

Pharmacomicrobiomics:
Investigating the Relationship between the Gut Microbiome and Drug Disposition

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Abstract

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Pharmacomicrobiomics is an emerging field that aims to understand how variation in the microbiome can impact drug disposition, action, and/or toxicity. In this dissertation, we used a humanized mouse model, multi-omics approaches, *in vitro* systems, and clinical samples to investigate the relationship between the gut microbiome and drug-metabolizing enzymes and transporters. Specifically, we focused on cytochrome P450 3A4 (CYP3A4), which is responsible for the metabolism of up to 40% of clinically used drugs. Chapter 2 examines how differences in microbial conditions impacted CYP3A4 expression in a pregnane X receptor-constitutive androstane receptor-cytochrome P450 3A4/3A7 (PXR-CAR-CYP3A4/3A7) humanized mouse model. We studied three different groups of male and female mice: conventional (CV), germ-free (GF), and conventionalized (GFCV) mice which were GF mice that were inoculated with sex-matched pooled human feces at 8 weeks ($n = 7$ per sex per group). As previous studies only investigated how the microbiome impacts the expression of Cyp3a11, the mouse orthologue of CYP3A4, this chapter's goal was to investigate whether the microbiome affected human CYP3A4. In summary, the presence of microbiome upregulated CYP3A4 expression by 7.6-fold in male mice ($p < 0.001$) but downregulated CYP3A4 expression by 1.69-fold in female mice (p

= 0.012). This highlights that there is both a sex-dependent and microbiome-dependent effect. We found a strong positive correlation between microbial alpha diversity and hepatic CYP3A4 expression ($R = 0.66$; $p = 0.0072$), in agreement with previous studies of Cyp3a11. Chapter 3 utilized multi-omics approaches (i.e., metagenomics, transcriptomics, metabolomics, and proteomics) to understand changes in the hepatic environment with alterations in microbial conditions and between sexes in a subset of the PXR-CAR-CYP3A4/3A7 mice in Chapter 2. Male CV mice had a more variable but less diverse microbiome compared to female CV mice. Additionally, principal components analysis (PCA) of the transcriptome and metabolome revealed that male CV mice displayed the greatest interindividual variability within the group, whereas the transcriptome and metabolome of each of the other groups (i.e., male GF, female CV and female GF mice) were more tightly clustered. Lastly, presence of a microbiome had a greater impact on drug metabolizing enzymes and transporters (DMET) gene expression in male mice (43.4% differentially expressed genes) compared to female mice (11% differentially expressed genes). Chapter 4 further expands on the PXR-CAR-CYP3A4/3A7 mouse work through a correlation analysis between CYP3A4 expression and the hepatic metabolome and subsequent *in vitro* testing of selected microbial metabolites in HepaRG cells. Microbial metabolites, such as short chain fatty acids, secondary bile acids, and tryptophan metabolites (i.e., indole derivatives) have been shown to induce CYP3A4 expression through nuclear receptor binding. Therefore, our goal was to identify other microbial metabolites that can also influence CYP3A4 expression. We identified three microbial metabolites with strong correlations to hepatic CYP3A4 expression: 1-deoxynojirimycin ($\rho = 0.72$, $p = 1 \times 10^{-4}$), betaine ($\rho = 0.66$, $p = 0.00056$), and L-pyroglutamic acid ($\rho = 0.62$, $p = 0.0015$). Physiologically-relevant concentrations (up to 150 μM of each microbial metabolite) were incubated in HepaRG cells for 72 hours, however no CYP3A4

induction was observed. In addition, CYP3A4 activity in pooled liver microsomes was not inhibited by any of the metabolites. Therefore, we concluded that the identified metabolites were not regulators of CYP3A4 under the conditions tested. To expand our findings into humans, the aim of Chapter 5 was to explore the relationship between normal variation in the gut microbiome and CYP3A activity. We used clinical samples collected from healthy male volunteers ($n = 7$) dosed with midazolam semi-simultaneously (2 mg orally at 0 h and 1 mg intravenously at 3 h). Midazolam and its primary metabolites (1'-hydroxymidazolam [1'-OH-MDZ] and 4-hydroxymidazolam [4-OH MDZ]) were quantified in plasma samples collected over 12 hours. Metrics related to CYP3A activity (clearance, hepatic and intestinal extraction ratios, and metabolite-to-parent AUC ratios) were calculated and correlated with microbial diversity. Microbial species richness was not correlated with clearance, hepatic extraction ratio or intestinal extraction ratio, however an inverse relationship was observed with the 1'-OH MDZ/MDZ AUC ratio ($R = -0.86$, $p = 0.024$) and 4-OH MDZ/MDZ AUC ratio ($R = -0.89$, $p = 0.012$). These results suggest that normal variation in the gut microbiome might not influence intestinal or hepatic CYP3A activity but may have an effect on glucuronidation as the parent-to-metabolite AUC ratios account for both formation clearance and metabolite clearance. Following metabolomics analysis of the plasma samples, we identified two tryptophan metabolites strongly correlated with hepatic CYP3A (i.e., hepatic extraction ratio), serotonin ($R = 1$, $p = 0.017$) and xanthurenic acid ($R = 0.86$, $p = 0.024$). HepaRG cells were treated with these metabolites and we found that 100 nM of serotonin or xanthurenic acid induced *CYP3A4* expression by ~2.0-fold ($p < 0.01$). However, when tested in a PXR reporter assay, these metabolites did not appear to be PXR agonists. In conclusion, this dissertation revealed that differences in microbial conditions more strongly influenced CYP3A4 expression in a humanized mouse model in a sex-dependent

manner and the effect on CYP3A metrics in humans was less apparent. We identified two metabolites (i.e., serotonin and xanthurenic acid) as potential inducers of CYP3A4. Findings from this dissertation contribute to further understanding the relationship between the gut microbiome and drug disposition, specifically through CYP3A4 regulation.

Dedication

To Meliana Tjiptoboesono, my mother and fiercest supporter.

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Chapter 1

Introduction

1.1. Specific Aims

The gut microbiome is a dynamic ecosystem that plays a pivotal role in human health, influencing various physiological processes, including drug metabolism and transport. In recent years, there has been a growing interest in the field of pharmacomicrobiomics, which examines how the microbiome impacts drug disposition, activity, and toxicity. Drug disposition is primarily mediated by drug metabolizing enzymes and transporters (DMETs) and understanding the regulatory effects of the gut microbiome on these processes is essential for improving predictions of drug pharmacokinetics and addressing interindividual variability in drug responses.

Notably, research has demonstrated that alterations in the gut microbiome can significantly affect mouse orthologues of CYP3A4 (Cyp3a11). For instance, absence of a microbiome has been linked to lower Cyp3a11 expression and activity. Given that CYP3A4 is responsible for the metabolism of substantial fraction of clinically used drugs, these changes can impact drug clearance and consequently, overall pharmacokinetics. Furthermore, understanding the relationship between the gut microbiome and CYP3A4 activity is crucial for predicting potential drug-natural product interactions and for explaining some of the observed interindividual variability in pharmacokinetics. CYP3A4 expression can be mediated through nuclear receptors such as the pregnane X receptor (PXR) and/or the constitutive androstane receptor (CAR). Specifically, ligands can bind to these nuclear receptors and increase transcription of CYP3A4. We hypothesize that the gut microbiome influences CYP3A4 expression and activity through interactions of the microbial metabolites with specific nuclear receptors (i.e., PXR and/or CAR).

To fill the above gaps in knowledge and test our hypothesis, we will pursue these specific aims:

Aim 1: To investigate the impact of the gut microbiome in the regulation of CYP3A4 in quadruple humanized PXR-CAR-CYP3A4/CYP3A7 transgenic mice.

Aim 2: To explore the microbial and sex differences in quadruple humanized PXR-CAR-CYP3A4/CYP3A7 transgenic mice through a hepatic multi-omics approach.

Aim 3: To investigate the influence of microbial metabolites on the regulation of CYP3A4 *in vitro*.

Aim 4: To investigate whether normal variation in the microbiome contributes to interindividual variability of CYP3A in humans.

1.2. The Gut Microbiome

The gut microbiome has gained recognition as the "second genome" of humans due to its vast genetic diversity (Zhu et al., 2010). Interest in the gut microbiome has surged because of the critical role of the gut microbiome in human health and its association with numerous disease states (Guarner and Malagelada, 2003; Manos, 2022; Zhu et al., 2010). Factors such as changes in diet or the consumption of xenobiotics can profoundly influence microbiome composition, contributing to interindividual variability (Carmody and Turnbaugh, 2014; Lkhagva et al., 2021; Perler et al., 2023). However, even without intervention, the gut microbiome is highly variable between individuals. For example, across age and population, pronounced differences in microbial composition have been seen. Yatsunenکو et al. (2012) reported that the gut microbiome differed significantly between adults in the United States metropolitan area compared to those in Amazonas of Venezuela and rural Malawi. The study also reported that although host genetics also contributes to interindividual variation of specific microbial taxa and functions,

environmental exposures are major determinants of gut microbiome composition. Specifically, the microbiomes of genetically unrelated but co-habiting family members were more similar compared to genetically related non-cohabitating family members. In infancy, the microbiome can be shaped by the circumstances at birth (Penders et al., 2006). When fecal samples were collected from 1032 one-month old infants in the Netherlands, factors such as mode of delivery, type of feeding, gestational age, hospitalization, and antibiotic use by the infant were determined to be the most essential in shaping the gut microbiome composition. Overall, there is not a single defined “normal” microbiome as the gut microbiome is affected by intrinsic factors (e.g., age, sex, disease, etc.) and extrinsic factors (e.g., environment, diet, drugs, etc.).

There have been studies trying to pinpoint a specific marker of a normal, healthy gut microbiome. One specific marker is bacterial diversity, where a higher diversity is typically considered a good indicator of gut health (Van Hul et al., 2024). For example, athletes tend to have higher species diversity (Mohr et al., 2022). However, Roager et al. (2016) found that high diversity was associated with the circulation of potentially harmful degradation products suggesting diversity may be an imperfect marker. Another suggested marker of a healthy gut microbiome is the *Firmicutes/Bacteroidetes* (F/B) ratio (now denoted as *Bacillota/Bacteroidota* ratio). The F/B ratio was shown to be increased in individuals with obesity (Ley et al., 2006). However, the applicability of this ratio is limited, and a low F/B ratio does not necessarily indicate a healthy gut microbiome (Van Hul et al., 2024). Despite many efforts to identify a marker, there still lacks a consensus on what constitutes as a normal, healthy microbiome.

As mentioned previously, the microbiome is sensitive to changes. Dietary modifications, particularly those involving changes in fiber, fat, carbohydrate, and protein intake, can significantly impact microbial diversity (Perler et al., 2023). Xenobiotics like antibiotics are

known to disturb the gut microbiome. Jernberg et al. (2007) analyzed the long-term consequences of a 7-day clindamycin treatment on the fecal microbiome and found that *Bacteroides* did not return to its original level two years post-treatment. Changes in the gut microbiome from these different factors are not necessarily “good” or “bad”. They just demonstrate the elasticity of the gut microbiome and the various ways that interindividual or intraindividual variability can occur.

Several strategies, such as fecal microbiota transplant and biotics supplementation, have emerged to modulate the gut microbiome in ways that benefit host health. Fecal microbiota transplantation (FMT) involves transferring the microbiome of a healthy donor to a recipient in order to restore microbial health. FMT has been utilized as a treatment for *Clostridioides difficile* infection, a condition that typically arises from antibiotic-induced disruption of the microbiome, specifically overgrowth of *Clostridioides difficile* which can be pathogenic (Paaske et al., 2025). Transplantation of healthy donor stool can help re-establish microbial homeostasis, effectively curing the infection (Smits et al., 2013; Wang et al., 2019). Another well-known example is the use of biotics (i.e., prebiotics, probiotics, and postbiotics) where their ingestion aims to alter the gut microbiota in ways that confer health benefits (Gibson et al., 2017; Hill et al., 2014; Salminen et al., 2021). For example, several clinical trials that have studied the benefits of biotics on treatment of inflammatory bowel disease (IBD) (reviewed in Estevinho et al., 2024; Kavita et al., 2024; Kennedy et al., 2024). Overall, with increasing numbers of studies using FMT or biotics to treat various diseases, the microbiome has become a therapeutic target.

1.3. The Effect of Microbial Manipulation on Drug Disposition

1.3.1. What are pre-, pro-, and postbiotics?

The International Scientific Association for Probiotics and Prebiotics (ISAPP) is a non-profit organization that focuses on advancing the science of biotics. A panel of experts achieved a consensus on the definition and scope of each type of biotics. Firstly, prebiotics were defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). Prebiotics are substrates such as oligosaccharides that do not contain microorganisms but are utilized by live host microorganisms. The most extensively studied prebiotics are non-digestible oligosaccharides, fructans, and galactans (Rastall and Gibson, 2015). Probiotics were defined by ISAPP as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2013). Probiotics are administered as the bacteria or foods containing live microorganisms. Examples include foods like kimchi (Park et al., 2014), supplements for conditions like metabolic syndrome (Wastyk et al., 2023), or topical applications (Myles et al., 2018). Lastly, ISAPP defines postbiotics as “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” (Salminen et al., 2021). For example, inactivated lactic acid bacteria for the treatment of *Helicobacter pylori* infection (Canducci et al., 2000) and heat-inactivated *Bifidobacterium bifidum* MIMBb75 for inflammatory bowel syndrome (Andresen, Gschossmann, and Layer, 2020) have been studied as potential therapeutics. Though prebiotics, probiotics and postbiotics can provide health benefits and can be generally found in food and supplements, they differ in their mechanisms of action as well as their effects on the microbiome.

1.3.2. Probiotics and drug pharmacokinetics in animal models

As mentioned before, biotics consumption has the ability to alter gut microbiome composition. This change in the gut microbiome through biotic consumption is hypothesized to influence drug pharmacokinetics (PK). The impact of prebiotics and postbiotics on drug PK remains largely unexplored. In contrast, several studies have examined how different strains of probiotics influence drug disposition in animal models. These findings suggest that probiotics may have strain-specific effects on drug exposure and metabolism with implications for both therapeutic efficacy and safety.

Several probiotics have been shown to increase drug exposure or area-under-the curve (AUC). For example, a study looked at the effect of probiotic treatment on gliclazide (a type 2 diabetes drug) PK on healthy and diabetic rats ($n = 10$; Al-Salami et al., 2008). Specifically, treatment with *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacterium lactis* increased the exposure of gliclazide in diabetic rats (25% increase in AUC, $p < 0.05$) but decreased exposure in healthy rats (60.4% decrease in AUC, $p < 0.01$). The authors concluded that probiotic treatment in diabetic rats can be useful as an adjunct therapy as it can both help increase gliclazide exposure and lower blood glucose. A group based in Indonesia studied the effect of a probiotic (*Lactobacillus plantarum* IS-10506) on the hypertension drug, amlodipine in rabbits (Saputri et al., 2018). Overall, the authors saw a higher amlodipine exposure in the probiotic-treated group compared to control group ($p < 0.05$). In rats treated with direct intestinal administration of nifedipine and 4 weeks oral pre-treatment of *Lactobacillus casei* Shirota or control ($n = 6-7$), probiotic administration led to a significant increase in intestinal bioavailability (0.62 ± 0.07 vs. 0.89 ± 0.04 , $p < 0.05$; Kato et al., 2007). As no change in the fraction of mucosal uptake (F_a) was seen, they hypothesized that this increase was due to increased passage through

intestinal mucosa. As nifedipine is metabolized by CYP3A, there is a possibility that the underlying mechanism may be due to inhibition of intestinal CYP3A.

Other probiotics showed the opposite effect and decreased drug exposure. For example, in mice ($n = 6$), treatment with *Lactobacillus reuteri* K8 reduced AUC of oral acetaminophen (APAP) by 68.4% compared to control ($p < 0.01$; Kim et al., 2018). The authors suggested that K8 probiotic inhibited absorption of acetaminophen from the GI tract by either increasing metabolism or conjugation of APAP.

The influence of probiotics on drugs also seems to be both microbial strain and drug specific. For example, rats ($n = 3$) were treated with either a saline solution or an *Escheria coli* strain Nissle 1917 (EcN) or *E. coli* strain ATCC 25922 for seven days before treatment with a single oral dose of the antiarrhythmic drug amiodarone (Matuskova et al., 2014). AUC₀₋₃₀ of amiodarone was increased after EcN administration (9.31 ± 0.65 vs. 6.52 ± 0.60 h· μ g/mL) but not after *E. coli* ATCC 25922 (7.37 ± 0.53 vs. 6.74 ± 0.56 h· μ g/mL). This observed phenomenon may be due to the decrease in intestinal pH due to EcN which can facilitate movement of amiodarone through the mucosal layer. The lack of effect by the ATCC 25922 strain suggests that the effect of *E. coli* is strain specific. Another study was done using the probiotic VSL#3, which contains eight strains of bacteria, including *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *Bulgaricus*, *L. paracasei*, *L. plantarum*, *B. breve*, *B. infantis*, *B. longum*, and *Streptococcus thermophilus* (Zhang et al., 2024). In rats ($n = 9$), there were significant alterations in the bioavailability of several drugs and the gut microbiome. VSL#3 increased omeprazole bioavailability by 269.9%, but decreased tolbutamide and chlorpropamide bioavailability by around 28.1% and 27.4%, respectively. This shows that the impact of the probiotic can depend

on the drug being studied and their disposition pathways (e.g., transport, metabolism and excretion).

