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Physiologically based kinetic (PBK) modeling as a new approach methodology (NAM) for predicting systemic levels of gut microbial metabolites

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ABSTRACT

There is a clear need to develop new approach methodologies (NAMs) that combine *in vitro* and *in silico* testing to reduce and replace animal use in chemical risk assessment. Physiologically based kinetic (PBK) models are gaining popularity as NAMs in toxico/pharmacokinetics, but their coverage of complex metabolic pathways occurring in the gut are incomplete. Chemical modification of xenobiotics by the gut microbiome plays a critical role in the host response, for example, by prolonging exposure to harmful metabolites, but there is not a comprehensive approach to quantify this impact on human health. There are examples of PBK models that have implemented gut microbial biotransformation of xenobiotics with the gut as a dedicated metabolic compartment. However, the integration of microbial metabolism and parameterization of PBK models is not standardized and has only been applied to a few chemical transformations. A challenge in this area is the measurement of microbial metabolic kinetics, for which different fermentation approaches are used. Without a standardized method to measure gut microbial metabolism *ex vivo/in vitro*, the kinetic constants obtained will lead to conflicting conclusions drawn from model predictions. Nevertheless, there are specific cases where PBK models accurately predict systemic concentrations of gut microbial metabolites, offering potential solutions to the challenges outlined above. This review focuses on models that integrate gut microbial bioconversions and use *ex vivo/in vitro* methods to quantify metabolic constants that accurately represent *in vivo* conditions.

1. Introduction

The human gut microbiome is increasingly recognized as an important site of xenobiotic metabolism with the potential to influence chemical bioavailability, bioactivity, and toxicity through the activation or inactivation of xenobiotics from diverse sources (Koppel et al., 2017a; Collins and Patterson, 2020). Prominent examples include the formation of potent xenoestrogens like α-zearalenone from the mycotoxin zearalenone (Gratz et al., 2017; Mendez-Catala et al., 2020), from the soy isoflavone daidzein (Kobayashi et al., 2013), 8-prenylnaringenin from the hop polyphenol isoxanthohumol (Possemiers et al., 2006, 2005), the production of beneficial urolithins from pomegranate ellagitannins (Tomás-Barberán et al., 2017), and the transformation and degradation of pharmaceuticals like Levodopa, Digoxin, and Irinotecan (Maini Rekdal et al., 2019). While much research has focused on identifying biotransformation products and the enzymes and microbes responsible, little is known about their impact on toxico-/pharmacokinetics. These aspects are influenced by the rate of gastrointestinal absorption of the parent compound, which is determined by its physicochemical parameters and may limit substrate availability to intestinal bacteria, as well as by the rate of biotransformation, which is mostly dependent on the microbiome composition related enzymatic activity and thus subject to large inter-individual variations. Therefore, a comprehensive understanding of these aspects is required to accurately predict the impact of gut microbiome-chemical interactions on host health. Despite their potential importance, microbiome-regulated reactions are not yet considered in chemical risk and safety assessment. The latter currently relies heavily on animal testing, especially on rodents. However, animal studies are resource-intensive, raise ethical concerns, and the translation of experimental results to humans is limited due to inter-species differences. Consequently, there are international efforts to shift from this approach towards the use of New Approach Methodologies (NAMs)

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(Krewski et al., 2020), which are defined as any method or combination of techniques that provide information for chemical risk assessment while minimizing or entirely eliminating the need for animal use (Stucki et al., 2022; Wambaugh JCB et al., 2019). In the context of research on gut microbiome-chemical interactions, NAMs can provide important information to better understand the role of the gut microbiome in chemical toxicity and the consequences of these interactions for human health. Physiologically based kinetic (PBK) models are computational tools used to simulate chemical absorption, distribution, metabolism, and excretion (ADME) based on *in silico* and *in vitro* data (Zhuang and Lu, 2016), allowing prediction of substance and metabolite levels in, e.g., blood, urine, or specific tissues of interest. By describing and predicting the toxicokinetics of substances and their metabolites without animal testing, these NAMs are key to facilitating quantitative *in vitro* to *in vivo* extrapolation (QIVIVE). The latter allows the extrapolation of *in vitro* toxicodynamic data to *in vivo* data that can be used for (human-relevant) risk assessment. Classically, PBK models focus mainly on hepatic and intestinal tissue metabolism while ignoring the metabolic potential of the gut microbiome, which is a methodological gap (Zhuang and Lu, 2016). Metabolic kinetic constants of microbially driven reactions can be determined experimentally *ex vivo/in vitro*, as for other metabolic organs. Different methods for performing these experiments are currently in use without much standardization and evaluation. The relationship between gut microbial metabolism of orally ingested substances and human physiology has several key facets. Specifically, microbial metabolites may have different properties than the parent compound, potentially causing toxicity in the host (Zimmermann et al., 2019a). To date, numerous xenobiotics are known to undergo chemical biotransformation by the gut microbiome, resulting in metabolites with toxicological properties that are potentially distinct from those of the parent compounds. These cases often became apparent when trying to understand the toxicological properties of the parent substances qualitatively or quantitatively. For example, the case of melamine revealed a missing link between its ingestion and the formation of melamine-cyanurate kidney stones, a relationship that could not be explained by co-exposure to cyanuric acid (Zheng et al., 2013). Another notable example is the unexpected deaths of 18 patients treated with sorivudine and 5-fluorouracil, where the unanticipated formation and activity of the gut microbial metabolite bromovinyluracil inhibited dihydropyrimidine dehydrogenase and thus interfered with the metabolism of 5-fluorouracil (Okuda et al., 1998). Also, the microbiome can reactivate toxic xenobiotics that have already been detoxified (via conjugation) by hepatic metabolism and are excreted via the bile (Collins and Patterson, 2020). Through this pathway, the gut microbiome is known to influence not only orally administered xenobiotics, but also intravenously administered pharmaceuticals (Wallace et al., 2010). To date, many xenobiotics are known to be chemically modified by the gut microbiome, and the metabolites often have different toxicological properties than the parent compound (Koppel et al., 2017b).

