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Exploring drug metabolism by the gut microbiota: modes of metabolism and experimental approaches

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Abbreviations:

cgr: Cardiac glycoside reductase

CYP450: Cytochrome P450

H.pylori: *Helicobacter pylori*

HFA: Human flora-associated

HMP: Human Microbiome Project

LDL: Low-density lipoproteins

MDM: Microbiome-Derived Metabolism

MetaHIT: Metagenomics of the Human Intestinal Tract

NIH: National Institutes of Health

NSAID: Nonsteroidal anti-inflammatory drugs

PBPK: Physiologically based pharmacokinetic modeling and simulation

p-cresol: Para-cresol

SHIME: Simulated human intestinal microbial ecosystem

SN-38G: SN-38 glucuronide

Th1: 1 T helper

Th17: 17 T-helper

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Abstract:

Increasing evidence uncovers the involvement of gut microbiota in the metabolism of numerous pharmaceutical drugs. The human gut microbiome harbours 10-100 trillion symbiotic gut microbial bacteria that utilize drugs as substrates for enzymatic processes to alter host metabolism. Thus, microbiota-mediated drug metabolism can change the conventional drug action course and cause inter-individual differences in efficacy and toxicity, making it vital for drug discovery and development. This review focuses on drug biotransformation pathways and discusses different models for evaluating gut microbiota role in drug metabolism.

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Significance statement:

This review emphasizes the importance of gut microbiota and different modes of drug metabolism mediated by them. It provides information on *in vivo, in vitro, ex vivo, in silico* and multi-omics approaches for identifying the role of gut microbiota in metabolism. Further, it highlights the significance of gut microbiota mediated metabolism in the process of new drug discovery and development as a rationale for safe and efficacious drug therapy.

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1. Introduction

Metabolism is the aggregate of all the chemical processes that occur in the body, and it comprises of anabolism and catabolism by enzymatic conversion of one chemical entity to another(Ritter *et al.*, 2018). Drug metabolism involves an enzyme catalyzed biochemical process that transforms lipophilic drugs into more polar and readily excretable metabolites, leading to the termination or alteration of the drugs biological activity(Katzung, 2012). Drug metabolizing enzymes are present in abundance in the liver and play a crucial role in the metabolism of xenobiotics. They are also present in other sites such as kidneys, mucosa of the gastrointestinal tract, lungs, brain and skin but contribute less to the drug metabolism(Krishna and Klotz, 1994). In addition to the metabolic enzymes present in host cells, similar enzymes produced by intestinal microorganisms are implicated in the metabolism of several drug compounds. Gut microbiota is the multitude of bacteria, archaea, eukarya and viruses colonizing the adult human gastrointestinal tract with an approximate count of 100 trillion, that outnumbers the microbial count associated with body surfaces and is nearly 10 times greater than the total number of somatic and germ cells in the human body(Bäckhed *et al.*, 2005). The collective genome of gut microbiota is termed microbiome and is approximately 100 times the number of genes compared to the human genome(Gill *et al.*, 2006). Moreover, recent estimates show that the number of human cells ($3*10^{13}$) and bacterial cells ($3.8*10^{13}$) in the human body is of the same order, and the total mass occupied by the bacterial cells accounts for about 0.2 Kg(Sender *et al.*, 2016).

Research in the field of metabolic capabilities of gut microbes has expanded concerning their effect on the efficacy and toxicity of drugs(Sousa *et al.*, 2008). Co-evolving with the host, the microbiota is now viewed as a virtual organ with properties worthy of being combined with the host physiology(Evans *et al.*, 2013). The United States NIH started an initiative, HMP, to

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understand the gamut of human genetics and physiological differentiation, the microbiome, and all aspects that affect the distribution transformation of the microorganisms in the body. It also involves the study of microflora related to human health and diseases. Another well-known European Union project on MetaHIT targets the gut microbiota in human health and associated metabolic activities of the microorganisms. Hence, the HMP and MetaHIT project are logical extensions of the Human Genome Project to characterize microbial populations colonizing healthy individuals(Turnbaugh *et al.*, 2007; Metagenomics of the Human Intestinal Tract | METAHIT Project | FP7 | CORDIS | European Commission, n.d.). This review discusses gut microbes, their role in drug metabolism, and the experimental approaches used to elucidate them.

2. Gut Microbiota: composition and physiological functions

Fetal gut was previously considered sterile, but recent studies have indicated that early microbial exposure begins *in utero* as demonstrated by the distinct low diversity microbial compositions found in the placenta and meconium(Rautava *et al.*, 2012; Nuriel-Ohayon *et al.*, 2016). By the age of 2-5 years, gut microbiota in a child resembles that of an adult concerning diversity and composition. The gut microbial composition is shaped by the mode of delivery, type of infant feeding, gestational age, antibiotic use, environment, lifestyle, and host genetics(Rodríguez *et al.*, 2015). The upper gut has an antimicrobial climate due to gastric acid and bile salts and the slow rate of movement of intestinal contents, thus making the large intestine the prime site for microbial colonization (Macfarlane and Macfarlane, 2009). Gram-positive *Firmicutes* and gram-negative *Bacteroidetes* constitute the dominant bacterial phyla found in the gut whereas, methanogenic archaea, eukaryotes like yeasts and viruses, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* are the minor bacterial phyla that are identified in the

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gut(Eckburg *et al.*, 2005; Lozupone *et al.*, 2012). Disruption of healthy gut microbiota (dysbiosis) has been implicated in the pathogenesis of various inflammatory and metabolic disorders; nevertheless, healthy gut microbiota plays an essential role in human physiology by carrying out numerous metabolic functions such as fermentation of indigestible carbohydrates, biosynthesis of vitamin K and vitamin B_{12}, production of hormonal mediators and neurotransmitters, metabolism of bile salts and xenobiotics. Microbes also perform protective functions like providing resistance to colonization by pathobionts, modulating intestinal barrier functions, and promoting immune homeostasis(LeBlanc *et al.*, 2013; Natividad and Verdu, 2013; Bäumler and Sperandio, 2016; Pickard *et al.*, 2017; Molinero *et al.*, 2019).

3. Drug metabolism by the gut microbiota

An emerging need to consider drug metabolism by gut microbiota as a crucial factor in drug discovery and development is unveiling. Drugs metabolized by gut microbiota may cause inter-individual differences in the drug response. Moreover, drug administration by diverse routes can encounter metabolism by gut microbes; for example, orally administered drugs remain unabsorbed in the upper gut. Instead, the intestinal microbes act upon a modified-release preparation that reaches the large intestine for their metabolism. Drugs that bypass the absorption process can still reach the gut microbiota through biliary excretion and undergo metabolism. In addition, rectally administered drugs are prone to microbial metabolism due to their proximity to the gut microbes(Sousa *et al.*, 2008). The potpourri of microbial species metabolize many drugs leading to altered bioavailability, toxicity, and adverse drug reactions affecting therapeutic efficacy referred to as MDM (Microbiome-Derived Metabolism).