Although certain probiotics have shown to influence drug PK, there are those that did not have an effect under the conditions studied. For instance, when adult male mice ($n = 3$) were administered amoxicillin orally, no significant PK differences were observed between control mice and those pre-treated with the probiotic *Saccharomyces boulardii* CNCM I-745 (Florastor®; Selig et al., 2020). No significant changes were observed in C_{max} (17.11 ± 1.36 vs. 14.3 ± 0.55 $\mu\text{g/mL}$) and $AUC_{0-\infty}$ (17.44 ± 2.25 vs. 13.79 ± 0.233 $\text{h} \cdot \mu\text{g/mL}$). In a rat study ($n = 5-8$) where sulfasalazine and a probiotic mixture (*Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Streptococcus salivarius*) were co-administered, no significant changes in PK parameters of sulfasalazine or its metabolite sulfapyridine were observed (Lee et al., 2012).

In summary, these studies illustrate the complex and varied effects of probiotics on drug pharmacokinetics. The strain-specific and drug-specific nature of these effects underscores the need for further research to fully understand how probiotics may influence drug absorption, metabolism, and therapeutic outcomes.

1.3.3. Probiotics and drug efficacy in humans

Some studies have been done in humans to investigate whether probiotics can increase treatment efficacy. For example, the effect of probiotic-based colonoscopy preparation was tested in children aged 2-15 years (Kesavelu, 2020). Addition of the *Bacillus coagulans* probiotic with a sennoside indicated a successful bowel preparation, with a Boston Bowel Preparation Scale of 2.93 ± 0.25 and 96.67% of patients had an accessible terminal ileum. However, this study did not have a control group for reference. Separately, in a 3-month randomized, double-blind, placebo-

controlled clinical trial, 82 Parkinson's disease patients were divided into either a probiotic (n = 48; *Bifidobacterium animalis* subsp. *Lactis* Probio-M8 (Probio-M8)) or placebo (n = 34) group (Sun et al., 2022). Both groups received treatments for their Parkinson's disease symptoms with benserazide (a DOPA decarboxylase inhibitor) and dopamine agonists. A few efficacy markers were improved in the Probio-M8 compared to placebo group, such as decrease in Hamilton Anxiety Scale scores (change = 4.48 vs. 1.06, $p = 8.6 \times 10^{-10}$) and increase in Parkinson's Disease Sleep Scale (change = 9.94 vs. 1.44, $p = 0.095$). These results suggest that co-administration with Probio-M8 may augment the efficacy of the Parkinson's disease treatments. Lastly, in a randomized, double-blind, and placebo-controlled trial of 159 patients with breast cancer, patients were divided into either a probiotic (*Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*; n = 80) or placebo (n = 79) group (Zhang et al., 2022). All patients were receiving standard chemotherapy. The probiotic group saw a decreased incidence of chemotherapy-related cognitive impairment compared to control (35% vs. 81%, $p < 0.001$), suggesting that probiotics can reduce adverse effects of breast cancer therapy. Outside of direct administration of probiotics, other studies have been done to show the association between treatment efficacy and certain bacteria. A notable example is *Akkermansia muciniphila*, where its abundance has been shown to be positively correlated to clinical response of Programmed Death-1 (PD-1)-based immunotherapy that uses checkpoint inhibitor drugs (e.g., pembrolizumab, atezolizumab, etc.) to block the PD-1 protein on T-cells from binding to programmed cell death ligand 1 (PD-L1) on cancer cells (Derosa et al., 2022; Routy et al., 2017).

The manipulation of the gut microbiome through probiotic administration has the potential to synergize drug efficacy. These human studies demonstrate that the gut microbiome

and drugs have a complex relationship and should be investigated further to improve therapeutics.

1.4. Interactions between Drugs and The Gut Microbiome

The relationship between the gut microbiome and drugs is bidirectional, with each capable of influencing the other (Dempsey and Cui, 2019; Spanogiannopoulos et al., 2016). Drugs can exert antimicrobial effects or stimulate the growth of certain bacterial species, ultimately altering the composition of the gut microbiome (Baldanzi et al., 2026; Kinney et al., 2000). Conversely, the microbiome can influence drug PK and pharmacodynamics (PD), with these interactions broadly categorized into direct and indirect mechanisms (Dempsey and Cui, 2019; Spanogiannopoulos et al., 2016; Figure 1.1).

1.4.1. Direct Interaction

As orally administered drugs can be absorbed into the gastrointestinal tract, they can encounter and interact with gut microbial species (Peter et al., 1974). This interaction is classified as a direct interaction. Direct interactions between drugs and the gut microbiome encompass three primary processes: drug metabolism, enterohepatic recirculation, and bioaccumulation (Klünemann et al., 2021; Spanogiannopoulos et al., 2016). A review by Scheline in 1973 proposed that the gut microbiome can act as its own metabolic organ. Specifically, microbes are thought to be able to metabolize certain drugs and other xenobiotics. Historically, it was assumed that only drugs that reach the distal gut have significant interactions with microbes (Sousa et al., 2008). However, emerging reports on the different effects of the gut microbiome on drug PK have suggested that the microbiome plays a bigger role than initially

expected (Enright et al., 2016). This section will explore the three major types of direct interactions mentioned.

Metabolism of Drugs. The gut microbiome can directly metabolize drugs through processes like reduction and hydrolysis (Haiser and Turnbaugh, 2013). The consequences of this microbial drug metabolism fall into three categories: (1) drug inactivation, (2) prodrug activation, and (3) production of toxic metabolites (Spanogiannopoulos et al., 2016). For drug inactivation, a notable example is the reduction of digoxin by *Eggerthella lenta*, which inactivates the drug into dihydrodigoxin (Dobkin et al., 1983). Specifically, when *E. lenta* was co-cultured with fecal microbiomes of five individuals, digoxin reduction was enhanced from ~20% to 95.39%, highlighting the role *E. lenta* played in digoxin metabolism (Haiser et al., 2014). For prodrug activation, the gut microbiome converts prodrugs from their inactive form into an active therapeutic form and improves absorption and bioavailability particularly for drugs with poor solubility (Wilson and Nicholson, 2017). Prontosil undergoes azo-reduction in the intestine and the active antimicrobial agent, sulfanilamide, is a result of microbial metabolism of the prodrug (Gingell and Bridges, 1973). Furthermore, rats treated with antibiotics before prontosil administration had reduced formation of sulfanilamide and urine metabolite recovery decreased from 81% to 43% (Gingell, Bridges, and Williams, 1971). Lastly, irinotecan is an example of drug-induced toxicity mediated by the microbiome (Wiseman and Markham, 1996). SN-38 is an active metabolite of irinotecan and is detoxified through glucuronidation in the liver, forming SN-38G. However, bacterial enzymes in the gut can hydrolyze SN-38G back to the toxic SN-38, which can cause significant side effects and dose-limiting toxicities. Common drug-related adverse events include diarrhea, nausea, and vomiting. *In vitro* studies showed that physiologically relevant SN-38 concentrations reflecting plasma concentrations after a single

irinotecan infusion resulted in significant cytotoxicity (Mathijssen et al., 2002). Overall, drug metabolism by the gut microbiome can result in varying effects, both positive and negative, and should be a factor to consider in the drug development process.

Enterohepatic recirculation. Irinotecan also undergoes enterohepatic circulation. With enterohepatic recirculation, the drug is conjugated in the liver with glucuronide or sulfate and the conjugated metabolite is excreted into the bile where in the intestine, the conjugated metabolite can be deconjugated by gut microbes back into the parent form for reabsorption (Figure 1.2; Roberts et al., 2022). Enterohepatic recirculation can appear as additional peaks in the plasma concentration vs. time profile and leads to altered PK (i.e., prolonged half-life and increased AUC; Kim et al., 2015). In the case of irinotecan, this deconjugation leads to formation of the active metabolite, SN-38, which causes intestinal toxicity (Wiseman and Markham, 1996).

Bioaccumulation. Bioaccumulation is a recently recognized phenomenon wherein gut bacteria sequester drugs intracellularly without chemically altering the drug. Bioaccumulation was identified in experiments where 25 bacterial strains were cultured with 15 selected oral drugs (Klünemann et al., 2021). After 48 hours of co-culture with the drug, in 17 of the drug-bacteria combinations, drugs were depleted in supernatant but recovered from total culture extracts. They hypothesized that those drugs were accumulating in the bacterial cells. The authors further studied duloxetine, an antidepressant, and saw that bioaccumulation led to two major outcomes: reduced drug bioavailability as the sequestered drug failed to reach systemic circulation and changes in bacterial metabolism due to drug interactions with bacterial enzymes.

1.4.2. Indirect Interaction

Direct interactions between gut microbes and drugs are straightforward while indirect interactions have intermediate steps. The indirect interaction between the gut microbiome and drugs can be classified into two categories: (1) competition and (2) influence on nuclear receptors (Dempsey and Cui, 2019; Spanogiannopoulos et al., 2016). In both cases, the gut microbes do not exert its effect on the drug itself, but rather produce a metabolite that can overall influence drug PK. This type of interaction can be harder to elucidate as it involves complex host-microbial interactions.

Competition. An example of competition is the interaction between the bacterial-derived metabolite *p*-cresol with APAP. *p*-Cresol is a product of tyrosine metabolism and is metabolized by SULT1A1 to *p*-cresol sulfate (Gryp et al., 2017). APAP is metabolized by SULT1A1 into an inactive metabolite, APAP-sulfate (Clayton et al., 2009). *p*-Cresol can competitively inhibit SULT1A1, forcing APAP to be metabolized through other pathways. Increased metabolism of APAP by CYP2E1 forms a toxic metabolite, N-acetyl-*p*-benzoquinone imine (NAPQI), which can cause drug-induced liver injury (Clayton et al., 2009; Spanogiannopoulos et al., 2016). Therefore, individuals taking APAP with intrinsically high *p*-cresol levels or exposed to external factors that result in elevated *p*-cresol levels could have altered APAP PK.

Influence of Host Receptors. Nuclear receptors are ligand-activated transcription factors that regulate cellular processes (Mangelsdorf et al., 1995). An example of a nuclear receptor that is associated with drug metabolizing enzyme and transporter expression is the pregnane X receptor (PXR). PXR is expressed in the liver, intestine, and kidney (Pavek, 2016). Activation of PXR leads to heterodimerization with retinoid X receptor (RXR), binding to sites in the promoter

region of specific drug metabolizing enzymes (e.g., CYP3A4), and increased CYP3A4 expression (Pavek, 2016).

For example, lithocholic acid (LCA), a PXR ligand, is formed by the metabolism of the primary bile acid chenodeoxycholic acid (CDCA) or the secondary bile acid ursodeoxycholic acid (UDCA) by bacterial 7-dehydroxylation in the intestine (Hofmann, 2004). LCA can enter the systemic circulation and reach the liver where it can activate PXR, leading to upregulation of cytochrome P450 3a11 (Cyp3a11) expression in mice (Staudinger et al., 2001). Therefore, endogenous or exogenous factors that affect the microbiome and result in changed microbial metabolite levels, could impact the PK of xenobiotics that rely on CYP3A4 metabolism or other DMETs regulated by PXR.

1.5. Interaction between Microbial Metabolites and Nuclear Receptors

1.5.1 Microbial metabolites

Microbial metabolites can be broadly classified into three categories: (1) those directly produced by bacteria from dietary components, (2) those synthesized by the host and subsequently modified by gut bacteria, and (3) those synthesized de novo by gut microbes (Postler and Ghosh, 2018). The three most extensively studied groups of microbial metabolites include bile acids (BAs), short-chain fatty acids (SCFAs), and tryptophan metabolites.

Primary BAs synthesized in the liver are metabolized by bacteria in the gut into secondary BAs, such as deoxycholic acid (DCA) and LCA, which are the predominant bile acids in human feces (Klaassen and Cui, 2015). Bile acids are not only crucial for lipid digestion but also exhibit antimicrobial properties and are thought to play a regulatory role in shaping the gut microbiome (Stacey and Webb, 1947). They activate multiple different nuclear receptors like

PXR (Staudinger et al., 2001), the constitutive androstane receptor (CAR; Guo et al., 2003), the vitamin D receptor (VDR; Makishima et al., 2002), and the farnesoid X receptor (FXR), which is involved in the regulation of bile acid synthesis (Clausel et al., 2005).

SCFAs, including acetate, propionate, and butyrate, are end-products of the fermentation of dietary fibers by gut microbes (den Besten et al., 2013). SCFAs have been shown to play diverse roles, ranging from influencing host energy metabolism to modulating immune responses (den Besten et al., 2013; Kim, 2023). Their potential health benefits have been investigated in relation to various diseases, including obesity, cancer, and diabetes (Xiong et al., 2022).

Tryptophan metabolism occurs through three major pathways: 5-hydroxytryptamine (i.e., serotonin), kynurenine, and indole pathways (Agus et al., 2018). Several metabolites of tryptophan metabolism are ligands for the aryl hydrocarbon receptor (AhR), and their binding has been associated with anti-inflammatory effects and other health benefits (Roager and Licht, 2018). Dysregulation of tryptophan metabolism has been implicated in various diseases, suggesting that restoration of these pathways may alleviate symptoms (Agus et al., 2018).

These three microbial metabolite groups have been studied as potential regulators of drug disposition through activation of nuclear receptors. Metabolites that have been studied are summarized in Table 1.1.

1.5.2. Bile Acids (BA)

In 2001, Staudinger et al. (2001) identified LCA as an activator of PXR and inducer of Cyp3a11 in mice. Specifically, in wild-type mice, the administration of 0.5% LCA significantly increased protein expression of Cyp3a11 compared to vehicle-treated mice in the liver but the induction was not seen in PXR^{-/-} mice which led to the suggestion that LCA is an activator of

PXR. In a subsequent study, Toda et al. (2009) supplemented germ-free (GF) mice with increasing doses of LCA (0.01% and 0.05%) and found that hepatic *Cyp3a11* expression increased and was nearly comparable to *Cyp3a11* in conventional (CV) mice. PXR expression was much higher in LCA-supplemented GF mice compared to CV mice. Separately, LCA was also shown to activate human VDR with an EC₅₀ of 8 μ M (Makishima et al., 2002).

Interestingly, PXR required at least a 10-times greater concentration of LCA for activation, indicating that LCA binds VDR more strongly. Matsubara et al. (2008) further confirmed these results where they introduced siRNA of human VDR but not human PXR into their cell lines and led to reduced LCA activation of the CYP3A4 reporter gene in both HepG2 and LS174T cells. Furthermore, when mice were dosed with LCA at 100 mg/kg/day for 3 days, Cyp3a induction was only observed in small intestine but not liver, contradicting previous studies.

Apart from LCA, other BAs have been studied as potential regulators of DMETs. For example, Matsubara et al. (2008) studied chenodeoxycholic acid (CDCA) and cholic acid (CA) *in vitro*. Specifically, they saw that CDCA weakly induced *CYP3A4* in HepG2 cells but not LS174T cells, and CA had no effect. Another study found that acetylated deoxycholic acid (DCA) was a potent ligand of PXR *in vitro* (Carazo et al., 2017). Specifically, acetylated DCA activated PXR with an EC₅₀ of 32.1 μ M and induced *CYP3A4* mRNA expression by ~7-fold in HepaRG cells.

1.5.3. Short Chain Fatty Acids (SCFA)

In human pluripotent stem cell-derived liver organoids, mixed SCFA treatment (1:1:1 acetate:propionate:butyrate) led to increased CYP3A4 expression, although its effect on nuclear receptors was not investigated (Mun et al., 2021). SCFAs have been shown to activate the aryl

hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptor- α (PPAR α). In an intestinal epithelial cell line, the SCFA butyrate at 2 mM was shown to induce AhR and subsequently CYP1A1 expression (Marinelli et al., 2019). In rat hippocampus, 3-hydroxy-(2,2)-dimethyl butyrate was found to be a ligand of PPAR α with an EC₅₀ of 32.85 μ M (Roy et al., 2016).

1.5.4. Tryptophan metabolites

In vitro, indole 3-propionic acid (IPA) was a more potent agonist for mouse PXR compared to human PXR (EC₅₀ = 0.55 μ M and 120 μ M, respectively; Venkatesh et al., 2014). Interestingly, the activation of PXR, as monitored by a PXR reporter gene in 293T cells, was stronger when 1 mM of indole was combined with increasing concentrations of IPA. Another study investigated metabolites of tryptophan and found that indole (EC₅₀ = 1485 μ M), tryptamine (EC₅₀ = 83.15 μ M), indole-3-pyruvate (EC₅₀ = 98.53 μ M), indole-3-acrylate (EC₅₀ = 42.25 μ M), indole-3-acetamide (EC₅₀ = 99.95 μ M) were AhR agonists and led to CYP1A1 induction in hepatic and intestinal cell lines (Vyhldalová et al., 2020). The other metabolites tested (i.e., indole-3-acetate, indole-3-aldehyde, indole-3-ethanol, indole-3-lactate, indole-3-propionate) were shown to be inactive. More recently, indoxyl sulfate, which is a uremic toxin, was found to induce CYP3A expression and activity in HepaRG, DPX-2, and primary human hepatocyte cells (Togao et al., 2025). However, interindividual variability was observed in the response to indoxyl sulfate treatment in three separate hepatocyte donors. A small inhibitory effect at higher concentrations was observed in one donor and further testing with human liver microsomes indicated weak CYP3A inhibition at 500 μ M indoxyl sulfate.

