



eBook

# GUT MICROBIOTA: A DISREGARDED SUBJECT IN THE TOXICOLOGICAL RISK ASSESSMENT

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## INTRODUCTION

The human gut houses  $10^{14}$  microorganisms that play a crucial role in physiological functions such as immunomodulation, metabolic functions, hemodynamics, electrophysiology, mental status, mood, behavior, production of neuroactive molecules, and brain development [1]. Microbiota refers to the community of microorganisms present in species such as humans. The microbiome is the collective genomes of all the microorganisms present in a species [2].

The alterations in the functional metabolites generated from gut microbiota play an essential role in the pathogenesis of various human ailments; for example, lipopolysaccharides (LPS) and peptidoglycan are the metabolites generated from gut microbiota that act as pro-inflammatory agents [3]. The short-chain fatty acids (SCFA) and bile acids modulate the secretion of glucagon-like peptide (GLP-1), which is linked to insulin secretion and glucose metabolism [4]. The tryptophan metabolites, such as indole-3-acetic acid and indole-3-propionic acid, are linked to immunity and irritable bowel syndrome (IBS) [5]. The bacterial metabolite trimethylamine N-oxide (TMAO) formed from choline and L-carnitine directly correlates with incidences of cardiovascular diseases [6]. The gut bacterial fermentation products of protein, such as polyamines and N-nitroso compounds, are known to exert carcinogenic potential [7]. In addition, gut microbiome-metabolized products are considered essential sources of fat-soluble vitamins (thiamine, riboflavin, vitamin B6, and vitamin B12, among others) and micronutrients (vitamin A, vitamin D, iron, and zinc, among others). They are known to be pivotal in the central nervous system by acting as neurotransmitters or precursors [8]. Thus, the gut microbiota has a profound role in the health and disease of animals and humans. Any alterations in the gut microbiota can lead to systemic alterations in different pathologies, as highlighted in a few examples above. However, so far, there is no provision or consideration for evaluating the impact of xenobiotics, such as pharmaceuticals, on the normal gut microbiota and associated health complications, both in the pre-clinical and clinical phases of development. With this background, this article focuses on leveraging gut microbiota's impact on health and disease and its considerations in the toxicological risk assessment of various xenobiotics intended for human use, such as pharmaceuticals.

## IMPORTANCE OF MICROBIOTA IN HEALTH

The microbiome is coevolved in humans and healthy individuals. The microbiome is said to be in the symbiosis or eubiosis state, in which the microbiome is intact with its functions, such as digestion and absorption of food and xenobiotics, eliciting the immune response, protecting the body from external microorganisms, and producing the essential metabolites required for normal functioning of the body [9]. Any alterations in the normal microbiota are called dysbiosis, which is scientifically well-established for being linked with various disease states such as inflammatory bowel diseases (IBS) [10, 5], neuropsychiatric disorders [11, 12], cancers [13, 14], cardio-metabolic disorders [15, 16, 17], liver diseases [18, 19] and so on.

Furthermore, an experimental study was performed using young (3 months), old (18 months), and aged male C57BL/6J mice (24 months) to explore the role of gut microbiota on the gut-brain axis, gut-retinal axis, and associated inflammatory pathways. Initially, fecal pellets were collected from all the animals, and the inherent gut microbiota associated with the animals were depleted



by giving a cocktail of broad-spectrum antibiotics orally for three days (100  $\mu$ L each of vancomycin 5 mg/mL, metronidazole, 10 mg/mL, ampicillin 1000mg/L, neomycin 500 mg/L). The fecal pellets collected from these mice (young, old, and aged) before antibiotic treatment (Baseline or Pre-Abx) were used to prepare the fecal slurry, which is used for the fecal microbial transfer (FMT). The fecal samples collected at baseline (pre-Abx), post-anti-biotic (post-Abx) treatment, and post-FMT were subjected to metabolomic analysis [20].

The results revealed that post-Abx, the aged animals developed retinal and CNS inflammation through alterations in the gut microbiota and associated detrimental effects on the gut-brain axis and gut-retinal axis; surprisingly, these pathological changes noticed in the aged animals were reversed upon doing FMT from the young animals. On the other hand, upon doing FMT from aged animals, the normal healthy young animals developed CNS and retinal inflammation through alterations in the gut microbiota [20]. Thus, this experiment demonstrated that age-related changes in the gut microbiota have a pivotal role in the pathogenesis of CNS and retinal inflammation.

