMR	antimicrobial Resistance
ATEI-The	Alexander Technological Educational Institute of Thessaloniki
ECDC	European Centre for Disease Prevention and Control
ECTS	European Credit Transfer and Accumulation System
EMA	European Medicines Agency
EU-FORA	European Food Risk Assessment Fellowship Programme
ICTAL	Institute of Food Science and Technology
SOPs	standard operative procedures
WGS	whole genome sequencing
WHO	World Health Organization
WMS	whole metagenome sequencing

EU-FORA SERIES 1

Animal & Plant Health Agency

EFSA Journal

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e160812

Livestock Health and Food Chain Risk Assessment

Animal and Plant Health Agency, United Kingdom
R Condoleo, P Gale, A Adkin, H Roberts and R Simons

Abstract

The EUFORA fellowship programme 'Livestock Health and Food Chain Risk Assessment' was proposed by the Animal and Plant Health Agency (APHA), a British governmental institution responsible for safeguarding animal and plant health in the UK. The working programme, which was organised into four different modules, covered a wide range of aspects related to risk assessment including identification of emerging risks, risk prioritisation methods, scanning surveillance, food production exposure assessment and import risk assessment of animal and human infectious diseases. Over the course of the year, the Fellow had the opportunity to work for international projects with experts in these disciplines. This allowed for significant opportunities to 'learn-by-doing' the methods and the techniques that are employed to assess animal health and food safety risks. Moreover, he consolidated his knowledge by attending several training courses and academic lessons, submitting scientific papers to peer-reviewed journals and conferences, giving presentations and using modelling software.

Keywords: livestock health, food chain, risk assessment

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: Animal and Plant Health Agency, United Kingdom, Condoleo R, Gale P, Adkin A, Roberts H and Simons R, 2018. Livestock Health and Food Chain Risk Assessment. EFSA supporting publication 2018;16(8):e160812, 10 pp. https://doi.org/10.2903/j.efsa.2018.e160812

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

The working programme 'Livestock Health and Food Chain Risk Assessment' was proposed for the EUFORA fellowship programme by the Animal and Plant Health Agency (APHA). APHA is a UK government agency that is responsible for 'safeguarding animal and plant health for the benefit of people, the environment and the economy' at the national level (APHA, 2018). To meet this goal, the Agency carries out numerous activities such as providing high-level laboratory services for private and public bodies, and support and technical advice for UK institutions and scientific research. Among these activities, animal health and food safety risk assessment represent two of the most important/strategic areas, as shown by the fact that many projects at APHA within these disciplines are regularly funded by national or European Union (EU) institutions. The Department of Epidemiological Sciences (DES), and in particular, its Biomathematics and Risk Research workgroup (BRR), is the core area within the agency for risk assessment activities, which are routinely performed over the year. The Fellow was part of the BRR group over the duration of the fellowship. He had the opportunity to collaborate with the staff (around 14 staff skilled in the disciplines of statistics, modelling and risk assessment) as well as to attend several events such as presentations, training courses and project-related meetings.

The working programme covered a wide range of aspects related to risk assessment and was organised into four different modules. A main supervisor was responsible for the general monitoring of the programme, while a specialist supervisor tutored the Fellow for each module. Some of the programme activities were conducted with the support of other organisations such as the Department of Environment, Food and Rural Affairs (DEFRA) and the Royal Veterinary College (RVC).

Module 1 was titled 'Principles and Terminology of Quantitative and Qualitative Risk Assessment'. It can be considered the foundational part of the whole project and it had the scope to consolidate the knowledge of the Fellow regarding the aims, structure and basic methodologies of animal health and food risk assessment.

The second module mainly focused on the different risk ranking methods ('Hazard Identification and Risk Prioritisation Methods'). Using evidence-based and objective criteria, risk ranking techniques are frequently employed to identify and prioritise those animal diseases or food hazards that merit specific and timely attention from the risk manager and consequently help them decide how best to allocate the resources available to prevent and/or control interventions. In the UK, several tools that prioritise those pathogens of highest risk have been designed and are regularly updated and maintained. The outputs feed into specific contingency plans within the Outbreak National Response (Del Rio Vilas et al., 2013; Gibbens et al., 2016). Some of the Fellow's tasks were exploring and understanding the criteria and applying these models in practice, giving him a good basis for the subsequent development of his own model. A further part of the module programme consisted of investigating the horizon-scanning methods routinely undertaken to ensure emerging issues are captured.

Module 3, 'Food Production Exposure Assessment', was devoted to studying the behaviour of food-borne pathogens along the food chain. For this purpose, a 'farm-to-fork' simulation developed for EFSA by a consortium led by APHA was used by the Fellow to acquire and analyse knowledge regarding the methods and basic processes that determine the exposure modelling.

Finally, module 4, 'Food Import Risk Assessment', included both qualitative and quantitative methodologies frequently used by risk assessors to estimate the risk of importing a livestock or zoonotic pathogen along with foodstuffs from foreign countries.

2. Description of work programme

2.1. Aims

The work programme for the Fellow comprised four modules, each with different aims:

- Module 1: To obtain an understanding of the basic principles of both qualitative and quantitative risk assessment and how to go about implementing some of the methods in practice.
- Module 2: To acquire a full understanding of the tools available to rank risks that threaten animal and public health, and knowledge of how they can be incorporated into a working schedule at a national level. As a deliverable, it was expected to build an own-risk ranking tool and submit a scientific manuscript to a scientific journal.
- Module 3: To gain an appreciation of the different risk assessment models and techniques available to assess various risk questions that occur in the political area associated with food-

borne risks and evaluation of the efficiency of control measures. As a deliverable, a country-specific risk assessment for *Salmonella* based on data from each member state was expected to be created.

- Module 4: Food import risk assessment: to gain a substantial understanding of the tools that can be developed to assess spatial risks and threats, learn spatial risk assessment techniques and determine the availability of public data sets that aid European risk assessments.

2.2. Activities/methods

Module 1: Principles and Terminology of Quantitative and Qualitative Risk Assessment

As an initial task, this preliminary module planned a literature review of the most important documents regarding the standard methodologies internationally adopted for animal health and safety food risk assessment. In particular, important international guidance and widely recognised books were selected and became the object of study for the Fellow (OIE, 2004; Codex Alimentarius, 2007; Vose, 2008). Over the year, the Fellow had periodic opportunities to discuss the different components of the risk assessment process with the supervisors and the other risk analysts from the BRR staff (e.g. aim/question, framework, modelling techniques, biomathematics, probability distributions, etc.).

