**TITLE:** Relevance of gut microbiome research in food safety assessment

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**ABSTRACT**

The gut microbiome is indispensable for the host physiological functioning. Yet, the impact of non-nutritious dietary compounds on the human gut microbiota and the role of the gut microbes in their metabolism and potential adverse biological effects have been overlooked. Identifying potential hazards and benefits would contribute to protecting and harnessing the gut microbiome’s role in supporting human health. We discuss the evidence on the potential detrimental impact of certain food additives and microplastics on the gut microbiome and health endpoints, with a focus on underlying mechanisms and causality. We provide recommendations for the incorporation of gut microbiome science in food risk assessment and identify knowledge and tools needed to fulfill the gaps.The incorporation of gut microbiome endpoints to safety assessments, together with well-established toxicity and mutagenicity studies, might better inform the risk assessment of certain contaminants in food, and/or food additives.

**KEYWORDS:** epithelial barrier; gut microbiota; metabolism; risk assessment; xenobiotic**.**

**MAIN TEXT**

Diet influences the composition and function of the human gut microbiome. Resident microbes and their activities impact many biological processes, such as metabolism, immunity, and the functioning of extra-intestinal organs (*e.g.,* brain, cardiac, liver, kidney)1. The human gut is densely colonized by microorganisms, specially by bacteria, belonging to more than 1,000 different species that together with their collective genetic material and by-products form the so-called gut microbiome. The gut microbiota is established at birth through contacts with the mother and the environment, starting a complex symbiotic interaction with the different host body’s systems. It can contribute to the host’s health status through multiple pathways, including the host response to challenges from pathogens, drugs, industrial chemicals, environmental pollutants, and food components. The use of partially-digestible food additives and chemical contaminants has highly increased in recent years and, therefore, their impact on human health requires constant updates. Classical risk assessment approaches applied to foods paradoxically consider non- or even partially-absorbable dietary compounds as inert upon excretion. This scenario overlooks the role that the gut microbiota and its metabolites play in bio-transformation and modulation of the biological activity of dietary compounds, as well as in their absorption to the bloodstream2. Given the absence of a specific legal framework that considers microbiomes as an additional criterion for risk assessment, there is a lack of globally accepted methodologies and guidance to comprehensively address the impact of the gut microbiome changes on humans, animals and the environment. This is in sharp contrast with the fact that many microbially-processed compounds and/or derived metabolites are transported along the gut epithelium to the bloodstream. Indeed, the gut microbiome acts at the interphase between the dietary exposure and the host and may play a significant part in determining the potential risks or benefits of non- or partially-absorbable dietary compounds.

Recent international initiatives have aimed to better understand the role of human microbiomes and their interactions with other microbial ecosystems in global health. The main goals of these initiatives were to define how the human microbiome affects health3-7, and to identify dietary components that may harm the gut microbiome (or be modulated by) and the underlying molecular mechanisms. This article focuses on the interplay between dietary xenobiotics and the gut microbiome with potential impact on human health. It also describes a series of research elements that could be part of safety studies to inform risk assessment and suggests research priorities to address knowledge gaps.

**The gut microbiome is a critical modulator of the catabolism of dietary components**

Gut bacteria produce and/or make available a plethora of metabolites (short-chain fatty acids (SCFAs), phenolic compounds, *etc*.) from dietary compounds, as well as micronutrients (*e.g.*, vitamins). Some of those microbially produced metabolites are known to exert beneficial effects on immune maturation and function and epithelial barrier integrity, helping, for example, to fight against gastrointestinal infections8. Gut microbes are also being increasingly recognized for their ability to communicate with the brain and modulate its function, through the production of neurotransmitters (*e.g.*, histamine, gamma-aminobutyric acid, serotonin, dopamine)9, and other bioactive metabolites (*e.g.*, indoxyl sulphate, indole-3-acetic acid, indole-3-propionic acid, 4-ethylphenol-sulfate), some of which are derived from (aromatic) amino acid catabolism10, 11. These metabolites can act locally in the gut affecting the enteric nervous system or reach distant organs affecting brain function12.

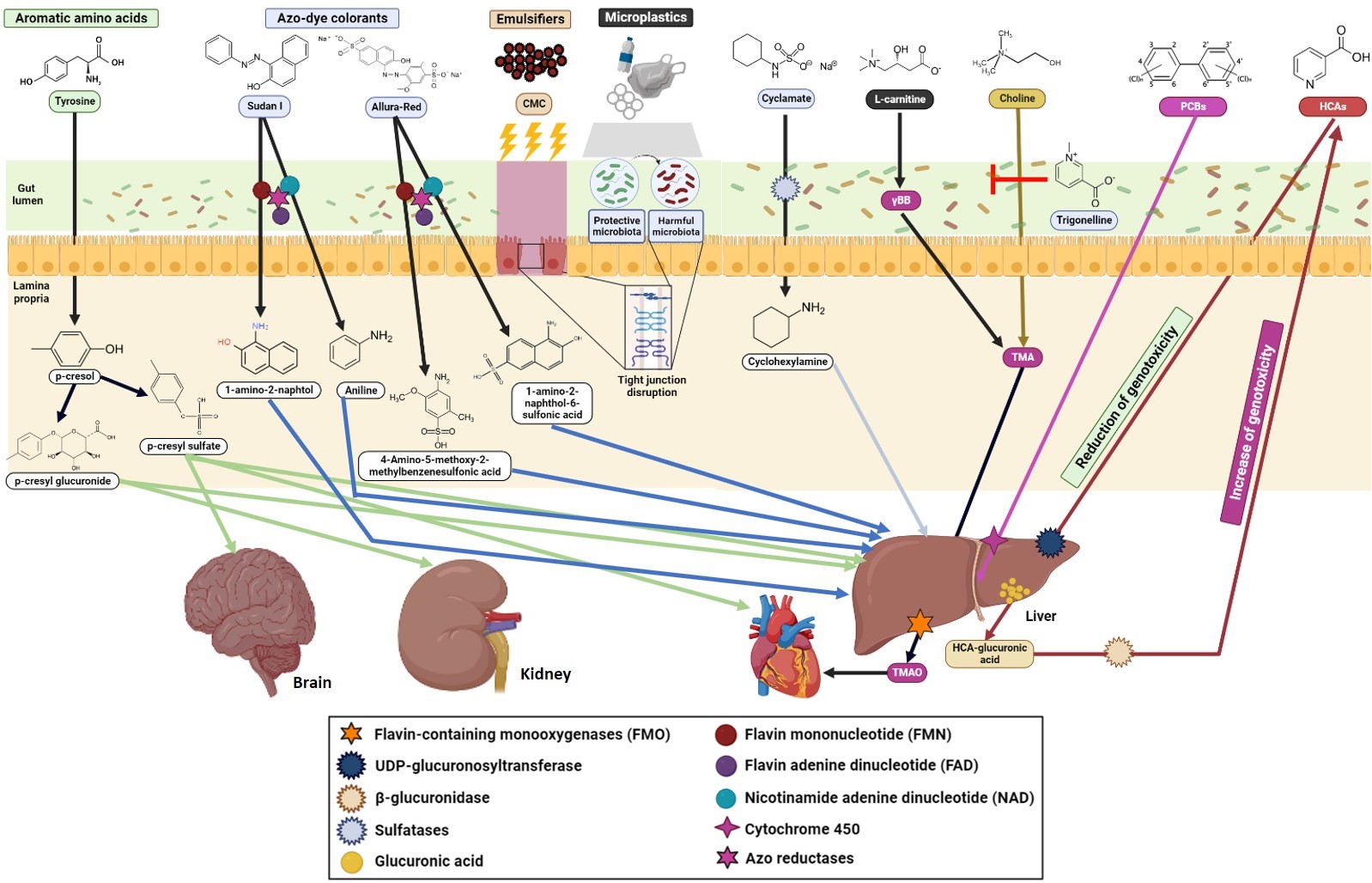
Conversely, the human gut microbiome may also undermine the host’s health by producing hazardous diet-derived metabolites. As an example, choline, betaine, or L-carnitine are bio-converted into trimethylamine (TMA) in the gut, which is further oxidized in the liver by flavin-containing monooxygenases resulting in trimethylamine N-oxide (TMAO)13 (**Figure 1**). The characterization of several microbial gene clusters, critical to this multi-step transformation pathway, linked microbial L-carnitine/choline catabolism of red meat with cardiovascular disease risk14, 15. Likewise, a range of xenobiotics used in agriculture, food, and pharmaceutical industry are known to interact with the gut microbiome, and could negatively influence human health through different mechanisms16-18, as shown in **Figure 1**.