Although most studies focus on the indole pathway and there are no studies on the kynurenine pathway, a study was done where rats were fed a tryptophan-free diet and serotonin concentrations were measured in the brain and plasma (Kot, Pilc, and Daniel, 2012). A tryptophan-free diet for 3, 7, or 21 days led to increases in plasma serotonin but not brain serotonin after a week but a reduction in both brain and plasma serotonin after three weeks. The authors observed different patterns in the CYP activity at 3, 7, and 21 days. After 3 days, activity of CYP2C11 decreased, while CYP2B, CYP2C6, and CYP3A activity increased. Interestingly, after one week, the activities of CYP2B and CYP2C11 decreased while CYP2A, CYP2C6, and CYP3A increased. At three weeks, CYP2C11 activity decreased but the other CYP isoforms increased. The authors concluded that a tryptophan deficit in the diet may lead to alterations in drug metabolism.

1.5.5. Other microbial metabolites

Although the focus has been mostly on SCFAs, BAs, and tryptophan metabolites, other microbial-related metabolites may play a role in CYP3A4 regulation. In a study looking at diet and phosphatidylinositol 3-kinase anticancer synergy, the authors found that when mice were fed a soy protein diet, hepatic *Cyp3a11* expression was induced compared to mice fed a casein protein diet (Roichman et al., 2025). The authors identified microbial metabolites of soyasaponins, soyasapogenols A and B, that were responsible for the induction. The influence of soyasapogenols were determined to be PXR-mediated with an EC₅₀ of 4 µM for soyasapogenol A and 5.7 µM for soyasapogenol B.

1.6. Regulation of Cyp3a by The Gut Microbiome

1.6.1 Cytochrome P450 3A4

The cytochrome P450 3A (CYP3A) subfamily is the most abundant and highly expressed P450 in the small intestine and liver, responsible for the metabolism of 30-40% of clinically used drugs like statins, antivirals, and immunosuppressants (Paine et al., 2006; Shimada et al., 1994; Zanger et al., 2013). In humans, the CYP3A subfamily consists of 4 isoforms, CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4, the most abundant CYP3A in adults, can vary as much as 40-fold between individuals when compared in both liver and small intestine tissues (Zanger et al., 2013; Williams et al., 2002; Lamba et al., 2002). The variability in hepatic and intestinal CYP3A4 expression and/or activity can arise from several known factors such as drug-drug interactions (Olkola et al., 1994), inflammation (Molanaei et al., 2018), and pregnancy (Fashe et al., 2023) and factors yet to be fully elucidated. Variability in CYP3A4 can cause differences in bioavailability and drug clearance and may impact the efficacy and/or toxicity of drugs, especially drugs with a narrow therapeutic index, which are drugs with a small difference between the effective and toxic doses (Lamba et al., 2002; Kuehl et al., 2001; Thummel et al., 1996). Multiple animal studies have emerged suggesting that the gut microbiome can influence Cyp3a and possibly contribute to interindividual variability.

1.6.2. Effect of microbiome manipulation on Cyp3a in animals

Animal models such as antibiotic-treated and germ-free (GF) animals have been employed to explore the relationship between the gut microbiome and Cyp3a (Table 1.2; Figure 1.3). Each type of model has different advantages and drawbacks. For example, antibiotic treatment is relatively inexpensive, but not all bacteria are eliminated, while germ-free models

are free of all microorganisms, but developmental defects exist (Kennedy, King, and Baldrige, 2018). Additionally, some groups use specific pathogen-free (SPF) animals as a control group while others use a conventional (CV) group. SPF animals are those that have been screened for specific pathogens while CV animals are thought to have a more unrestricted, natural microbiome (Dobson et al., 2019). Sequencing data on microbiome composition can aid in translation of findings as confounding factors can exist between types of animals studied and even the geographical location of research groups.

Antibiotic-treated animals. When 6-week-old Sprague-Dawley male rats ($n = 6-7$) were treated with ciprofloxacin, hepatic *Cyp2c11* and *Cyp3a1* mRNA expression was decreased (2-fold and 5-fold, respectively, $p < 0.05$; Xie et al., 2003). Similarly, in 10-week-old C57BL/6 male mice ($n = 4$) that were treated with vancomycin and polymyxin B, liver *Cyp3a11* protein expression was decreased to 0.116-fold ($p < 0.001$; Kuno et al., 2016). Lastly, 6-to-8-week-old Sprague-Dawley male rats ($n = 6$) were treated with an antibiotic cocktail of ampicillin, metronidazole, neomycin, and vancomycin every 2 days for a total of four different time points (1, 2, 3, and 4 weeks; Wang et al., 2023). Interestingly, hepatic CYP3A protein expression between the antibiotic-treated rats vs. control at the 1-week time point was unchanged and CYP3A expression was not quantified at other timepoints.

Germ-free animals. Decreased *Cyp3a* expression has been consistently reported in GF animals compared to controls. Firstly, Toda et al. (2009) explored the differences in *Cyp450* between 9-week-old male GF and SPF IQI mice ($n = 3-5$). They found that GF mice had lower mRNA expression of *Cyp3a11* (~5-fold, $p < 0.01$), *Cyp3a16* (~10-fold, $p < 0.01$), *Cyp3a25* (~2-fold, $p < 0.05$), *Cyp3a41* (~10-fold, $p < 0.05$), *Cyp3a44* (5-fold, $p < 0.01$) and a 1.4-fold lower *Cyp3a* protein level in the liver ($p < 0.05$) compared to SPF mice. In 2 to 3-month-old male

C57BL/6 mice, GF mice had an 87% decrease in hepatic *Cyp3a11* mRNA expression and undetectable hepatic Cyp3a11 protein expression compared to CV mice ($p < 0.05$; Fu et al., 2017). In the same group of mice, GF mice had lower *Cyp3a11* expression in the duodenum (60%, $p < 0.05$) and ileum (86%; $p < 0.05$) compared to CV mice. An age dependent change in Cyp3a11 was noted in male C57BL/6 mice that were studied from 1 to 90 days of age ($n = 4$; Selwyn et al., 2015b). No difference was noted in hepatic *Cyp3a11* mRNA expression at 1 to 30 days of age, but at 90 days of age, GF mice had an 80% decrease in *Cyp3a11* mRNA expression, ~2-fold decrease in Cyp3a11 protein expression, and 2.5-fold lower hepatic Cyp3a activity compared to CV mice ($p < 0.05$ for all comparisons). In a separate study of 10-week-old C57BL/6 mice ($n = 3$), GF mice had 0.172-fold lower Cyp3a11 hepatic protein expression compared to SPF mice ($p < 0.001$; Kuno et al., 2016). Lastly, liver microsomes from 10-week-old male GF and SPF BALB/c mice ($n = 5$) were prepared and incubated with midazolam (Togao et al., 2020). The intrinsic clearance ($CL_{int} = V_{max} / K_m$) for 1'-hydroxymidazolam formation and 4-hydroxymidazolam formation were lower in GF mice compared to SPF mice (4.27-fold and 3.20-fold, respectively, $p < 0.001$). Studies have conclusively demonstrated that the absence of the microbiome in male GF animals leads to reduced Cyp3a expression and activity, indicating that the gut microbiome can play a regulatory role on Cyp3a.

Conventionalization. To confirm the effect of the microbiome, follow-up studies have employed a conventionalization method in which the microbiome is restored either through probiotic administration, re-exposure to the conventional environment, or fecal transplantation of CV or SPF mouse feces to GF mice. Selwyn et al. (2016) employed two of these techniques in their study in 3-month-old male C57BL/6 mice ($n = 6-8$). When GF and CV mice were administered the probiotic VSL#3, hepatic Cyp3a11 mRNA and protein expression were not

altered compared to their GF and CV counterparts. However, when GF mice were conventionalized through re-exposure of conventional environment for 2 months (GF+CV), hepatic Cyp3a11 mRNA, protein, and activity approached CV levels ($p < 0.05$ for each comparison). Togao et al. (2021) employed a fecal transplantation method to conventionalize 8-week-old male GF BALB/c mice ($n = 6$) mice and observed that the degree of restoration varied depending on the mouse donor. Specifically, they used three different mouse donors (Jcl, Slc, and Crj). Although all three fecal transplants resulted in significantly higher Cyp3a activity compared to control ($p < 0.001$), there were subtle differences in Cyp3a activity restoration with the Slc mice having a significantly higher hepatic Cyp3a activity compared to Jcl and Crj mice ($p < 0.001$ and $p = 0.002$, respectively). Lastly, when 5-week-old male GF BALB/c mice ($n = 8$) were inoculated with two separate human donor feces, hepatic Cyp3a activity was restored in both groups compared to control but differed in fold-change (Togao et al., 2023).

These studies highlight the influence of the gut microbiome on Cyp3a enzymes. The variations in findings between different models emphasize the complexity of microbiome-enzyme interactions, impact of gut microbiome's composition and the need for further research to delineate the specific microbial populations responsible for these effects.

1.6.3. Sex differences

Although the previously discussed studies were all conducted in male mice, a few studies explored the microbial regulation of Cyp3a in female mice. Sex differences in Cyp3a isoform expression exist in mice which makes studying the microbial regulation of Cyp3a between sexes complicated. For example, in female C57BL/6 mice ($n = 4$), GF mice had a higher though not significant expression of hepatic *Cyp3a11* mRNA than CV mice at 30 days, but ~5-fold lower

expression at 90 days ($p < 0.05$; Selwyn et al., 2015b). This age-related expression difference was not noted in male mice (i.e., male GF mice had lower *Cyp3a11* mRNA expression at all ages). Jourová et al. (2020) reported that in 2-month-old BALB/c mice ($n = 5$), hepatic *Cyp3a11* mRNA expression was 21-fold higher in female SPF mice vs. male SPF mice, but there was no sex difference in GF mice. Interestingly, they observed ~15-fold lower *Cyp3a11* expression in female GF vs. female SPF mice ($p < 0.05$). When they quantified Cyp3a activity in liver microsomes, Cyp3a activity decreased by 3.3-fold in male GF vs. male SPF mice ($p < 0.05$) and by 3.5-fold in female GF vs. female SPF mice. The effect of pregnancy was tested in 8-to-15-week-old female C57BL/6 mice ($n = 5-6$; Han et al., 2020). Cyp3a activity was reduced ~4-fold in pregnant GF vs. pregnant CV mice ($p < 0.05$) but no difference was observed in non-pregnant GF and CV mice. Overall, these studies demonstrate that sex is a complicating factor in the interaction between the gut microbiome and Cyp3a.

Human studies indicate that women generally have higher bacterial diversity compared to men (Yoon and Kim, 2021). The sex differences may be partially attributed to variations in sex-specific hormones. Shin et al. (2019) found that higher testosterone levels in men and elevated estradiol levels in women were associated with increased microbial diversity. Furthermore, in population studies, women have higher CYP3A4 activity than men (Tanaka, 2003). Several studies have suggested that the higher CYP3A4 activity in females is due to activation of PXR and CAR by sex hormones (Woldbold et al., 2003; Timsit and Negishi, 2007; Waxman and Holloway, 2009). As of yet, the association between microbial diversity and CYP3A4 expression in humans has not been studied.

1.6.4. Limitations of animal studies

Despite the advantages of animal models in studying the microbiome, several limitations exist in the translatability of the results to humans. First, interspecies differences exist in the regulation of drug metabolizing enzymes. For example, in humans, rifampicin is a potent inducer of PXR that leads to an increase in CYP3A4 levels (Backman, Olkkola, Neuvonen, 1996). However, pregnenolone 16 α -carbonitrile (PCN) is the prototypical mouse PXR ligand and Cyp3a11 inducer (Cheng et al., 2010; Guzelian et al., 2007). One way that researchers have overcome the difference in PXR ligand binding is by creating a humanized mouse model where mouse PXR, CAR and Cyp3a genes are replaced with human orthologs (i.e., PXR-CAR-CYP3A4/3A7 mouse model). When comparing the humanized PXR-CAR-CYP3A4/3A7 mice to wild-type mice, the humanized mouse model was able to capture CYP3A4 clinical inhibition and induction within the same magnitude as expected with a few exceptions (Ly et al., 2017). For example, rifampicin treatment did not change hepatic Cyp3a in the wild-type mice while a 3.3-fold increase in CYP3A4 was observed in PXR-CAR-CYP3A4/3A7 mice. Another limitation is that some human microorganisms do not thrive in the mouse gut. A review by Lundberg (2018) summarized the challenges of human microbial colonization in animal models and explained how difficult it can be to study human-specific microbial species in animals. Lastly, a universal issue is that GF mice are not equivalent to CV mice without a microbiome. The breeding and housing of GF mice result in GF mice having different anatomical values compared to CV mice, such as an enlarged cecum (Coates, 1975). GF mice also have reduced oxygen consumption and body fat compared to CV mice (Bäckhed et al., 2004). These differences in anatomy and physiology could be confounding factors in the regulation of Cyp3a.

1.7. Conclusions

The gut microbiome is highly variable between individuals and its composition and diversity can be modified by approaches such as biotic administration. Manipulation of the gut microbiome by probiotics has been shown to impact PK in both animal models and humans. This interplay demonstrates the potential regulatory role that the microbiome has on DMETs with an observable impact on drug PK. Several types of interactions between the gut microbiome and drugs have been explored, highlighting their complex relationship (Figure 1.1). Notably, microbial metabolites have been shown to activate nuclear receptors and alter the expression of downstream drug metabolizing enzymes and transporters (Table 1.1). Specifically, three major groups of microbial metabolites (bile acids, short chain fatty acids, and tryptophan metabolites) have been investigated and demonstrated activation of certain nuclear receptors, leading to induction of downstream genes. One of these genes is the drug metabolizing enzyme, CYP3A4. As CYP3A4 is responsible for the metabolism of the majority of drugs on the market, it is important to elucidate factors that can lead to alterations in CYP3A4 activity and understand the mechanisms underlying CYP3A4 regulation. Several animal studies have suggested that the gut microbiome is potentially one of those factors and therefore needs to be further studied as a regulator of CYP3A4. Most studies concluded that the absence or reduction in the gut microbiome led to a decreased Cyp3a expression and activity and restoring the microbiome through conventionalization methods led to a partial restoration in Cyp3a (Table 1.2; Figure 1.3).

Although a lot of work has been done to study the relationship between the gut microbiome and CYP3A4, several knowledge gaps still exist. First, no study to date has been done to investigate the impact of the gut microbiome on human CYP3A4 activity. A clinical study with midazolam as a probe substrate could be done to identify whether normal variation or

manipulation of the gut microbiome (e.g., through biotic supplementation) influences CYP3A4 activity. The sex differences seen in the microbial regulation of Cyp3a in animal models also remain unexplained. Mechanistic studies should be done to try to unravel this phenomenon. Another knowledge gap is that most studies on microbial metabolites and CYP3A4 are focused on the three major groups so work should be done to identify other possible microbial metabolites that can regulate CYP3A4. Lastly, the impact of microbial metabolites on CYP3A4 has only been studied *in vitro* and in animal models. There needs to be clinical studies done to validate these findings in humans.

In conclusion, the gut microbiome can play a role in drug disposition and action, specifically through regulation of CYP3A4. Therefore, the goals of pharmacomicrobiomics for CYP3A4 encompass understanding the contribution of the microbiome to CYP3A4 variability, the mechanisms behind microbial regulation of CYP3A4 and the identification of regulatory microbial metabolites.

1.8. References

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1.9. Tables and Figures

Table 1.1. Microbial metabolites that have been shown to have an impact on nuclear receptors.

Metabolite	Group	Nuclear receptor	<i>In vitro</i> data	Mouse data
Lithocholic acid	Bile acid	PXR	EC50 = ~80 μ M (Makishima et al., 2002)	Hepatic Cyp3a11 induction (Staudinger et al., 2001; Toda et al., 2009)
		VDR	EC50 = 8 μ M (Makishima et al., 2002)	Intestinal Cyp3a11 induction (Matsubara et al., 2008)
Acetylated DCA		PXR	EC50 = 32.1 μ M, CYP3A4 induction in HepaRG cells (Carazo et al., 2017)	N/A
Acetate: Propionate: Butyrate = 1:1:1	Short chain fatty acid	PXR	CYP3A4 induction in stem cell-derived liver organoids (Mun et al., 2021)	N/A
Butyrate		AhR	AhR and CYP1A1 induction in HT-29 cells (Marinelli et al., 2019)	N/A
3-hydroxy-(2,2)-dimethyl butyrate		PPAR α	N/A	EC50 = 32.85 μ M (Roy et al., 2016)
Indole 3 -propionic acid	Tryptophan	PXR	EC50 = 0.55 μ M for mPXR, EC50 = 120 μ M for hPXR (Venkatesh et al., 2014)	N/A
Indole		AhR	EC50 = 1485 μ M (Vyhlídalová et al., 2020)	N/A
Tryptamine		AhR	EC50 = 83.15 μ M (Vyhlídalová et al., 2020)	N/A
Indole-3-pyruvate		AhR	EC50 = 98.53 μ M (Vyhlídalová et al., 2020)	N/A
Indole-3-acrylate		AhR	EC50 = 42.25 μ M (Vyhlídalová et al., 2020)	N/A
Indole-3-acetamide		AhR	EC50 = 99.95 μ M (Vyhlídalová et al., 2020)	N/A
Indoxyl sulfate		PXR	Induction and inhibition of CYP3A (Togao et al., 2025)	N/A
Serotonin		N/A	N/A	Potential influence on CYP450s

Soyasapogenol	Soyasaponins	PXR	EC50 = 4 μ M for soyasapogenol A and 5.7 μ M for soyasapogenol B (Roichman et al., 2025)	Induces hepatic <i>Cyp3a11</i> (Roichman et al., 2025)
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Table 1.2. Summary of animal studies studying the impact of the microbiome on Cyp3a.