The working programme included attendance at the risk assessment lectures organised during the MSc in Veterinary Epidemiology at the Royal Veterinary College. The training, concerning both qualitative and quantitative risk assessment, consisted of an initial theoretical part followed by a group practical session. The first practical session (about qualitative risk) requested attendees to estimate the annual risk of introduction of Crimean Congo haemorrhagic fever virus (CCHFV) into the UK from Spain and the eventual risk of human infection. The second practical consisted of a series of case studies for which the Fellow was asked to quantify the risk of importing an exotic animal disease and explore potential mitigation measures using quantitative techniques.

Over the fellowship period, the Fellow also participated in several training courses and presentations at APHA headquarters related to risk assessment. In particular, he attended the National Emergency Epidemiology Group (NEEG) Annual Meeting during which, together with other APHA operators, he acquired knowledge regarding the application of risk assessment and management procedures during an ongoing veterinary emergency. The Fellow was also invited to be an observer at meetings of the Human–Animal Infections and Risk Surveillance Group (HAIRS) and the Veterinary Risk Group (VRG) from DEFRA. The two groups include members of the most important health, agricultural and environmental agencies in the UK, and meet periodically to identify, discuss and assess potential emerging zoonotic diseases (HAIRS) and new animal health threats (VRG). For these reasons, the meetings were an excellent opportunity for the Fellow to see risk assessment methodologies applied to real situations.

Finally, as suggested by the Fellow, a part of the module was dedicated to training him in the use of R software, which is widely employed to create quantitative simulations. Since several members of the BRR group are able to code in R, many risk analysts contributed to the training and communicated their experiences of previous risk assessment projects.

Module 2: Hazard Identification and Risk Prioritisation Methods

The first part of the module was dedicated to an extensive literature review of risk ranking methods and tools used in the field of food and animal health risk assessment. In this respect, the European Food Safety Authority (EFSA) has published two comprehensive opinions regarding the development of a risk ranking framework on biological hazards, the performance of the most common tools, and assessment of uncertainty in this context (EFSA BIOHAZ Panel, 2012, 2015). Some tools such as Risk Ranger, iRisk and the ECDC Burden of Communicable Diseases in Europe toolkit were studied in detail and their application simulated through case studies based on data from the Fellow's country of origin.

Furthermore, beyond the food safety aspects, the working programme also included aspects related to animal health. In this regard, the EFSA web seminar on rapid risk assessment tools for animal disease outbreaks was particularly useful. This gave the Fellow an overview of some models used in different EU countries to evaluate the importance of potential exotic diseases (EFSA, 2017).

Nevertheless, the module was mainly focused on the exploration of the several tools/frameworks that are routinely used to prioritise the impact of endemic or non-endemic disease on the UK. One of those, D2R2, aims to rank several endemic pathogens based on the available evidence and data. Five different areas are assessed (public health, welfare of animals, interests of the wider economy, environment and society, and international trade) and an additional module regarding potential

post-mitigation measures. D2R2 includes a long list of diseases (endemic and exotic) that are ranked separately for each of the potential animal species involved (Gibbens et al., 2016).

ETHiR is a risk ranking framework used mainly for emerging or new threats/vulnerabilities, with the goal of estimating their probability of occurrence and economic impact. It is meant for new threats, described as the 'risk resulting from a newly identified hazard to which a significant exposure may occur or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard'. ETHiR uses a scoring system to assess several factors (e.g. public health impact, public concern, potential countermeasure, etc.) and it is used by the Veterinary Risk Group in the UK (Kosmider et al., 2017).

Finally, particular attention was given to the IDM risk of incursion tool (Roberts et al., 2011; EFSA, 2017). This ranks many exotic diseases (34 for the August 2017 revision) on the basis of their estimated probability of entry into the UK. To perform this, a score is attributed to each disease, weighting the potential import routes (live animals trade, food trade, etc.), using available data (e.g. volume of trade from an infected area) or expert opinions. Risk of incursion is periodically updated by DEFRA to monitor the risk of importing disease, and the reports are sent to APHA, policymakers and a wider government audience including the Cabinet Office and UK Border Agency (Roberts et al., 2011). The present version of the risk of incursion tool uses a Microsoft Excel® spreadsheet (Microsoft Corporation, Redmond, WA, USA, v. 2010). One of the tasks of the Fellow was to convert the tool to R language to simplify the updating process and make it possible in the future to download the data of interest from defined sources and automatically update the results (SPARE Project Group, 2016).

A further task of the Fellow was to develop a version of the tool (using his country as a case study) that supported the authorities in border surveillance for those pathogens with higher probabilities of entry. A selection approach, taken from the EU SPARE project (SPARE, 2016; Horigan et al., 2018), was applied to produce a list of infectious diseases that are non-endemic to Italy and subject to World Organisation for Animal Health (OIE) notification. The world was divided into different areas and OIE data used to assign a score related to the prevalence of each disease in the animal population. Several possible pathways for pathogen introduction were considered: trade in live animals, foodstuffs, and germplasm; vectors; and the movements of people and wild animals. For each pathogen, a score was calculated to estimate the probability of incursion of the pathogen through each potential pathway. The scores were based on data retrieved from EU databases or published scientific research. The volume of live animal and foodstuff imports to Italy from a particular area were retrieved from COMEXT (2018) and TRACES (European Commission, 2018) to estimate the potential likelihood of entry of the different pathogens through an associated commodity. Information regarding aircraft/ship/truck movements (EUROSTAT, 2018), certain types of commodities (COMEXT, 2018) and wind streams were used to evaluate the probability of entry of an infected vector. The tool used data from EUROSTAT regarding annual people movement in Italy and from the DG SANTE website (https://ec.europa.eu/info/departments/health-and-food-safety_en) for information on collection centres authorised to import semen and embryos. Dispersal of terrestrial wild animals and bird migration were assessed, considering, respectively, the proximity of the studied areas to Italy and the different flight paths used by migratory birds (EFSA AHAW Panel, 2005; Stroud et al., 2004). No score was attributed to a pathogen when a pathway was considered of negligible importance because it was not a biologically plausible means of transmission. Finally, an algorithm was proposed to calculate an overall risk score for each pathogen:

$$\sum_{p=1}^{n} \text{Pathway (d, p)} = \frac{\sum_{i=1}^{n} \text{Area status (i, d)} \times \text{score (i, s)}}{\text{Pathway maximum risk}}$$

where Pathway is the overall disease score of the p pathway for the given pathogen, d; Area status is the disease score for that pathogen in the area, i; Score is the value attributed to an area for the species/commodities/risk factor, s, that can potentially cause the entry of the given pathogen. The score was standardised by dividing by the *Pathway maximum risk* that was defined for each pathway as the highest score among the considered diseases. Figure 1 shows a schematic representation of the method applied.

