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

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## Abstract

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



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

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# 1. Introduction

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

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

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

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

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

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

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

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

## 2. Description of work programme

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

### 2.1. Aims

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

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

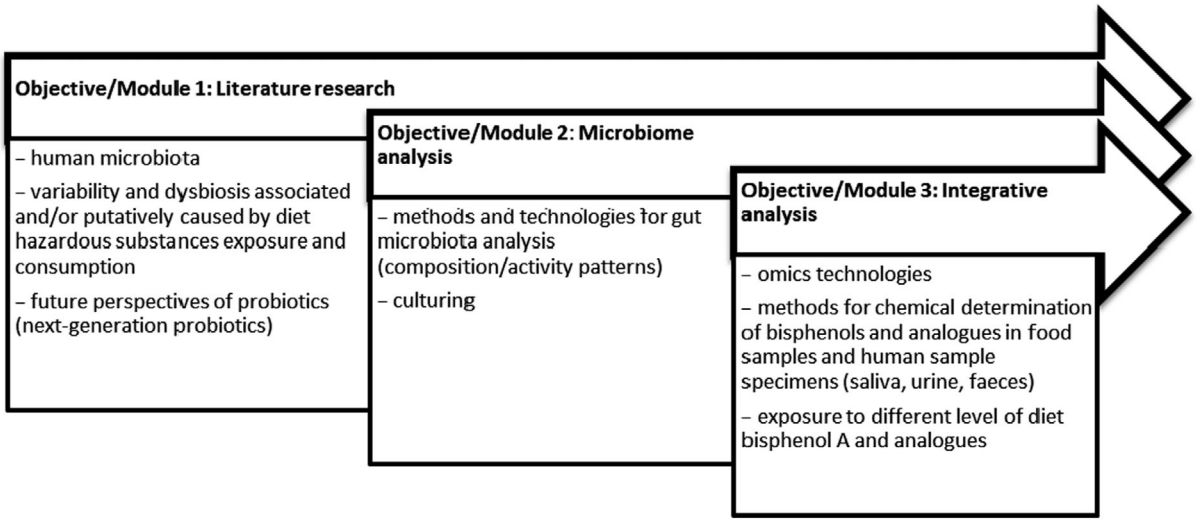


**Figure 1:** Diagram of proposed work programme as timeline

## 2.2. Activities/Methods

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

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

Safety assessment and regulatory framework

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

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

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

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

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

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

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

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

### 2.2.2. Objective/Modules 2 and 3: Practical work

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

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

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

**Table 2:** Different genomic analyses for evaluation of microbial communities

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

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

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

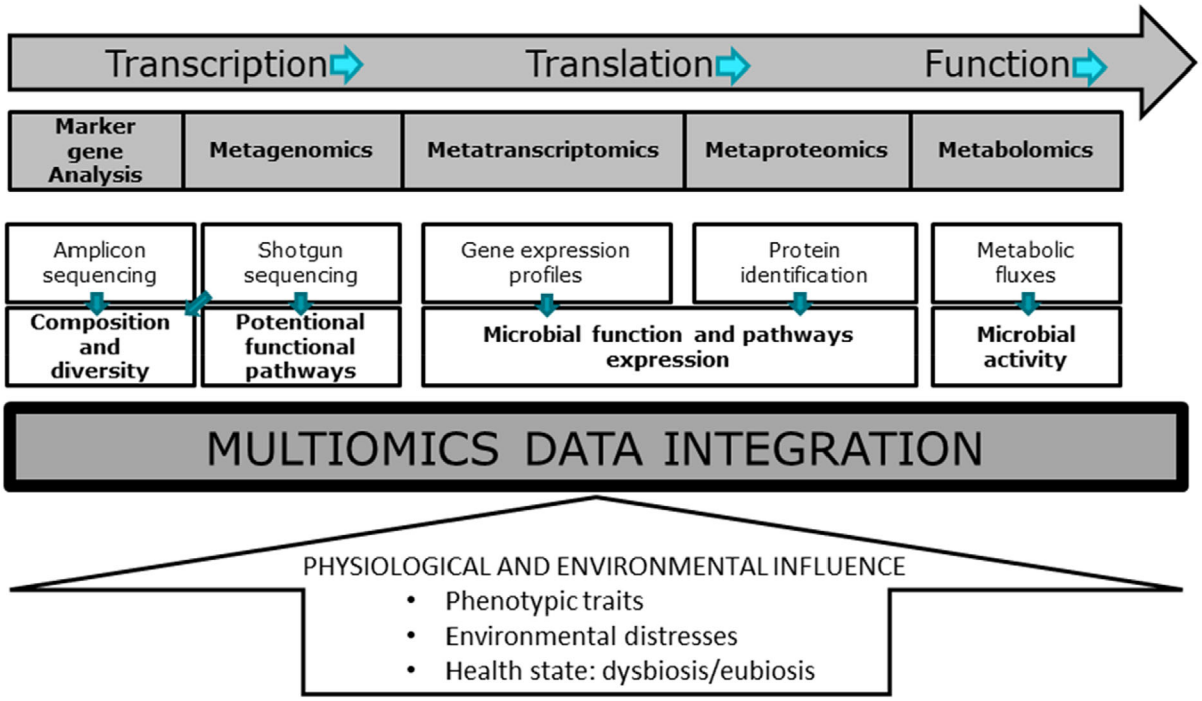