Study	Animal Model	Comparison	Impact on Cyp3a
<i>Antibiotic treatment</i>			
Xie et al., 2003	6-week-old Sprague-Dawley male rats (n = 6-7)	Ciprofloxacin-treated vs. Control	Ciprofloxacin treatment reduced hepatic <i>Cyp3a1</i> mRNA expression by ~5-fold ($p < 0.05$)
Kuno et al., 2016	10-week-old C57BL/6 male mice (n = 4)	Vancomycin and polymyxin B treated vs. SPF	Vancomycin and polymyxin B treatment reduced Cyp3a11 liver protein expression by 0.116-fold ($p < 0.001$)
Wang et al., 2023	6-to-8-week-old Sprague-Dawley male rats (n = 6)	Antibiotic cocktail (Ampicillin, metronidazole, neomycin, and vancomycin) vs. Control	No difference in hepatic CYP3A protein expression between the antibiotic-treated vs. control rats at the 1-week time point
<i>Germ-free</i>			
Toda et al., 2009	9-week-old IQI male mice (n = 3-5)	GF vs. SPF	GF mice had a lower mRNA expression of <i>Cyp3a11</i> (~5-fold, $p < 0.01$), <i>Cyp3a16</i> (~10-fold, $p < 0.01$), <i>Cyp3a25</i> (~2-fold, $p < 0.05$), <i>Cyp3a41</i> (~10-fold, $p < 0.05$), <i>Cyp3a44</i> (5-fold, $p < 0.01$) and a 1.4-fold lower ($p < 0.05$) Cyp3a protein level in the liver
Kuno et al., 2016	10-week-old C57BL/6 male mice (n = 3)	GF vs. SPF	GF mice demonstrated a 0.172-fold lower ($p < 0.001$) Cyp3a11 hepatic protein expression compared to SPF mice
Selwyn et al., 2015a	2 to 3-month-old C57BL/6 male mice (n = 3)	GF vs. CV	In the liver, GF mice had an 87% decrease in <i>Cyp3a11</i> mRNA expression, and an undetectable Cyp3a11 protein expression compared to CV mice ($p < 0.05$)

Selwyn et al., 2015b	90-day-old C57BL/6 male mice (n = 4)	GF vs. CV	GF mice had an 80% decrease in <i>Cyp3a11</i> mRNA expression and ~2-fold decrease in Cyp3a11 protein expression compared to CV mice ($p < 0.05$). Cyp3a enzyme activity in liver microsomes also showed a 2.5-fold lower activity in GF mice ($p < 0.05$)
Selwyn et al., 2015b	90-day-old C56BL/6 female mice (n = 4)	GF vs. CV	GF mice had a ~5-fold lower <i>Cyp3a11</i> hepatic mRNA expression compared to CV mice ($p < 0.05$)
Fu et al., 2017	2 to 3-month-old C57BL/6 male mice (n = 3)	GF vs. CV	GF mice demonstrated a lower <i>Cyp3a11</i> mRNA expression in the duodenum (60%, $p < 0.05$) and ileum (86%; $p < 0.05$)
Togao et al., 2020	10-week-old BALB/c male mice (n = 5)	GF vs. SPF	Intrinsic clearance ($CL_{int} = V_{max} / K_m$) of both 1'-OH (4.27-fold) and 4-OH midazolam (3.20-fold) were consistently lower in GF vs. SPF mice ($p < 0.001$)
Jourová et al., 2020	2-month-old BALB/c male and female mice (n = 5)	GF vs. SPF	Hepatic Cyp3a activity decreased by 3.3-fold ($p < 0.05$) in GF male mice and decreased by 3.5-fold in GF female mice
Han et al., 2020	8-to-15-week-old C57BL/6 female mice (n = 5-6)	Pregnant and non-pregnant GF vs. CV	<i>Cyp3a11</i> mRNA expression: 0.6-fold change ($p = 0.953$) in non-pregnant mice and a 0.3-fold change ($p = 0.131$) in pregnant mice Cyp3a activity: ~4-fold ($p < 0.05$) reduction in activity in GF pregnant mice but no differences in non-pregnant mice
Conventionalization			
Selwyn et al., 2016	3-month-old C57BL/6 male mice (n = 6-8)	a. VSL#3 treatment b. GF+CV vs. GF	a. When both GF and CV mice were administered the probiotic VSL#3, hepatic Cyp3a11 mRNA and protein expression were not altered b. Hepatic Cyp3a11 mRNA (~6-fold) and protein expression (~2-fold) and activity (~2.5-fold) were higher in GF+CV vs. GF mice ($p < 0.05$)

Togao et al., 2021	8-week-old BALB/c male mice (n = 6)	Transplanted GF vs. GF	Although all three donations resulted in a significantly higher hepatic Cyp3a activity compared to control ($p < 0.001$), there were subtle differences in Cyp3a activity restoration (~6-fold vs. ~10-fold vs. ~11-fold)
Togao et al., 2023	5-week-old BALB/c male (n = 8)	Inoculated GF (2 donors) vs. GF	Cyp3a activity was restored in both groups but had a higher fold change in one recipient over the other (~7-fold, $p < 0.001$ vs. ~4-fold, $p < 0.01$) compared to control

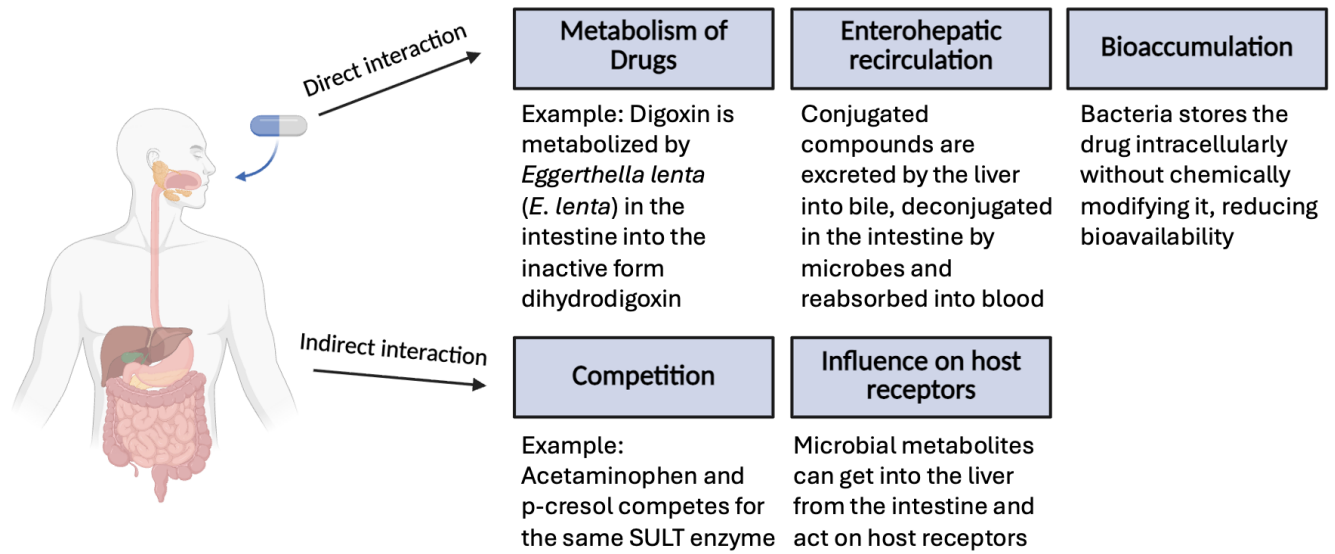


Figure 1.1. Direct and indirect interactions between drugs and the gut microbiome.

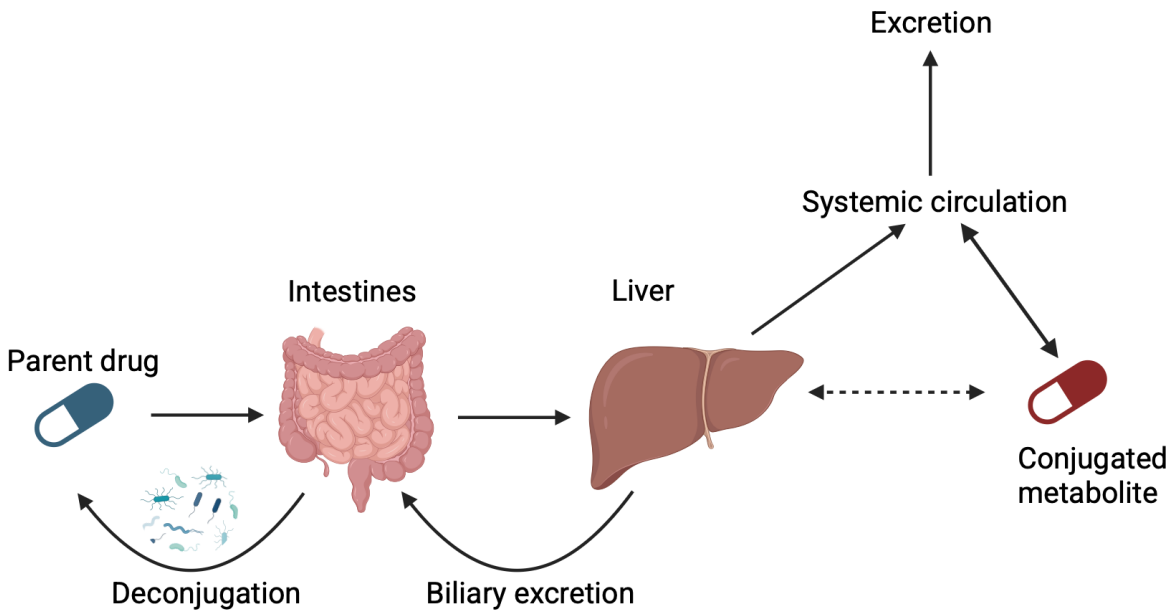


Figure 1.2. Enterohepatic recirculation where the parent drug is conjugated in the liver and is excreted into the bile. In the intestine, the conjugated metabolite can be deconjugated by gut bacteria into its parent form allowing reabsorption, thereby increasing its overall bioavailability and half-life.

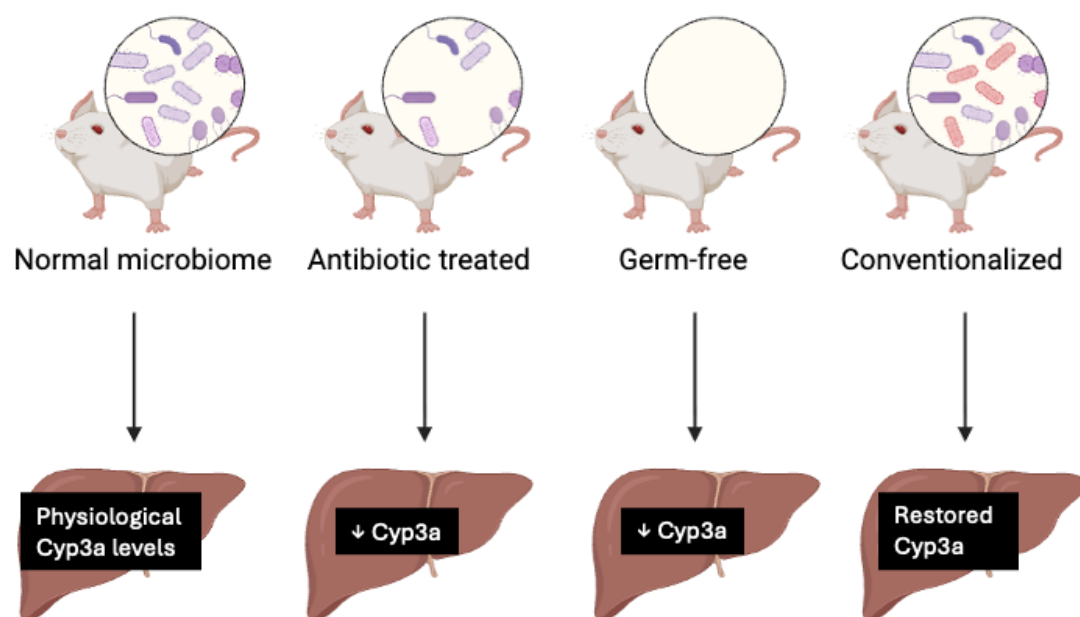


Figure 1.3. Influence of microbiome manipulation on Cyp3a in animal models.

Chapter 2

Impact of the Gut Microbiome on Hepatic CYP3A4 in Humanized PXR-CAR-CYP3A4/3A7 Mice

The work presented in this chapter was published in:

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2.1. Introduction

The gut microbiome is composed of microorganisms whose roles vary from commensal to pathogenic (Bengmark, 1998). Because of the microbiome's variability and function, it has been implicated in a wide range of diseases from gastrointestinal to neurological etiologies (Loh et al., 2024; Nagao-Kitamoto et al., 2016; Sekirov et al., 2010). Pharmacomicrobiomics is an emerging field that investigates the contribution of the microbiome to drug disposition, action, and toxicity. The interaction between the microbiome and drugs can manifest in several ways and is typically classified as either a direct or indirect interaction (Demsey and Cui, 2019; Klünemann et al., 2021; Spanogiannopoulos et al., 2016). A form of a direct interaction is metabolism of drugs by a gut bacterium (e.g., inactivation of digoxin by *Eggerthella lenta*; Dobkin et al., 1983). Conversely, an indirect interaction can manifest through the activation of a transcription factor by a microbial metabolite leading to upregulation of drug metabolizing enzymes and transporters (DMET; Staudinger et al., 2001). The upregulation of DMETs could lead to altered metabolism and pharmacokinetics (PK) of a drug.

Cytochrome P450 3A4 (CYP3A4) is highly expressed in the intestine and liver and is involved in the metabolism of roughly 30-50% of all drugs on the market (Burk et al., 2004). Changes in CYP3A4 can have a clinical impact on drug PK. For example, in pregnancy, CYP3A4 is induced resulting in increased metabolism of drugs that are primarily metabolized by CYP3A4 and reducing drug exposure and efficacy (Hebert et al., 2008; Sachar et al., 2019). For drugs that need to achieve targeted concentrations, interindividual variability in CYP3A4 can make dosing challenging. Although many factors are known to influence CYP3A4 activity (e.g., concomitant medications; Hernández-Lorca et al., 2025), the role of the gut microbiome in the regulation of CYP3A4 is not fully understood.

Previous studies have investigated the influence of the gut microbiome on DMETs in conventional (CV) and germ-free (GF) mice (Selwyn et al., 2015a; Togao et al., 2020). These studies concluded that the microbiome impacts mouse hepatic DMET expression and activity, including Cyp3a. Additionally, Cyp3a expression was restored when the GF mice were conventionalized through exposure to a conventional environment, mouse feces, or human feces, further confirming the microbiome's role in Cyp3a expression (Selwyn et al., 2016; Togao et al., 2021; Togao et al., 2023). Although most of these studies were conducted in male mice, Selwyn et al. (2015b) found that age and sex can affect the expression of DMETs. For example, at postnatal day 90, the absence of microbiome downregulated the mRNA expression of Cyp3a11, which was unchanged in earlier developmental time points in male WT mice. For female mice, the expression of Cyp3a11 at day 30 was higher in GF compared to CV mice, but this trend was inversed at day 90 (Selwyn et al., 2015b). Additionally, over the course of 27 months, male CV mice had consistently higher expression of Cyp3a11 than female CV mice (Fu et al., 2012). Therefore, sex and age differences as well as the microbiome (CV vs. GF) influenced Cyp3a11 expression.

Several studies have shown that microbial metabolites are ligands of pregnane X receptor (PXR) and increase the transcription of Cyp3a in mice (Staudinger et al., 2001; Kuno et al., 2018; Toda et al., 2009). For example, when GF mice were supplemented with increasing doses of lithocholic acid (LCA), the mRNA expression of both *PXR* and *Cyp3a11* increased. Specifically, at a dose of 0.05% LCA in their diet, *Cyp3a11* expression in GF mice was nearly comparable to that of CV mice and *PXR* expression was higher than that in the CV mice (Toda et al., 2009). Additionally, a mixture of short chain fatty acids was shown to induce CYP3A4 expression in liver organoids (Mun et al., 2021). More recently, indoxyl sulfate also showed both

inhibition and induction of CYP3A4 in hepatocytes (Togao et al., 2025). Therefore, it is likely that CYP3A4 expression can be influenced by microbial metabolites, especially bile acids and short chain fatty acids.

Inter-species differences exist between mice and human nuclear receptors, specifically the PXR, which is responsible for regulation of CYP3A4 (Lehmann et al., 1998). Most notably, rifampicin is a potent ligand of human PXR but not of mouse PXR (Lee et al., 2015). This adds complexity to the investigation of regulatory effects on downstream genes. To address this issue, a humanized mouse model (PXR-CAR-CYP3A4/3A7) was developed and its ability to replicate the effects of rifampicin was confirmed (Lee et al., 2015; Holmstock et al., 2013). The humanized mouse model predicted the magnitude of CYP3A4-mediated drug-drug interactions within 2-fold (Ly et al., 2017). Additionally, due to the cross-talk between CAR and PXR in the regulation of CYP3A4, it was also noted that using a double humanized mice provides more value in predicting drug disposition compared to a single nuclear receptor humanized mice (Lee et al., 2015). Thus, this humanized mouse model allows for the study of regulatory effects of the microbiome on human genes.

The goal of this study was to use the PXR-CAR-CYP3A4/CYP3A7 humanized mouse model to study the modulatory effects of microbiome on the expression of CYP3A4, and to test conventionalization of GF mice using sex-matched pooled human fecal samples. We aimed to compare the microbiome composition and diversity between CV, GF, and conventionalized (GFCV) humanized mice, and determine the impact of the microbiome composition on CYP3A4 expression.