**The gut microbiome is impaired by certain dietary xenobiotics**

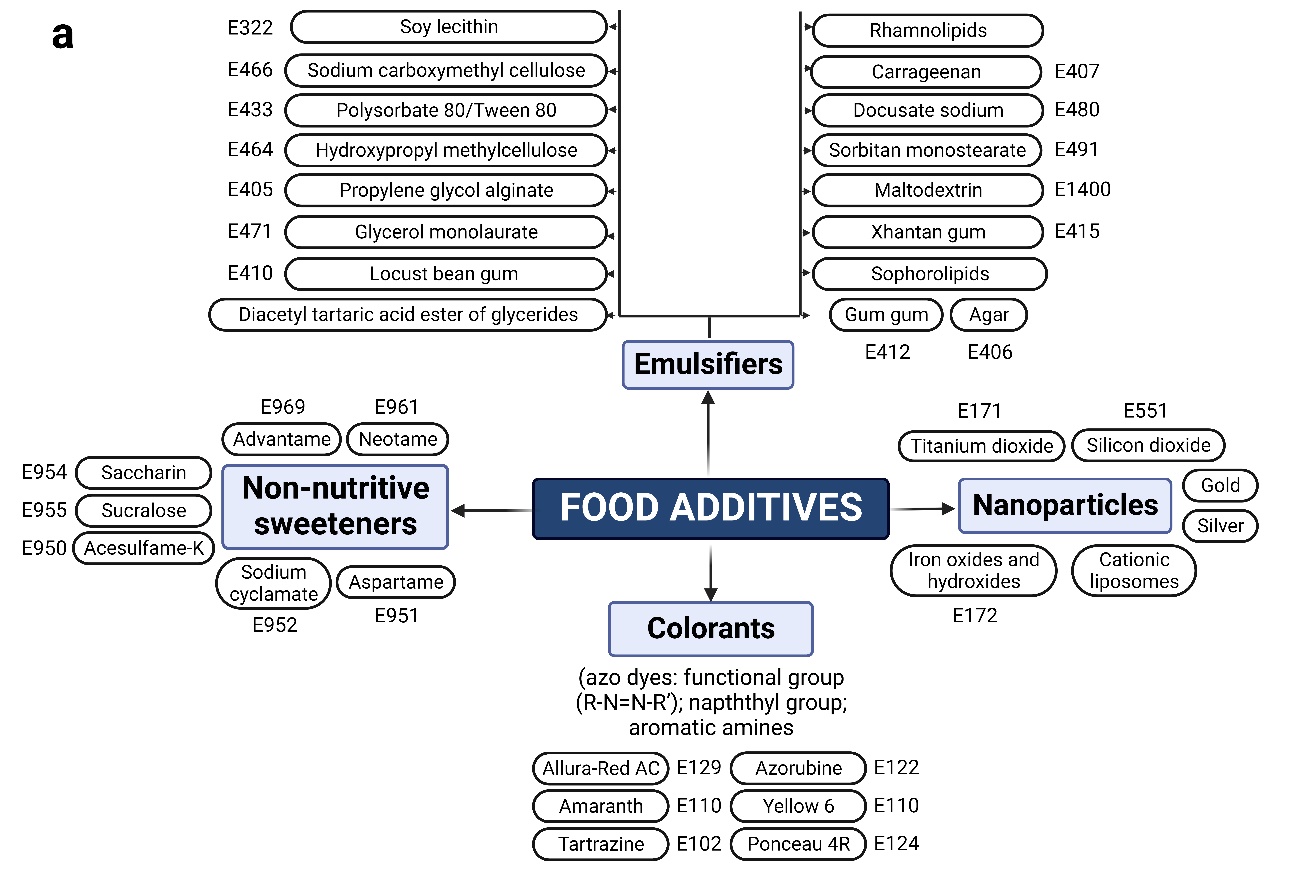
The gut microbiome can be disturbed by certain dietary xenobiotics, such as additives (**Figure 2a**) and chemicals present in food (**Figure 2b**). Here, we address the interaction of the gut microbiome with synthetic colorants, non-nutritive sweeteners, emulsifiers, and microplastics, as well as their influence on host biological functions and health. According to current dietary habits and lifestyle patterns, a high level of human exposure to these xenobiotics from infancy and through the lifespan is anticipated19. This is partly because food additives are increasingly present in modern and Westernized diets20 and microplastics are ubiquitous, constituting a global and emerging concern21.