It is apparent that the gut microbiome can strongly influence the effects of orally ingested dietary xenobiotics and drugs, indicating the need to include gut microbial metabolism in PBK models. Microbiome-competent PBK models represent a specialized approach tailored to chemicals that exhibit significant interactions with the gut microbiome, in which their metabolic fate and toxicological profile are altered. Selection criteria for the use of this methodology are based on observed (qualitative or quantitative) differences in metabolite formation between the host and the microbiota, underscoring the need for assessment. In the case of a de novo, animal-free risk assessment, the formation of metabolites by the intestinal microbiome should be screened for as a first tier to decide whether or not further research into this direction is needed. Ultimately, when performing a safety or risk assessment of chemicals without the use of animal experimentation, chemicals should be screened for gut microbial metabolism. In the case of the formation of intestinal microbial metabolites, their (differential) toxicity should be assessed, and quantitative *in vitro* kinetic data should be generated and integrated into the respective PBK model. When evaluating the interaction between chemicals and gut microbiome, particularly compounds that are food-born and have oral exposure routes are important. Given the current gaps in understanding of structural determinants of such interactions, a screening approach is recommended. Initially, this could be qualitative, determining whether metabolites are formed. Subsequent quantitative assessments could measure the extent of transformation. In the case of metabolite formation, their (differential) toxicity should be assessed. If biotransformation products show different toxicity profiles than the parent compound, further detailed assessments may be appropriate. Taken together, we suggest a screening strategy that allows for the collection of sufficient data to later refine assessments based on chemical structure, thereby optimizing the balance between thoroughness and practicality in chemical safety assessments. Although many compounds are known to be metabolized by the gut microbiome with significant consequences for the host, the extent to which the microbiome contributes to the metabolism and toxicity of the vast majority of compounds remains unclear. In addition, the metabolic activities of the gut microbiome may represent a missing link in PBK models that do not incorporate microbial interactions and, as a result, do not accurately predict kinetic behaviors observed *in vivo*. In cases where host and microbial metabolism perform the same chemical transformation, it is important to evaluate the effect of microbial transformation independent of host metabolism to determine their respective contributions to overall metabolism. Microbiome-competent PBK models serve as predictive tools estimating the kinetics of metabolite formation and aid in the safety assessment of the compounds by predicting systemic concentrations of both parent compounds and gut microbial metabolites. Additionally, they can serve as an approach to provide additional data to incorporate the assessment of gut microbial metabolism into chemical risk assessment. Integrating gut microbial metabolism into chemical risk assessment marks a critical shift towards more comprehensive NAM-based safety assessments. However, the implementation of microbial metabolites in risk assessment is limited due to a lack of established methods to quantitatively assess bacterial biotransformation *in vivo*, the complexity of the host-microbiome interactions, and large microbial composition differences, both within and across species. The development and evaluation of corresponding methods that are applicable to chemical risk assessment is an emerging field with ongoing research across various projects and laboratories (e.g., PARC) (Marx-Stoelting et al., 2023). This mini-review aims to provide an overview of the state of the art and to highlight the potential of microbiome-competent PBK models to stimulate further investigation. Furthermore, by highlighting these interactions, it aims to encourage further research to better understand the impact of the microbiome on chemical safety. In this mini-review, we discuss predictions of established models, approaches used to implement gut microbial metabolism in PBK models, and the experimental challenges in obtaining metabolic kinetic constants (Fig. 1).

2. An overview of microbiome-competent PBK models, their *in silico* methodology, and the challenges of using PBK models in chemical risk assessment

Extending PBK models to account for the contribution of gut microbial metabolism is necessary to confidently predict bioactive compounds of interest that are consumed orally and may be metabolized by the gut microbiome or undergo biliary excretion and/or enterohepatic circulation (EHC). Most existing microbiome-competent PBK models use *ex vivo/in vitro* fecal fermentation methods in which stool samples of donors are incubated with compounds of interest to obtain the required kinetic parameters. The methods used to measure and incorporate these kinetic parameters into PBK models are discussed in detail below. Three important factors to account for microbial metabolism in PBK models are: first, determining the rate of chemical transformation by gut bacteria; second, scaling the reaction rate parameter to accurately represent