2.2. Materials and Methods

2.2.1. Materials

Ammonium bicarbonate (ABC; 98% purity), dithiothreitol (DTT), iodoacetamide (IAA), Pierce trypsin protease (MS-grade), stable isotope-labeled peptides, bovine serum albumin (BSA), hydrochloric acid (HCl), protease inhibitor cocktail, potassium phosphate dibasic and monobasic anhydrous, and Qubit Protein Assay Kit were obtained from Thermo Fisher Scientific (Waltham, MA). Acetone, sucrose, Trizma[®] base, and analytical reagent-grade 3-nitrophenylhydrazine (3NPH)·HCl (97%) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC)·HCl were purchased from Sigma Aldrich (St. Louis, MO). Optima MS-grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Pooled male and female human feces were purchased from BioIVT (Westbury, NY). Molecular biology reagents included: E.Z.N.A.[®] Stool DNA kit from Omega Bio-Tek Inc. (Norcross, GA), RNA Bee reagent from Tel-Test Inc. (Friendswood, TX), High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Waltham, MA), Sso Advanced Universal SYBR Green Supermix from Bio-Rad (Hercules, CA). Pierce Mem-PER Plus Membrane Protein Extraction Kit was purchased from Thermo Fisher Scientific and BCA Protein Assay Kit from Pierce Biotechnology. The P450-Glo CYP3A4 Luciferin-IPA Assay and NADPH regeneration system were purchased from Promega (Madison, WI).

2.2.2. Animals

All animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington

(PROTO202000114 / 4035-07). Animals were maintained on 14-hr light/10-hr dark cycles and autoclaved Breeder Chow #5021 (LabDiet, St. Louis, MO) and autoclaved non-acidified water were provided *ad libitum*. Mice were housed with autoclaved Enrich-N'Pure bedding (The Andersons Inc., Maumee, OH).

Humanized PXR-CAR/CYP3A4/3A7 mice were purchased from Taconic Bioscience (Germantown, NY). This model was generated by replacing mouse *Nr1i2* and *Nr1i3* genes that encode for the nuclear receptors PXR and CAR with the corresponding human orthologous genes, and seven mouse *Cyp3a* genes (*Cyp3a11*, *Cyp3a16*, *Cyp3a25*, *Cyp3a41*, *Cyp3a44*, *Cyp3a57*, *Cyp3a59*) with approximately 125 kb of genomic human DNA comprising CYP3A4 and CYP3A7. Mouse *Cyp3a13* was not knocked-out. The germ-free (GF) colony was generated by rederivation of the conventional (CV) humanized PXR-CAR/CYP3A4/3A7 mice by the University of Washington Gnotobiotic Core. GF mice were housed in the Gnotobiotic Animal Core in isolators.

The conventionalized mice (GFCV) were generated by moving GF mice (8 weeks old) from the Gnotobiotic Animal Core to a conventional animal facility and inoculating the mice by oral gavage with pooled human feces screened to IAW AABB standards (0.2 mL of 10% PBS suspension). Female GF mice were inoculated with pooled female human feces (2 donors: Caucasians aged 59 and 46 years) while male GF mice were inoculated with pooled male human feces (2 donors: Hispanic aged 47 years and Caucasian aged 65 years). After inoculation, GFCV mice were housed for 4-6 weeks in the CV environment prior to study procedures.

2.2.3. Animal procedures

A total of 42 mice (12-14 weeks) were used in this study. Three groups with differing

microbial backgrounds were studied: conventional (CV), germ-free (GF), and conventionalized (GFCV) with each group consisting of 7 males and 7 females.

On the day of tissue collection, mice were anesthetized with isoflurane, and blood was collected via cardiac puncture and immediately centrifuged ($1,500 \times g$ for 10 min at 4°C) to isolate plasma. Liver, colon, and other tissues were removed and washed in cold PBS. The colon contents were collected for microbial sequencing. All plasma, tissue, and colon contents were snap-frozen on dry ice and stored at -80°C until analysis. Bacterial DNA extraction and hepatic RNA isolation were performed with the entire set of mice ($n = 42$), whereas metagenomics shotgun sequencing, proteomics, and microsomal activity were determined in a subset of mice ($n = 24$).

2.2.4. Bacterial DNA Extraction

Total genomic bacterial DNA was extracted from the colonic contents of male and female CV, GF, and GFCV mice ($n = 7$ per group) as well as the male and female pooled human feces used for conventionalization of the GF mice ($n = 1$ each). The E.Z.N.A.[®] Stool DNA kit was used according to the manufacturer's instructions. The concentration of total DNA was determined using a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY).

2.2.5. qPCR Quantification of Bacterial DNA

With the isolated bacterial DNA from the colonic contents of all mice ($n = 7$ per group, 42 total), universal bacterial abundance was determined using universal bacterial primer pairs (Table 2.1) as published before (Selwyn et al., 2016). Each sample was analyzed in technical triplicates. Bacterial DNA from GF mice indicated a low microbial composition and hence, GF

samples were not analyzed by metagenomics shotgun sequencing. Cq values that appeared as blank were assumed to be ~40 for graphing purposes (Figure 2.1).

2.2.6. Metagenomics Shotgun Sequencing

Metagenomics shotgun sequencing was performed on colonic bacterial DNA of CV and GFCV mice (n = 4 per sex per group; 16 total) and the male and female pooled human feces (n = 1 each). Samples were shipped to Diversigen (New Brighton, MN) for sequencing. Sequences were aligned to a curated database containing all representative genomes in RefSeq for bacteria with additional metagenomically assembled genomes (MAGs) and cell-cultured genomes. Only high-quality MAGs (completeness > 90% and contamination < 5% via checkm) were considered. Alignments were made at 97% identity against all reference genomes. Every input sequence was compared to every reference sequence in the Diversigen Venti database using fully gapped alignment with BURST (Al-Ghalith and Knights, 2020). Ties were broken by minimizing the overall number of unique Operational Taxonomic Units (OTUs). For taxonomy assignment, each input sequence was assigned the lowest common ancestor that was consistent across at least 80% of all reference sequences tied for best hit. The taxonomy data were based on the Genome Taxonomy Database (GTDB r95). Samples with fewer than 10,000 sequences were discarded. OTUs accounting for less than one-millionth of all strain-level markers and those with less than 0.01% of their unique genome regions covered (and < 0.1% of the whole genome) at the species level were discarded. Abundance was normalized by centered-log ratio transformation. All plots were created using ggplot2 in R version 2024.04.1+748.

2.2.7. Hepatic RNA Isolation

Livers from CV, GF, and GFCV mice (n = 7 per group) and banked wild-type (WT) C57BL/6 male and female mice (n = 5 per sex; 10 total) were prepared for RNA isolation. RNA was isolated using RNA Bee reagent following the manufacturer's protocol. RNA concentration was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) at 260 nm. RNA quality was assessed by running the samples on formaldehyde-agarose gel electrophoresis with visualization of 18S and 28S rRNA bands under ultraviolet light and confirmed by an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA).

2.2.8. RT-qPCR Quantification of Liver

Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions on the isolated hepatic RNA from CV, GF and GFCV mice (n = 7 per sex per group; 42 total) and C57/BL6 wild type (WT) mice (n = 5 per sex; 10 total). The cDNA products were amplified by qPCR using the Sso Advanced Universal SYBR Green Supermix in a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The quantified transcripts were: *CYP3A4*, *CYP3A7*, *hPXR*, *hCAR*, *Cyp3a11*, *Cyp3a13*, *mPXR*, and *mCAR*. The specific primer sequences are noted in Table 2.1. Each sample was analyzed in technical triplicates. Data were normalized to the housekeeping gene *Gapdh* using the $\Delta\Delta C_q$ method and were expressed as % of *Gapdh*.

2.2.9. Hepatic Protein Isolation, Digestion, and Proteomic Quantification

The hepatic membrane proteins of the GF, CV, and GFCV mice (n = 4 per sex per group; 24 total) were isolated using a Pierce Mem-PER Plus Membrane Protein Extraction Kit. Protein

concentration was determined using the BCA Protein Assay Kit. Samples were sent to Precision Quantomics (Spokane, WA) for digestion and proteomic quantification. The membrane protein samples were digested with trypsin following a previously described protocol (Sharma et al., 2023). Briefly, an 80 μ L sample (1 mg/mL membrane protein) was combined with 30 μ L of ABC buffer (100 mM), 10 μ L of DTT (250 mM), and 20 μ L of BSA (0.02 mg/mL). The mixture was incubated at 95°C for 10 min with gentle shaking at 300 rpm to facilitate denaturation and reduction. After cooling to room temperature, 10 μ L of IAA (500 mM) was added, and the sample was kept in the dark for 30 min. Ice-cold acetone (1 mL) was added, and the sample was vortexed and stored at -80°C for 1 hr, followed by centrifugation at $16,000 \times g$ for 15 min at 4°C. The supernatant was discarded, and the pellet was washed with cold methanol (0.5 mL) and centrifuged again under the same conditions for 10 min. The supernatant was removed, and the pellet was air-dried for 30 min at room temperature. The dried protein was reconstituted in 60 μ L of 50 mM ABC buffer and digested with a freshly prepared trypsin solution (20 μ L in 50 mM ABC buffer, with a protein:trypsin ratio of 25:1) at 37°C for 16 hr with gentle shaking at 300 rpm. The digestion was stopped by adding 5 μ L of 5% formic acid. The sample was vortexed, centrifuged at $10,000 \times g$ for 10 min at 4°C, and stored at -80°C until LC-MS analysis.

Global proteomics analysis was acquired on the Easy Spray 1200 series nanoLC coupled with Q-Exactive HF mass spectrometer (Thermo Fisher Scientific) using Acclaim PepMap™ trap column (75 μ m $\times$ 2 cm, 3 μ m) (Thermo Fisher Scientific) connected with analytical PepMap™ RSLC C18 column (75 μ m $\times$ 250 mm, 2 μ m) (Thermo Fisher Scientific). The mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in 80% acetonitrile (B), was run in a gradient mode (%B) of 2-6% (0-10 min), 6-30% (10-110 min), 30-100% (110-115 min), and 100% (115-131 min) with a flow rate of 300 nL/min. Data were acquired in the

positive ion mode using data-independent acquisition (DIA) feature of Xcalibur software (Thermo Fisher Scientific, Version 4.3.73.11). Global proteomics data were processed using DIA-NN software and the quantitative values for CYP3A4 were determined using the total protein abundance (TPA) method (Sharma et al., 2023). CYP3A7 was not selected because of low mRNA expression, and nuclear receptor (i.e., PXR and CAR) abundance could not be determined using this membrane protein preparation protocol.

2.2.10. Liver Microsome Preparation and CYP3A4 Activity Determination

Microsomes were isolated from the livers of CV, GF, and GFCV mice (n = 4 per sex; 24 total) for determination of CYP3A4 activity. Livers were homogenized in ice-cold Sucrose-Tris buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5). The homogenates were centrifuged at 10,000 x g for 20 min at 4°C. The supernatants were removed and centrifuged at 100,000 x g for 60 min. Sucrose-Tris buffer containing a protease inhibitor cocktail was used to resuspend the microsomal pellet. Protein concentration was determined using the Qubit Protein Assay Kit. Microsomes were stored at -80°C until analysis.

CYP3A4 enzyme activity was determined using the Promega P450-Glo CYP3A4 Luciferin-IPA Assay per the manufacturer's instructions with minor modifications. Briefly, luciferin-IPA (final concentration: 3 µM) was added to 20 µg of liver microsomal protein with KPO₄ buffer (total volume: 12.5 µL). Water (12.5 µL) was added, and the mixture was vortexed and pre-incubated at 37°C for 10 min. The reaction was initiated through the addition of 25 µL of 2X NADPH-regeneration system and incubated at 37°C for 30 min. An equal volume (50 µL) of luciferin detection reagent was added and the mixture was vortexed and incubated at room temperature for 20 min. Luminescence was quantified using a SpectraMax i3x Microplate

Reader (Molecular Devices, San Jose, CA). Net luminescence was calculated by subtracting the negative control from the values. All analyses were run in triplicate.

2.2.11. Bile Acid and Short Chain Fatty Acid Quantification

Fifty-four bile acids (BAs) were measured in liver samples and quantified using LC-MS/MS as previously described (García-Cañaveras et al., 2014). Briefly, a mixture of five stable isotope-labeled internal standards was spiked into the samples. Around 30 mg of liver tissue were homogenized and extracted with methanol, centrifuged at $3,000 \times g$ for 5 min at 4°C, and the supernatant was collected. The supernatants were evaporated to dryness and reconstituted with 50 µL methanol/water (1:1 v/v) and 10 µL aliquots were injected for analysis. Samples were analyzed on a Waters Acquity I-class UPLC coupled to a Waters Xevo TQS-micro MS with chromatographic separations performed on a Waters XSelect® HSS T3 (2.1 × 150 mm, 2.5 µm) column. Protein pellets were preserved and analyzed using the Pierce™ BCA Protein Assay. Calibration curves were generated, and concentrations were calculated from the curves. Data were normalized using the total protein weight.

Ten short chain fatty acids (SCFAs) were measured in plasma samples and quantified using LC-MS/MS as described previously (Han et al., 2015). Briefly, samples were extracted using methanol, then centrifuged and the supernatants were collected. The fatty acids were derivatized using 40 µL supernatant mixed with 20 µL of 3-nitrophenylhydrazine (200 mM) in 50% aqueous acetonitrile and 20 µL N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (120 mM) in 50% aqueous acetonitrile with 6% pyridine. The mixture was incubated at 40°C for 30 minutes. After incubation, the mixture was diluted to 2 mL with 10% aqueous acetonitrile. A 500 µL aliquot was mixed with 500 µL of the isotope-labeled internal standard mixture containing

standard fatty acids reacted with isotopically labeled derivatization agents using the described procedure. For the analysis, 20 μ L of each mixture was analyzed using a Waters Acquity I-class UPLC coupled to a Waters Xevo TQS-micro MS. Chromatographic separations were performed on a Waters BEH C18 (2.1 $\times$ 50 mm, 1.7 μ m) UPLC column. Concentrations were determined by comparing the SCFA peak area for each sample to a corresponding standard calibration curve. If no peaks were seen or if the observed peak was too low to measure, values were reported as not detected (ND).

2.2.12. General Statistical Analysis

Group differences in alpha diversity, mRNA expression, protein expression and activity were assessed using multi-way ANOVA followed by Tukey's post-hoc analysis for significance testing. Beta diversity differences were evaluated using PERMANOVA. Correlation analyses were performed using Spearman's correlation. Where appropriate, p-values were adjusted for multiple comparisons using the false discovery rate.

2.3. Results

2.3.1. Confirmation of gene expression in humanized PXR/CAR/CYP3A4-3A7 CV mice

The hepatic gene expression of selected human and mouse genes in humanized PXR-CAR-CYP3A4/3A7 CV mice (n = 7 per sex) and banked wild-type C57BL/6 mice (n = 5 per sex) were compared using RT-qPCR (Figure 2.2). As expected, the human genes *CYP3A4*, *CYP3A7*, *hPXR*, and *hCAR* were expressed in humanized mice, but were undetectable in WT mice. In humanized CV mice, *CYP3A4* was highly expressed, *hPXR* and *hCAR* were moderately expressed, and *CYP3A7* was detectable, but at very low levels. Female humanized CV mice

exhibited roughly 2-fold lower *CYP3A4* ($p < 0.001$) and 1.4-fold and 1.8-fold lower *hPXR* and *hCAR*, respectively ($p = 0.01$ for each), compared to male humanized CV mice. *CYP3A7* expression was comparable in male and female humanized CV mice.

As expected, the knocked-out mouse genes, *Cyp3a11*, *mPXR*, and *mCAR*, were not detected in CV mice. *Cyp3a11* was highly expressed and *mPXR* and *mCAR* were moderately expressed in WT mice. *Cyp3a11* was 1.92-fold lower in female WT mice compared to male WT mice ($p = 0.0005$), *mPXR* was comparable between sexes, and *mCAR* was 1.77-fold higher in female WT mice compared to male WT mice ($p = 0.024$). The expression of *Cyp3a13*, which was not knocked-out in the CV mice, was comparable in male and female WT and CV mice.

2.3.2. Gut microbiome composition and diversity

To confirm general colonization of bacteria in CV and GFCV mice and the absence of bacteria in GF mice, qPCR with a universal bacterial primer was performed on total genomic bacterial DNA isolated from the colonic contents of mice (Figure 2.1). Samples that had undetected levels were set to a Cq value of 40. As expected, GF mice did not have measurable microbial signals in their intestinal content.

To investigate differences in the gut microbiome, alpha and beta diversity were calculated using the Shannon Index and Bray-Curtis Index, respectively. Shannon Index quantifies species evenness and richness in microbial composition within a sample, whereas Bray-Curtis Index assesses similarity between samples or groups. The groups compared included CV and GFCV mice ($n = 4$ per sex per group) and the pooled human feces used to inoculate the GFCV mice ($n = 1$ per sex).

As shown in Figure 2.3a, the alpha diversity of male and female CV mice was higher

than that of their male and female GFCV counterparts ($p = 0.025$ and 0.003 , respectively). Additionally, the pooled human feces and CV groups exhibited a comparable overall Shannon Index. Of note, male CV mice had a larger range of values for the Shannon Index compared to other groups. Beta diversity analysis revealed that the overall microbial composition of CV mice and pooled human feces were similar but differed from male and female GFCV mice ($p = 0.001$; Figure 2.3b).