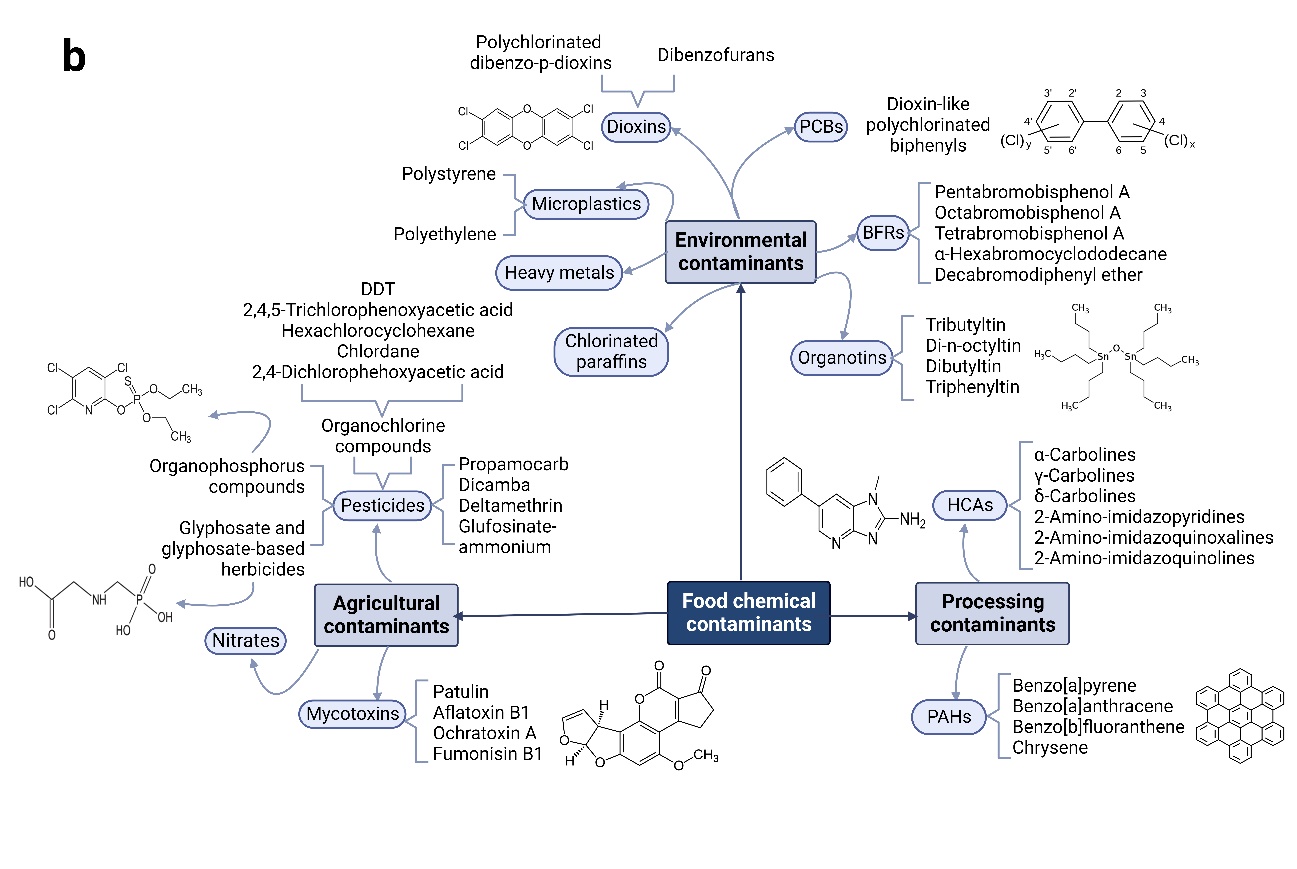
*Synthetic colorants*

Artificial food colorants or dyes are substances made from petroleum (or crude oil) that enhance the color of processed foods. They are widely used in the food and pharmaceutical industries to increase the appeal and acceptability of their products. The use of synthetic colorants in dietary products has significantly increased over the past 50 years22. These compounds can be metabolized in the gut releasing aromatic amines which are potentially carcinogenic and mutagenic23,24. The synthetic food colorant Allura

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**Figure 1. Overview of selected gut microbiome–dependent metabolic pathways of ingested xenobiotics with potential detrimental effects on different aspects of human physiology.** *The gut microbiome can modify the toxicokinetic and toxicodynamic of xenobiotics through different mechanisms by: i) direct metabolism through the reduction/increase of the compound activity (e.g., azo-dye colorants or PCBs) or re-activating inactive metabolites (e.g., HCAs) mediated by specific enzymes (e.g., azo-reductases, nitro- and nitrate-reductases, β-glucuronidases, β-glycosidases, sulfohydrolases, flavin-containing monooxygenases, β-lyases, organophosphorus hydrolases); ii) indirect influence of host metabolic and transport pathways in the liver and gut (e.g., alkaloids as trigonelline inhibiting gut microbial choline utilization and TMA-forming ability); iii) inhibition (e.g., dietary emulsifiers) or promotion of bacterial growth affecting the composition and function of the gut microbiome; iv) up-regulation of genes associated with virulence factors. CMC: carboxymethylcellulose; γBB: γ-butyrobetaine; HCAs: heterocyclic amines; PCBs: polychlorinated biphenyls; TMA: trimethylamine; TMAO: trimethylamine N-oxide.*

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**Figure 2. Reported (a) food additives and (b) chemical and/or microbial contaminants in food that could exert potentially detrimental effects on/by the human gut microbiome***. Most of collected studies have been conducted in vitro or using murine models and their findings need to be treated with caution when translating them to human due to the complexity of replicating host-microbiome interrelationship. These limitations need consideration when using data for human health risk assessments. PCBs: polychlorinated biphenyls; BFRs: brominated flame retardants; HCAs: heterocyclic amines; PAHs: polycyclic aromatic hydrocarbons.*

Red (AR) is widely used in many countries and has been shown to enhance the susceptibility of mice to colitis via different mechanisms. First, azo-reductases produced by gut commensal bacteria (*e.g.*, *Bacteroides ovatus* and *Enterococcus faecalis*) can release sulfonated aromatic amines leading to intestinal inflammation in IL-23 transgenic mice25. A similar metabolic pathway has been described for Sudan I (**Figure 1**) and Yellow 6 colorants. On the other hand, exposure to AR also enhances susceptibility to colitis via colonic secretion of serotonin, microbial alterations and disruption of the epithelial barrier function. Indeed, AR exposure only induces mild colitis in naïve germ-free mice suggesting a pivotal role of the microbiota in mediating colorant-dependent inflammation26. However, the effects of these colorants on humans need to be further investigated.