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However, poor attention is given to understanding the pharmacokinetics of drugs affected by MDM(Javdan *et al.*, 2020). The potential benefit of MDM is the activation of certain prodrugs, such as the conversion of azo drugs prontosil and neoprontosil to an active sulphanilamide moiety(Gingell *et al.*, 1969). Oral co-administration of anti-viral drug sorivudine and anti-cancer drug 5-fluorouracil (5-FU) was the cause of acute deaths in eighteen patients. This lethality resulted from soaring levels of 5-FU due to the inhibition of hepatic enzyme-dihydropyrimidine dehydrogenase responsible for the metabolism of 5-FU by (E)-5-(2-bromovinyl)uracil, a metabolite of sorivudine produced by gut microbiota(Okuda *et al.*, 1998).

4. Modes of drug metabolism by the gut microbiota

Gut microbiota can cause drug metabolism in several ways, including direct interaction with the drug or indirect action by intermeddling with the host metabolism. Figure 1 depicts the different modes of drug metabolism by gut microbiota.

4.1 The direct effect of gut microbiota on drug metabolism

The gut microbiota expresses an extensive array of drug-metabolizing enzymes like oxidoreductases, hydrolases, and lyases(Koppel *et al.*, 2017). The activity of these enzymes leads to alteration of bioactivity of the drug, such as activation, deactivation or reactivation of drug, or conversion of the drug to a toxic metabolite(Wilkinson *et al.*, 2018; Hitchings and Kelly, 2019).

4.1.1 Activation of drug

A prodrug is an inactive drug that converts into an active form after metabolism. Although the liver performs most of the metabolic processes, the enzymes produced by the gut microbiota also actuate the conversion of the prodrug to its active form. For

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instance, prodrugs protonsil and neoprotonsil are transformed into the active sulfanilamide moiety by azoreductases, a gut microbial enzyme(Gingell *et al.*, 1969). Substantial conversion of the neoprotonsil to sulfanilamide occurs in the gut, as evidenced by the excretion of a significant percentage of unchanged drug in bile after its intraperitoneal injection. Further, antibiotic-treated rats had less sulfanilamide excretion, implicating gut microbial enzymes role in azo-reduction of the drugs(Gingell *et al.*, 1971). Azoreductases cleave Azo bond in sulfasalazine, balsalazide and olsalazine releasing sulfapyridine and 5- amino salicylic acid (5-ASA). 5-ASA is an active moiety responsible for anti-inflammatory activity and used in the treatment of ulcerative colitis(Peppercorn and Goldman, 1972; Crouwel *et al.*, 2021). Although probiotic treatment increased the azoreductase activity and plasma concentration, it failed to affect the pharmacokinetic parameters of sulfasalazine(Lee *et al.*, 2012). Thioguanine (TG) and mercaptopurine (MP) are used in treatment of lymphoblastic leukaemia as immunomodulating agents. E. *coli* strain DH5α in the gut microbiota metabolizes TG and MP to 6-TGN by hypoxanthine phosphoribosyl transferase (HPRT)(Movva *et al.*, 2016). The immunosuppressive action occurs due to interaction between DNA and phosphorylated 6-thioguanine nucleotide (6-TGN), an active metabolite of thiopurines, during replication(de Boer *et al.*, 2018; Crouwel *et al.*, 2021). In addition, colonic microbiota improved the chronic colitis by TG even in the absence of host-mediated conversion of TG to 6-TGN by HPRT, suggesting that the local gut microbiota mediated transformation can help in targeted therapy for Crohn's and ulcerative colitis(Oancea *et al.*, 2017). Another interesting recent example is Chinese herbal medicine, berberine,

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which undergoes drug activation upon conversion to oxyberberine by gut microbiota that helps treat gastric colitis(Li *et al.*, 2020).

4.1.2 Deactivation of drug

The active form of the drug can lose its therapeutic efficacy due to the deactivating action of gut microbial enzymes. Lindenbaum *et al.* performed an experiment in volunteers which showed that the formation of dihydrodigoxin, a reduced inactive metabolite of cardiac glycoside digoxin, was subject dependent(J. Lindenbaum *et al.*, 1981). Further experimentation in volunteers displaying substantial levels of reduced metabolites showed that co-administration of erythromycin reduces the excretion of the dihydrodigoxin(John Lindenbaum *et al.*, 1981). After examining hundreds of microbial isolates, *Eggerthelalenta*, a common anaerobic inhabitant of the gut, was the sole microbe responsible for this type of reduction. However, the mere presence of this microorganism in the gut cannot guarantee the inactivation of digoxin to dihydrodigoxin in *in vivo*(Dobkin *et al.*, 1982; Saha *et al.*, 1983). An investigation later showed that a two-gene *cgr1* and *cgr2* operon was present in the strain of *E. lenta*, reducing digoxin and their expression was necessary for the reduction reaction. Digoxin upregulates the *cgr* operon, whereas arginine inhibits it. Arginine repressed the expression of cgr operon, thus inhibiting digoxin reduction, which is explicated by higher digoxin levels in the serum and urine of gnotobiotic mice. And these mice are colonized with digoxin reducing *E. lenta* strain on a high protein diet. The serum and urine levels of digoxin in mice colonized by non-reducing strains remain unaffected. This was a unique case where dietary intervention reverses the metabolism by gut microbes(Haiser *et al.*, 2013).

4.1.3 Conversion of a drug to a toxic metabolite

Metabolizing enzymes produced by gut microbiota can form toxic intermediates, although rare, but can lead to adverse drug reactions. For example, a study was performed on rats to investigate the involvement of gut microbiota in the metabolism of a hypnotic drug-nitrazepam and its teratogenic effect. Administration of large doses to pregnant rats displayed teratogenic effects. Even though nitrazepam was co-metabolized by liver and gut microbiota, antibiotic treatment sharply decreased the excretion of reduced metabolites and diminished the fetal aberrations(Takeno and Sakai, 1991). The investigation later concludes that nitrazepam-related teratogenicity depended on the reduction step, which converted nitrazepam to 7-aminonitrazepam. The reduction was catalyzed by an enzyme, nitroreductase, expressed by the gut bacterium *Clostridium leptum*(Rafii *et al.*, 1997). There is unclear evidence about the role of gut microbiota in cisplatin-induced liver toxicity, but co-administration of antibiotics and cisplatin resulted in the reduction of hepatoxicity; thus, confirming the gut microbiota role in cisplatin-induced liver toxicity(Gong *et al.*, 2021).