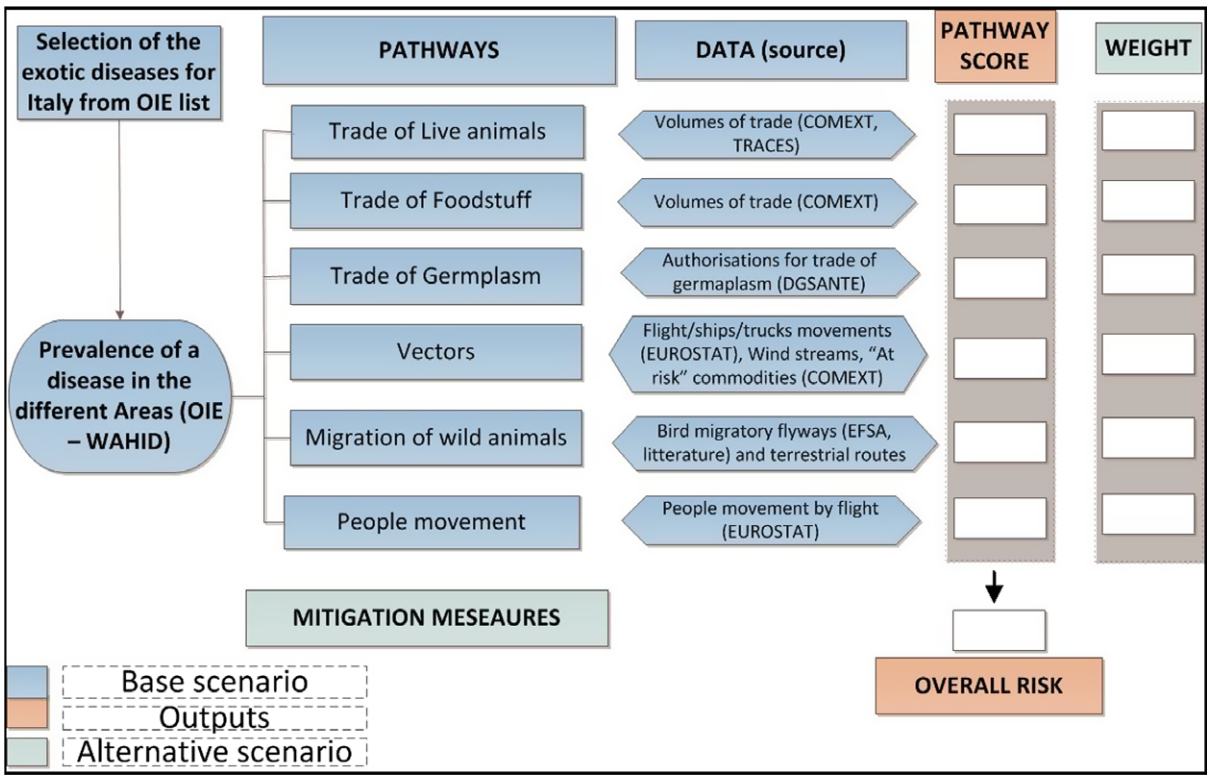

Figure 1: Schematic representation of risk ranking approach

The last part of module 2 was dedicated to exploring horizon scanning methods. Horizon scanning is a routine activity that aims to improve the situational awareness of decision-makers across the government, thus allowing governments to be more anticipatory in their responses to natural hazards and reduce the impacts of future disasters. In the UK, in addition to APHA, three further agencies (Met Office, British Geological Survey and Public Health England) work in coordination to produce periodic reports on new, emerging or deteriorating situations regarding global natural/climate disasters, animal health and human public health issues. The Fellow was instructed on the structure and layout of the report, the information sources usually consulted by APHA and the methodology used by them to complete all parts of the report. He gave a presentation on 'Identification of animal health emerging risks in the UK' during a summer school organised by EFSA and the University of Parma (16 May 2018, Parma, Italy).

Module 3: Food Production Exposure Assessment

The third module 'Food Production Exposure Assessment' involved studying and properly running a 'farm-to-consumption' simulation to understand how the different basic processes of a quantitative microbiological risk assessment (QMRA) could be applied to modelling the variations in prevalence and concentration of a pathogen (Nauta, 2002). To this purpose, a QMRA was employed on '*Salmonella* in Slaughter and Breeder Pigs' developed for EFSA by a European Consortium led by APHA (Hill et al., 2016a,b; Simons et al., 2016; Snary et al., 2016a,b; Swart et al., 2016a,b; Vigre et al., 2016a,b). The model deals with four European countries and, as main output, it calculates the risk of human salmonellosis associated with the consumption of fresh pork cuts, minced meat and fermented sausage. The first task of the Fellow was to understand the workings of the simulation and the technical solutions that were adopted to model the exposures. The second task was to find specific information for the country-specific parameters of the simulation to generate an alternative scenario concerning Italy. Whenever possible, the retrieved data were manipulated to generate probability distributions. For this reason, the Fellow learnt what distributions are usually adopted to model certain type of biological events/phenomena in a risk assessment process and how to select them based on data availability and widely accepted scientific criteria. In addition, several tools were applied to fit the data (e.g. R software, Matlab scripts). Finally, the simulation was implemented using the new data and the results compared with those of the other countries.

Module 4: Food Import Risk Assessment

To meet the objective of module 4 'Food Import Risk Assessment', the Fellow collaborated with several members of the BRR group on two different projects. The first was a qualitative risk assessment commissioned by DEFRA. Working alongside colleagues who were performing the risk assessment for the UK, the Fellow performed a risk assessment for his own country. It focused on the risk of introduction of transmissible spongiform encephalopathies through unusual domestic animals such as camels. The second project involved collaborating on one of the tasks within COMPARE, an EU-funded Horizon 2020 project. The task in question concerned the development of a generic and spatial quantitative risk assessment framework that could estimate the risk of introduction and spread of exotic diseases into new areas that would be applicable to any disease, pathway or area. The Fellow worked with others in BRR to develop the food importation pathway and estimate the risk of importing a zoonotic or animal disease through the importation of foodstuffs, based upon pathways that had previously been developed within COMPARE. To do this, the Fellow acquired and refined skills in data extraction and comprehension, understanding of the risk assessment framework specifically within a generic setting, and modelling quantitative import risks using R software.