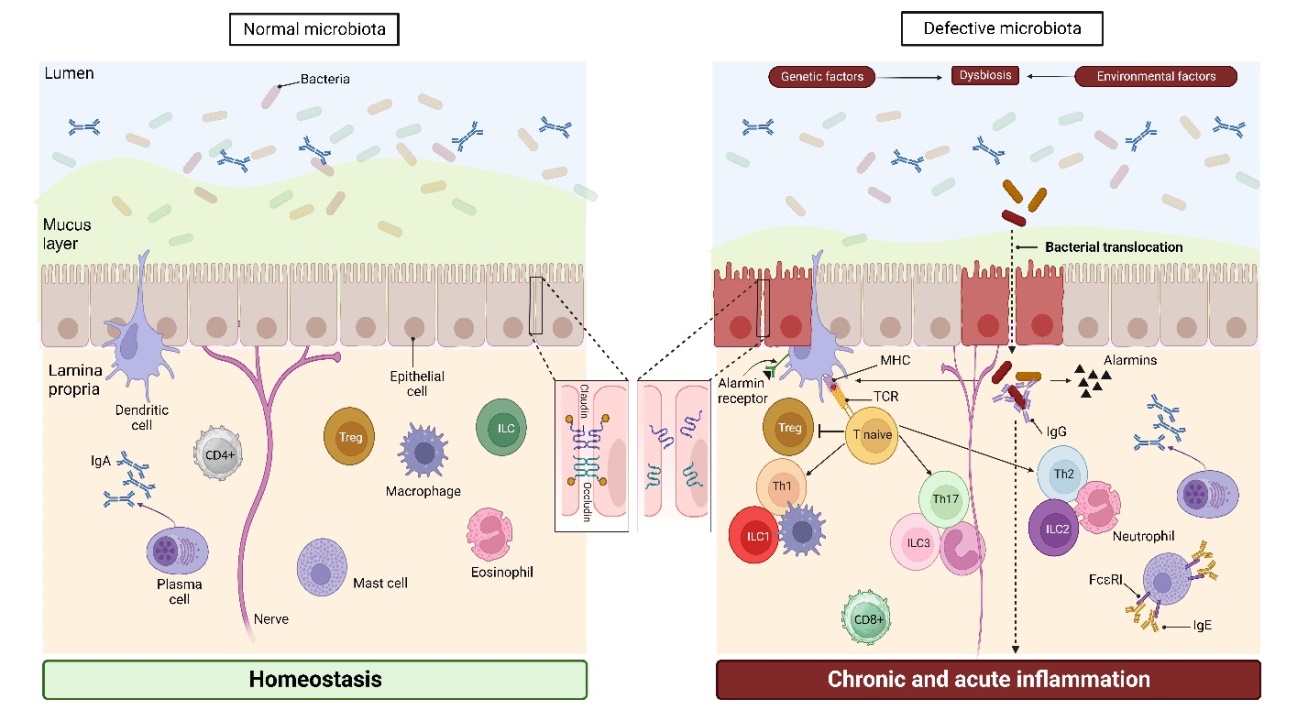
*Non-nutritive sweeteners (NNS)*

NNS are sugar substitutes that contain few to no calories but have a higher intensity of sweetness per gram. NNS have been broadly incorporated into foods such as beverages, frozen desserts, yogurts, snacks, and candies, as are thought to contribute to reducing overall caloric intake. The most common NNS are saccharin, aspartame, sucralose, and cyclamate. Although NNS have gained increasing attention to combat obesity by providing a sweet taste without the extra calories, there is controversy over the safety of consuming NNS. A study showed that consumption of commonly used NNS drives the development of glucose intolerance through induction of compositional and functional alterations to the intestinal microbiota, with saccharin being the most deleterious27. Pathways overrepresented in saccharin-consuming mice include those involved in glycan degradation. Glycans are fermented to form, among others, SCFAs. These pathways markedly enhanced energy harvest and their enrichment was previously associated with obesity, although SCFAs are also linked to beneficial immune and metabolic effects. An increase on glycosaminoglycan sulfatases and glycoside hydrolases degradation pathway gene expression was also observed in fecal samples of saccharin-consuming mice. Sulfohydrolases may catalyze the transformation of cyclamate into cyclohexylamine28, a metabolite responsible for the potential carcinogenic effect of cyclamate.

In a randomized-controlled trial (RCT) on the effects of NNS in 120 healthy individuals, saccharin and sucralose consumption significantly impacted glucose tolerance, unlike aspartame and stevia. Microbiome changes were highly correlated with the alterations observed in individuals´ glycemic responses. Thus, the glycemic responses from conventionalized gnotobiotic mice with microbiomes from multiple top and bottom responders of each of the NNS-supplemented groups were largely reflecting those of their respective human donors. These findings suggest that gut microbiome alterations in humans following consumption of certain NNS could lead to metabolic person-dependent alterations29.

*Emulsifiers*

Emulsifiers are detergent-like food additives used to mix two substances that typically separate when they are combined. Emulsifiers play a crucial role in the food and beverage industry by stabilizing mixtures of immiscible substances. A RCT showed an association between carrageenan consumption and relapse in patients with ulcerative colitis in remission. An increase in the pro-inflammatory markers IL-6 and fecal calprotectin was also associated to carrageenan intake30. It was hypothesized that this detrimental effect might be due to carrageenan interaction with the microbiome and specific immune responses, as well as to the direct activation of inflammatory pathways in the colonic epithelial cells. A recent study showed that carrageenan impacts bacteria-derived SCFAs that could be the cause of intestinal inflammation31. Carrageenan-mediated inflammation is also attributable to its unique chemical structure, which is based on a D-galactose (Gal) backbone alternating α-1,3 to β-1,4 linkages sulphated at up to 40%32. Interestingly, α-1,3-galactosidic bonds have been associated with human rejection of vascularized organ transplants from pigs mediated by Gal-specific human IgM and IgG33, 34. Moreover, the disaccharide galactose-α-1,3-galactose is responsible for the α-gal syndrome, an allergic syndrome that typically presents as a delayed hypersensitivity due to the ingestion of red meat35, 36.