4.1.4 Reactivation of drug

Enterohepatic recycling includes biliary excretion followed by intestinal reabsorption of the drug or its metabolites. In biliary excretion, drugs and metabolites are secreted and concentrated in the bile. Later, they are released from the gall bladder and drained into the intestine, where reabsorption occurs. Figure 2 represents the enterohepatic recycling of drugs, including those released by the de-conjugating action of bacterial enzymes. Reabsorption may convert the inactive form of the drug or its metabolite to an active form(Dobrinska, 1989). Enterohepatic cycling causes the plasma drug concentration-time profile to rise after specific time intervals, termed the “multiple peaking” phenomenon,

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and it significantly affects pharmacokinetic parameters(Malik *et al.*, 2016). One of the plausible causes of intestinal injury is NSAIDs such as diclofenac, where the cleavage of one of its metabolites,diclofenac-1-β-O-acyl glucuronide to diclofenac by the action of bacterial β-glucuronidase in the gut. Recurrent exposure of enterocytes to the resulting product of glucuronide hydrolysis (aglycone) could cause local tissue injury and marks the beginning of enteropathy(Saitta *et al.*, 2014). LoGuidice *et al*. undertook a study in mouse models of NSAID enteropathy and demonstrated that administration of selective bacterial β-glucuronidase inhibitors reduces the exposure of intestinal mucosa to the aglycone and provided protection against diclofenac-induced enteropathy(LoGuidice *et al.*, 2012). Anti-cancer drug irinotecan (CPT-11) is an excellent example of the reactivation of drugs caused by gut microbial enzymes. SN-38 is the prodrug irinotecan's active metabolite, glucuronidated in the liver to SN-38G and secreted in the bile. After reaching the intestine, β-glucuronidases of gut bacteria reconvert SN-38G to SN-38, responsible for the irinotecan-induced severe diarrhoea(Takasuna *et al.*, 1996).

4.2 Effect on drug metabolism via host function modulation

4.2.1 Binding of the drug to microbial cells

Bacterial cells express adhesive proteins called adhesins and are responsible for binding to the host cells. Adhesins are also known to interact and directly bind to the drug molecules, decreasing microbial binding to host cells and altering drug pharmacokinetics. Parkinson’s disease is characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of intracellular aggregates of α-synuclein(Poewe *et al.*, 2017). Levodopa, an immediate precursor of dopamine, is given to patients to relieve the symptoms. A small percentage of levodopa that escapes

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peripheral decarboxylation crosses the blood-brain barrier and is taken up by functioning dopaminergic neurons. Niehues *et al.* designed an *in vitro* study to test the hypothesis that the presence of *H.pylori* in patients with Parkinson's disease treated orally with levodopa can affect the plasma level of levodopa(Pierantozzi *et al.*, 2001; Narozanska *et al.*, 2014). The study revealed that adsorption of levodopa to bacteria surface is possible, and such interactions with levodopa results in the blocking of the proteins with adhesive properties, causing a lowered adhesion of *H.pylori* to gastric epithelial cells(Niehues and Hensel, 2009). The interaction between levodopa and *H.pylori* affects the pharmacokinetics of levodopa, i.e., it reduces the absorption and lowers plasma levels. Direct chemical transformation of levodopa by intestinal microbes has also been reported in patients with parkinsonism and rat models(Sandler *et al.*, 1969; Goldin *et al.*, 1973; Niehues and Hensel, 2009).

4.2.2 Alteration in the absorption of drugs caused by gut microbiota

Gut microbiota and their metabolites can change the local microenvironment in the human gut, altering the absorption of drugs(Enright *et al.*, 2016). Zou et al. screened 136 drug excipients for their inhibitory potential of the intestinal transporter, OATP2B1. Out of the 24 potent inhibitors of the OATP2B1 identified, 8 of them are azo dyes. Administration of FD&C Red No. 40 in mice, a red azo dye, decreased plasma levels of fexofenadine, a substrate for Oatp2B1, due to Oatp2B1 inhibition. However, the gut microbial isolates from diverse unrelated healthy humans metabolized azo dye to inactive metabolites that lack OATP2B1 inhibition. Thus, altering drug absorption by the gut microbiome proved beneficial in this case(Zou *et al.*, 2020).

4.2.3 Competition of microbial metabolites with the drug or host metabolites for host metabolic enzymes

Competition for active sites of host metabolic enzymes amongst drug or its metabolites and gut microbial metabolites varies the drug's therapeutic efficacy. Metabolism of acetaminophen occurs by three means: a) conjugation with glucuronide catalyzed by UDP-glucuronosyltransferases, b) sulfate conjugation catalyzed by sulfotransferases, and c) oxidative metabolism through the CYP450enzymes(McGill and Jaeschke, 2013). The oxidative metabolic product formed primarily by the CYP2E1, N-acetyl-p-benzoquinone imine, is highly reactive in nature as it covalently binds to the thiol groups on proteins causing cellular oxidative stress(Vermeulen et al., 1992). As a result, it rapidly conjugates with intracellular glutathione producing a non-toxic glutathione conjugate and excreted as cysteine and mercapturic acid conjugates(Larson, 2007). p-cresol is a product of tyrosine fermentation produced by anaerobic gut microbes belonging to Coriobacteriaceae and Clostridium clusters XI and XIVa(Smith and Macfarlane, 1997; Saito et al., 2018). Clayton et al. used acetaminophen to test the applicability of pharmacometabonomic study in man. The research done on 99 healthy male volunteers inferred that pre-dose urine level of p-cresol sulfate was inversely related to the post-dose ratio of acetaminophen sulfate to acetaminophen glucuronide. Thus, competition between p-cresol and acetaminophen for the active site of sulfotransferase reduces the effective systemic capacity of the individual to produce acetaminophen sulphate(Clayton et al., 2009). Moreover, computational chemistry has also established that cresols compete with acetaminophen for aryl sulfotransferase active site(DiGiovanni et al., 2013). This competition may

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lead to a toxic build-up of acetaminophen in case of acetaminophen overdose and result in hepatotoxicity. Another study deduced that the total excretion of acetaminophen conjugates remain constant to a great extent in germ-free and conventional housed mice, i.e. lowered sulfonation was compensated by improved glucuronidation(Possamai *et al.*, 2015). However, this subject matter requires further investigation.

4.2.4 Modification of host gene expression

Despite the absence of direct contact between the liver and gut microbiota, they modulate hepatic gene expression of crucial genes playing a vital role in drug metabolism(Björkholm *et al.*, 2009). Gene expression studies conducted on microarrays from germ-free (GF) and conventionally raised (CV) specific pathogen-free mice demonstrated differential expression of 112 genes among them, affecting the liver metabolic functions. In GF mice, genes regulated by constitutive androstane receptors showed higher expression. In contrast, CYP2B9 and CYP4A14 expressed to a lower extent in CV mice. The enhanced metabolism with shorter-term pentobarbital-induced anesthesia further ascertained differential expression in GF compared to CV mice(Björkholm *et al.*, 2009). Moreover, immunoblotting experiments in GF and CV rats revealed that gut microbiota moderately affects the levels of Phase II xenobiotic-metabolizing enzymes in the large intestine and liver but remain unaffected in the small intestine(Meinl *et al.*, 2009). mRNA profiling in the four intestinal sections and liver for the expression of xenobiotic-processing genes (XPGs) in CV and GF mice elucidated that the GF mice expressed 116 XPGs in at least one intestinal section, but 133 XPGs were unaffected. Also, the liver and intestine of GF mice showed

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downregulation of CYP3A that may result in altered metabolism of xenobiotics(Zd *et al.*, 2016; Fu *et al.*, 2017). Administration of a VSL3, a commercial probiotic mixture containing eight live strains of bacteria to CV and GF mice was investigated. In CV mice, VSL3 enhanced the mRNAs expression for CYP4V3, alcohol dehydrogenase 1, and carboxyesterase 2a, and declined for multiple phase II glutathione-S-transferases, whereas, in GF mice it reduced the mRNAs expression for UDP-glucuronosyltransferases 1a9 and 2a3(Selwyn *et al.*, 2016). In conclusion, gut microbiota modulates the expression of XPGs leading to altered xenobiotic metabolism.