At the phylum level, eight major phyla were identified with the ninth group being unclassified (noted as “Other”; Figure 2.4a). The bacterial composition of male GFCV mice differed markedly from that of male CV mice and the male pooled human sample, with notable differences such as a higher proportion of unclassified bacteria (25%, 6.0%, and 5.9%, respectively) and lower representation of *Desulfobacterota* (0.02%, and 0.49%, respectively) and *Proteobacteria* (0.13%, 9.8%, and 8.9%, respectively). Similarly, the bacterial profile of female GFCV mice differed from that of female CV mice and the female pooled human sample. Specifically, female GFCV mice lacked *Desulfobacterota* (Figure 2.4a), but had the largest proportion of *Verrucomicrobiota* (23.2%) compared to the other groups. Lower proportions of *Verrucomicrobiota* were noted in female CV mice (3.1%), male and female pooled human fecal samples (4.7% and 4.4%, respectively), and was almost absent in male CV (0.002%) and male GFCV (0.16%) mice. Lastly, *Campylobacterota* was absent (0%) in all groups except male CV (0.011%) and female CV (0.008%) mice.

A total of 308 bacterial species were identified among the samples. In male and female CV mice as well as in the pooled human samples, the top 20 species accounted for approximately 70% of the overall bacterial composition. In contrast, in GFCV mice, these species make up only about 40% of the bacterial composition. For example, the species

Lactobacillus Johnsonii, was differentially abundant in CV mice and pooled human feces compared to GFCV mice (Figure 2.5). This is consistent with the alpha diversity matrix which had indicated low alpha diversity (evenness and richness) in GFCV mice. Additionally, as demonstrated in Figure 2.5b, GFCV mice possess the highest number of unique bacteria (84 for male, 99 for female) that do not overlap with the other comparison groups. This analysis confirmed what was seen with the beta diversity matrix which indicated that GFCV mice had a different microbial composition compared to CV mice and the pooled human species.

2.3.3. Hepatic mRNA expression

The hepatic mRNA expression of *CYP3A4*, *CYP3A7*, *hPXR* and *hCAR* was measured in GF, CV, and GFCV mice (n = 7 per group per sex; Figure 2.6). Compared to male GF mice, male CV mice exhibited 7.61-fold higher *CYP3A4* ($p < 0.001$) and 1.68-fold higher *hPXR* ($p = 0.022$) while no differences were observed for *hCAR* and *CYP3A7*. *CYP3A4*, *CYP3A7*, *hPXR* and *hCAR* did not differ between male GFCV and male GF mice. The conventionalization of male GFCV mice using male pooled human fecal samples did not appear to restore expression of these 4 genes. Compared to female GF mice, female CV mice had 1.69-fold lower *CYP3A4* ($p = 0.012$) with no differences in *CYP3A7*, *hPXR* and *hCAR*. Female GFCV mice had 2.71-fold lower *CYP3A4* compared to female GF mice ($p < 0.001$) and no differences in *CYP3A7*, *hPXR* and *hCAR*.

Sex differences were seen for a few of the target genes. *CYP3A4* was 4.32-fold higher in female vs. male GF mice ($p < 0.001$) and 3.00-fold lower in female vs. male CV mice ($p < 0.001$), while there was no significant difference in GFCV mice ($p = 0.07$). No sex differences were observed for *CYP3A7* expression in GF, CV or GFCV mice. For both *hPXR* and *hCAR*,

there were no sex differences in GF and CV mice, but *hPXR* and *hCAR* was 1.79-fold higher ($p = 0.0261$) and 2.67-fold higher ($p = 0.006$) in female vs. male GFCV mice, respectively. Overall, the pattern of expression of *CYP3A4*, *hPXR* and *hCAR* in GF, CV and GFCV mice differed by sex.

2.3.4. Hepatic protein expression

Hepatic CYP3A4 was estimated by proteomics analyses as shown in Figure 2.7. CYP3A7, hPXR, and hCAR were not measured. Consistent with mRNA expression, male CV mice had 2.68-fold higher CYP3A4 compared to male GF mice (80.7 ± 17.2 vs. 30.1 ± 17.1 pmol/mg; $p < 0.001$). Although no difference was noted in CYP3A4 between male GF and GFCV mice, the GFCV mice had the lowest CYP3A4 levels (5.8 ± 1.9 pmol/mg total protein). Compared to female GF mice (107.6 ± 18.8 pmol/mg), CYP3A4 expression was 1.63-fold lower in female CV mice (66.0 ± 6.4 ; $p = 0.003$) and 2.91-fold lower in GFCV mice (37.0 ± 5.1 pmol/mg; $p < 0.001$). CYP3A4 expression was 3.57-fold higher in female GF mice ($p < 0.001$) and 6.34-fold higher in female GFCV mice ($p = 0.0324$) compared to their male counterparts, but no sex difference was noted in CV mice. Hepatic protein expression of CYP3A4 followed similar trends to *CYP3A4* mRNA expression (Figures 2.6 and 2.7.a) and protein and mRNA values were strongly correlated (Spearman's $\rho = 0.91$, $p < 0.001$; Figure 2.7b).

2.3.5. Hepatic activity

CYP3A4 activity in mouse liver microsomes was determined using a P450-Glo luciferin-IPA assay (Figure 2.8a). The variability in CYP3A4 activity within each group was larger than variability noted for CYP3A4 mRNA and protein expression. No differences in CYP3A4 activity

were observed between the GF, CV and GFCV groups. Protein expression and activity were weakly correlated (Spearman's correlation = 0.46, $p = 0.026$; Figure 2.8b).

2.3.6. Correlation between bacterial species and CYP3A4 protein expression

CYP3A4 protein expression was strongly correlated with alpha diversity (Spearman's correlation = 0.66, $p = 0.0072$; Figure 2.9a). To determine which bacterial species may be influencing CYP3A4 expression, a correlation analysis was conducted. A total of 57 bacterial species of the mouse gut microbiome were significantly correlated ($p < 0.001$) with CYP3A4 protein expression; 12 species had positive correlations, and 45 species had negative correlations with CYP3A4 (Table 2.2). The top bacterial species with a positive or negative correlation with CYP3A4 expression are presented in Figure 2.9b. For *Acetatifactor muris* (Spearman's $\rho = 0.85$) and *Actualibacter muris* (Spearman's $\rho = 0.87$), data were anchored by low CYP3A4 and low bacterial abundance in GFCV mice and high CYP3A4 and high bacterial abundance in CV mice. In contrast, *Anaerotruncus colihominis* (Spearman's $\rho = -0.9$) and *Intestinimonas timonensis* (Spearman's $\rho = -0.9$) displayed a greater distribution of mice throughout the correlation.

2.3.7 Bile Acid and Short Chain Fatty Acid Quantification

A total of 25 bile acids (BAs) were quantified in the liver samples of GF, CV, and GFCV mice ($n = 4$ per group; Table 2.3). To identify which BA might be influencing CYP3A4, a correlation analysis was conducted, and five BAs were found to be positively correlated with CYP3A4 protein expression. These BAs were: tauroolithocholic acid (Spearman's $\rho = 0.57$, $p = 0.004$), taurochenodeoxycholic acid (Spearman's $\rho = 0.62$, $p = 0.001$), 7- α -OH-glycochenodeoxycholic acid (Spearman's $\rho = 0.71$, $p = 0.006$), and taurodeoxycholic acid

(Spearman's $\rho = 0.61$; $p = 0.007$), and taurocholic acid (Spearman's $\rho = 0.55$, $p = 0.006$). No BAs were identified with a negative correlation with CYP3A4 protein expression.

Only four short chain fatty acids (SCFAs) were quantifiable in the plasma samples of GF, CV, and GFCV mice (Table 2.4). The concentrations of all four SCFAs did not differ significantly between groups. There were no significant correlations between the concentrations of each short chain fatty acid and CYP3A4 protein expression.

2.4. Discussion

This is the first study to investigate the role of the gut microbiome in the regulation of CYP3A4 in humanized PXR-CAR-CYP3A4/3A7 mice. We confirmed the deletion of selected mouse genes (*Cyp3a11*, *mPXR*, and *mCAR*) and replacement with human genes (*CYP3A4*, *CYP3A7*, *hPXR*, and *hCAR*) in the humanized mice (Figure 2.2).

In male mice, the CV mice had a higher expression of *CYP3A4* mRNA (7.61-fold; $p < 0.001$) and CYP3A4 protein (2.68-fold; $p < 0.001$) compared to GF mice. In female mice, the CV group had 1.69-fold lower *CYP3A4* mRNA and 1.63-fold lower protein expression than the GF group which was the opposite of what was seen in male mice (Figures 2.6 and 2.7). CYP3A4 activity was unchanged between groups in male and female mice, likely due to high sample variability (Figure 2.8). The pattern of increased expression in male CV mice compared to microbiome-deficient mice was seen in other studies looking at *Cyp3a11*, a mouse orthologue of CYP3A4, in CV vs. GF or antibiotic-treated C57BL/6 and BALB/c mice (Selwyn et al., 2015a; Togao et al., 2020; Kuno et al., 2016). A similar pattern was seen for *Cyp3a13*, *Cyp3a16*, *Cyp3a25*, *Cyp3a41*, and *Cyp3a44* in IQI mice (Toda et al., 2009). As CYP3A7 is the fetal isoform of CYP3A4, we did not expect CYP3A7 to be highly expressed in these mice (Kitada et

al., 1985).

CYP3A4 was 4.32-fold higher in female GF mice ($p < 0.001$) and 3.00-fold lower in female CV mice ($p < 0.001$) compared to their male counterparts, while *CYP3A4* expression was comparable in male and female GFCV mice ($p = 0.07$). Conflicting data have been reported on the sex-related *Cyp3a11* differences in CV vs. GF mice. Although one study showed that hepatic *Cyp3a11* expression was consistently higher in male CV mice compared to female mice, another reported that *Cyp3a11* and *Cyp3a13* expression was higher in female CV mice compared to male mice (Fu et al., 2012; Jourová et al., 2020). It has also been shown that the differences between female CV and GF mice's *Cyp3a11* and *Cyp3a44* expression can be age dependent (Selwyn et al., 2015b). The authors hypothesize that this difference in female mice may be due to sex-specific hormones and may be STAT5b-mediated as suggested by Waxman and O'Connor (2006).

A unique aspect of this study was the recolonization of GF mice with sex-matched pooled human fecal samples. Although the overall bacterial abundance in CV and GFCV mice did not differ, metagenomics shotgun sequencing revealed major differences. Overall, GFCV mice demonstrated lower alpha diversity (Figure 2.3a), and the GFCV bacterial composition differed from its CV counterparts and the pooled human feces in beta diversity (Figure 2.3b), and phylum and species composition (Figure 2.4). In addition, CYP3A4 protein expression was correlated with alpha diversity (Figure 2.9a). Successful fecal microbiota transplantation (FMT) of human feces into mice has been performed in several studies (Togao et al., 2023; Huang et al., 2024; Liu et al., 2023). However, to our knowledge, this is the first study to conduct FMT of human feces into this humanized mouse model. The incomplete microbiome restoration in GFCV mice could be due to several factors: influence of environmental conditions like housing or food, differential

survival of human microbes in the mouse gut, limited exposure to pooled samples, among others. Lastly, though the human pooled male and female donor microbiota compositions were similar, we observed sex-dependent conventionalization following FMT as GFCV bacterial composition differed between male and female mice (Figure 2.3b). Other studies have shown sex-specific effects of FMT. For example, when male and female GF mice were transplanted with the same feces from an adult man, significant sex differences were observed in the microbiome composition and diversity (Wang et al., 2016). This suggests that sex plays a role in the establishment of a microbiome following FMT.

Differences in expression of various bacteria were noted. For example, male CV and male GFCV mice were the only groups with a very low expression of the phylum *Verrucomicrobiota* (< 1% of total composition), and male and female CV mice were the only ones with a noticeable presence of the phylum *Desulfobacterota* (> 5% for both, < 1% in other groups; Figure 2.4a). One study showed that mice fed with regular chow vs. a high-fat, low-fiber (Hf-Lf) diet had differences in microbial composition and the difference also varied depending on circadian rhythm (as measured by the zeitgeber timescale (ZT)). Specifically, *Verrucomicrobiota* differed in chow-fed male and female mice at ZT12 and ZT16 while *Desulfobacterota* had an increase in expression in male Hf-Lf mice at ZT0 and ZT12 (Munyoki et al., 2023). We did not record sample collection times for this study to monitor for potential circadian rhythm changes. At the species level, the composition of bacterial species in GFCV mice was uniquely different from CV mice and pooled human species (Figure 2.4b).

Male GFCV and GF mice had comparable levels of CYP3A4 mRNA and protein expression, whereas female GFCV mice had 2.71-fold lower *CYP3A4* mRNA ($p < 0.001$) and 2.91-fold lower CYP3A4 protein ($p < 0.001$) compared to female GF mice. Minor differences

between male GF, CV and GFCV mice were noted in *hPXR* and *hCAR*, but no differences were seen in female mice (Figure 2.6). The GFCV mice did not show a restored expression of CYP3A4 compared to CV mice. This was unexpected as other studies reported restored expression of *Cyp3a11* following conventionalization with mouse fecal samples (Selwyn et al., 2016; Togao et al., 2021; Togao et al., 2023). The differences in male and female GFCV mice might be explained by sex-dependent variation in the pooled human fecal samples used for inoculation, differences in establishing a restored microbiome (though the overall DNA content were comparable), or underlying sex-related differences in the GF mice.

Although the conventionalization of the GF mice with human feces did not result in restoration of CYP3A4 expression (Figure 2.6 and 2.7) and activity (Figure 2.8), previous studies showed that when different types of conventional mouse feces were transplanted into GF mice, the Cyp3a activity was differed between the GFCV groups (Togao et al., 2021). This suggests that variations in the microbiome can lead to differences in Cyp3a. Togao et al. showed that when two different human donor feces were used for fecal transplantation, the resultant Cyp3a activity level differed in the GFCV mice (Togao et al., 2023).

Given the distribution of CYP3A4 expression across the mouse groups, we performed a correlation analysis of bacterial species and CYP3A4 (Table 2.2 and Figure 2.9 for top correlations). We hypothesized that the sex-dependent regulation of CYP3A4 might be attributable to differences in the gut microbiome, specifically abundance of distinct microbes, between groups. The top two strongest positively correlated species were *Acutalibacter muris* and *Acetatifactor muris*. Not much is known about *Acutalibacter muris* but it is a strictly anaerobic bacteria (Lagkouvardos et al., 2016). *Acetatifactor muris* was first identified in the intestine of obese mice (Pfeiffer et al., 2012). Several papers have described that it is altered with

different interventions like dicaffeoylquinic acids, CD1d, and Baichanting Compound (Huang et al., 2025; Lee et al., 2019; Lu et al., 2024). The top two strongest negatively correlated species were *Anaerotruncus colihominis* and *Intestinimonas timonensis*. *Anaerotruncus colihominis* has been isolated from human feces (Lawson et al., 2004) and implicated in multiple sclerosis (Bianchimano et al., 2022). No papers have been published regarding the relationship between *Intestinimonas timonensis* and disease states. Overall, none of these four microbes have been studied in relation to DMETs. We hypothesize that microbes with a positive correlation could produce metabolites that upregulate CYP3A4 and conversely, microbes with a negative correlation could produce metabolites that repress CYP3A4 expression. We found 53 other microbes that were correlated with CYP3A4 expression that could potentially be important. However, no study to date has shown a direct link between bacterial species and CYP3A4 regulation. Only *Lactobacillus thamnusus* was found to induce Cyp3a1/2 in rats and the authors hypothesized that this induction was mediated through increased production of microbial metabolites leading to activation of PXR (Liu et al., 2021). Future work will need to be performed to confirm these results.

Due to the previously reported influence of microbial metabolites on CYP3A4, BAs and SCFAs were quantified in liver and plasma, respectively and correlated with CYP3A4 protein expression. Although no SCFAs were identified as having a significant correlation, five BAs were shown to have a positive correlation. Taurocholic acid and taurochenodeoxycholic acid are conjugated forms of the primary BA cholic acid and chenodeoxycholic acid, respectively. 7- α -OH-glycochenodeoxycholic acid is a metabolite of the glycine conjugated form of chenodeoxycholic acid. All three of these metabolites are likely not products of the gut microbiota (Chiang, 2004). On the other hand, taurodeoxycholic acid and tauroolithocholic acid

are taurine conjugates of the secondary BA deoxycholic acid and lithocholic acid which are microbial metabolites (Chiang, 2004). The unconjugated form of lithocholic acid was below detection in all samples and deoxycholic acid was not detected in 3 out of 6 groups (Table 2.3). It was recently reported that some of these BAs were altered in patients with Crohn's Disease compared to controls and these patients had lower 4 β -hydroxycholesterol plasma concentrations, an endogenous biomarker for CYP3A4 activity (Wilson et al., 2020). Another recent finding demonstrated that soyasapogenols, which are the microbial metabolites of soyasaponins present in chow, induces hepatic Cyp3a11 in mice (Roichman et al., 2025). This suggests that other microbial metabolites outside of SCFAs and BAs can also influence CYP3A4 expression.