**Figure 2:** Multiomics data integration approach for elucidating the role of microbiota

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

### 2.2.3. EU-FORA Fellowship additional activities and trainings

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

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

and participation at congresses (Annex A):

- Abstract acceptation and iPoster presentation at the World Microbe Forum 2021, 20–24 June 2021 (online worldwide); in collaboration with the team (Ana López-Moreno, Alfonso Torres-Sánchez, Ángel Ruiz-Moreno, Pilar Ortiz, Marina Úbeda, Jesús Pardo, Margarita Aguilera; Faculty of Pharmacy, University of Granada, Granada and 'José Mataix Verdú' Institute of Nutrition and Food Technology (INYTA), Granada).
  Title: *Safety Assessment Criteria Implemented for the Gut Microbiota Taxa with Potential Use in Metabolization of Dietary Endocrine Disruptors*.
- Oral communication at THE EFSA-OBEMIRISK WORKSHOP Granada meeting Action: OBEMIRISK-Knowledge platform for assessing the risk of Bisphenols on gut microbiota and its role in obesogenic phenotype: looking for biomarkers (Granada, Spain; 14–15 October 2021) in collaboration with the team (Ana López-Moreno, Alfonso Torres-Sánchez, Ángel Ruiz-Moreno, Pilar Ortiz, Antonis Ampatzoglou, Agnieszka Gruszecka-Kosowska, Margarita Aguilera; Faculty of Pharmacy, University of Granada, Granada and 'José Mataix Verdú' Institute of Nutrition and Food Technology (INYTA), Granada):
  *Title: Metagenomic analysis of children gut microbiota: challenges and standardization*
- Abstract acceptation and Poster presentation at the EFFoST conference 2021, from 1 to 4 November 2021, Lausanne; Switzerland in collaboration with the team (Ana López-Moreno, Alfonso Torres-Sánchez, Ángel Ruiz-Moreno, Pilar Ortiz, Antonis Ampatzoglou, Agnieszka Gruszecka-Kosowska, Margarita Aguilera; Faculty of Pharmacy, University of Granada, Granada and 'José Mataix Verdú' Institute of Nutrition and Food Technology (INYTA), Granada):
  *Title: Safety assessment of Bacillus sp. AM1 isolated from human gut microbiota, with the ability to metabolize dietary endocrine disruptors, as potential product used in food production chain*