4.2.5 Immunomodulation

Gut microbiota can impact the efficacy of chemotherapeutic drugs by translocation and immunomodulation. For instance, the anti-cancer drug cyclophosphamide caused the shortening of small intestinal villi and disrupted the intestinal barrier function(Alexander *et al.*, 2017). These disruptions led to the translocation of several commensal gram-positive bacteria such as Lactobacillus johnsonii, Lactobacillus murinus and Enterococcus hirae into the secondary lymphoid organs in the mice(Viaud *et al.*, 2013). that resulted in stimulation of the differentiation of naive $CD4^+$ T cells to cause accumulation of type Th17 and type Th1 cell responses. GF mice and mice treated with antibiotic vancomycin deplete gram-positive bacteria that lowers the Th17 response and inhibit the antitumor effect of cyclophosphamide(Viaud *et al.*, 2011, 2013). Effectiveness of gut microbiota in immune checkpoint blocked cancer therapy is proven in periclinal models and cancer patients for *Akkermansia*

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muciniphila, *Bacteroides fragilis*, *Bifidobacterium spp*. and *Faecalibacterium spp*(Gopalakrishnan *et al.*, 2018; Routy, Gopalakrishnan, *et al.*, 2018; Routy, Le Chatelier, *et al.*, 2018). The immune checkpoint blocked therapy targets cytotoxic T lymphocyte protein-4, blockade of programmed death-1 (PD-1) protein, and programmed death-ligand 1 (PD-L1)(Sivan *et al.*, 2015; Farrokhi *et al.*, 2019). Table 1 summarises the gut microbiota biotransformation reactions.

5. Experimental approaches to analyze the metabolism of drugs by gut microbiota

Different approaches are utilized to analyze gut mediated metabolism. Table 2 gives examples of drug metabolism investigated using different approaches. Figure 3 elucidates the experimental approaches to analyze the metabolism of drugs by gut microbiota.

5.1 *In vivo* approaches

5.1.1 Murine models

Since the beginning of research in gut microbiota, mice models have been widely used due to their anatomical, physiological and genetic similarities to humans. Mice models allow approaches that require invasive sampling methods that would be considered unethical in human subjects. They have added advantages such as small size, high reproductive rate, and low maintenance cost(Hugenholtz and de Vos, 2018). Similar to humans, gut microbiota in mice is also dominated by *Bacteroidetes* and *Firmicutes* phyla. Nevertheless, there lies a variation in the plethora of microorganisms at the genera level. *Prevotella*, *Faecalibacterium*, and *Ruminococcus* genera have a relatively higher abundance in the human gut, whereas *Lactobacillus*, *Alistipes*, and *Turicibacter* genera occupy a relatively higher proportion of mouse gut

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microbiota(Nguyen *et al.*, 2015). Animal models cannot accurately recapitulate human microbial repertoire, curbing direct extrapolating results from conventionally raised mice to humans.

Germ-free mice are reared in an isolator to ensure the complete absence of detectable microbes. Also, germ-free mice have enlarged caecum, reduced villous thickness and also show under-developed intestine-associated lymphoid tissue(Taguer and Maurice, 2016; Sun *et al.*, 2019). Difficulty in generating and maintaining germ-free mice has led to an alternative antibiotic use to deplete mice gut microbiota. Some antibiotics can target a particular subset of microorganisms, such as metronidazole and clindamycin deplete anaerobes(Kennedy *et al.*, 2018). Gnotobiotic mice, colonized by one or more defined microbial species, simplify the complex host-microbial interactions. Other animals such as rats, pigs, dogs and guinea pigs serve as an alternative to mice experiments. Thus, comparing metabolites in gnotobiotic mice and conventionally raised mice provides valuable insights into drug metabolism by gut microbes. HFA (Human flora-associated) animals were used to understand the role of human intestinal microbes in drug metabolism. They were created by inoculating germ-free animals with human faecal matter(Hirayama and Itoh, 2005). However, using the HFA animal models has several drawbacks; factors such as genetics and diet of the recipient animals have been found to affect colonization of the rodent gut by human bacterial communities. For instance, HFA mice display a lower *Firmicutes* to *Bacteriodetes* ratio than donor human or HFA rats(Wos-Oxley *et al.*, 2012). However, despite its flaws, the establishment of human gut flora into the intestines of animals is

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known to yield a stable model with a better resemblance to microbial metabolism in the human gut.

5.1.2 Other animal models

Invertebrate *Caenorhabditis elegans*, a free-living bacterivorous nematode, is a simplified *in vivo* model to study drug metabolism by microbiota. This model was used to show that bacterial ribonucleotide metabolism can cause activation of fluoropyrimidine drugs like 5-fluorouracil(Scott *et al.*, 2017). A vertebrate zebrafish also serves as a model of intermediate gut microbial diversity that is more complex than invertebrates and less complex than that of mammals. Some significant advantages of this alternative testing model include their small size, high fecundity, external fertilization, rapid development, transparency and low cost. In addition, microbe-free embryos of zebrafish can be colonized with a required strain of microbial species by simple immersion in water(axenic zebrafish) and used as an *in vivo* model to study the effects of drug metabolism by microbiota(Catron *et al.*, 2019).

5.2 *In vitro* approaches

5.2.1 Cell-culture

In vivo studies utilizing human and animal models are inappropriate for routine and large-scale screening of xenobiotic metabolism by gut microbiota due to ethical, economic and time restrictions. *In vitro* models such as culturing representative strains of gut microbiota with the drug have been used to study microbial drug bio-transformation. Although both *in vitro* and *ex vivo* studies require laboratory experimentation setup, they differ in the source of the microbial sample used. *In vitro*

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studies use microbial samples that are isolated from an organism instead of laboratory microbial strains(*Read "Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy" at NAP.edu*, 2021). *In vitro* models allow differentiation of microbial xenobiotic metabolism from that of host metabolic activities. *In vitro* fermentation models can range from simple static batch cultures to multistage continuous cultures systems that utilize chemostats to match the dynamic equilibrium of the gut. Culture models require suitable culture media and environmental conditions such as the anaerobic chamber, temperature and pH controls(Payne *et al.*, 2011). Drugs are introduced into the fermentation systems, and aliquots removed at defined time points are quantitatively analyzed for the depletion of substrate and appearance of metabolites to determine the rate and extent of drug metabolism by gut microbiota. In such models, adaptation to specific culture conditions may cause deviation in the bacterial composition over time(Payne *et al.*, 2011). These model designs prevent the extension of information regarding the drug's pharmacokinetics in the upper gastrointestinal tract; besides, they neglect the interactions between the host tissue and microbiota, which are essential for the drugs co-metabolized by the host and microbes(Hu *et al.*, 2019). An *in vitro* fermentation model for investigating exopolysaccharides effect on gut microbiota showed that exopolysaccharides are transformed into short-chain fatty acids. Furthermore, 16S rDNA sequencing elucidated that exopolysaccharides increased the abundance of *Ruminococcus*, *Dorea*, *Butyricicoccus*, and *Blautia*(Zhu *et al.*, 2021).