****Other emulsifiers such as carboxymethylcellulose (CMC) and polysorbate (P) 80 have been shown to induce low-grade inflammation and metabolic syndrome in wild-type mice, and robust colitis in predisposed mice. Emulsifier-induced metabolic syndrome was associated with microbiota alterations, bacterial encroachment and increased pro-inflammatory response. Bacterial invasion was marked by a significant thinning of the inner mucus layer due to the accelerated breakdown of mucus by proteolytic activity, whilst increased intestinal permeability correlated with pro-inflammatory markers37 (**Figure 3**).

**Figure 3. Key molecular events underlying host-gut microbiome interplays following exposure to potentially harmful dietary xenobiotics**. *Metabolism, inflammation, immune responses, and intestinal barrier integrity are all crucially influenced by the gut microbiome. Evidence based on a limited number of research studies shows that exposure to certain dietary xenobiotics may alter gut homeostasis (left side) in different ways. These include affecting gut microbial composition, reducing the mucus layer, causing rupture of tight junctions, epithelial damage, and release of alarmins, increasing intestinal permeability, favoring translocation of commensal bacteria into the lamina propria, activation of dendritic cells and sensory neurons, etc. (right side). These processes are typical of altered host-microbiota interactions in chronic inflammatory diseases, such as IBD, and others (e.g., metabolic dysregulations, autoimmune diseases, hypersensitivity reactions) as compared to normal gut microbiota maintaining homeostasis (left side). All of them represent threads to gut homeostasis which dampen T-regulatory responses and lead to different types of innate (eosinophils, neutrophils, mast cells, innate lymphoid cells (ILC)) and adaptive inflammation (Th1, Th2, Th17) including IgA and IgG response against commensal bacterial, and ultimately to disease manifestations.*

Furthermore, a RCT revealed that individuals who consumed CMC had increased postprandial abdominal discomfort and disturbed gut microbiota composition in a way that reduced its diversity. CMC-fed subjects experienced fecal metabolome changes, such as decreases in SCFAs and free amino acids. Two CMC-consuming subjects also showed an increased microbiota translocation into inner mucus layer, a characteristic of altered host-microbiota interactions in chronic inflammation, as well as other alterations in microbiota composition38. More recently, P20 and P80 have shown dose-dependent cytotoxicity and impaired epithelial barrier function and integrity on human gut epithelial cells in Caco-2 cells and human intestinal organoids39. Biological processes including inflammation and oxidative stress, among others, were upregulated at the cellular level in response to both emulsifiers, thus creating a local environment prone to allergic sensitization or other inflammatory diseases40.

*Microplastics as emerging contaminants in food*

Xenobiotics, such as pesticides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls or dioxins (**Figure 2b**) are formed in industrial processes or used in plant and animal farming to boost production, reduce food waste and ensure an adequate food supply. Some of these compounds, such as dioxins, activate the Aryl hydrocarbon receptor (AhR) and Pregnane X receptor, both of which are key on intestinal homeostasis and xenobiotic/infection clearance. AhR activation leads to release of antimicrobial peptides in the intestine that potentially modify microbial composition41. This receptor has been linked to chronic intestinal inflammation42, 43. Overall, chemical contaminants in food are known to persist in the environment (air, soil, and water)44 and their bioaccumulation in the agri-food chain could represent a health risk leading to alterations in the human gut microbiome composition and function. Although studies with pesticide exposure report some degree of microbial disturbances, there are important limitations that should be considered when interpreting the results of these studies and using their data for human health risk assessments45.

Microplastics and other plastic-associated contaminants are recognized as emerging and ubiquitous contaminants with unknown health implications46, 47. Microplastics are widely distributed due to the overuse of plastics nowadays, easily ingested with our diet and may accumulate in various organs due to their small sizes (< 5 mm diameter) and low degradation rate. Despite the lack of current legislation to regulate microplastics and nanoplastics as food contaminants, the oral route represents an important exposure pathway in humans48, 49. Indeed, they have been detected in human feces, colon, and placenta. Based on the metadata analysis of 59 publications, Senathirajah et al.50 estimated the amount of microplastics that humans may ingest, which can serve as a basis for future investigations and risk assessment. On average, humans could be ingesting 0.1–5 g of microplastics weekly. Moreover, a qualitative analysis identified over 10,000 chemicals in plastics51. The exposure to microplastics is especially relevant for infants where different intake sources have been identified, including baby feeding bottles52, breastmilk storage bags53, baby teats54, or breastmilk itself55. A provocative study has shown a higher concentration of fecal microplastics in inflammatory bowel disease (IBD) patients than healthy individuals. Intriguingly, the concentration of microplastics positively correlated with the disease severity, suggesting these particles as potential triggers of IBD pathology. Authors also reported that patients with a higher abundance of fecal microplastic consumed more plastic-packaged products56. Based on *in vitro* and *in vivo* studies, microplastics could alter microbiome composition and function, barrier dysfunction, as well as induce immune responses57, 58 (**Figure 3**). However, further evidence is needed to elucidate whether microbial alterations are the cause or consequence of either microplastic consumption or host’s response to the particles59. Moreover, microplastics could also act as carriers by transporting contaminants (*e.g.*, heavy metals, phthalate esters), antibiotics, or pathogens, which could directly or indirectly affect the gut microbiota and contribute to the accumulation of hazardous substances, change exposure pathways, and share detrimental mechanisms60-62. Consequently, the co-exposure of microplastics together with other chemicals could generate additional risks that need to be further investigated.