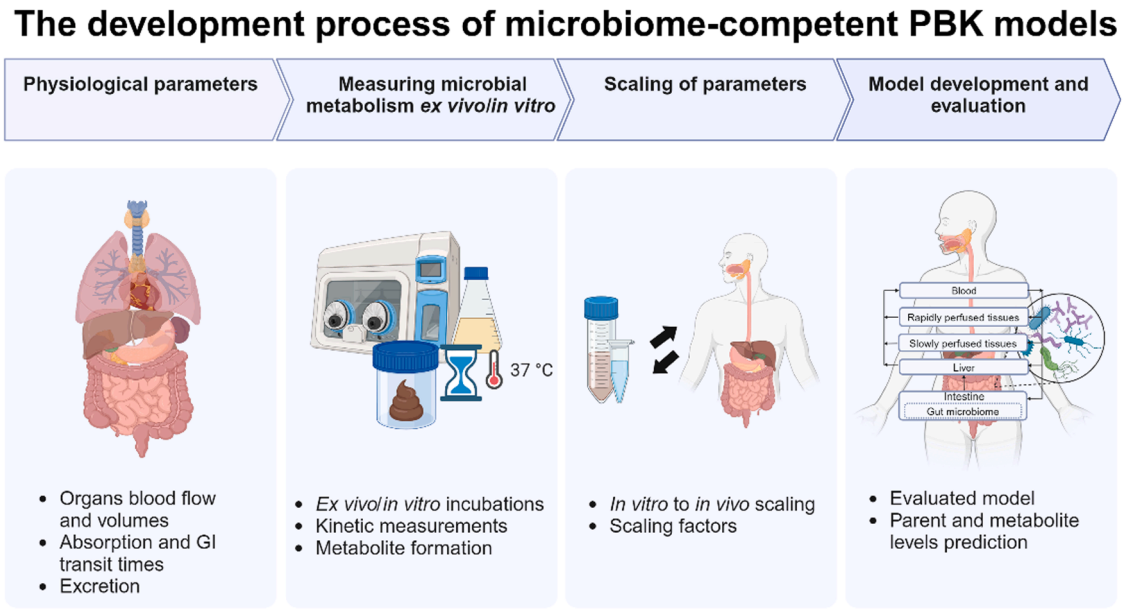

Fig. 1. A methodology for constructing and validating a physiologically based model that includes quantification of physiological parameters, ex vivo/in vitro metabolic profiling, parameter scaling, and predictive evaluation of pharmacokinetic behavior.

the gut environment; third, accounting for biliary excretion and enterohepatic circulation of modeled chemicals. All currently published PBK models that have included the gut microbiome as a metabolic compartment (Zimmermann et al., 2019b; Wang et al., 2020, 2022; Liu et al., 2022; Kostantini et al., 2022; Mendez-Catala et al., 2021) have been evaluated and summarized in Table 1. Various search terms such as, PBK modeling (and derivations thereof, such as PBPK modeling, kinetic modeling) and gut microbial metabolism (and derivations thereof, such as intestinal microbial metabolism) or gut microbiota (and derivations thereof, such as intestinal microbiome, gut microbiome)'' were used to find relevant studies. The search yielded a total of 12 studies, of which 3 were no primary research articles and were excluded. The remaining publications were studied, and all papers describing a PBK model with the gut microbiome as a metabolic compartment were included. All included studies predicted systemic concentrations of both the parent compound and gut microbial metabolites formed after ingestion of the parent compound.

2.1. Microbiome-competent PBK models for drug and dietary compound metabolism

Using data from germ-free and colonized mice, Zimmermann et al. (2019b) established a PBK model in the presence and absence of gut microbiome for brivudine and its hepatotoxic metabolite bromovinyluracil (BVU). By building twin models based on physiological and chemical parameters obtained from germ-free and conventional mice, the authors predict host and microbial contributions to the formation of BVU and show that most of the predicted serum concentrations are attributable to microbial metabolism. Although it was already known that the microbiome converts brivudine to BVU, the added value of their models was to quantify the amounts of BVU produced by the gut microbiome. In addition, their model was also parametrized for two additional drugs to distinguish host and microbial metabolism. While this approach can be used to (better) quantify data obtained from animal experimentation retrospectively, it should be noted that current efforts

Table 1
An overview of methods to obtain quantitative kinetics for gut microbial metabolism for use in existing microbiome-competent PBK models.

Study	Subjects/Samples	Storage conditions	Media	*Ex vivo/in vitro* incubation conditions
Separating host and microbiome contributions to drug pharmacokinetics and toxicity (Zimmermann et al., 2019b)	Germ-free & conventional mice	/	/	No *ex vivo/in vitro* incubations
Use of physiologically based kinetic modeling to predict rat gut microbial metabolism of the isoflavone daidzein to S-equol and its consequences for ERα activation (Wang et al., 2020)	20 female & 20 male rat fecal samples	Dissolved in 10% (v/v) glycerol in PBS and diluted to 20% w/v in PBS, stored at −80 °C	PBS	6% of fecal slurry for 35 min of anaerobic incubations (solvent and negative control included)
Use of physiologically based pharmacokinetic modeling to predict human gut microbial conversion of daidzein to S-equol (Wang et al., 2022)	15 human fecal samples	Diluted 1:5 (w/v) in 10% glycerol PBS, filtered using filter tubes, and stored at −80 °C	PBS	Pretest to distinguish S-equol producers and nonproducers 60 mg/mL for 1 h anaerobic incubations (solvent and negative control included)
Use of physiologically based kinetic modeling-based reverse dosimetry to predict *in vivo* Nrf2 activation by EGCG and its colonic metabolites in humans (Liu et al., 2022)	9 female & 5 male fecal samples	Dissolved in 10% glycerol in PBS for a final storage concentration of 20% (w/v) fecal samples	PBS	15-, 30-, and 50-min incubations to derive the microbial metabolic constants 20 mg/mL feces (solvent and negative control included)
Usefulness of optimized human fecal material in simulating the bacterial degradation of sulindac and sulfinpyrazone in the lower intestine (Kostantini et al., 2022)	3 female & 3 male fecal samples	Diluted 1:3.8 (w/v) with saline diluted to 25% w/v and sieved through a 350 μm metal mesh, stored at −70 °C	Saline	Incubated for 2 h for the bacteria to be regenerated, and samples were pooled final 8.3% of the fecal sample in saline.
PBK model-based prediction of intestinal microbial and host metabolism of zearalenone and consequences for its estrogenicity (Mendez-Catala et al., 2021)	Rat & human fecal samples	Dissolved in 10% glycerol in PBS to obtain a final concentration of 20% (w/v) fecal slurry, stored at −80 °C	PBS	5% fecal slurry in PBS incubated for 5 h