5.2.2 Simulators

Complex simulators of the intestine, such as the SHIME reactor have been developed

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to mimic the gut microbial environment. SHIME is a validated five-stage model in which a two-step fill-and-draw system simulates the small intestine while the latter three reactors mimic the large intestine. When inoculated with microorganisms, each reactors hosts intestinal region-specific distinctive microbial communities. All the reactors are connected in series employing pumping systems and placed under well-defined and controlled conditions(Molly *et al.*, 1993). One validation test for the SHIME model involves converting prodrug sulfasalazine to its active moiety 5-aminosalicylic acid. Results stated that only a small percentage of 5-aminosalicylic acid was released up to the end of vessel 2 (small intestine). At the same time, a complete transformation happened at the beginning of vessel 3 (large intestine)(Molly *et al.*, 2009). The disadvantage of this model is that the bacterial miscellany in the colon mucosa is under-represented in the faecal inoculum(Durbán *et al.*, 2011). Therefore, an up-gradation, mucosal-SHIME (M-SHIME), was introduced in the SHIME model, which had an advantage over the older SHIME model. This optimization of SHIME considers the luminal microbiota and the colon mucosa-associated microbial communities by incorporating mucin-covered microcosms(Van den Abbeele *et al.*, 2012).

5.2.3 Microfluidic devices (organ-on-chip systems)

Organ-on-a-chip models consist of continuous perfusion of microchambers that are amenable to the inhabitation of cultured living cells to simulate tissue- and organ-level physiology(Bhatia and Ingber, 2014). Gut-on-a-chip models comprised two hollow chambers separated by an extracellular matrix coated by a porous membrane of polyester or polycarbonate and lined with human intestinal epithelial cells. In some

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studies, the microenvironment of the cells is recreated by control of culture medium flow rate through the microchannels and, in other studies, by applying cyclic strain to cause peristalsis like deformations(Kim *et al.,* 2012). A handful of microbiota-related studies have been done using microfluidic devices that demonstrate the ability of differentiated epithelial cells to sustain the growth of intestinal inhabitants. *Lactobacillus rhamnosus* GG host-pathogen interactions and interaction among components of the intestine, the immune system and bacteria(Kim *et al.*, 2010, 2012; HJ Kim *et al.*, 2016). These models with fluidic control can permit controlled delivery of desired concentration of drugs dissolved in medium to the intestinal epithelium co-cultured with microbes to investigate microbiota-based drug metabolism in a biomimetic system(Tanaka *et al.,* 2006; Vickerman *et al.*, 2008). However, replicating all the intestinal wall layers and co-culturing total microbiota rather than single microbial species or microbial consortia are some of the challenges yet to be explored(Lee *et al.*, 2019). Shah *et al.* presented a modular, microfluidics-based model HuMix (human–microbial crosstalk) that allows co-culturing of human and microbial cells under representative gastrointestinal human-microbe interface conditions. The individual transcriptional responses from human epithelial cells co-cultured with *Lactobacillus rhamnosus* GG under anaerobic conditions inside HuMix are in accordance with *in vivo* data. In addition, human epithelial cells co-cultured with *Bacteroides caccae* and LGG elicit a transcriptional response differing from a co-culture containing only LGG, suggesting the use of HuMix in molecular interactions between host and host microbiome(Shah *et al.*, 2016).

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5.3 *Ex vivo* approaches

In contrast to *in vitro* studies, *ex vivo* studies use biological material comprising microbes in an artificial setting. For example, multiple studies utilize *ex vivo* fermentation systems inoculated with human or animal faecal homogenates or animal caecal to screen drug metabolism by microbes(E *et al.*, 2018). This allows drug screening against a greater diversity of microorganisms present in the faecal/caecal contents.

5.3.1 Fecalase or caecalase assay

This research tool makes use of a cell-free extract of faecal or caecal contents, fecalase or caecalase assays. Lysis of bacterial cells in faecal suspension by passing through a homogenizer or sonication followed by removing cell debris by centrifugation yields a stable extract that retains the enzyme fraction of bacteria(Tamura *et al.*, 1980). The fecalase assay assesses the conversion of amlodipine to pyridine metabolite. The pyridine metabolite concentration increased with incubation time, suggesting the role of gut microbiota in amlodipine metabolism and further confirmed by a pharmacokinetic study demonstrating an increase in bioavailability of amlodipine in antibiotic-treated mice compared to control mice(Yoo *et al.*, 2016). However, diet and physiological factors have affected fecalase and caecalase activity, leading to intra- and inter-individual differences(Yeo *et al.*, 2012).

5.3.2 Organoids

Scientific advancement has led to the development of three-dimensional *ex vivo* multicellular tissue constructs derived from human stem cells containing organ-specific cell types termed organoids(de Souza, 2017; Min *et al.*, 2020). Intestinal and gastric organoids have been used to examine the crosstalk between infectious

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pathogens such as *Salmonella typhimurium*, *Helicobacter pylori*, rotavirus and the luminal epithelium(Finkbeiner *et al.*, 2012; Bartfeld *et al.*, 2015; Forbester *et al.*, 2015). Engineered gastrointestinal organoids microinjected with microbiota can decipher the mechanism of drugs action and microbiota-based metabolism. Cell culture and simulator models are insufficient to represent host cell impact on microbiota physiology that can be overcome using the organoid model(Hill *et al.*, 2017). The intestinal epithelial organoid is a novel model to study host-microbiota interactions. Investigation on the effect of short-chain fatty acids produced by commensal gut bacteria with the metabolites of *Akkermansia muciniphila and Faecalibacterium prausnitzii* revealed that *Akkermansia muciniphila* metabolites affect transcription factors and genes (Fiaf, Gpr43, histone deacetylases, and peroxisome proliferator-activated receptor-gamma) associated with cellular lipid metabolism and growth. In contrast, *Faecalibacterium prausnitzii* has a weak effect on host transcription(Lukovac *et al.*, 2014).

5.4 *In silico* approaches

Technological advances have resulted in efficacious computational tools to predict drug pharmacokinetics in the human body. Despite the difficulty in modelling complex biochemical reactions, multiple computational tools have been developed to predict microbial xenobiotic metabolism. The complexity arises from the non-specificity of many microbial enzymes for substrates and vast microbiota diversity at the species and community level(Klünemann *et al.*, 2014). PBPK models describe the whole-body drug kinetics by using compartments corresponding to the body's organs connected by a circulating blood system(Zhuang and Lu,

2016). However, PBPK models were unable to account for microbial metabolism until the recent inclusion of the microbiome component in the intestinal compartment. Therefore, a PBPK model was devised that accurately predicts and separates brivudine's host and microbiome mediated metabolism to its hepatotoxic metabolite bromovinyluracil. For constructing the model, the kinetic parameters of the drug and its metabolite were obtained in various compartments over time in the presence and absence of microbial drug metabolism(Zimmermann *et al.,* 2019b). Besides, a generalized approach was developed by building models parametrized with drug and metabolite kinetics in germ-free and conventionally raised mice for two additional drugs, sorivudine and clonazepam(Zimmermann *et al.,* 2019b). An example of a searchable resource is MicrobeFDT, in which networks of food compounds and drugs with similar structures are created and linked to microbial enzymes with known toxicities. Based on the postulation, if a microbial enzyme acts on one compound from a group, it may act on related compounds found in the group(Guthrie *et al.,* 2021). A PBPK model-based study indicated that hydrolysis of intestinal glucuronide impacts the pharmacokinetics of aglycone. A PBPK model was developed to investigate the effect of intestinal glucuronide on the pharmacokinetics of an active compound SN-38 glucuronide considering liver and gut as the major eliminating organs. This two-compartment model suggested that hydrolysis of glucuronide in the gut increased the local intestinal exposure to the SN-38, but the systemic exposure was insignificant(Wu, 2012; Guthrie and Kelly, 2019). Thus, results from *in silico* prediction tools can complement and improvise the experimental setup of *in vitro* studies.