3. Conclusions

The working programme 'Livestock Health and Food Chain Risk Assessment' was an opportunity for the Fellow to consolidate and broaden his knowledge of risk assessment. Several aspects of qualitative risk assessment were examined in depth (e.g. identification of the pathways and consequent tree scenario definition) and practical means/tools such as risk tables, procedures and report layouts were provided and applied to simulate risk case situations (modules 1 and 4). Studying the elements of animal health risk assessment was a relevant part of the programme, and the Fellow not only acquired the theoretical bases but he also had the opportunity to apply them in practice (modules 1, 2 and 4). In particular, the different phases of import risk analysis (OIE, 2004) were examined in detail and combined with risk prioritisation methods to create a risk ranking tool (module 2). The applied methodology was presented in a poster at the 2018 EFSA Conference, while a full description of the tool and its results will be reported in a manuscript currently in preparation.

Regarding quantitative risk assessment, because the Fellow already possessed a basic understanding of the theoretical background and had practical experience in the application of quantitative risk assessment, the project aimed mainly to strengthen some specific aspects such as the different sensitivity analysis approaches (Patil and Frey, 2004), convergence testing, uncertainty assessment and modelling techniques (modules 1 and 3). In this regard, the simulation of *Salmonella* in pigs (EFSA *Salmonella* in Pigs QMRA Consortium, 2010) was shown to be a suitable case study because, in contrast with several other QMRAs, its complexity means it includes a wide range of elements/situations that can be present in a food risk assessment (module 3). Furthermore, the setting of a new alternative country-based scenario allowed the Fellow to become familiar with data gathering and application of curve fitting techniques in the field of probability distribution. To provide an exhaustive representation of the quantitative risk assessment, different frameworks were also practically applied to estimate the risk of importing zoonotic disease via food trade (module 4).

Finally, as mentioned above, some activities, such as learning a new modelling software, were not explicitly planned by the programme, but could be added because of the Fellow's pre-existing knowledge and the need to enhance the core programme. At the end of the period, the Fellow was able to use the R software functions that are commonly employed in risk simulation, and he used this software language to replicate a risk ranking tool (IDM Risk of incursion, Roberts et al., 2011) and a previously published QMRA implemented using a different language (modules 1 and 2).

References

APHA (Animal & Plant Health Agency), 2018. Available online: https://www.gov.uk/government/organisations/animal-and-plant-health-agency

Codex Alimentarius, 2007. Principles and guidelines for the conduct of microbial risk management (MRM). Available online: http://www.fao.org/docrep/004/y1579e/y1579e05.htm

COMEXT, 2018. Bulk download data. Available online: http://ec.europa.eu/eurostat/estat-navtree-portlet-prod/BulkDownloadListing?sort=1&dir=comext

Del Rio Vilas VJ, Voller F, Montibeller G, Franco LA, Sribhashyam S and Watson E, Hartley M, Gibbens JC, 2013. An integrated process and management tools for ranking multiple emerging threats to animal health. Preventive Veterinary Medicine, 108, 94–102. https://doi.org/10.1016/j.prevetmed.2012.08.007

EFSA (European Food Safety Authority), 2017. Webinar: rapid risk assessment tools for animal disease outbreaks. Available online: https://www.efsa.europa.eu/en/events/event/171127

EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare), 2005. Opinion on animal health and welfare aspects of Avian Influenza. EFSA Journal 2005;3(9):266, 21 pp. https://doi.org/10.2903/j.efsa.2005.266

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2012. Scientific Opinion on the development of a risk ranking framework on biological hazards. EFSA Journal 2012;10(6):2724, 88 pp. https://doi.org/10.2903/j.efsa.2012.2724

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2015. Scientific Opinion on the development of a risk ranking toolbox for the EFSA BIOHAZ Panel. EFSA Journal 2015;13(1):3939, 131 pp. https://doi.org/10.2903/j.efsa.2015.3939

EFSA Salmonella in Pigs QMRA Consortium, 2010. Quantitative microbiological risk assessment on *Salmonella* in slaughter and breeder pigs: final report. EFSA supporting publication, 437 pp. Available online: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2010.EN-46

European Commission, 2018. TRACES: TRAde control and expert system. Available online: https://ec.europa.eu/food/animals/traces_en

EUROSTAT, 2018. Database. Available online: http://ec.europa.eu/eurostat/web/international-trade-in-goods/data/database

Gibbens JC, Frost AJ, Houston CW, Lester H and Gauntlett FA, 2016. D2R2: an evidence-based decision support tool to aid prioritisation of animal health issues for government funding. Veterinary Record, 179, 21–26. https://doi.org/10.1136/vr.103684

Hill AA, Simons RRL, Kelly L and Snary EL, 2016a. A farm transmission model for *Salmonella* in pigs, applicable to E.U. member states. Risk Analysis, 36, 461–481. https://doi.org/10.1111/risa.12356

Hill AA, Simons RL, Swart AN, Kelly L, Hald T and Snary EL, 2016b. Assessing the effectiveness of on-farm and abattoir interventions in reducing pig meat-borne Salmonellosis within E.U. Member States. Risk Analysis, 36, 546–560. https://doi.org/10.1111/risa.12568

Horigan V, De Nardi M, Simons RRL, Bertolini S, Crescio MI, Estrada-Peña A, Léger A, Maurella C, Ru G, Schuppers M, Stärk KD, 2018. Using multi-criteria risk ranking methodology to select case studies for a generic risk assessment framework for exotic disease incursion and spread through Europe. Preventive Veterinary Medicine, 153, 47–55. https://doi.org/10.1016/j.prevetmed.2018.02.013

Kosmider R, Gibbens J, R A, 2017. Identification, assessment and management of new and re-emerging animal-related risks: UK perspective. The Veterinary Record, 181, 67. https://doi.org/10.1136/vr.104258

Nauta MJ, 2002. Modelling bacterial growth in quantitative microbiological risk assessment: Is it possible? International journal of food microbiology, 73, 297–304.

OIE (World Organisation for Animal Health), 2004. Handbook on Import Risk Analysis for Animals and Animal Products. Volume 1. Introduction and qualitative risk analysis. OIE, Paris, France. 57 pp.

Patil SR and Frey HC, 2004. Comparison of sensitivity analysis methods based on applications to a food safety risk assessment model. Risk Analysis, 24, 573–585.