## GUT MICROBIOME-MEDIATED TOXICITY

The toxicity associated with gut microbiome or chemical-gut microbial interactions is broadly classified into two types 1) *toxicant modulation of the microbiome (TMM)* and 2) *microbiome modulation of the toxicant (MMT)*. The changes in the microbiota after exposure to the test substance are called TMM. At the same time, the biotransformation of the exposed chemical, either more or less toxic, in the presence of microbial enzymes or microbial metabolites, is termed MMT. In MMT, different groups of bacterial metabolizing enzymes have been discovered, such as  $\beta$ -lyases, sulfatases,  $\beta$ -glucuronidases, nitro-reductases, and azo-reductases. They have unique roles in the metabolic modifications of test chemicals.

The evidence suggests that gut microbiota metabolizes the xenobiotics more extensively and effectively than any other organ system in the body. Hence, microbiota-mediated enzymatic modifications of the test chemical are considered the first mechanism by which toxicity of a xenobiotic is induced either more or less. The other one is bioavailability. The gut microbiome has a significant role in the absorption of xenobiotics and, therefore, is considered a deciding factor in the induction of toxicity in the host [21].

One of the classic examples of microbiome-modulated toxicity is cyclamate-induced carcinogenicity. Cyclamate is one of the widely used artificial sweeteners in Europe. However, it was banned in the UK and US due to the carcinogenic potential of one of its metabolites named, cyclohexamine. In an *ex vivo* experiment, various tissues were pre-incubated with cyclamate to see the conversion; interestingly, the conversion of cyclamate to cyclohexamine was evident only in the lower gut contents, and the conversion was negligible in other tissues, including the liver, kidney, spleen, and blood. In support of this, an *in vivo* study conducted on rats using radio-labeled cyclamate revealed similar results. In another experiment, rats were administered cyclamate in drinking water for several months, and the rats became converters; however, the conversion ability was lost when antibiotics were co-administered along with cyclamate [21, 23,

24]. Thus, all the available evidence strongly suggests that gut microbiota has a significant role in the formation of cyclohexamine from cyclamate. Hence, the gut microbiota has a pivotal role in cyclamate's carcinogenicity.



**Table 1. Examples of gut-microbiome-mediated toxicity through TMM and MMT mechanisms.**

Examples of gut microbiome-mediated toxicity (MMT) in experimental animals			
Xenobiotic	Microbiome-induced alteration	Organism/ Test system	Effects
Chloramphenicol	Amine formation	<i>Enterobacteria</i>	Bone marrow aplasia [27]
Balsalazide	Azoreduction → mesalamine	<i>Pseudomonas aeruginosa</i>	Anorexia, nausea, and skin rash [28, 29]
Risperidone	Ring cleavage → 9-hydroxy-risperidone	Rats and dogs	Symptoms of Parkinson's disease [30]
Pyrene	Hydroxylation → 1-hydroxypyrene	Human gut microbiota	Endocrine disruption [31]
Benzo(a)pyrene	Hydroxylation → 7-hydroxybenzo(a)pyrene	Human gut microbiota	Endocrine disruption [31]
Nitrazepam	Nitroreduction → 7-aminonitrazepam	Sprague-Dawley rats	Teratogenic [32]
Examples of the toxicant-induced altered microbiome (TMM) in experimental animals			
Xenobiotic	Toxicant-induced microbiome alteration or dysbiosis	Test system	Effects
Glyphosate	General gut flora depletion	Mice	Anxiety and depression [26]
Ethanol (Oxidative metabolite)	↓ Bacteroides, Bifidobacterium and Ruminococcus. ↑ Streptococcus and other minor species.	Humans	Rectal cancer [33, 34]
Arsenic	↑ Bacteroidetes and ↓ Firmicutes	IL-10 <sup>-/-</sup> mice	Dysbiosis of the Gut [35]

In this case, the parent chemical was not carcinogenic; however, the gut microbiota has generated a toxic metabolite from the parent chemical; it is an example of microbiome-modulated toxicity.

Similarly, melamine is used in infant formulas and pet food. In 2008, melamine grabbed a lot of attention from the media and the public due to its toxic effects, such as mortality and nephrotoxicity in animals and children. The nephrotoxicity was due to one of the toxic metabolites, 'cyanuric acid', formed due to the activity of gut microbiota. Interestingly, these toxic effects observed in rats were reversed upon antibiotic administration. Thus, it was confirmed that the reported toxicity was due to the toxic metabolite formed from melamine rather than melamine alone. Particularly, *Klebsiella terrigena* has significantly increased the toxic metabolite formation from melamine. The renal toxicity of melamine is mediated by the GI microbiota [25].

On the other hand, the TMM is yet another mechanism in which perturbation of the gut microbiome by some toxicant exposure may significantly impact normal health. As highlighted earlier, alterations in normal gut flora have been linked to various human ailments. For example, exposure to valproic acid, antibiotics, arsenic, lead, cadmium, nickel, and silver nanoparticles has been scientifically proven to cause alterations in the gut microbiome.