5.5 Multi-omics approach

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An ever-increasing knowledge of the microbiome impact on drug efficacy, disposition and toxicity, pharmacomicrobiomics (microbiome-drug interactions) were pioneered as an extension of pharmacogenetics(Mariam *et al.,* 2010; Aziz *et al.,* 2018; Sharma *et al.,* 2019). Genetic diversity of the gut microbial communities has come to light due to the advent of next-generation sequencing techniques such as 16S rRNA gene amplicon sequencing and shotgun metagenomics(Sanschagrin and Yergeau, 2014). Metagenomics, in addition, gives a peek at the potential functional capabilities of the microorganisms (Haiser and Turnbaugh, 2013). Nevertheless, the mere abundance of the gene cannot guarantee its expression. Meta-transcriptomics, the sequencing of gene transcripts and meta-proteomics, the measurement of expressed proteins also form part of pharmacomicrobioics. Apart from solely detecting the microbial species present in large numbers, these techniques also distinguish the metabolically active gut microbes(Ursell and Knight, 2013; Xiong *et al.*, 2015). Targeted or untargeted metabolomics and metabonomic analysis coupled with *in vivo* and *in vitro* experimental designs have proven valuable to determine the gut microbial contribution to drug metabolism(Aura *et al.*, 2011; Yip and Chan, 2015). Metabolomic analysis has been used for targeted analysis of drug metabolites and to discover unique biomarkers that have helped predict the host response or the host metabolism of a drug. For instance, the pre-dose level of bacterial metabolite, *p*-cresol, influences the hepatic sulfate conjugation of acetaminophen(Clayton *et al.*, 2009). A high throughput screening led to the identification of bacterial gene products that metabolize drugs. Integrated data obtained from relevant omic approaches such as untargeted metabolomics and metagenomics supports identifying the gene products. Moreover, the study demonstrated the ability of 76 gut bacterial species to metabolize 271 drugs belonging to a wide range of chemical classes(Zimmermann *et al.,* 2019a). Even though the omic methods provide valuable

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information, using these techniques in elucidating drug metabolism by gut microbiota still has a long way to go.

6. Toxicological assessment of drug metabolites

As discussed earlier, microbial action may result in drug toxicity, and a meticulous study has been done on the intestinal toxicity caused by the activity of gut microbes on the anti-cancer drug irinotecan.SN-38, the active metabolite of the prodrug irinotecan, is glucuronidated in the liver to SN-38G and secreted in the bile. In the intestine, bacterially-derived β-glucuronidases converts SN-38G to SN-38, responsible for the irinotecan-induced severe diarrhoea(Takasuna *et al.*, 1996). The toxicity of drug metabolites produced by gut microbes is mainly evaluated by comparative clinical observations and histological examination of tissues of germ-free/antibiotic-treated and conventionally raised/HFA animals. Other tools such as toxicity predicting software and omics technology for studying toxico-microbiomics are also used(Abdelsalam *et al.*, 2020). The toxicity of short-lived reactive metabolites is analyzed by trapping the electrophilic metabolites with nucleophiles like glutathione and cyanide ions followed by the mass spectrometric analysis adducts formed(Tang and Lu, 2010). Manipulation in the gut microbial composition or metabolic activity can lower the production of toxic metabolites or improve the therapeutic outcomes. Wallace and co-author demonstrated that the administration of selective bacterial β-glucuronidase inhibitor protected mice from irinotecan-induced toxicity without killing the bacteria. This inhibitor would benefit cancer patients as it will selectively inhibit the bacterial β-glucuronidase and prevent the formation of SN-38 that kills the microbiota essential for human health; hence, it will alleviate irinotecan-induced toxicity(Wallace *et al.*, 2010).

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7. Impact of drugs on the gut microbiome

Several drugs such as proton pump inhibitors, statins, ACE inhibitors have demonstrated the ability to change the gut environment, thus affecting the composition, growth and functions of gut microbial communities(Weersma *et al.*, 2020). For instance, metformin affects the composition and function of gut microbiota. Germ-free mice treated with gut microbiota from metformin-treated individuals showed lower blood glucose levels than those treated with gut microbiota from placebo-treated donors. This showed that metformin-altered gut microbiota improves glucose metabolism(Wu *et al.*, 2017). High-throughput screening of over 1000 drugs covering a wide range of therapeutic classes was performed against 40 gut microbial strain isolates. Around 835 drugs acted upon molecular targets in human cells, and the rest were anti-infectives. Using drug concentrations similar to that estimated to be found in the gut for many drugs, the study showed that 27% of non-antibiotic suppressed the growth of at least one of the tested microbial strains(Maier *et al.*, 2018). Thus, the interplay between gut microbiota and drugs is complex and bidirectional.

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8. Conclusion

Although gut microbiota-mediated drug metabolism was first discovered in the mid-20th century, research in this field has been challenging owing to the extensive and variable repertoire of gut microbes in individuals and the complicated mechanisms by which the microbes carry out drug metabolism. Furthermore, Microbially-derived drug metabolites can have a different potency than parent drugs or, in some cases, even a toxic potential, thus causing deviations from the expected therapeutic outcomes of the drug. Integrating experimental, computational, and multi-omic approaches will deepen our understanding of gut microbial composition and recognize gut microbiota-mediated drug metabolism. Evaluation of drug metabolism by gut microbiota in new chemical entity drug discovery and development is rational for safe and efficacious drug therapy. Once the role of gut microbes in drug metabolism is established, the host-microbiota symbiotic relation exploration will help develop refined and personalized drug therapies with maximum therapeutic benefits and minimal toxicological effects.

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Figure legends

Figure 1: The gut microbiota can perform metabolism of drugs by different modes of action: a) activation of drug which includes conversion of prodrug to its active form, b) deactivation of drug which results in loss in therapeutic efficacy of drug by inactivating it, c) conversion of drug to a toxic metabolite leading to an adverse drug reaction, d) reactivation of drug through enterohepatic recycling, e) direct binding of drug to microbial cells due to adhesive proteins on the microbial cell surface, f) competitive binding of microbial metabolite with the drug for host enzyme, g) modification of the host gene expression and altered expression of crucial genes necessary for drug metabolism, h) immunomodulation or translocation of microbes that stimulates differentiation of immune cells and develop autoimmunity.

