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

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Abstract

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

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

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1. Introduction

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

1.1. Exposome, xenobiotics and endocrine disruptors

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

1.2. Gut microbiota and metabolic disorders

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

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

1.3. Toximicrobiomics

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

1.4. Next-generation probiotics

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

2. Description of work programme

2.1. Aims

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

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

2.2. Methodologies

2.2.1. Omics characterisation of gut microbiota

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

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

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

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

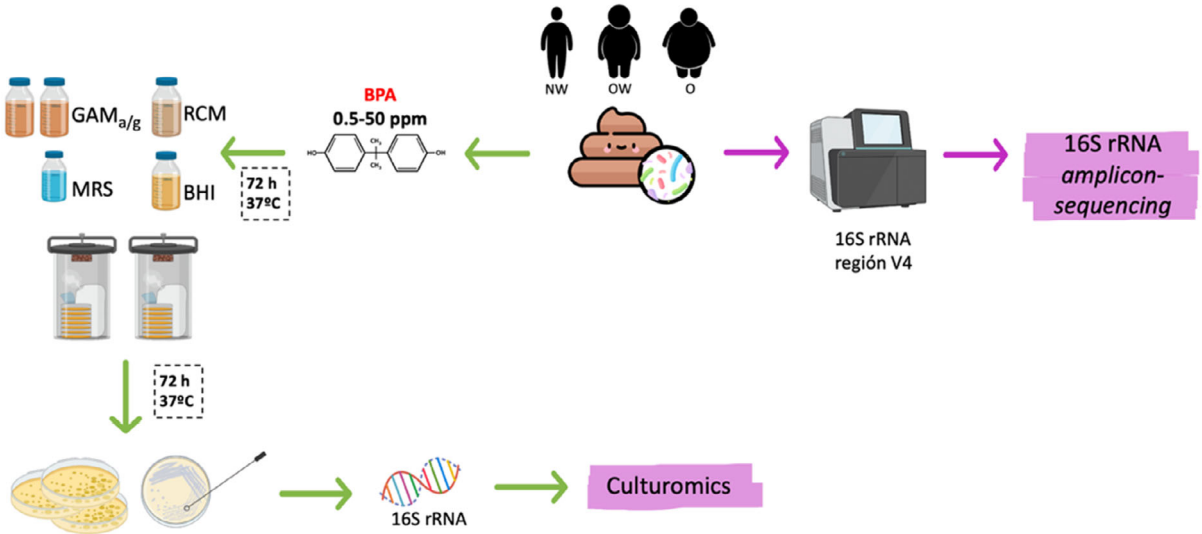

Figure 1: Detailed protocol for the Omics characterisation of gut microbiota of the study population

2.2.2. Characterisation of potential NGP

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

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

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

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

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

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

2.2.3. Inflammatory assessment of BPA and NGP

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

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

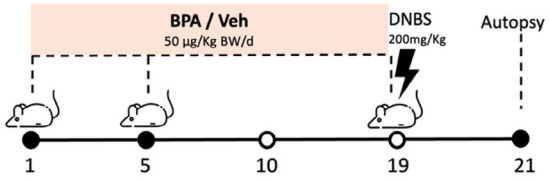

Figure 2: DNBS-induced colitis in mice treated orally with BPA to assess the immunological effects of BPA

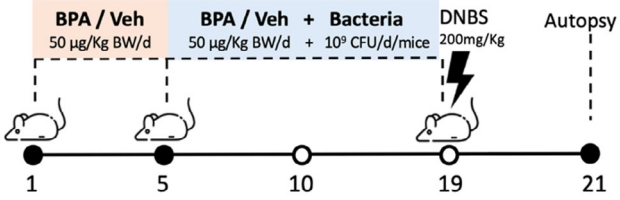

Figure 3: DNBS-induced colitis in mice treated orally with BPA and selected potential NGP to assess the potential anti-inflammatory properties

3. Outcomes

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

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

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

4. Conclusion

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

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Abbreviations

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

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

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

- Metagenomic analysis of the gut microbiota in children in relation to bisphenol A exposure. Ruiz-Rodríguez A; Cerk K; **López-Moreno A**; Rivas A; Monteoliva-Sánchez M; Suárez A; Aguilera M. XIX Reunión Taxon, 2022, Mallorca, Spain. Oral communication.
- Study of the gut microbiota resistant to endocrine disruptors in children with obesity and potential use as Next-generation probiotics. **López-Moreno A**; Moreno MA; Martín R; Monteoliva-Sánchez M; Aguilera M. XIX Taxon, 2022, Mallorca, Spain. Oral communication.
- Gut microbiota dynamics influenced by bisphenol A tolerant taxa in childhood obesity using culturomics and amplicon-sequencing. **López-Moreno A**; Cerk K; Aguilera M; Ruiz-Rodríguez A. XXIX Congreso Sociedad Española de Microbiología, 2023, Burgos, Spain. **Poster communication - Best poster award.** FEMS Meeting Attendance Grants.
- Dissertation of the thesis during the EU-FORA programme.