There are several limitations to this study. As this was a pilot study with limited funding, metagenomic sequencing, proteomics analyses, and CYP3A4 activity determination were performed on a subset of each group (n = 4 per group), but qPCR analyses were performed on all samples (n = 7 per group). As discussed previously, the protocol for conventionalization of GF mice with pooled human feces may not have resulted in adequate recolonization as the single exposure to human feces may not have been sufficient. When different dosing schedules for FMT of human feces into mice were compared (once-a-week vs. twice-a-week, and one dose FMTs vs. two dose FMTs), FMT of once-a-week seemed to have the best outcome in terms of colonization and diversity (Wrzosek et al., 2018). However, the authors concluded that regardless of dosing frequency, human bacteria were detected in the mouse feces. Subsequent studies in our lab could explore the sex-dependent differences in conventionalization, time needed to establish a new microbiome in GFCV mice, differences in mouse vs. human fecal inoculation for conventionalization, or whether the GF humanized mouse is more challenging to conventionalize. As SCFA concentrations were measured in plasma, differences in intestinal

SCFA are not fully represented which may have confounded our findings. Attempts to isolate high quality RNA from the intestinal tissue were not successful. We were unable to assess changes in CYP3A4 expression in intestinal tissues that would provide additional support for the regulation of CYP3A4 by the microbiome. Finally, it is unknown whether the observed impact of the gut microbiome on hepatic CYP3A4 is PXR- and/or CAR-mediated and further studies with PXR or CAR knockout mice may be necessary to elucidate the mechanism. Despite the limited sample size, the use of a humanized mice model offers increased confidence in translation of the results to humans.

2.5. Conclusion

Overall, variation in microbiome composition and diversity contributed to differences in hepatic CYP3A4 expression. As the human fecal microbiome transplant to sex-matched GF mice did not restore CYP3A4 expression, we further hypothesized that the regulation of CYP3A4 by the gut microbiome is driven by specific microbes and/or microbial metabolites. We found that several bacterial species, five bile acids, but no short chain fatty acids were correlated with CYP3A4 expression. Additionally, the regulation of CYP3A4 by the gut microbiome may be sex-specific and confounded by other factors. Further work should be done to elucidate the mechanism of CYP3A4 regulation by the microbiome.

2.6. References

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2.7. Tables and Figures

Table 2.1. Primer sequences for RT-qPCR.

Gene	Forward Sequence	Reverse Sequence
<i>CYP3A7</i>	TTTCCATTCTTACCCCAATTCT	GAGGCGACCTTCTTTTATCTGT
<i>CYP3A4</i>	GTGTGTTTCCAAGAGAAGTTACAAA	GTATCTTCGAGGCGACTTTCTTT
<i>hPXR</i>	GCTTTCGAGCTGTGTCAACTG	TGCAGTGTCTTCCAAGCAGTA
<i>hCAR</i>	TTCATCCATCACCAGCCCTTG	TTGATGTCTGCGAAGTGTGTG
<i>Cyp3a11</i>	TGGGACTCGTAAACATGAACTT	GTGCCTAAAAATGGCAGAGGTT
<i>Cyp3a13</i>	CTTTCCATTCTTACCCCAGT	GCGATTCTCTTTCATTCGTTCT
<i>mPXR</i>	CCTCACTGTTGGAAGACCCTG	TGGAAGGGGGTTGGTAGTTC
<i>mCAR</i>	GTCCCATCTGTCCGTTTGCT	GCCAACATTTAGACACTTCTGC
<i>Universal Bacteria</i>	GTGSTGCAYGGYTGTCGTCA	ACGTCRTCCMCACCTTCCTC
<i>Gapdh</i>	GGCAAATTCAACGGCACAGT	GTCTCGCTCCTGGAAGATGG

Table 2.2. Bacterial species significantly correlated with CYP3A4 protein expression ($p < 0.05$).

Spearman's R Correlation	Species
> 0.80	<i>Acutalibacter muris</i> (0.87); <i>Acetatifactor muris</i> (0.85)
0.70 to 0.79	<i>Lactobacillus johnsonii</i> (0.74); <i>Eubacterium J plexicaudatum</i> (0.71)
0.60 to 0.69	<i>Muribaculum intestinale</i> (0.69); <i>Bacteroides caecimuris</i> , <i>Adlercreutzia caecimuris</i> (0.65); <i>Duncaniella muris</i> (0.64); <i>Lactobacillus crispatus</i> (0.60)
0.50 to 0.59	<i>Paramuribaculum intestinale</i> (0.59); <i>Faecalibaculum rodentium</i> (0.50)
-0.50 to -0.59	<i>Dorea scindens</i> (-0.51); <i>Ruthenibacterium lactatiformans</i> (-0.54); <i>Agathobaculum desmolans</i> (-0.55); <i>Eutepia gabavorous</i> (-0.57); <i>Faecalimonas nexilis</i> , <i>Gemmiger formicilis</i> , <i>Clostridium Q symbiosum</i> (-0.58); <i>Faecalimonas umbilicata</i> (-0.59)
-0.60 to -0.69	<i>Anaerotignum lactatifermentans</i> (-0.60); <i>Dorea longicatena</i> (-0.61); <i>Lachnospira eligens B</i> (-0.65); <i>Fournierella massiliensis</i> ; <i>Blautia A obeum</i> (-0.67); <i>Eisenbergiella massiliensis</i> (-0.68)
-0.70 to -0.79	<i>Enterocloster bolteae</i> , <i>Roseburia hominis</i> , <i>Eisenbergiella tayi</i> , <i>Mediterraneibacter A butyricigenes</i> (-0.73); <i>Pseudoflavonifractor capillosus</i> , <i>Anaerobutyricum hallii A</i> , <i>Longicatena innocuum</i> , <i>Eubacterium G ventriosum</i> (-0.74); <i>Emergencia timonensis</i> (-0.75); <i>Harryflintia acetispora</i> , <i>Ruminococcus B gnavus</i> (-0.76); <i>Roseburia inulinivorans</i> , <i>Intestinimonas butyriciproducens</i> (-0.77); <i>Enterocloster clostridioformis A</i> , <i>Mediterraneibacter torques</i> (-0.78), <i>Roseburia intestinalis</i> (-0.79)

-0.80 to -0.89	<i>Frisingicoccus caecimuris</i> (-0.80); <i>Lachnoclostridium A edouardi</i> , <i>Dorea formicigenerans</i> (-0.83); <i>Blautia A wexlerae</i> , <i>Faecalibacterium prausnitzii D</i> , <i>Angelakisella massiliensis</i> , <i>Angelakisella massiliensis</i> (-0.85); <i>Sellimonas intestinalis</i> , <i>Fusicatenibacter saccharivorans</i> , <i>Bariatricus comes</i> , <i>Flavonifractor plautii</i> (-0.88); <i>Oscillibacter welbionis</i> , <i>Enterocloster citroniae</i> , <i>Enterocloster clostridioformis</i> (-0.89)
> -0.90	<i>Anaerotruncus colihominis</i> , <i>Intestinimonas timonensis</i> (-0.90)

Table 2.3. Concentrations of bile acids in mouse liver and correlation with CYP3A4 (n = 4 per sex per group).¹

Bile Acid	Abbreviation	Concentration (pmol/g protein)						Correlation with CYP3A4 protein (p-value)
		Male			Female			
		GF	CV	GFCV	GF	CV	GFCV	
Cholic acid	CA	3.7 ± 0.05	100 ± 148	9.5 ± 8.2	4.8 ± 1.0	27 ± 18	338 ± 553	0.065 (NS ²)
Taurocholic acid	TCA	1114 ± 615	3415 ± 892	860 ± 149	2233 ± 899	3467 ± 4132	2868 ± 960	0.55 (0.0063)
Chenodeoxycholic acid	CDCA	6.9 ± 0.6	11 ± 12	5.2 ± 1.2	7.6 ± 2.3	6.8 ± 2.7	23 ± 29	0.12 (NS)
Glycochenodeoxycholic acid (7α-oh)	GCDCA	0.16 ± 0.03	0.6 ± 0.07	ND ⁵	0.2 ± 0.002	0.4 ± 0.2	0.04 ± 0.01	0.71 (<0.001)
Taurochenodeoxycholic acid	TCDCa	116 ± 18	388 ± 114	77 ± 19	162 ± 69	233 ± 191	169 ± 42	0.62 (0.0014)
Deoxycholic acid	DCA	ND	11 ± 14	ND	ND	7.6 ± 7.3	6.2 ± 7.1	0.33 (NS)
Taurodeoxycholic acid	TDCA	ND	153 ± 48	ND	ND	155 ± 108	24 ± 4.2	0.61 (0.0066)
Ursodeoxycholic acid	UDCA	5.8 ± 2.0	13 ± 19	3.3 ± 1.3	7.2 ± 1.9	13 ± 11	33 ± 40	0.23 (NS)
Taurolithocholic acid	TLCA	0.9 ± 0.2	15 ± 4.7	0.5 ± 0.2	0.9 ± 0.4	6.7 ± 4.6	0.7 ± 0.4	0.57 (0.0043)
Tauroursodeoxycholic acid (7β-oh)	TUDCA	81 ± 33	172 ± 39	69 ± 17	104 ± 38	256 ± 215	177 ± 53	0.44 (0.033)
α-Muricholic acid	αMCA	5.0 ± 1.2	16 ± 22	4.7 ± 1.8	6.8 ± 1.2	7.8 ± 3.5	39 ± 39	0.20 (NS)

Tauro- α -muricholic acid	T α MCA	7859 $\pm$ 4599	2777 $\pm$ 757	3431 $\pm$ 359	7613 $\pm$ 2485	4031 $\pm$ 5301	8504 $\pm$ 3865	0.036 (NS)
β -Muricholic acid	β MCA	548 $\pm$ 288	85 $\pm$ 109	255 $\pm$ 114	529 $\pm$ 85	129 $\pm$ 82	744 $\pm$ 423	-0.11 (NS)
ω -Muricholic acid	ω MCA	2.6 $\pm$ 0.2	16 $\pm$ 17	4.1	2.3 $\pm$ 0.8	55 $\pm$ 35	3.0 $\pm$ 0.9	0.049 (NS)
Tauro- ω -muricholic acid	T ω MCA	10766 $\pm$ 6168	4265 $\pm$ 1328	5108 $\pm$ 735	10899 $\pm$ 3488	5626 $\pm$ 7302	12142 $\pm$ 5032	0.047 (NS)
Hyocholic acid	HCA	3.3 $\pm$ 2.0	2.2 $\pm$ 1.6	2.3 $\pm$ 0.9	2.5 $\pm$ 0.5	2.0 $\pm$ 0.7	1.9 $\pm$ 0.7	-0.027 (NS)
Taurohyocholic acid	THCA	11 $\pm$ 7.6	12 $\pm$ 4.0	14 $\pm$ 12	8.5 $\pm$ 3.7	31 $\pm$ 33	10 $\pm$ 4.0	0.11 (NS)
Hyodeoxycholic acid	HDCA	6.1 $\pm$ 1.9	12 $\pm$ 17	3.7 $\pm$ 1.3	7.1 $\pm$ 1.7	12 $\pm$ 11	31 $\pm$ 36	0.20 (NS)
Taurohyodeoxycholic acid (6 α -oh)	THDCA	72 $\pm$ 32	159 $\pm$ 37	61 $\pm$ 15	94 $\pm$ 35	240 $\pm$ 206	164 $\pm$ 51	0.44 (0.033)
Murocholic acid	MuroCA	2.0 $\pm$ 0.7	16 $\pm$ 21	ND ¹	1.7 $\pm$ 0.7	11 $\pm$ 10	6.6 $\pm$ 3.5	-0.078 (NS)
Dehydrocholic acid	DHCA	3.9 $\pm$ 1.6	2.2 $\pm$ 1.0	2.6 $\pm$ 0.7	4.1 $\pm$ 0.9	1.9 $\pm$ 1.4	3.0 $\pm$ 0.5	0.071 (NS)
Isoodeoxycholic acid	isoDCA	1.8 $\pm$ 0.7	0.3	1.0 $\pm$ 0.3	1.9 $\pm$ 0.7	2.0 $\pm$ 1.2	2.0 $\pm$ 0.5	0.16 (NS)
3-dehydrocholic acid	3-DHCA	15 $\pm$ 6.4	11 $\pm$ 8.0	16 $\pm$ 10	12 $\pm$ 3.5	23 $\pm$ 16	20 $\pm$ 16	-0.0040 (NS)
12-dehydrocholic acid	12-DHCA	7.4 $\pm$ 1.5	124 $\pm$ 174	7.8 $\pm$ 2.1	6.9 $\pm$ 1.8	7.8 $\pm$ 4.0	12 $\pm$ 5.7	0.14 (NS)
5 α -cholanolic acid-3 α -ol-6-one		ND	6.1 $\pm$ 7.6	ND	ND	ND	3.6	-0.5 (NS)

¹ Of the 54 compounds analyzed, the following were not detected: 3,12-diketocholanolic acid, 3,6-diketocholanolic acid or 3,7-diketocholanolic acid, 3-ketocholanolic acid, 3 α ,12 α , 23-nordeoxycholic acid, 3 α -hydroxy-12 ketolithocholic acid, 3 α -hydroxy-6,7-diketocholanolic acid, 3 α -hydroxy-7,12-

diketocholelonic acid, 3 α -hydroxy-7-ketolithocholic acid, 5-cholelonic acid-3 β -ol, 5 α -cholelonic acid-3,6-dione, 5 β -cholelonic acid-3 α , 6 α -diol-7-one, 5 β -Cholelonic Acid-3 β , 12 α -diol, 8(14),(5 β)-cholelonic acid-3 α , 12 α -diol, 9(11), (5 β)-cholelonic acid-3 α -ol-12-one, allolithocholic acid, glycocholic acid, glycoursocholelonic acid, isolithocholic acid, lithocholelonic acid, tauroursocholelonic acid, ursocholelonic acid, glycodehydrocholic acid, glycodeoxycholic acid, glycohyocholic acid, glycohyodeoxycholic acid, glycolithocholic acid, glycoursodeoxycholic acid, lithocholic acid, and taurodehydrocholic acid

² NS: not significant

³ BLOQ: below limit of quantification

⁴ --: not determined

⁵ ND: not detectable

Table 2.4. Short Chain Fatty Acid Concentrations and Correlation with CYP3A4 (n = 4 per sex per group).

Short Chain Fatty Acid	Concentration (μM)						Correlation with CYP3A4 protein (p-value)
	Male			Female			
	GF	CV	GFCV	GF	CV	GFCV	
Acetic acid	785 ± 73	1808 ± 421	1095 ± 124	735 ± 111	1619 ± 213	1022 ± 268	0.046 (NS ¹)
Propionic acid	50 ± 8.3	55 ± 7.0	42 ± 5.0	44 ± 4.2	54 ± 14	51 ± 4.5	0.100 (NS)
Isobutyric acid	5.5 ± 1.0	4.8 ± 0.9	4.1 ± 0.8	4.1 ± 0.5	5.2 ± 1.6	4.5 ± 0.6	-0.056 (NS)
Butyric acid	144 ± 28	182 ± 36	192 ± 32	151 ± 42	178 ± 69	156 ± 23	-0.06 (NS)

¹ NS: not significant

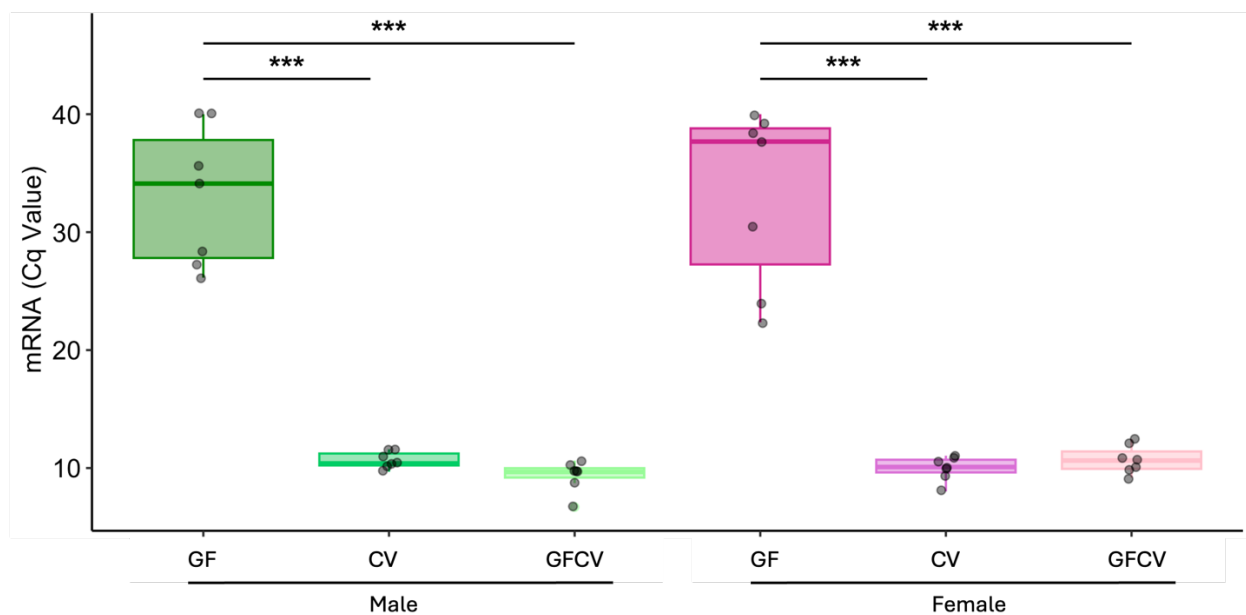


Figure 2.1. Cq values of universal bacteria in the colon contents of male and female GF, CV, and GFCV mice (n = 7 per sex per group). Significance was determined by two-way ANOVA and Tukey's Posthoc analysis [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)].