Figure 2: Reactivation of drug by gut microbiota induced enterohepatic cycling. Enterohepatic recycling includes two processes- i) biliary excretion followed by ii) intestinal absorption of drug or its metabolite. In the former part, drugs and metabolites which are formed due to action of liver enzymes are the inactivated or conjugated form of the drug and are secreted and concentrated in the bile; in the later part the drug or the metabolites are released from the gall bladder and drained into the intestine where the action of microbial enzymes converts the inactivated or conjugated for of drugs to its active or deconjugated form. This activated or deconjugated form of drug can be absorbed through the hepatic portal vein and get back into systemic circulation.

Figure 3: Different experimental approaches to analyze the metabolism of drugs by gut microbiota

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Table 1: Biotransformation reactions performed by gut microbiota

Type of reaction	**Drug exemplar**	**Class of drug**	**Produced drug metabolites**	**Outcome**	**Microorganism**	**Reference**
Azo-bond reduction	Sulfasalazine	Anti-inflammatory	5-Aminosalicylic acid and sulphapyridine	Activation of prodrug	Unknown	(Peppercorn and Goldman, 1972)
Alkene double bond reduction	Digoxin	Cardiac glycoside	Dihydrodigoxin	Reduction of therapeutic efficacy	*Eggerthella lenta*	(J. Lindenbaum *et al.*, 1981)
Carbonyl double bond reduction	Tacrolimus	Immuno-suppressant	9-Hydroxytacrolimus	Low and variable exposure to the immunosuppressant	*Faecalibacterium prausnitzii*	(Guo *et al.*, 2019)
Nitro reduction	Nitrazepam	Hypnotic	7-Aminonitrazepam	Produces 7-acetylaminonitrazepam having	*Clostridium leptum*	(Rafii *et al.*, 1997)

				teratogenic potential		
N-oxide reduction	Loperamide oxide	Anti-diarrheal	Loperamide	Activation of prodrug	Unknown	(Lavrijsen *et al.*, 1995)
Sulfoxide reduction	Sulindac	NSAID	Sulindac sulfide	Activation of prodrug	Aerobes *Escherichia coli*, *Enterobacter* and anaerobes *Clostridia*	(Strong *et al.*, 1987)
Hydrolysis	Sorivudine	Anti-viral	(E)-5-(2-bromovinyl) uracil	Lethal effects due to combination of Sorivudine and 5-fluorouracil	*Bacteroides* species *B. Vulgatus, B.Thetaiotaomicron, B. fragilis, B. uniformis and B. eggerthii*	(Nakayama *et al.*, 1997; Okuda *et al.*, 1998)
Proteolysis	Calcitonin	Synthetic	Unknown	Degradation of	Unknown	(Sousa *et al.*,

		hormone		peptide drug causing loss of therapeutic efficacy		2008)
De-conjugation	Irinotecan metabolite	Anti-cancer	SN-38	Intestinal toxicity	Unknown	(Takasuna *et al.*, 1996)
De-hydroxylation	Levodopa	Anti-parkinsonian	m-Tyramine and m-hydroxyphenyl acetic acid	Reduced therapeutic activity	Unknown	(Sandler *et al.*, 1969; Goldin *et al.*, 1973)
De-methylation	Imipramine	Tricyclic anti-depressant	Desipramine	Conversion to active metabolite causing variation in response to imipramine therapy	Aerobes *Escherichia coli* *Klebsiella pneumoniae* and anaerobes *Fusobacterium fusiforme*	(Clark *et al.*, 1983)

Acetylation	5-Aminosalicylic acid	Metabolite of sulfasalazine	N-Acetyl-5-aminosalicylic acid	Therapeutic inactivation of the drug	Unknown	(Allgayer *et al.*, 1989)
De-acetylation	Aspirin	NSAID and anti-thrombotic	Salicylic acid and hydroxylated salicylic acid	Decrease in anti-thrombotic effect of the drug	Unknown	(IS Kim *et al.*, 2016)
De-amination	5-Fluorocytosine	Anti-fungal	5-Fluorouracil	Possible cause of toxicity of the drug	Unknown	(Harris *et al.*, 1986)
Oxidation	Lovastatin	HMG-CoA reductase inhibitor	Hydroxylated and hydroxy acid metabolites	Inter-individual variability in pharmacokinetics	Unknown	(Yoo *et al.*, 2014)
De-nitration	Glyceryl trinitrate	Anti-anginal	Glyceryl-1,3-dinitrate, glyceryl-1,2-dinitrate, glyceryl-1-	Decrease in the therapeutic activity of the drug	Unknown	(Sousa *et al.*, 2008)

			mononitrate, glyceryl-2-mononitrate			
Thiazole ring opening	Levamisole	Anthelmintic	Levametabol I, II and III	The activity of metabolites not reported	*Bacteroidetes* and *Clostridium spp*. under anaerobic conditions	(Shu *et al.*, 1991)

Table 2: Examples of investigation of drug metabolism by gut microbiota using different approaches

Sr. No.	**Type of study**	**Drug**	**Class of drug**	**Result**	**Reference**
1	*In vivo* murine	Deleobuvir	Hepatitis C	Plasma exposure of reduced metabolite of deleobuvir (CD	(McCabe *et al.*,

	model			6168) was 9-fold lower in pseudo-germ free rats than control rats	2015)
2	*In vivo* nematode model *C. elegans*	Doxorubicin	Anti-cancer	Reduced toxicity of doxorubicin was attributable to its deglycosylation by capsulated bacilli *Raoultellaplanticola* under anaerobic conditions	(Yan *et al.*, 2018)
3	*In vitro*	Flucytosine	Anti-fungal	Extensive conversion of flucytosine to fluorouracil can occur in the human intestinal microflora by *E. coli*. Hence, fluorouracil exposure and fluorouracil-related toxicity may occur in the flucytosine-treated patient	(Vermes *et al.*, 2003)
4	*Ex vivo*	Epacadostat (EPA)	Anti-cancer	Amidine metabolite of EPA (M11) and N-dealkylated form of M11 (M12) were formed in negligible quantities when EPA was incubated with human microsomes from multiple tissues, hepatocytes, recombinant human cytochrome P450s. Whereas, M11 was formed when EPA was incubated with human faecal homogenates, and the amount of M11 depleted upon addition	(Boer *et al.*, 2016)

				of antibiotic ciprofloxacin to the faecal homogenates which confirmed the role of gut microbiota in its metabolism	
5	*In silico*	Brivudine	Anti-viral	PBPK model accurately predicts and separates the host and microbiome mediated metabolism of brivudine to its hepatotoxic metabolite bromovinyluracil	(Zimmermann *et al.*, 2019b)
6	Multi-omic	Simvastatin	HMG-CoA reductase inhibitor	Metabolomic study revealed that the levels of bacterially derived bile acids assist in predicting the efficacy of simvastatin in lowering LDL cholesterol and raise the possibility of competition between simvastatin and bile acids for SLCO1B1 transporter may influence both the pharmacokinetics and pharmacodynamics of simvastatin, and possibly the risk of muscle toxicity	(Kaddurah-Daouk *et al.*, 2011)