## 2.3. Results and discussion

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

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

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

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

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

## 3. Conclusions

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

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

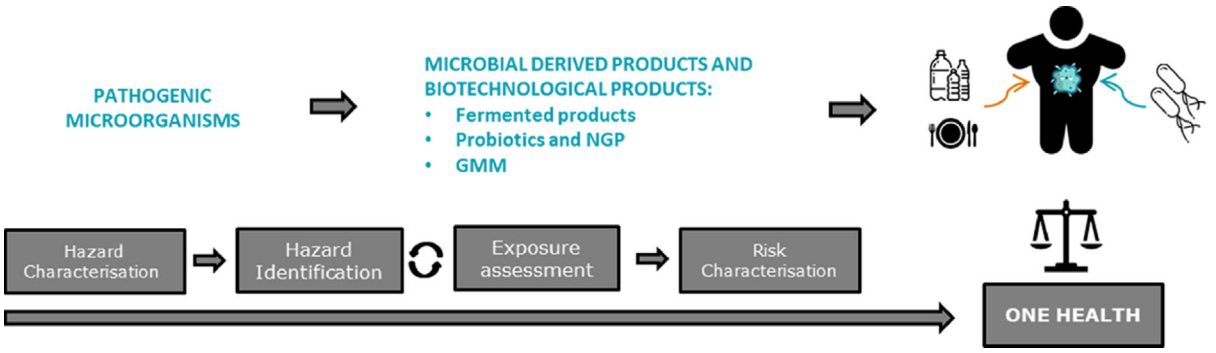


**Figure 3:** Evolution of microbial risk assessment under One Health approach

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

Disclaimer

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

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## Abbreviations

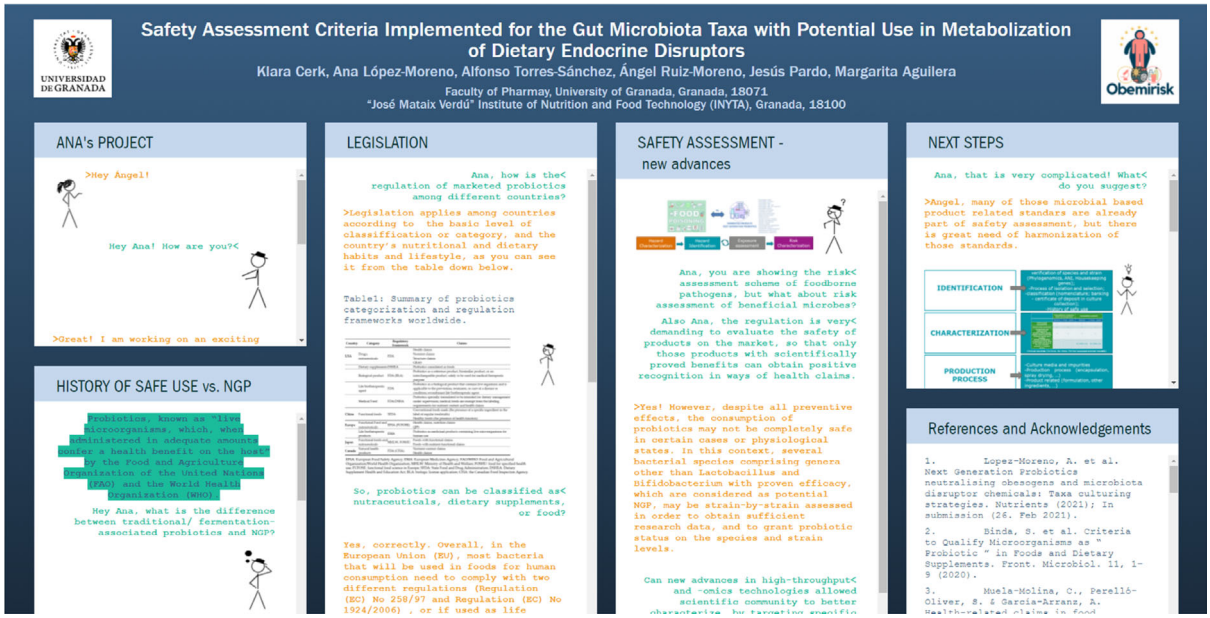
| | |
|---|---|
| AGES | Agency for Health and Food Safety |
| AMR | antimicrobial resistance |
| ANI | alignment-based calculation of average nucleotide identity |
| ASV | amplicon sequence variant |
| BPA | bisphenol A |
| BfR | Federal Institute for Risk Assessment |
| BLA | biologic license application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

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

# Annex A – Posters presented at conferences:

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

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




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

Title: Safety assessment of *Bacillus* sp. AM1 isolated from human gut microbiota, with the ability to metabolize dietary endocrine disruptors, as potential product used in food production chain.