are aimed to predict these results entirely from *ex vivo/in vitro* experiments. In addition, the absence of a microbiome in germ-free mice influences other metabolic pathways compared to colonized mice and may lead to inaccurate conclusions if the microbiome is assumed to be the only variable different between conventional and germ-free mice.

Other studies have used an alternative method to determine gut microbial metabolism, i.e., using fecal samples to conduct fermentation experiments. Mendez-Catala et al. (2020) first established an anaerobic fecal fermentation model to quantify kinetic constants for gut microbial metabolism, and the results were used for interspecies comparisons. Using pig, rat, and human fecal samples, the authors investigated microbial metabolism of the mycotoxin zearalenone. The models showed species differences in the host metabolism and gut microbiota metabolism of zearalenone to α-zearalenol and β-zearalenol, respectively, highlighting the importance of establishing species-specific models. Using the *ex vivo/in vitro* fermentation method of Mendez-Catala et al. (2020) with rat fecal samples, Wang et al. (2020) parameterized a PBK model to predict the toxicokinetics of the soy isoflavone daidzein and its microbial activation product S-equol. The metabolism of daidzein to S-equol was investigated using anaerobic incubations of rat fecal samples, and the host metabolism of these substances was evaluated using rat hepatic and intestinal S9 fractions. Model estimates were consistent with available toxicokinetic data for both daidzein and S-equol in rats. In addition, the C_{max} of unconjugated daidzein and S-equol were consistent with the plasma concentrations measured *in vivo* after oral administration of daidzein to rats. The results indicate that the microbial metabolism of daidzein has only a minor effect on the total systemic concentration of daidzein. The inclusion of gut microbial metabolism was required for the successful prediction of the plasma concentration of S-equol, demonstrating the importance of including gut microbial metabolism in the PBK model and highlighting the importance of including gut microbial metabolism in chemical risk assessment. As far as chemical safety assessment is concerned, this is currently not taken into account due to a lack of data and validated test methods. Therefore, it is a goal to develop reliable tools to fill this gap, which we want to help with this mini-review. Subsequently, the rat model was translated into a human PBK model by adapting the physiological characteristics of the model and measuring the metabolism using human-derived fecal samples and pooled fecal slurry from different individuals (Wang et al., 2022). The model was evaluated by comparing daidzein levels to the C_{max} levels of free daidzein measured *in vivo*, whereas the evaluation of S-equol was based on the reported *in vivo* urinary excretion of S-equol. The anaerobic incubations of the fecal samples revealed that there are producers and non-producers of S-equol within the donor population, as would be expected based on human intervention studies. This approach is advantageous because it can account for the inter-individual differences in S-equol production observed in humans (Setchell and Cole, 2006). Circulating concentrations of the parent compound daidzein were predicted to be slightly lower in S-equol producers, indicating a moderate impact of microbial metabolism on isoflavone bioavailability. However, of the 15 participants in the study, only 6 were S-equol producers, making the sample size relatively small to draw population-wide conclusions. Importantly, an interspecies comparison revealed that the rate of S-equol formation is hundreds of times higher in rats than in humans, emphasizing the importance of species-specific experiments. The distinction between producers and non-producers in this study is useful, and similar approaches could be used to investigate, for example, sex differences in microbial metabolism. Given the differences in gut microbial composition, considering sex separately could contribute to a more comprehensive assessment of microbial kinetic parameters (Kim et al., 2020). A recent study by Liu et al. (2022) developed a PBK model for the (predominantly) tea polyphenol epigallocatechin gallate and its colonic metabolites, gallic acid, and pyrogallol. Pooled human fecal samples from 5 males and 9 females were used to assess chemical transformation rates. The model predicted that systemic metabolite levels would be sufficient to activate the Nrf2 signaling pathway, which is associated with the reported health benefits of green tea. The microbial metabolites were found to be more potent activators of Nrf2 than the parent compound, highlighting the importance of microbial metabolism in the dietary response. The present studies demonstrate the importance of microbiome-competent PBK models and their various purposes, including the comparison of metabolic processes across different species and modeling microbial transformations that are either the same or different for the host and microbes.