One of the typical examples of TMM is glyphosate-induced toxicity. Glyphosate is one of the most widely used herbicides for agricultural purposes. It is known to act by inhibiting the shikimic acid pathway in herbs and is assumed to have extremely low toxicity in animals since this pathway is absent in animals. However, in experimental studies, glyphosate has caused depression and anxiety in the experimental animals; mechanism-based studies revealed that gut microbiota has a shikimic acid pathway, and glyphosate has caused depression and anxiety in animals by causing dysbiosis of the gut microbiota. Thus, toxicant-induced altered gut microflora significantly impacts health [26].

Examples of gut microbiome-mediated toxicity through TMM and MMT mechanisms are summarized in **Table 1**.

### STRATEGIES FOR CONSIDERATION OF MICROBIOTA IN TOXICOLOGICAL RISK ASSESSMENTS

It is crucial to evaluate and understand what the drug/test substance does to the gut microbial community and what gut microbiota does to the drug/test substance. There are step-by-step approaches for evaluating gut microbiota-associated toxicities.

The following approaches can be used to evaluate microbiome-correlated toxicities [29]:

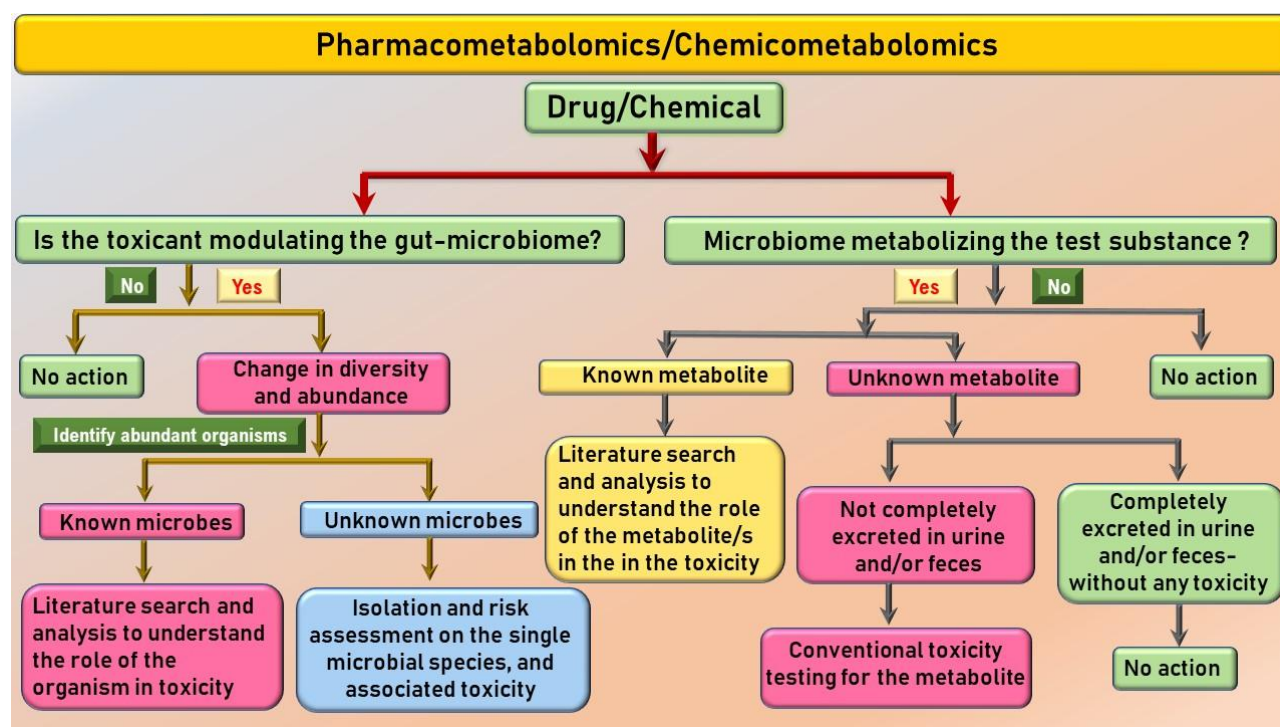
1. *In silico* analysis/predictions: This can be performed using databases such as the Mechanism of Action of the Human Microbiome (MAHMI), Microbiome Database (MDB), Human Microbiome Project (HMB), Association Between Microbiota and Disease (Amadis), Disbiome, microbiota—active substance interactions (MASI) and so on.
2. *In vitro* cell cultures: The microbial cells isolated from various sites were cultured under diverse conditions to mimic the gut microbiome. This test system was used for both MMT and TMM studies.
3. Pre-clinical testing: This is the most conventional method, which considers all the possible interactions of the host-microbiome. In this test, the substance-mediated toxicities via different mechanisms involving the microbiome will be evaluated in experimental animals.
4. Gnotobiotic animal models: The germ-free animals were infected with the organism of interest and subjected to testing.
5. Human studies: This involves exposing healthy human volunteers to the test substance and evaluating the associated health risks mediated through MMT and TMM mechanisms.