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Figure 1

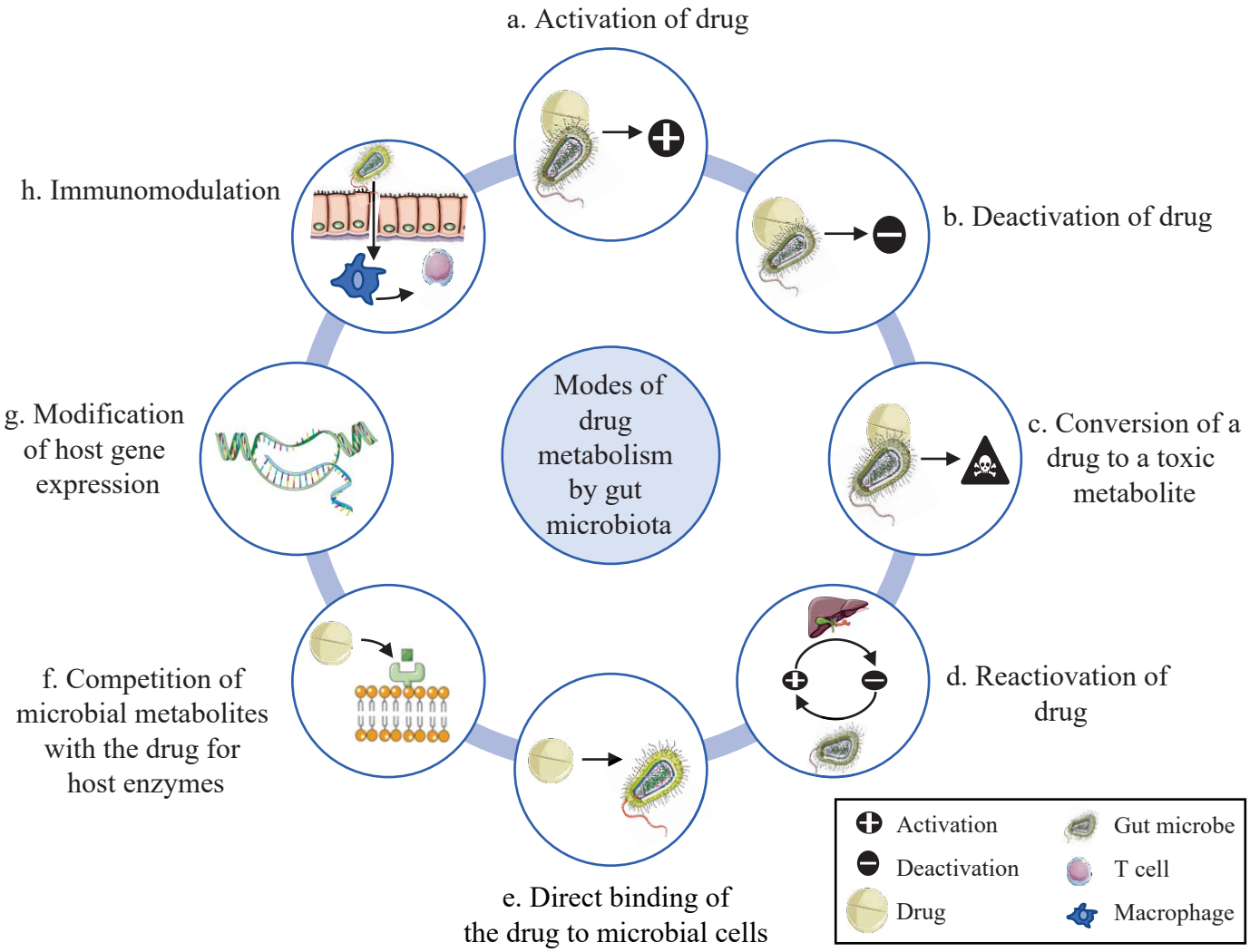

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Figure 2

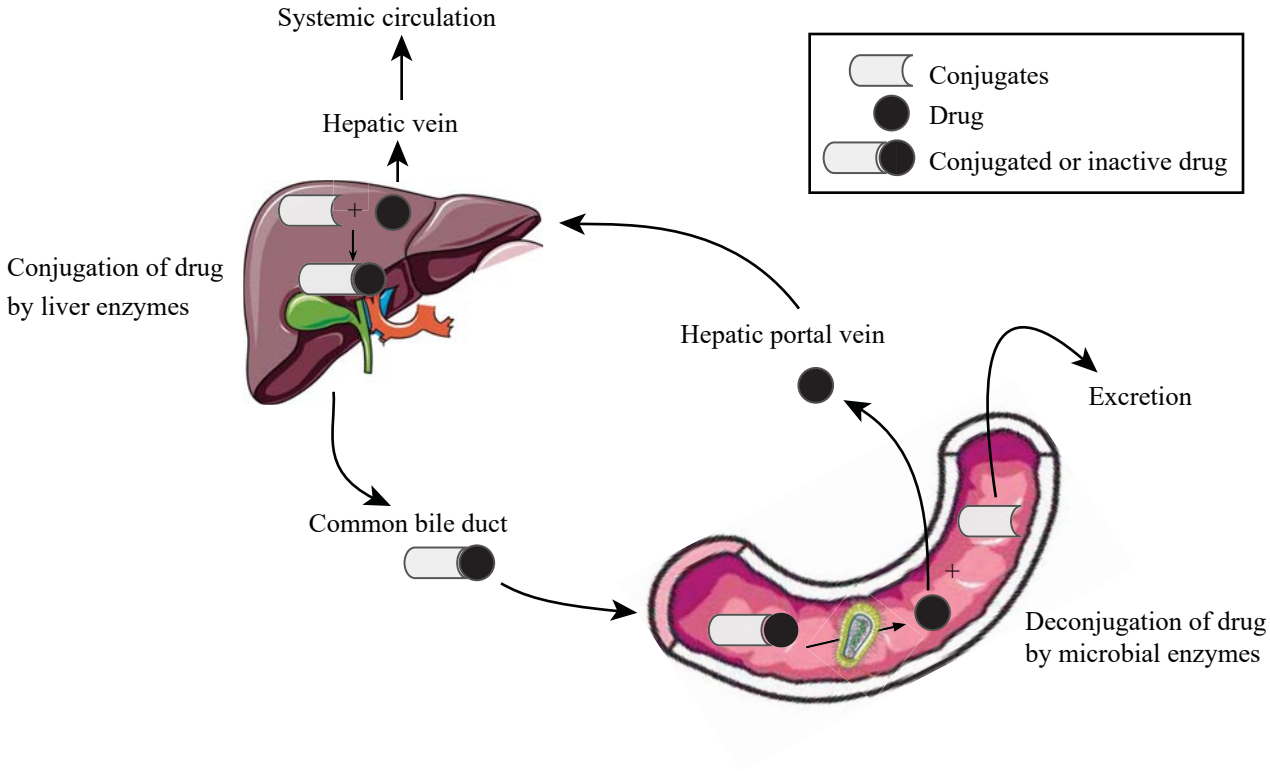

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Figure 3