2.2. Quantitative scaling of metabolic kinetic constants

An essential step in using reaction rates from *ex vivo/in vitro* fecal fermentation is scaling to *in vivo* conditions to achieve accurate predictions. For a microbiome-competent PBK model, the physiology of the species of interest must be adequately described. One way to scale results from *ex vivo/in vitro* microbial fermentation assays is to normalize biotransformation rates to fecal mass, expressed as a certain fraction of the body weight. However, according to the literature, the exact proportion of gut microbial mass in a human varies by nearly 10-fold, causing scaling factors to vary substantially. For example, Wang et al. (2022) used a scaling factor of 0.0140 kg/kg BW, while Liu et al. (2022) and Mendez-Catala et al. (2021) used a fecal fraction of 0.0018 kg/kg BW. The former was based on the relative weight of small and large intestine contents, as reported by Brown et al (1997). In contrast, the latter was based on a median wet fecal mass of 128 g/day in 116 healthy individuals (Rose et al., 2015). However, the study reported a wide range of fecal mass from 51 to 796 g/day. Additionally, a dry fecal mass of 29 g/day was reported in the study. To eliminate variation due to fecal water content, the use of dry mass for scaling *ex vivo/in vitro* incubations may be a useful approach. In addition to fecal weight, reactions can be scaled relative to the total (qPCR-quantified) bacterial load in each individual fecal sample. Van Dongen et al. (2022) investigated the scaling of microbial degradation reactions of fructoselysine and carboxymethyllysine relative to both fecal weight and amount of bacterial cells and showed that both scaling methods yield comparable results. Quantification by qPCR estimates the total number of bacteria per host, but there is a large variation in bacterial load per human, leading to substantial variation and uncertainty. Furthermore, species-specific reactions cannot be accounted for by total bacteria values, introducing another layer of error. Additionally, the human gut microbiome consists of 10–100 trillion microbial cells (Turnbaugh et al., 2007), primarily bacteria, making it difficult to accurately scale for modeling based on bacterial counts. Another factor to consider when scaling is intestinal passage time. Substantial interindividual differences exist, ranging from 10 to 73 hours of whole gut transit time (Lee et al., 2014). This has implications for intestinal uptake of substances, but also contact times with the microbiome, potentially leading to differences in the amounts of metabolites formed.

2.3. The complexity of modeling the gastrointestinal tract

Another difference between microbiome-competent models published to date is the conceptual modeling of the gastrointestinal tract. Depending on the substance of interest, microbial conversion may occur in the small intestine, colon, or both (Koppel et al., 2017b). The contents of these two organs differ in total volume, physiological properties (e.g., pH, oxygen content), metabolite levels (Folz et al., 2023), bacterial density, and microbial composition (Christian, 2014). Some dietary chemicals, such as choline and hydrolyzed proteins, are absorbed in the small intestine, resulting in only low amounts reaching the colon, while other chemicals are released only after reaching the colon, for example, intracellular plant metabolites being released after microbial enzymes break down cell walls (Folz et al., 2023). Nevertheless, most PBK models implement microbial metabolism based on the colon because it contains most of the bacteria of the gut microbiome (Turnbaugh et al., 2007). The inclusion of a separate small intestine in PBK models would be favorable,

considering the potentially higher concentrations of parent chemicals in the small intestine (in the case of substances that are taken up in the small intestine). It is assumed that small intestinal microbes are ultimately also transferred to the large intestine and the feces; if these microbes are still active and present, the feces may retain some representative characteristics. From a practical point of view, there are examples of the small intestine and colon being applied as separate compartments in PBK models and even a segmentation of the small and large intestine (Mendez-Catala et al., 2021). However, accurate parameters for regions of the small intestine for both intestinal absorption and microbial activity are scarce due to the physical inaccessibility of these sites *in vivo*.

2.4. The role of enterohepatic circulation in gut microbial metabolism

Another variable to consider when modeling microbially modified chemical kinetics is EHC. The process of EHC includes chemicals being conjugated by the liver, secreted into the intestine via bile, deconjugated by microbial enzymes, and subsequently reabsorbed via the intestinal walls. This creates a cyclic flow (Malik et al., 2016) that increases the residence time of the affected chemicals in the organism, but also increases the concentration in the intestines available to microbes (Klaassen and Cui, 2015; Folz et al., 2021). Thus, chemicals that undergo EHC are more likely to be significantly affected by gut microbial biotransformation. EHC is included in some of the PBK models, as recently reviewed by Talevi *et al.* (2021). Briefly, the authors describe two main ways of implementation: models in which the biliary excretion into the gut is either simplified to a continuous function or time delays between biliary excretion and reabsorption are assumed (Talevi and Bellera, 2021). More recently, a model was published in which EHC was included as a fraction of the glucuronide entering the small intestine via bile. The EHC value was set in the range from 0 to 1 and was adjusted by 0.002 increments until the predicted C_{max} value was matched to *in vivo* concentrations of the parent compound. Furthermore, to evaluate the EHC and model predictions, the results were compared to an additional human intervention study (Aichinger et al., 2023). However, difficulties arise when adding both gut microbial metabolism and EHC to PBK models as multiple organs are involved, and parameterization becomes complex. This has led to the use of simplified models, e.g., models for zearalenone and daidzein, which have only considered microbial metabolism, although both are known to be affected by EHC (Wu et al., 2021; Fujitani et al., 2019). Expanding PBK models to include both gut microbial metabolism and EHC requires further investigation and optimization.