Pertaining to the *in vitro* and *in vivo* experimental approaches, the fecal samples must be collected and assessed for abundance and variability in the microbiota to understand the xenobiotic-induced alterations in gut microbiota and associated effects. Any observed alteration should be scientifically correlated for possible adverse reactions or diseases. For example, overexpression of *Fusobacterium nucleatum* bacteria was reported to cause colon tumorigenesis



in rodents, and decreased abundance of *Akkermansia muciniphila* was reported in obesity and diabetes, and the latter was reversed upon supplementing *A. muciniphila*.

Conversely, pharmacometabolomics/chemometabolomics is the branch of metabolomics that deals with quantifying and analyzing metabolites formed from drugs/chemicals, respectively; this helps identify the metabolites, map the metabolic pathways, and explore their possible role in the drug/chemical-induced toxicities. If the metabolites formed are well-known, the toxicity profile can be assessed from the available literature on the metabolites, while in the case of unknown metabolites, a conventional toxicological risk assessment must be performed for the new metabolite/s identified [36]. The Pharmacometabolomics/Chemicometabolomics approach has been illustrated in **Figure 1**.



**Figure 1. Pharmacometabolomics/Chemicometabolomics approach**

### CONSIDERATIONS OF MICROBIOTA DURING THE CONDUCT OF TOXICOLOGICAL STUDIES

As highlighted above, gut microbiota may represent substantial and incomprehensible effects in the *in vivo* toxicological assessments. The gut microbiota is one of the possible mechanisms postulated for the significant variations observed in the animal experimental outcomes, such as individual-to-individual and/or study-to-study variation [37]. However, despite being a significant factor in xenobiotics-mediated toxicity or being a target for xenobiotic-induced toxicity, the gut microbiota has not been considered while conducting toxicity studies, as none of the currently recommended guidelines or international standards mandates the consideration of gut microbiota-related effects in the design and conduct of pre-clinical studies and as a part of risk assessment.



The following approaches can be adopted before and during the pre-clinical toxicological experiments to fill the evidential gap and compensate for the possible variabilities that may arise from

- 1]. As much as possible, procure the animals with standardized microbiota. In case it is unavailable, obtain information on the origin of the species, such as litter, breeding, and barrier, which must be considered during the randomization of animals.
- 2]. Choose the animal model/species with the utmost bacterial diversity comparable to wild animals.
- 3]. Routine evaluation of gut microbiota, including the diversity and composition of animals receiving the xenobiotic treatments during the toxicity studies (before, during, and at termination)
- 4]. Evaluation of the impact of the xenobiotics on the gut microbiota and vice versa gives better clarity to correlate the role of gut microbiota in the toxicity of the xenobiotics on the *in vivo* systems

## SUMMARY

Based on the available scientific evidence, it can be concluded that gut microbiota has a significant role in xenobiotic-induced toxicity. However, as of now, the gut microbiota is not included as a part of toxicity testing either in any guideline or as a requirement in any regulatory authorities. In the absence of any guidelines and recommendations, the role of gut microbiota has been overlooked or disregarded.

The inclusion of gut microbiota in toxicological assessments of pharmaceuticals is the need of the hour. Gut microbiota has a significant role in the quality of life; hence, it must be included in the toxicological assessments. Further, gut microbiota-related assessments should be included as a part of regulatory requirements to determine/rule out any possibilities of test substance-induced perturbances of the normal gut microbiome.

**Literature Search Strategy:** A detailed literature search was performed using the search terms “Gut microbiota” in combination with ‘dysbiosis’, ‘toxicology,’ ‘disorders’, ‘mechanism’, ‘toxicity testing’, ‘toxicology risk assessment’, ‘strategies for toxicity testing,’ and so on. in the online databases such as PubMed/Medline, ScienceDirect, and Google Scholar. All the articles obtained from the above databases were initially screened with the “abstract” or “full-length copy,” and the papers fitting into the criteria were considered. Further, wherever additional data was required, the cross-references in the papers and reviews were searched.

At ClinChoice, we have a team of experienced and dedicated toxicologists who are expertise in the literature search, risk and hazard identification, and preparation of toxicological profiles, including cosmetics, pharmaceuticals, chemicals, and medical devices. Also, we have a team of



experts working on preparing and reviewing non-clinical electronic common technical documents (eCTD) for botanicals, chemicals, and pharmaceuticals for submission to regulatory approvals.



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