Roberts H, Carbon M, Hartley M and Sabirovic M, 2011. Assessing the risk of disease introduction in imports. Veterinary Record, 168, 447–448. https://doi.org/10.1136/vr.d1784

Simons RRL, 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

Snary EL, Swart AN and Hald T, 2016a. Quantitative microbiological risk assessment and source attribution for Salmonella: taking it further. Risk Analysis, 36, 433–436. https://doi.org/10.1111/risa.12605

Snary EL, Swart AN, Simons RRL, Domingues ARC, Vigre H, Evers EG, Hald T and Hill AA, 2016b. A quantitative microbiological risk assessment for *Salmonella* in pigs for the European Union. Risk Analysis, 36, 437–449. https://doi.org/10.1111/risa.12586

SPARE, 2016. SPatial Risk Assessment Framework for Assessing Exotic Disease IncuRsion and Spread Through Europe. Available online: https://www.spare-europe.eu/wp1-release-assessment

Stroud DA, Davidson NC, West R, Scott DA, Hanstra L, Thorup O, Ganter B and Delany S, 2004. Status of migratory wader populations in Africa and Western Eurasia in the 1990s. International Waders Studies, 15, 1–259. Available online: https://www.researchgate.net/profile/David_Stroud/publication/228588274_Status_of_migratory_wader_populations_in_Africa_and_Western_Eurasia_in_the_1990s/links/00b7d52acc83eb812d000000/Status-of-migratory-wader-populations-in-Africa-and-Western-Eurasia-in-the-1990s.pdf?origin=publication_detail

Swart AN, Evers EG, Simons RLL and Swanenburg M, 2016a. Modeling of *Salmonella* contamination in the pig slaughterhouse. Risk Analysis, 36, 498–515. https://doi.org/10.1111/risa.12514

Swart AN, van Leusden F and Nauta MJ, 2016b. A QMRA model for *Salmonella* in pork products during preparation and consumption. Risk Analysis, 36, 516–530. https://doi.org/10.1111/risa.12522

Vigre H, Barfoed K, Swart AN, Simons RRL, Hill AA, Snary EL and Hald T, 2016a. Characterization of the human risk of salmonellosis related to consumption of pork products in different E.U. countries based on a QMRA. Risk Analysis, 36, 531–545. https://doi.org/10.1111/risa.12499

Vigre H, Domingues AR, Pedersen UB and Hald T, 2016b. An approach to cluster EU member states into groups according to pathways of *Salmonella* in the farm-to-consumption chain for pork products. Risk Analysis, 36, 450–460. https://doi.org/10.1111/risa.12579

Vose D, 2008. *Risk Analysis: A Quantitative Guide*. 3rd Edition, John Wiley & Sons, Ltd, Chichester, UK. 752 pp.

Abbreviations

APHA	Animal and Plant Health Agency
BRR	Biomathematics and Risk Research workgroup
CCHFV	Crimean Congo haemorrhagic fever virus
DEFRA	Department of Environment, Food and Rural Affairs
DES	Department of Epidemiological Sciences
HAIRS	Human–Animal Infections and Risk Surveillance Group
NEEG	National Emergency Epidemiology Group
OIE	World Organisation for Animal Health
QMRA	Quantitative Microbial Risk Assessment
RCV	Royal Veterinary College
VRG	Veterinary Risk Group

EU-FORA SERIES 1

APPROVED: 6 July 2018

doi: 10.2903/j.efsa.2018.e160813

Risk ranking of chemical and microbiological hazards in food

Swedish National Food Agency (NFA), Uppsala, Sweden,
T Langerholc, R Lindqvist and S Sand

Abstract

Risk ranking is a versatile tool used to prioritise activities performed by public health regulatory bodies. It also allows efficient communication between all stakeholders in the process of risk analysis. However, risk ranking methods are still not optimal. Because of the different approaches employed in the risk assessment of microbiological agents and chemicals, it is difficult to rank them together using the same metrics. In our work, we first discuss differences and commonalities between chemical and microbiological risk assessment to provide a starting point for consideration of a common risk ranking platform. In the second part, we perform risk ranking of contaminants and regulated chemicals using the recently developed Risk Thermometer tool. In this approach, chemicals are not ranked solely on the basis of the margin of exposure between a reference value and the exposure, but also by considering the severity of the critical health effects used. The results show that ranking using both methods provides different results from the use of either method alone. Overall, specific chemical groups (i.e. heavy metals, pesticides, etc.) do not generally rank higher or lower, but individual compounds are scattered in the rankings from low to high. Risk ranking methods demand further development to gain wide acceptability and recognition.

Keywords: risk ranking, chemical hazards, microbiological hazards, risk thermometer

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: Swedish National Food Agency (NFA), Uppsala, Sweden, Langerholc T, Lindqvist R and Sand S, 2018. Risk ranking of chemical and microbiological hazards in food. EFSA Journal 2018;16 (8):e160813, 9 pp. https://doi.org/10.2903/j.efsa.2018.e160813

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

Public health institutions need to carefully optimise their workload and directions of activities owing to limited resources. In this context, risk ranking can help to set priorities. Ideally, ranking by risk assessors should be risk- and science-based to provide risk managers with clear input and exclude subjectivity. It should also provide a clear scientific input to risk communication.

Recently, Van der Fels-Klerx et al. (2018) reviewed risk ranking methods. Methods have been classified as quantitative, semi-quantitative and qualitative. Quantitative risk ranking is preferable because semi-quantitative and qualitative methods use different practices and metrics, and the results may not fully correlate with quantitative risk assessments. For chemical hazards, risk ratio, scoring, flow charts and risk matrices have been considered for ranking. Cost of illness (CoI), health-adjusted life years (HALY) and expert judgements were most commonly applied to microbiological hazards. There is a clear difference in preferred methods used for chemical and microbiological risk ranking because chemical and microbiological risk assessments (CRA and MRA) are based on different principles. This may prevent chemical and microbiological risks from being effectively ranked using the same platform and metrics. Specifically, chemical risk is assessed if the estimated exposure is below the established health-based guidance values (HBGV), while for microbiological risk, the probability or risk of disease (or similar) associated with the estimated exposure is assessed.

The use of a risk ratio and its variants has been the most commonly applied approach for risk assessment of chemicals, including ranking. By such ratios, the HBGVs, i.e. the tolerable daily intakes (TDI) for food contaminants and the acceptable daily intakes (ADI) for food additives, are compared with corresponding estimates of chemical exposure. The TDI and ADI are usually set by dividing a reference point (RP), derived from experimental data, by an overall adjustment factor (AF) of 100 to account for differences between animals and humans as well as differences in susceptibility within the human population. The (risk) ratio between the RP and the exposure is known as the margin of exposure (MOE). For compounds that are both genotoxic and carcinogenic, an MOE higher that 10,000 is generally regarded as of low concern (EFSA, 2005). For non-genotoxic compounds, an MOE of $>$ 100 would correspond to an exposure below the HBGV (provided that a default overall AF of 100 is applied in the establishment of the HBGV). The drawback with this type of method is that an extra safety margin specifically for the nature/severity of the critical effect is only applied in the case of genotoxic cancer risk. To address this issue, a tool called Risk Thermometer was developed by the Swedish National Food Agency (NFA; Sand et al., 2015). This method uses an extra AF that accounts for the severity of the critical health effect in a systematic manner, resulting in a modified risk ratio denoted as the severity-adjusted margin of exposure (SAMOE).