2.5. Novel approaches to microbial metabolism research

A NAM with growing interest is zebrafish models, which offer a new, promising way to assess toxicity (Becker et al., 2022). Zebrafish provide an alternative to animal testing, as zebrafish embryos are considered an unprotected life stage up to five days post-fertilization under EU Directive 2010/63/EU (Christian Lawrence et al., 2009). The zebrafish microbiome can perform xenobiotic reduction, hydrolysis, deglucuronidation, nucleophilic substitution, and lyase reactions, making it a good candidate for xenobiotic transformation studies. Advantages over rodent models include that axenic zebrafish are easy to grow and can be easily colonized by immersing them in water containing the desired microbes (Catron et al., 2019). However, this approach is unlikely to work for very strict anaerobes found in the human gut due to the presence of oxygen. Recently, it was shown that zebrafish microbiome plays a role in xenobiotic metabolism by exposing zebrafish larvae to the antimicrobial agent triclosan and supports the hypothesis that zebrafish microbiome can be used to study gut microbial metabolism (Weitekamp et al., 2019). Additionally, members of the bacterial communities of zebrafish models are relatively easy to culture *in vitro*, suggesting that they may be useful for *ex vivo/in vitro* microbial bioconversion studies. A classic PBK zebrafish model was first established in 2014 by Péry *et al.* to study adult zebrafish exposed to non-ionic organic chemicals in water. Their model is a good basis for zebrafish PBK modeling and provides highly relevant physiological information for zebrafish. Recently, a PBK model of perfluorooctanoic acid for zebrafish was established, providing mechanistic insights into zebrafish model development. In addition, the importance of determining PBK parameters for zebrafish has been highlighted (Khazaee and Ng, 2018). Parameters related to zebrafish physiology, particularly gut microbial metabolism, require further development and refinement. The applicability of zebrafish models to human chemical hazard assessment remains limited to date, and ongoing research is exploring the role of the gut microbiome in interspecies extrapolation challenges.

2.6. Application of PBK models in risk assessment

Identifying the challenges and knowledge gaps in PBK models is critical for their recognition in chemical risk assessment and regulatory applications. To address this knowledge gap, a survey of the modeling community by Paini et al. (2017) assessed the frequency of use and application of PBK models in regulatory assessments. According to the survey results, of the participants with experience in submitting PBK models for regulatory risk assessment, only 39% had experienced challenges in gaining regulatory acceptance. The main challenges include a lack of regulatory expertise in PBK modeling, difficulties in validating models due to limited scenario testing, a lack of user-friendly software for non-programmers, and differences in acceptance criteria between agencies and countries. Good modeling practice (GMP) has been described as a way to increase their acceptance. Briefly, GMP involves defining a simplified representation of human physiology in a conceptual model, translating it into differential equations, assigning parameters from peer-reviewed literature or standardized experiments, and evaluating and refining the model (Paini et al., 2017). In addition, careful evaluation of the selected parameters, the model code, and the validation of the results are required for regulatory acceptance of the models. While PBK models are becoming more widely accepted in risk assessment as regulatory guidelines emerge, health authorities remain cautious about using them to support regulatory decisions on chemical safety. Recently, a guidance document has been published that serves as a starting point for the use of PBK models in regulatory applications (OECD., 2021). The guidance document emphasizes the importance of standardizing the development of PBK models and encourages gaining confidence in their use for chemical risk assessment. Extending the PBK models to microbiome-competent PBK models holds great promise for risk assessment and allows for effective toxicokinetic predictions. Gut microbiome research is gaining increasing attention and is expected to play a relevant role in regulatory science with potential implications for risk assessment (Merten et al., 2020).

3. Quantitatively assessing gut microbial metabolism for PBK modeling

Accurately modeling the human gut microbiome *in vitro* and *in silico* is challenging due to the complexity of the bacterial communities present, the interaction of microbes with human cells *in vivo*, and the spatial variation along the length of the human intestinal tract, among other factors. One approach to measuring microbial transformation rates is to use isolated bacterial strains with chemicals of interest and attempt to scale the result to human conditions. An important obstacle in this approach is that not all gut microbes can be cultured *in vitro*. Furthermore, multiple bacteria are often involved in the metabolism of individual substances, and all relevant bacteria would need to be identified, co-cultured in some cases, and tested. In addition, scaling such data is complex due to often unknown microbial densities in the intestine and differences in microbial metabolism when coupled with a complex microbial community or presented with intestinal conditions, such as

nutrient availability, the presence of bile acids, or other (dietary) xenobiotics. However, this approach also holds promise for reconstructing complex microbial communities, for example, to represent the otherwise inaccessible small intestinal communities. Other options for generating and maintaining (small) intestinal communities are continuous cultures originally inoculated with fecal samples (Molly et al., 1994; Venema, 2015), which are relatively expensive and time consuming, in contrast to small batch incubations, and therefore less suitable for measuring kinetic rates suitable for PBK modeling. Current approaches to culture the complex human gut microbiome *ex vivo/in vitro* include the use of anaerobic conditions and culture media that mimic the gut environment and preserve the composition of the microbial community. However, the lack of well-established experimental approaches potentially leads to results biased by unbalanced, unrepresentative gut microbial composition and likely a correspondingly altered microbial activity. Therefore, we review different *ex vivo/in vitro* approaches to model the gut microbiome and how the results may affect metabolic constants derived for PBK modeling.