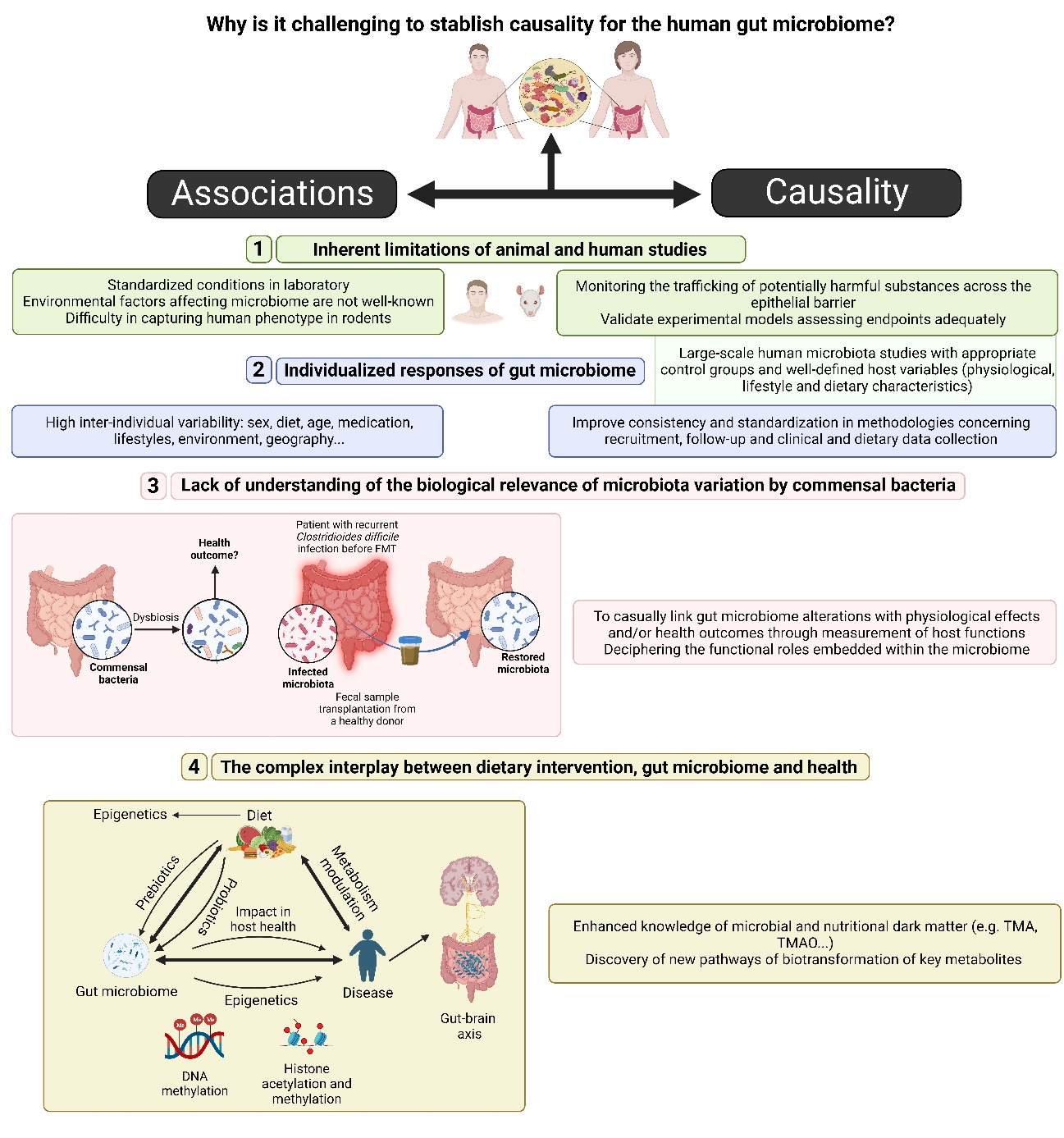
**The challenge of establishing the causal relationship of the gut microbiome with human health**

Discriminating causality from association in human gut microbiome represents one of the most significant and long-standing challenges in the microbiome field. Approaches to understand causation such as Koch’s postulates and Bradford Hill’s criteria have been traditionally considered. However, they are more suitable for the “one-pathogen” paradigm than for the human gastrointestinal tract, which harbors one of the most complex, interrelated, and abundant microbial ecosystems63. Hence, it is important to advance from the simplistic view of “one-compound, one-bacteria, one-disease” as it is not plausible that one specific food or component causes complex multi-factorial disorders by modifying individual bacterial species or strains. An example is the proposed mechanism linking the relationship between diet and IBD pathogenesis where various food components including fiber, red meat, fat, and food additives (*e.g.*, emulsifiers) interact with our microbiome to either strengthen or weaken intestinal barrier function64. Thus, gaining a better understanding of the multi-causal nature of diseases and their etiology is needed to identify the most effective and convenient targets for preventive interventions.

A number of relevant factors preventing the elucidation of causality in human gut microbiome research and corresponding actions to address this challenge are discussed below and summarized in **Figure 4**.

*The inherent limitations of animal and human studies in gut microbiome research*

Although experiments in rodent models for studying diet-microbiota interactions are very informative due to the capacity of controlling many variables (genetics, diet, microbiota), the translation of findings from animal models to humans needs to be carefully considered because of the difficulty in capturing human genetics, microbiome and phenotypic diversity65. Laboratory-animal studies are typically conducted with genetically homogeneous, in-bred animals maintained in standardized conditions, thus overlooking environmental and host factors that affect variability in human gut microbiome66.

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**Figure 4. Main factors preventing the elucidation of causality in gut microbiome research studies and actions for their improvement.** *The causal role for the effects of dietary compounds mediated by the human gut microbiome is yet to be established as many of the outcomes of in vitro, in vivo and/or pre-clinical studies fail to recapitulate what is found in clinical studies. A shift from associations (left side) to causal relationships (right side) between a disrupted gut microbiome and adverse health effects in the host following interactions with specific dietary stressors is needed.*

Observational studies are often used to establish associations between human exposure and health outcomes. Although epidemiological observations inform about real life events in humans, they have many limitations, such as the lack of objective ways to assess dietary compliance (also impacting in RCTs) and the difficulty of implementing a true control group67, 68. Most studies typically compare healthy individuals with patients, failing to account for the impact of shared environments and diets on the microbiota. This aspect could be better understood by including analysis of household controls and/or siblings69. In addition, there are many confounding factors mostly related to diet variations (*e.g.*, poor patient adherence to dietary regimes and large diversity of ingested food ingredients, diet recording approach, dietary changes influencing other host metabolism pathways in a microbiota-independent way) that preclude establishing a direct causal role between the intake of a specific dietary compound and a given health effect70. Vujkovic-Cvijin et al.71 listed a series of host variables (physiological, lifestyle, and dietary characteristics) that should be controlled in human microbiota studies to match comparison groups. Considering these variables would increase robustness and reproducibility of microbiome signatures associated with human disease. Further translational studies (*e.g.*, fecal microbiota transplantation (FMT) and interventions) are required, however, to ultimately prove causality.

Lastly, available animal and human model data sets are often small and normally use different assay methods which can be a limitation when trying to compare changes, particularly in risk assessment.

*Responses of the gut microbiome to diet are highly individual*

The high inter-individual variability of gut microbiota and its evolving capacity hamper efforts in identifying a universally “normal” or “healthy” gut microbiota. The composition and function of the human microbiome is unique in each individual and the responses toward specific food components may vary depending on the microbiome of each person. A variety of factors, including diet, age, sex, medications, lifestyle, environment, geography, or ethnicity, shape the human gut microbiome. The intra-host adaptation of the gut microbiota is thought to be shaped by age, immunity, and dietary habits in healthy individuals72, determining the individual human gut microbiota’s ability to metabolize specific dietary components.