This work has been performed within in the framework of the 'Risk ranking of chemical and microbiological hazards in food' project. Partners in the project are the NFA and the Finnish Food Safety Authority (EVIRA), and the project is supported by the European Food Safety Authority (EFSA).

2. Description of work programme

2.1. Aims

Objective 1

Analysis of CRA and MRA methods and principles for ranking risks in each area. To identify differences and commonalities in methods, concepts and data requirements for CRA and MRA as a starting point for exploring the possibility of developing a common platform for risk ranking of microbiological and chemical risks.

Objective 2

Application of the Risk Thermometer tool to rank chemicals for which EFSA has made risk assessments, and for which the required data are available on toxicity and exposure at the European level. The purpose of this study is to investigate how a large group of chemicals (e.g. regulated products such as pesticides and food additives and other substances like heavy metals, dioxins, diverse halogenated molecules and mycotoxins) become distributed between the five risk grades of the Risk Thermometer scale.

2.2. Activities/methods

Differences and commonalities between CRA and MRA

Literature searches were performed to identify and summarise important aspects of risk assessment and risk ranking methods for chemical and microbiological hazards. This included differences and similarities in terms of approaches (e.g. bottom–up, top–down, extrapolation methods), concepts (e.g. acute or chronic exposure), assumptions (including the use of assessment factors) and the necessary data requirements. Literature where simultaneous ranking of microbiological and chemical risks had been attempted was also identified along with the approaches used for those studies.

Risk ranking of chemicals using the Risk Thermometer tool

A case study was performed by applying the Risk Thermometer to chemicals for which EFSA has made risk assessments providing both toxicity and exposure data in the European Union (EU). A detailed description of the Risk Thermometer can be found in Sand et al. (2015). The required input parameters for this analysis are described below. Data on these parameters were mainly extracted from EFSA scientific opinions and reports.

1) Reference point (RP): RPs in terms of a benchmark dose (BMD), a no-observed-adverse-effect level (NOAEL) or the lowest-observed-adverse-effect level (LOAEL) were used. In cases where the BMD method had been applied, both the BMD and BMD lower level limit (BMDL) are preferably needed, and also the BMD upper level limit (BMDU), if available. The BMD was regarded as the geometrical mean of the BMDL and BMDU in cases when the BMDL and BMDU were provided, but not the BMD. If data on BMDL and BMDU were not available, a default 0.2 log uncertainty was applied to the BMD in both directions as a qualitative measure of uncertainty. When only the BMDL was available, a default 0.4 log uncertainty was applied as a qualitative measure of uncertainty.
2) Adjustment factors (AFs): Default AFs to account for intraspecies variability (3.16 for toxicokinetics and 3.16 for toxicodynamics) and interspecies variability (3.98 for toxicokinetics and 2.51 for toxicodynamics) were generally used when the RP was based on experimental data. Whenever available, chemical-specific AFs were applied according to the specific EFSA risk assessments used as a basis.
3) The severity factor (SF): The severity of the critical effect was classified according to a health effect classification scheme developed by the NFA (Sand et al., 2015). Based on this classification, the value-based SF was set by experts at NFA: the SF can adopt values of 1 (10^0), 3.16 ($10^{0.5}$), 10 (10^1), 31.6 ($10^{1.5}$) or 100 (10^2). A factor of 1 is given to 'mild' critical effects, like changes in non-specific biomarkers, while a factor of 100 is ascribed to cancer or severe developmental effects, for example.
4) Exposure (E): The mean and 95th percentile (if available) of exposure for European adults were considered. Upper-, middle- and lower-boundary results, which describe the impact of how concentration values below the limit of detection/quantification are treated, were used as part of the uncertainty analysis. The middle-boundary result was used as a point estimate, while the lower- and upper-boundaries were allowed to describe uncertainty. The geometrical mean of the lower and upper boundary results was used as a point estimate in cases when the middle-boundary result was not provided.

The Risk Thermometer is based on a severity-adjusted margin of exposure (SAMOE) approach. The SAMOE depends on the parameters described above in bullets 1–4, such that $SAMOE = RP/(AF \times SF \times E)$. Risk ranking is performed based on the SAMOE value, which is presented as a point estimate with a 90% confidence interval describing uncertainty. At the level of the Risk Thermometer, the SAMOE is classified into one of five risk classes corresponding to different levels of population health concerns (Sand et al., 2015).

As a comparative starting point in these analyses, a more traditional risk ratio was calculated: the ratio between the HBGV and the mean exposure. This analysis was limited to chemicals with established HBGVs.

A user interface for the Risk Thermometer tool has been developed in Matlab (MathWorks, Inc., USA). Results have been further evaluated using Excel® (Microsoft Corp. USA).

3. Conclusions

3.1. Differences and commonalities between CRA and MRA

This work is still in draft form and the main conclusions are not yet formulated since the analysis is ongoing. The information presented here is therefore to be considered provisional.

Risk assessment in combination with risk management and risk communication constitute the core of risk analysis. Risk assessment is science-based, with a clear separation between hazard and risk in both MRA and CRA. CRA and MRA follow the same basic four-step procedure of hazard identification, hazard characterisation, exposure assessment and risk characterisation.

Acute vs lifetime hazards

Since chemical and microbiological hazards substantially differ in their characteristics, persistence, survivability and adverse health effects, differences have emerged between CRA and MRA. For chemicals, the potential risks associated with low-level exposure over a long time period are usually of concern. In contrast, with supporting clinical data and case studies, it is easier to diagnose microbiological hazards causing an acute illness and to establish a link to the food chain. Microbes also do not build up in the body as many chemicals do (e.g. cadmium, dioxins). Exposure to specific microbes leads to a certain degree of immunity, decreasing or preventing the risk of re-infection.

Exposure assessment

Microbes can multiply in foods depending on food handling, storage and processing. Exposure assessment in MRA, therefore, requires a multistep analysis to provide an estimate of microbial contamination at the consumption level. However, some toxins secreted by microorganisms behave similarly to chemicals that are stable upon storage and treatment.

Threshold vs non-threshold

With the notable exception of genotoxic carcinogens considered to have a non-threshold dose–response at low doses, most chemicals are generally considered to follow threshold dose–response models. Microbial dose–response modelling in MRA generally uses non-threshold models that may include host, pathogen and epidemiologic parameters.