3.1. Microbiome sample collection, preparation, and preservation

Sample preparation and storage prior to metabolic analysis are critical to investigating microbial activity. For use in quantitative models, cryopreserved samples are often used instead of fresh feces as an alternative to overcome time constraints and to increase the number of tests that can be performed simultaneously on the same samples. For instance, Wang et al.(2020 & 2022) and Mendez-Catala et al. (2021) stored rat and human fecal samples at 20% w/v in anaerobic 10% v/v glycerol in PBS at −80 °C before performing fecal fermentations. Glycerol is used as a cryoprotectant to preserve the bacteria and protect them from ice crystals that would otherwise form during freezing (Perez-Burillo et al., 2021a). However, after thawing, glycerol may serve as a nutrient for some microbes and cause medium enrichment, which may favor the growth of certain bacterial species and cause a shift in microbial composition (Acha et al., 2005). To partially overcome this, samples are usually diluted before incubation and can be washed. The viability, composition, and activity of microbial species can be affected by both sample storage and the media used (Aguirre et al., 2015). In the fecal incubations cited above (Mendez-Catala et al., 2020, 2021), the authors reported having tested the effect of freezing on the metabolic reactions studied and did not observe any effect on the respective reactions. It should be noted that the incubations in the above studies were set up to be as short as possible (preferably less than 1 hour; if longer times were required, then linearity of the reaction rate over time was a prerequisite) to avoid microbial adaptation as much as possible. Recent work by Perez-Burillo et al. (2021b) found that the use of frozen samples can result in a different microbial community than when fresh samples are used but that the functionality of the communities is largely maintained. However, they only studied fermentation processes, and bacteria were given six days in nutrient media to recover from the freezing process. In the few published microbiome-competent PBK models, fecal samples have only been incubated with the compounds of interest for up to 24 hours without a prior adaptation period for the bacterial communities. In summary, fresh fecal samples provide the most accurate representation of gut microbial communities (Isenring et al., 2023) but freezing fecal slurries with a cryopreservative is also expected to provide biologically relevant results. Future chemical-specific studies are needed to determine to which extent freezing affects microbial activity rates. Additionally, the media used to culture microbial communities impacts the metabolic activity and community structure. Fecal slurries prepared in PBS represent a nutrient-poor minimal medium that can be used to follow the microbial metabolism of given compounds over a short period of time with limited bacterial growth and adaptation; this is considered to more closely mimic conditions in distal parts of the colon. However, the use of nutrient-rich media, such as those rich in carbon sources, vitamins, and gut-relevant chemicals, creates a less stressful environment for the bacterial communities and supports the growth of most microbial taxa (Macfarlane and Gibson, 1998). For longer-term exposures (up to 48 hours), nutrient-rich media are required, but these will selectively enrich communities and thereby alter microbiome composition. Overall, when assessing the kinetic rate of microbial metabolites produced during fecal fermentation, it seems advisable to minimize the duration and enrichment of incubations to closely mimic intestinal conditions and to determine reaction rates in samples as they are collected. For integration in PBK models, it is preferable to have Michaelis-Menten-like kinetic data, which means that the incubations need to be optimized first for linearity over time and over fecal concentrations.

3.2. Bacterial culturing and sample pooling strategies

In addition to the media type and storage conditions, the inoculation ratio (fecal content to total volume) impacts resulting chemical reaction rates. When measuring the microbial conversion of selected substrates, the inoculation ratio of feces to media is typically higher (10–80%) than when monitoring the growth of bacterial communities (0.1–1%). Published microbiome-competent PBK models often use less than 10% of the fecal content to measure microbial conversion rates. In addition to incubation times, these were chosen to a) be able to detect and quantify metabolites and b) to achieve linearity over the applied microbial mass. As reported by (Wang et al., 2020), at low fecal mass concentrations, there was a lag phase before metabolites were formed, whereas at higher concentrations, metabolism started immediately. Others have reported that the use of higher percentages of fecal inoculum could eventually result in more reliable measures of the microbial bioconversion capacity of the gut microbiome (Isenring et al., 2023). Culture conditions need to be optimized for each test compound, always with the goal of mimicking the gut environment as closely as possible. Since many experiments have to be performed to derive the desired Michaelis-Menten-like kinetic constants, batch fermentation of small volumes ultimately appears to be the most feasible option, while continuous models hold the potential to produce communities potentially representative of proximal parts of the intestinal tract.