For example, the biotransformation pathway L-carnitine→γ-butyrobetaine→TMA/TMAO mediated by the human gut microbiota (**Figure 1**) is induced by omnivorous dietary patterns and chronic L-carnitine exposure, whilst vegans/vegetarians produce lower TMAO levels than omnivores73. Similarly, the production of uremic *p*-cresol sulphate, which is generated by the gut bacterial metabolism of tyrosine (**Figure 1**), was also lower in vegetarians than in individuals consuming an unrestricted diet74. The ability of certain animals, including humans, to convert cyclamate into cyclohexylamine (**Figure 1**) appears to depend upon a continuous intake of cyclamate75. Several pathways involved in heterocyclic compound metabolism were enriched in saccharin-fed mice, suggesting that exposure to this NNS can be associated with an increase of saccharin-catabolizing bacteria76. A similar finding was observed following consumption of the food additive xanthan gum, a complex polysaccharide used as a stabilizer and thickening agent. Authors showed the ability to digest xanthan gum is common in human gut microbiomes from industrialized countries, revealing that the abrupt introduction of a new dietary component may drive changes in human microbiome with potential impacts on human health77.

Therefore, the notion of a “health” or “unhealthy” gut microbiota appears to be context dependent. Determining an optimum combination of gut microbes and microbiome features considered universally healthy is not feasible in the short/medium term78.

*The biological relevance of gut microbiome variation triggered by commensal bacteria is unclear*

A direct relationship between specific human gastrointestinal microbes and disease conditions has been firmly established only for a few diseases including peptic ulceration and gastric cancer linked to *Helicobacter pylori*, and antibiotic-associated diarrhea linked to *Clostridium difficile*65. This is likely because both are single invasive pathogens of the stomach (*H. pylori*) and intestine (*C. difficile*), whose colonization and pathogenic mechanisms are well-understood79, 80. Indeed, FMT is an effective therapy for reversing microbial dysbiosis and treating recurrent or refractory *C. difficile* infection (CDI)81; also, an orally administered microbiota-based therapeutic product for preventing the recurrence of CDI in adults has been recently approved82. Yet, the utility of FMT beyond the treatment of CDI is still limited and controversial83, and further knowledge of the underlying ecological dynamics is required to understand its potential for other applications. FMT has been less successful in restoring the disrupted gut microbial ecosystem in other diseases which etiologies involve networks of bacterial species and metabolites and/or in diseases where the microbiome is not so severely disrupted (*e.g.*, Crohn’s disease, metabolic syndrome) as in infections. Recent studies suggest, for example, that other factors such as the similarity between donor and baseline recipient microbiota, as well as the sex and age, could be determinants for successful FMT trials in the amelioration of metabolic syndrome markers84. The United States-Food and Drug Administration (US FDA) has developed a policy to help facilitate access to FMT for patients with CDI not responding to standard therapies85. In this context, promising advances based on the use of engineered native bacteria (*e.g., Escherichia coli*) as chassis to functionally manipulate the gut microbiome of conventionally raised mice have been recently developed86. Likewise, the relevance of the gut-lung axis through their respective microbiomes is increasing in respiratory allergy disorders87. A significant association between probiotics supplementation and reduced risk of respiratory allergy could not be found during a meta-analysis of data obtained from the randomized controlled trials of probiotics usefulness for the protection against asthma incidences in infants88. In addition, one must be very cautious for such treatments in preterm infants, as use of such probiotics might predispose highly vulnerable population, more susceptible to infection89.

Unlike above examples, most gut microbiome studies have established associations between changes in commensal microorganisms and different pathologies. These changes, however, are frequently described by vaguely used terms like “dysbiosis”, “imbalance of microbiota” or “reduced microbial diversity”, and do not always consider the causal links to physiological and/or health outcomes. Furthermore, the assumption that a decreased gut microbial diversity is generally associated with an unhealthy state has been challenged by several studies. For instance, a greater bacterial richness was found in feces from colorectal cancer patients compared to healthy individuals, partially due to the presence of oral cavity-associated species which are rarely found in the healthy gut90.

Since the mechanisms by which “dysbiosis” could cause disease are still under investigation and the significance of most microbiota fluctuations to disease remains speculative, the term dysbiosis alone does not indicate whether it is cause or consequence91, 92. Indeed, it is necessary to differentiate between harmless and harmful microbiome fluctuations understood by those that are dysfunctional, breaking the host-microbe symbiosis and adversely impacting human health. The term dysbiosis is also biased as it refers to deviations/differences from a “healthy baseline” which is not even close to being defined as previously mentioned. Therefore, the concept of dysbiosis has limited value in risk assessment unless supported by physiological and/or clinical outcomes and molecular mechanisms. The diversity and type of functions encoded by the microbiome may be more relevant than the alterations in the composition to define resilient or dysfunctional microbiome93. Consequently, the analysis of the metagenome and their products could provide more insights into the biological role of the microbiome than the current taxonomic analysis often performed94. However, unraveling functional roles encoded by the microbiome is much more expensive and complex than performing a taxonomic profiling95.

*The complex interplay between dietary intervention, gut microbiome, and health*

Associations between gut microbes, gene microbial paths and functions and the intake of specific nutrients, foods, and dietary patterns have been made by large-scale and high-quality metagenomic studies in well-phenotyped individuals96-98. Even though, the studies have been mainly focused on prokaryotic microorganisms so far and results are inherently biased towards the most abundant ones99. Furthermore, other microbes inhabiting the gut such as eukaryotic fungi and viruses have been overlooked until very recently95.

Moreover, the bulk of our current understanding of how diet affects health is limited to ~150 key nutritional components, representing only a minute subset of the total pool of distinct and definable biomolecules present in foods, estimated to be ~27,000. This incomplete biochemical profiling of food components, defined as “nutritional dark matter”100, adds uncertainty to the health implications of our diet and its interaction with the gut microbiome on human health.

Therefore, the generation of further knowledge on the relationship between the gut microbiome and its interactions with the dietary and food composition should help to unravel their role in human health. This could lead to the elucidation of new microbiota-derived metabolites and bio-transformation pathways with biological impacts. Likewise, in addition to the analysis of collective genes of microbes (metagenomics), their expression (metatranscriptomics) and possible epigenetic modifications (*e.g.*, DNA methylation or histones modification) could play a key role in the pathogenesis of gastrointestinal101-103 and metabolic diseases104. Interestingly, diet can influence the host epigenetic modifications through gut microbiome-derived metabolites105, 106.