Risk assessment vs safety assessment

CRA and MRA differ in what determines their fitness for purpose. When exposure assessment in CRA is below the HBGV, or the MOE > 10,000 in the case of genotoxic compounds, the risk of adverse effects is considered to be of low concern. The approach in MRA provides the probability of illness as a consequence of exposure. Overall, safety assessment in CRA and risk assessment in MRA are based on problems related to the consequences of exposure, which are mainly chronic and acute, respectively.

Variability and uncertainties

There is an array of variability and uncertainty sources in CRA and MRA. In CRA, variability within the human population and uncertainties in route-to-route extrapolation, duration of exposure in experimental animal studies, the dose–response curve, the nature and severity of the effects, extrapolation from animal species to humans, and concentrations of chemicals in commodities below the limit of detection or quantification are the main issues. In MRA, the main sources of variability include differences within the human population as well as the genetic variability of microbial strains. Additional sources of uncertainty include events along and within the food chain that predictive microbiology models are often used to describe. In both CRA and MRA, consumption data depend on dietary survey methods. In MRA, separate treatment of variability and uncertainty has become a common practice through the development of two-dimensional models and stochastic modelling. In CRA, uncertainty and variability are described at the level of exposure, whereas HBGVs are fixed upon application of uncertainty and variability factors (even though uncertainty may be addressed at the level of the RP derivation).

Exposure sources

CRA calculates a cumulative risk for a specific compound found in different foods, since the occurrence of chemicals is generally not limited to a single food. In contrast, MRA can be for one food and one pathogen, one food and several pathogens, or many foods or a group of similar foods and one pathogen, or many foods and many pathogens. Pathogen survival depends on physical and

processing parameters for the food across the farm-to-fork supply chain, requiring a separate calculation for each specific food. However, cumulative risks to several foods can also be calculated in an MRA.

3.2. Risk ranking of chemicals using the Risk Thermometer tool

Based on EFSA reports, we extracted data on toxicity and exposure for > 40 food chemical contaminants and > 25 regulated substances (pesticides, sweeteners, food colour additives).

Chemicals were first ranked in line with a traditional risk ratio method by dividing HBGVs by the corresponding (mean) exposure estimated for adults in the EU (Figure 1). Heavy metals (nickel, cadmium) rank the highest in risk (considering that the risk ratio indirectly relates to response/effect and risk), while aluminium, copper, organic and inorganic mercury rank lower. From all EFSA-evaluated mycotoxins with HBGVs, the T-2 + HT-2 group ranks the highest. Dithiocarbamates rank the highest from all pesticides considered, which may be because they are commonly used phytopharmaceutical substances. Dioxin, like polychlorinated biphenyls (PCBs) and nitrates, also rank high, next to nickel and cadmium. Glyphosate, despite recently drawing widespread public attention, ranks very low among all pesticides. Food colour additives (E 102, E 104, E 124) likewise rank much lower than the heavy metals.

In the next step, risk ranking of selected chemicals was performed using the Risk Thermometer (Figure 2). As described earlier, this tool uses an extra assessment factor (the SF) that depends on the critical health effect used for each chemical. Based on their SAMOE value, chemicals are classified into five classes.

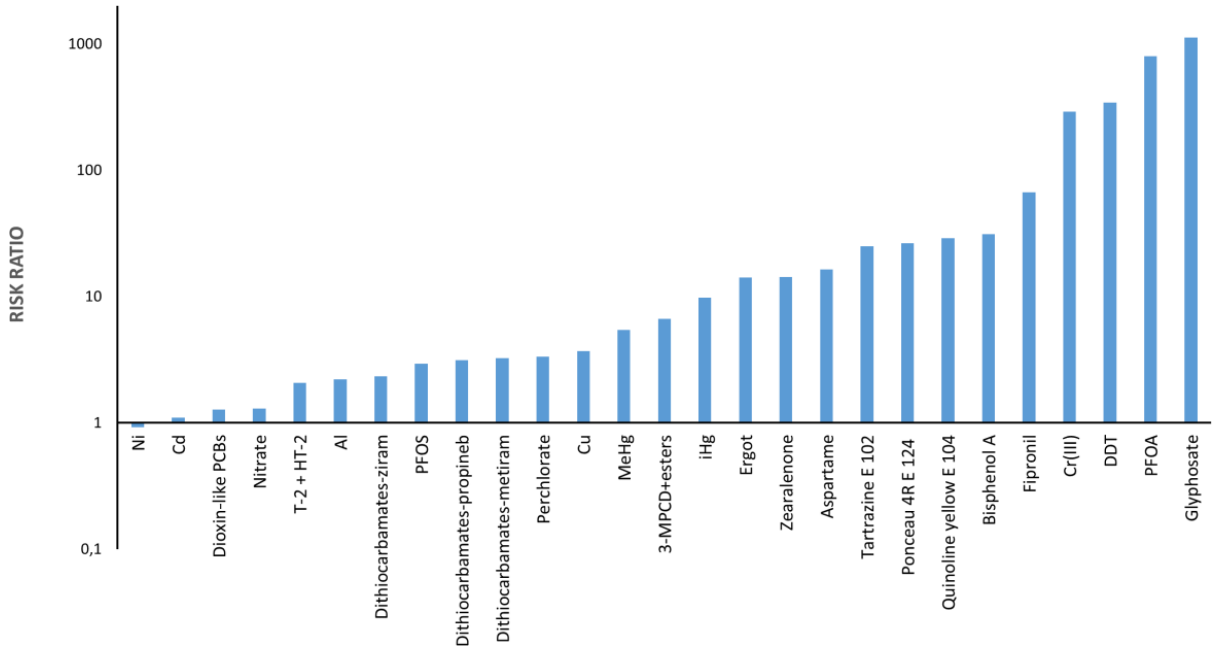

Figure 1: Risk ranking of selected contaminants and regulated substances using the risk ratio, HBGV:exposure. Mean European exposure for adults was applied in the calculations

The results in Figure 2 show that ranking of chemicals by the Risk Thermometer differs from the ranking obtained by the traditional risk ratio method. The genotoxic compound acrylamide ranks higher than methylmercury and the dithiocarbamate pesticide ziram. Since neurodevelopmental disorders and less-severe organ-specific changes are the critical effects of methylmercury and ziram, respectively, they are ranked differently compared with the results obtained by the risk ratio method. Glyphosate and the food colour Sunset Yellow (E 110) rank significantly lower, which is also observed in with ranking based on risk ratio.