In *ex vivo/in vitro* studies of gut microbial metabolism for PBK modeling, the use of pooled fecal samples is used to reduce inter-donor variability by having a mixture of multiple communities, allowing the same sample to be used in multiple sets of experiments and provide reproducible results. So far, all studies on microbiome-competent PBK models have measured biotransformation kinetics by pooling fecal samples from different donors to overcome inter-individual differences and thus obtain representative microbiome samples. Recent work by van Dongen et al. (2021) tested both pooled and individual human fecal slurries to derive quantitative kinetic information for the bacterial degradation of fructoselysine where the kinetic constants determined from the pooled samples were comparable to the average of the results from individual incubations. However, a recent review by Isenring et al. (2023) states that pooling fecal samples from multiple donors creates a new artificial community that, as such, is not representative of the human gut microbiome, and pooled communities may exhibit unpredictable activity. If the samples are treated primarily as sources of metabolic enzymes while trying to avoid enrichment and adaptation, this does not have to be of concern for these dedicated types of incubations. Other literature reports, for example, that short chain fatty acid (SCFA) production is stable and reproducible and comparable between individual samples and their pooled samples (Aguirre et al., 2014). Taken together, there is a lack of studies investigating the effect of sample storage and processing, highlighting the need to assess how sample handling affects the microbial bioconversion of substrates of interest.

3.3. Alternatives to fecal microbiome ex vivo/in vitro fermentation

Fecal samples are only partially representative of the gut microbiome because they only represent the bacterial species present in the distal colon. This results in a loss of regional variation of the intestinal tract when biotransformation kinetics are measured in fecal samples. However, *in vivo* experiments to measure chemical reaction rates are difficult due to the challenge of collecting multiple samples from the intestinal tract and the aforementioned efforts to avoid animal experimentation. A promising new approach to obtaining intestinal samples is through the use of an orally ingestible capsule device recently published by Shalon et al. (2023) that collects samples from the human intestine for *ex vivo/in vitro* analysis. The capsules are designed to open based on specific pH changes throughout the gastrointestinal tract and collect luminal contents from specific regions. The viability of the collected microorganisms was tested by culturing them under anaerobic conditions. It was observed that after 4 hours of bacterial culture on an agarose pad, 20–50% of the cells resumed growth. This study demonstrates a new, safe, and feasible method for collecting upper gastrointestinal luminal contents from humans that could be extended to use in *ex vivo/in vitro* microbial bioconversion experiments. However, a limitation of this approach is the time required to recover the capsule, which varied from 8 to 67 hours, consistent with the variable gastrointestinal transit times observed *in vivo* (Maurer et al., 2015). The longer recovery times may alter the bacterial communities and reduce the relevance of the local sampling, as communities are expected to change during this transit time. Additionally, the capsules collect small volumes of liquid (< 0.4 mL), making kinetic experiments difficult to perform directly on sample contents. Another alternative approach to using human stool samples for *ex vivo/in vitro* fermentations is to use small or large intestinal stoma effluent samples from patients with ileostomy or colostomy. This method provides a non-invasive access route to the small or large intestine. A recent study by Yilmaz et al. (2022) examined the ileum microbiome before and after stoma surgery and found that the ileostoma microbiome is representative of the intact small intestine. Even though stoma samples are not representative of the healthy physiological state, they can be used to advance PBK models as a personalized medicine approach for certain disease states. For example, Konstantini et al. (2022) established a PBK model that captured the microbial metabolism of sulfinpyrazone and sulindac not only in healthy subjects but also in ileostomy and colectomy patients. The bacterial degradation of sulfinpyrazone was measured in pooled bulk fecal material in samples from healthy volunteers. However, for the modeling of ileostomy and colostomy patients, the authors did not use stoma effluent for bacterial degradation but relied on an estimate of bacterial degradation. Using samples from ileostomy and colostomy patients for bacterial degradation, *ex vivo/in vitro* fermentations would provide a better representation of their gut microbial metabolic capacity. Gaining an understanding of gut microbial metabolism in different populations, such as in disease states, will advance PBK modeling toward personalized medicine. Identifying the fundamental processes that influence microbial activity *ex vivo/in vitro* and developing a standardized approach for the optimal conditions to obtain broadly applicable kinetic parameters will help to advance integrative PBK models and assess metabolite toxicity using NAMs.

4. Conclusion and outlook

The integration of gut microbial metabolism into PBK models is a promising NAM for the study of pharmaco-/toxicokinetics of health-relevant microbial metabolites that is rapidly advancing. However, it has to be acknowledged that attention is still needed in terms of method standardization. A primary challenge will be to understand the dynamics of microbial metabolic activity and to establish a gold standard for *ex vivo/in vitro* determination of metabolic constants for PBK models. Another layer of complexity is added because the gut microbiome is highly affected by multiple factors, including diet, sex, age, disease state, etc. Besides the factors shaping the gut microbial composition, the number and diversity of samples required to obtain population-representative results remains an open question. Differences in scaling methods lead to high conceptual variability among microbiome-competent PBK models. The high inter-individual variation in gut microbial composition and activity adds further complexity that has yet to be fully addressed in microbiome-competent modeling. However, microbiome-competent PBK models are promising tools for investigating food component safety and drug response, particularly for metabolites formed by microbial biotransformation of xenobiotics. We emphasize the importance of establishing coherent *ex vivo/in vitro* methods for measuring microbial biotransformation of compounds. Further research and advances in this area are needed to understand the potential of these models and to unravel the complex relationship between xenobiotic disposition, gut microbial metabolism, and human health.

CRediT authorship contribution statement

Maja Stevanoska: Writing – review & editing, Writing – original draft. **Georg Aichinger:** Writing – review & editing. **Jacob Folz:** Writing – review & editing. **Karsten Beekmann:** Writing – review & editing.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used DeepL, Grammarly and ChatGPT 3.5 in order to refine and detect grammar and spelling errors. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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