**Concluding remarks and future perspectives**

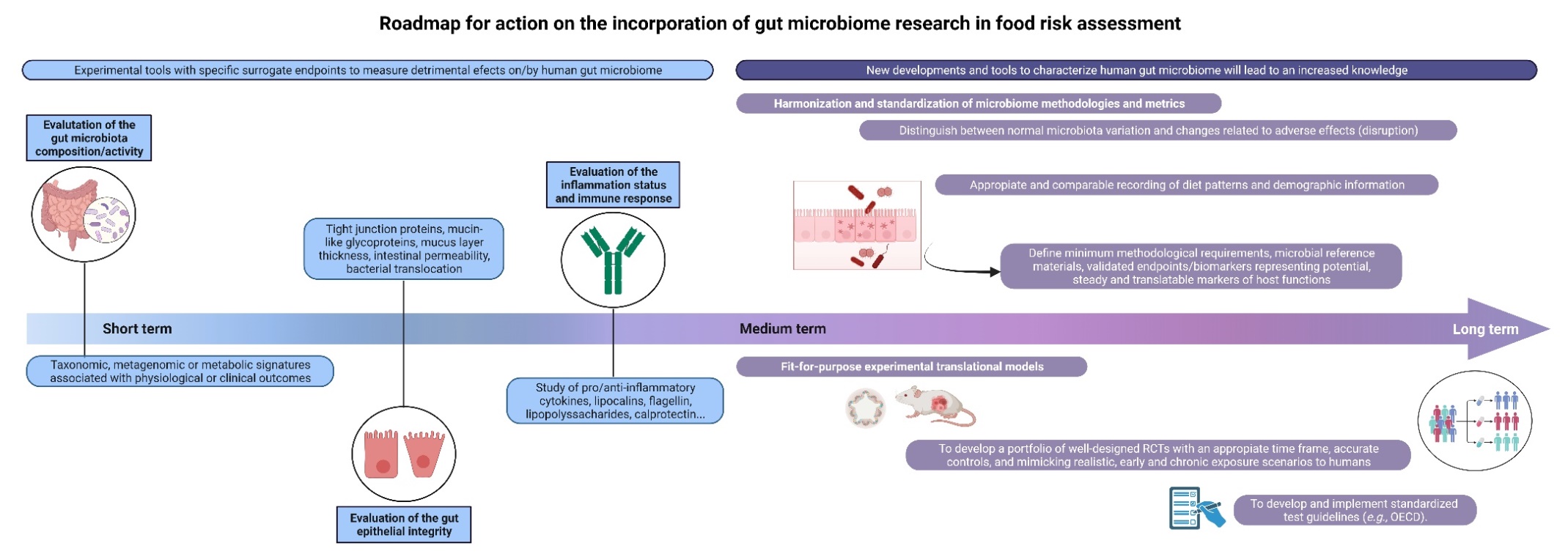
The detrimental impact of some dietary xenobiotics, such as food additives and chemical contaminants on human health, is partly mediated by the gut microbiome and its ability to be modified by and respond to these xenobiotics. In addition, the variation in the microbiome across individuals may explain why people show differential responses to these compounds. However, these potentially harmful xenobiotics are clinically under-studied and most of the collected research has been conducted either in *in vitro, ex vivo*, or rodent models. This scenario, together with the multiple factors involved in gut microbiome dynamics (high inter-individual variability, diet, ethnicity, *etc*.) and characterization (lack of consistency and standardization in microbiome analysis, sampling, or recruitment), often hampers the elucidation of how dietary modulators and the gut microbiome could interact leading to adverse health outcomes (**Figure 4**).

The safety risk assessment of specific food components of concern could need to integrate the effects on or mediated by the gut microbiome in the near future. These studies together with the well-established toxicity and mutagenic trials, could help to fully understand potential health hazards. A roadmap is needed to incorporate microbiome science in food risk assessment (**Figure 5**). This roadmap should encompass a prioritization strategy for dietary compounds that are increasingly present in modern diets and that have been identified as potentially harmful to the human gut microbiome and health. A series of experimental tools with specific microbiome-related health endpoints to measure gut microbiome perturbations could be incorporated as part of safety studies to inform risk assessment in the short/medium term (**Figure 5**). These studies, whose outcome might further support the safety assessment of certain food contaminants and/or additives, could include the evaluation of the:

* Gut microbiota composition and metabolism when causally linked with physiological and/or clinical outcomes and supported by molecular mechanisms.
* Gut epithelial integrity and permeability.
* Inflammation status and immune response.

There is also the need for the development of new approaches and tools to structurally and functionally characterize the human gut microbiome, providing enhanced knowledge to guide future research into the relationship between dietary components and the microbiome in risk assessment (**Figure 5**). We suggest focusing on the following key elements:

* Accelerate harmonization and standardization of microbiome methodology and metrics to obtain high-quality comparable evidence in future research studies.
* Assemble appropriate and consistent recording of diet patterns and demographic information.
* Define the type and levels of functional microbiota modulation that are biologically relevant.
* Identify indicators demonstrating the disruption of the gut microbiome (*i.e.*, minimum methodological requirements, microbial reference materials, and validated endpoints/biomarkers/metabolites that could represent potential, steady, and translatable markers of the host functions).
* Identify relevant translational research methods to connect *in vitro* and *in vivo* outcomes to the human context.
* Develop a portfolio of well-designed RCTs with appropriate time frames and time-series data, accurate controls, and mimicking realistic, early, and chronic exposure scenarios to humans.
* Develop and implement standardized test guidelines (*e.g.*, such as those of the OECD).



**Figure 5. Starting proposed roadmap for the future incorporation of gut microbiome research in food safety risk assessment.** *This roadmap provides recommendations for developing a harmonized approach to use gut microbiome research studies in regulatory decision-making process. Firstly, a battery of validated experimental tools, assays and endpoints could be incorporated in the short/medium term if international consensus is achieved. Secondly, there is a need for new developments and tools to address the identified gaps in the medium/long term. If these actions are successfully accomplished, the assembly and appraisal of the gathered information will be useful for the future development of standardized guidelines for the risk assessment of some specific stressors of the human gut microbiome*.

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**Disclaimer statement**

The conclusions, findings, and opinions expressed in this scientific paper reflect only the view of the authors and are not the official position of EFSA, US FDA, FSANZ or Health Canada.

**Ethics declarations - Competing interests**

The authors declare no competing interests.

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