Ranking of chemicals using a traditional risk ratio method has several drawbacks. For example, some non-genotoxic compounds do not have a TDI/ADI for diverse reasons. Also, genotoxic compounds do not have HBGVs; safety is assessed by MOE. Upon consideration of an additional uncertainty factor of 100 (that accounts for the process of carcinogenicity), genotoxic compounds with

MOEs > 10,000 are regarded as safe or to be of low concern (EFSA 2005). These two groups of compounds cannot be ranked with other compounds by the classical risk ratio ranking approach. The approach used in the Risk Thermometer allows calculation of SAMOE for all compounds, with or without set HBGVs, including genotoxic compounds. Besides accounting for the severity of effect, uncertainty is also addressed by the SAMOE/Risk Thermometer in contrast with traditional methods. The Risk Thermometer can therefore be regarded as an upgrade of risk ratio methods, providing more realistic results for a decision-making process.

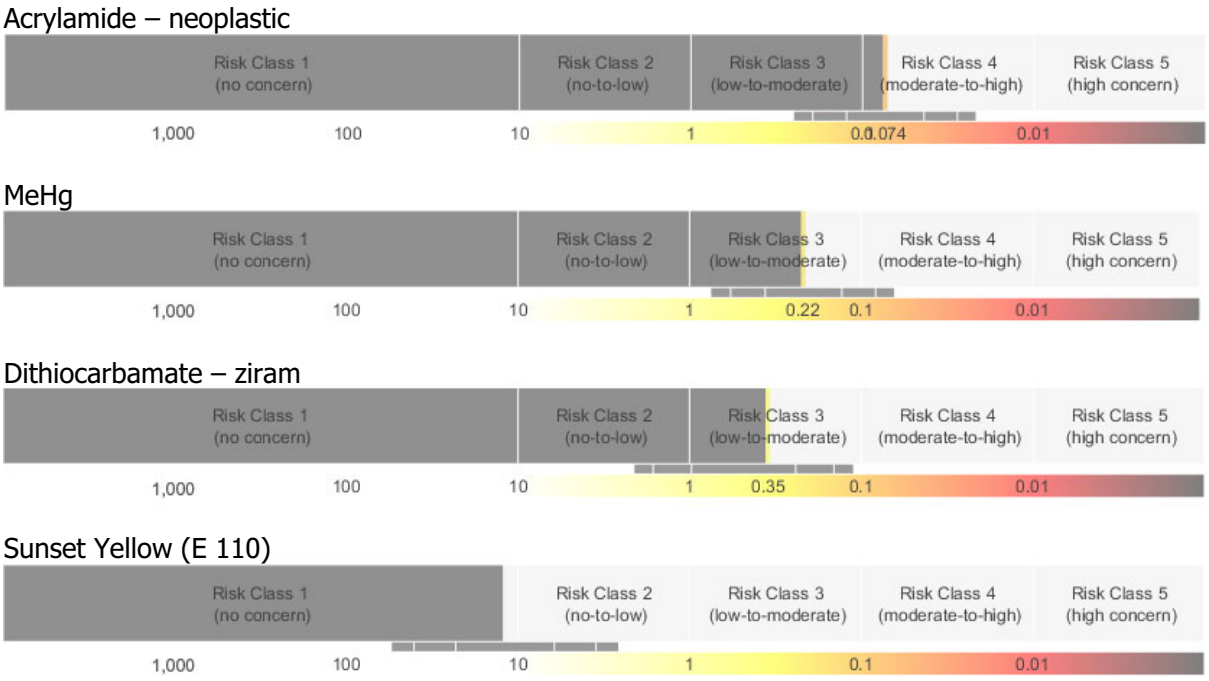

Figure 2: Risk ranking of selected contaminants and regulated substances using the Risk Thermometer. The grey bars describe the estimate (point estimate and 90% confidence interval) of the severity-adjusted margin of exposure (SAMOE) to the compound that places it in a particular Risk Class that is associated with a described level of health concern (Sand et al., 2015). Calculations are based on the European mean exposure

References

EFSA (European Food Safety Authority), 2005. Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic. EFSA Journal 2005;3(10):282, 31 pp. https://doi.org/10.2903/j.efsa.2005.282

Sand S, Bjerselius R, Busk L, Eneroth H, Färnstrand JS and Lindqvist R, 2015. The Risk Thermometer - a tool for risk comparison. Swedish National Food Agency, 2015, 59 pp. Available online: https://www.livsmedelsverket.se/globalassets/rapporter/2015/the-risk-thermometer.pdf

Van der Fels-Klerx HJ, Van Asselt ED, Raley M, Poulsen M, Korsgaard H, Bredsdorff L, Nauta M, D'agostino M, Coles D, Marvin HJP, Frewer LJ, 2018. Critical review of methods for risk ranking of food-related hazards, based on risks for human health. Critical Reviews in Food Science and Nutrition, 58, 178–193. https://doi.org/10.1080/10408398.2016.1141165

Abbreviations

ADI	allowed daily intake
BMD	benchmark dose level
BMDL	benchmark dose lower level limit
BMDU	benchmark dose upper level limit
CoI	cost of illness
CRA	chemical risk assessment
EVIRA	Finnish Food Safety Authority
HALY	health-adjusted life years

HBGV	health-based guidance values
LOAEL	lowest-observed-adverse-effect level
MOE	margin of exposure
MRA	microbiological risk assessment
NFA	Swedish National Food Agency
NOAEL	no-observed-adverse-effect level
PCB	polychlorinated biphenyl
RP	reference point
SAMOE	severity-adjusted margin of exposure
SF	severity factor
TDI	tolerable daily intake

Getting in touch with the EU

In person

All over the European Union there are hundreds of Europe Direct information centres. You can find the address of the centre nearest you at: https://europa.eu/european-union/contact_en

On the phone or by email

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

– by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),

– at the following standard number: +32 22999696 or

– by email via: https://europa.eu/european-union/contact_en

Finding information about the EU

Online

Information about the European Union in all the official languages of the EU is available on the Europa website at: https://europa.eu/european-union/index_en

EU publications

You can download or order free and priced EU publications at: https://publications.europa.eu/en/publications. Multiple copies of free publications may be obtained by contacting Europe Direct or your local information centre (see https://europa.eu/european-union/contact_en).

EU law and related documents

For access to legal information from the EU, including all EU law since 1952 in all the official language versions, go to EUR-Lex at: http://eur-lex.europa.eu

Open data from the EU

The EU Open Data Portal (http://data.europa.eu/euodp/en) provides access to datasets from the EU. Data can be downloaded and reused for free, for both commercial and non-commercial purposes.

TM-BC-18-001-EN-N

European Food Safety Authority

Via Carlo Magno 1A
43126 Parma
ITALY

Tel. +39 0521 036 111
www.efsa.europa.eu

Publications Office

ISBN 978-92-9499-056-3
doi:10.2805/307848