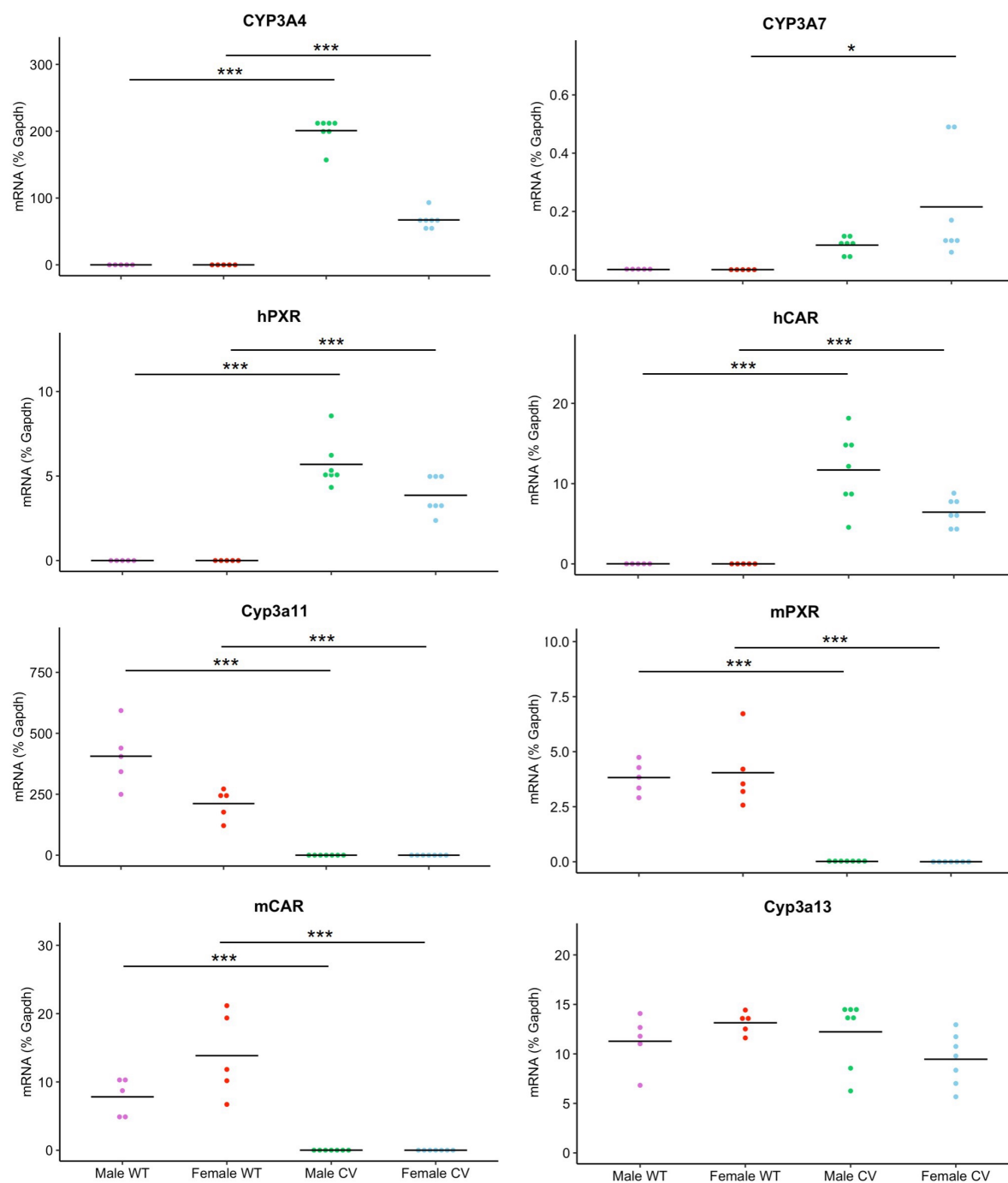


Figure 2.2. mRNA expression (expressed as % Gapdh) of human genes (CYP3A4, CYP3A7, hPXR, hCAR) and mouse genes (Cyp3a11, mPXR, mCAR, Cyp3a13) in the livers of male and female C57BL/6 (WT, n = 5 per sex) and humanized PXR-CAR-CYP3A4/3A7 (CV, n = 7 per

sex) mice. Significance was determined by multi-way ANOVA and Tukey's Posthoc analysis [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)].

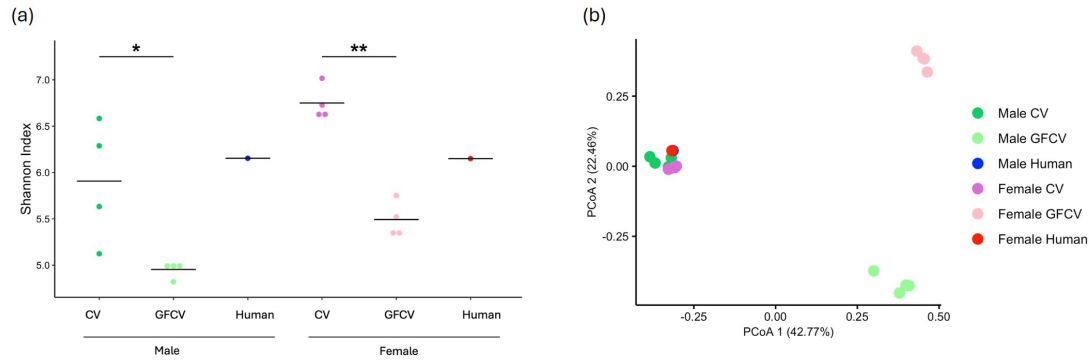


Figure 2.3. Results of shotgun metagenomics sequencing of colonic contents of male and female CV and GFCV mice ($n = 4$ per sex per group) and the pooled human feces used for conventionalization ($n = 1$ per sex). (a) Alpha diversity of each group as calculated by the Shannon Index. Significance was determined by multi-way ANOVA and Tukey's Posthoc analysis [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)]. (b) Beta diversity of all groups as calculated by the Bray-Curtis Index. Significance was determined by PERMANOVA ($p = 0.001$).

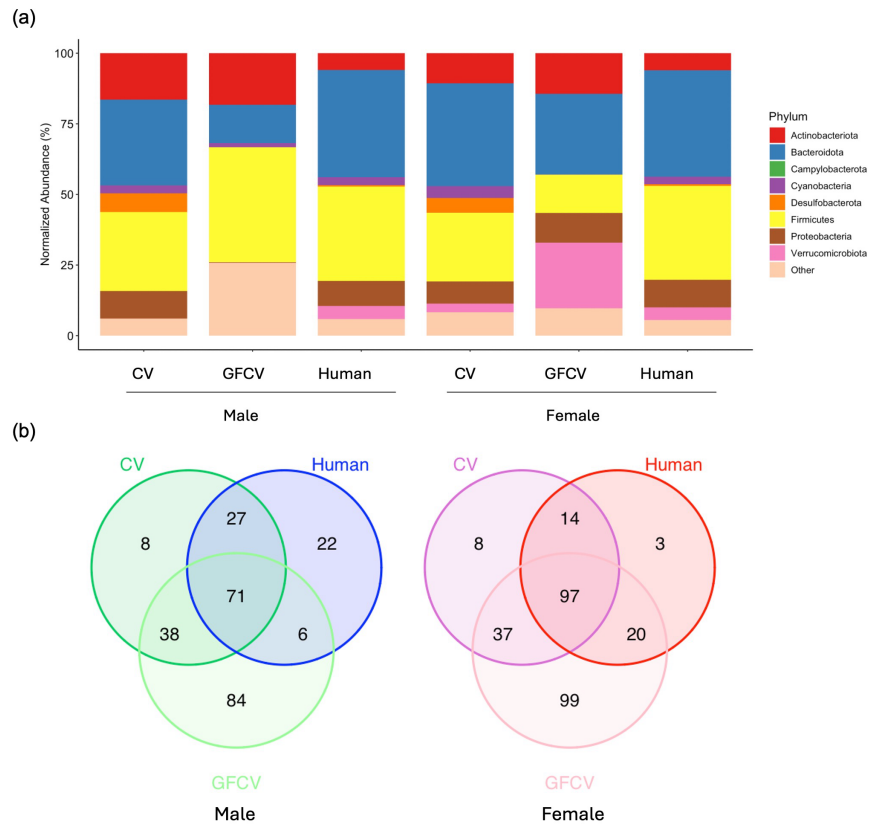


Figure 2.4. Bacterial expression in colonic contents of male and female CV and GFCV mice and the pooled human feces used for conventionalization. (a) Distribution of phylum in mice ($n = 4$ per sex per group) and pooled human feces ($n = 1$ per sex). (b) Venn diagrams demonstrating number of species that overlap between groups for male and female mice.

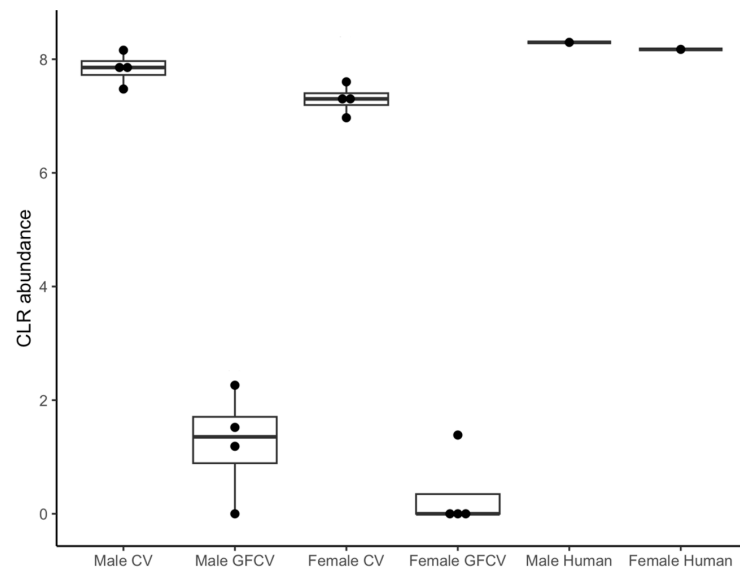


Figure 2.5. Abundance (centered log ratio; CLR) of *Lactobacillus Johnsonii* in the colon contents of male and female CV and GFCV mice (n = 4 per group) and pooled human feces (n = 1 each sex).

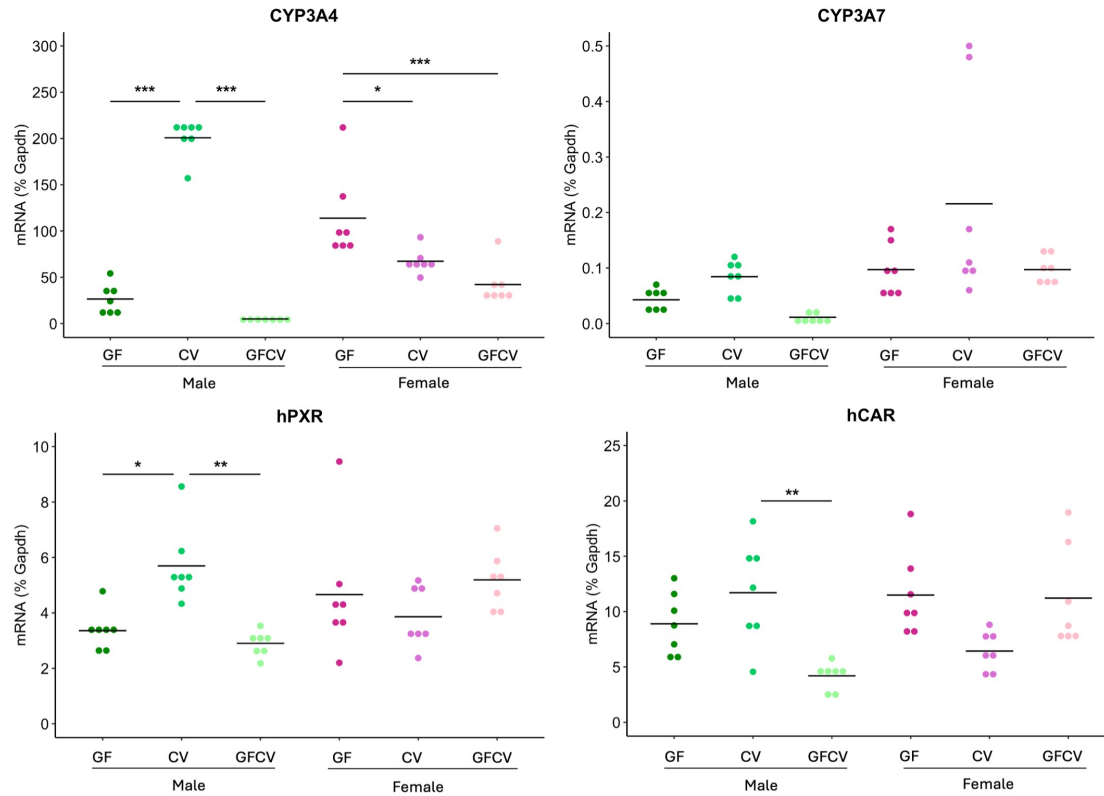


Figure 2.6. Hepatic mRNA expression of human genes (CYP3A4, CYP3A7, hPXR and hCAR) in male and female germ-free (GF), conventional (CV) and conventionalized (GFCV) mice (n = 7 per sex per group). Significance was determined by two-way ANOVA and Tukey's Posthoc analysis [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)].

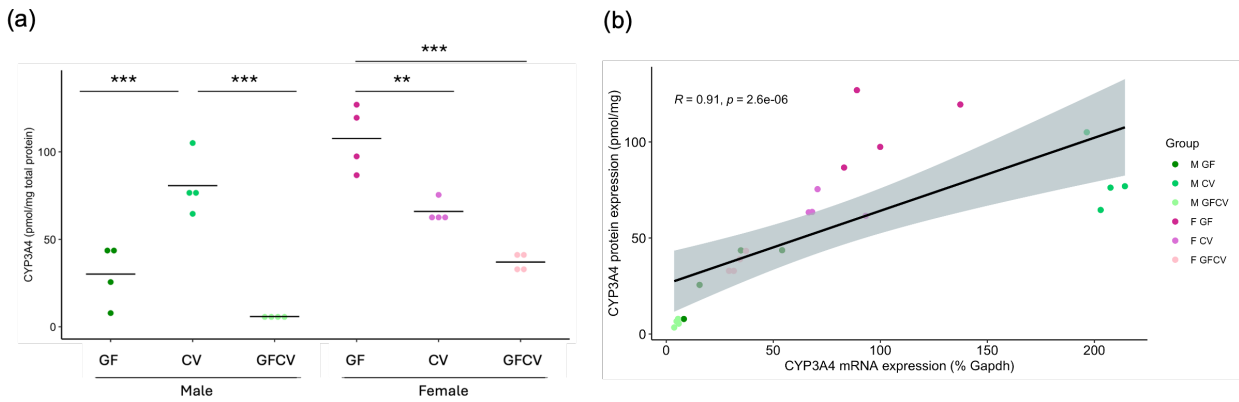


Figure 2.7. Hepatic CYP3A4 protein expression quantified using proteomics. (a) CYP3A4 protein expression in male and female germ-free (GF), conventional (CV) and conventionalized (GFCV) mice ($n = 4$ per sex per group). Significance was determined by two-way ANOVA and Tukey's Posthoc analysis [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)]. (b) Spearman correlation between CYP3A4 RNA and protein expression (Spearman's $R = 0.91$, $p = 2.6 \times 10^{-6}$) where shaded area represents 95% confidence interval.

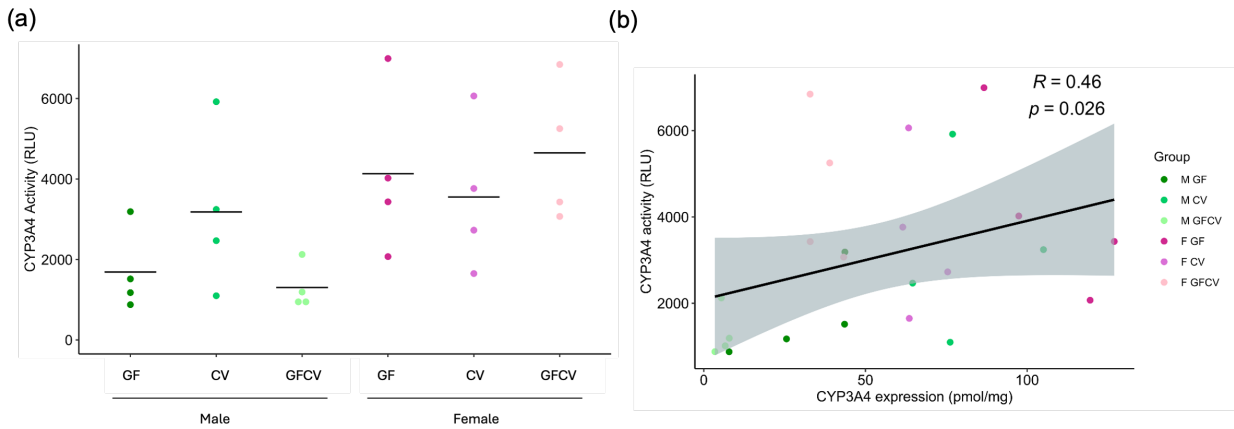


Figure 2.8. (a) Hepatic CYP3A4 activity as quantified by luminescence in male and female germ-free (GF), conventional (CV) and conventionalized germ-free (GFCV) mice ($n = 4$ per sex per group). Significance was determined by Two-way ANOVA and Tukey's Posthoc analysis. (b) Spearman correlation between CYP3A4 protein expression and activity (Spearman's $R = 0.46$, p -value = 0.026) where shaded area represents 95% confidence interval.

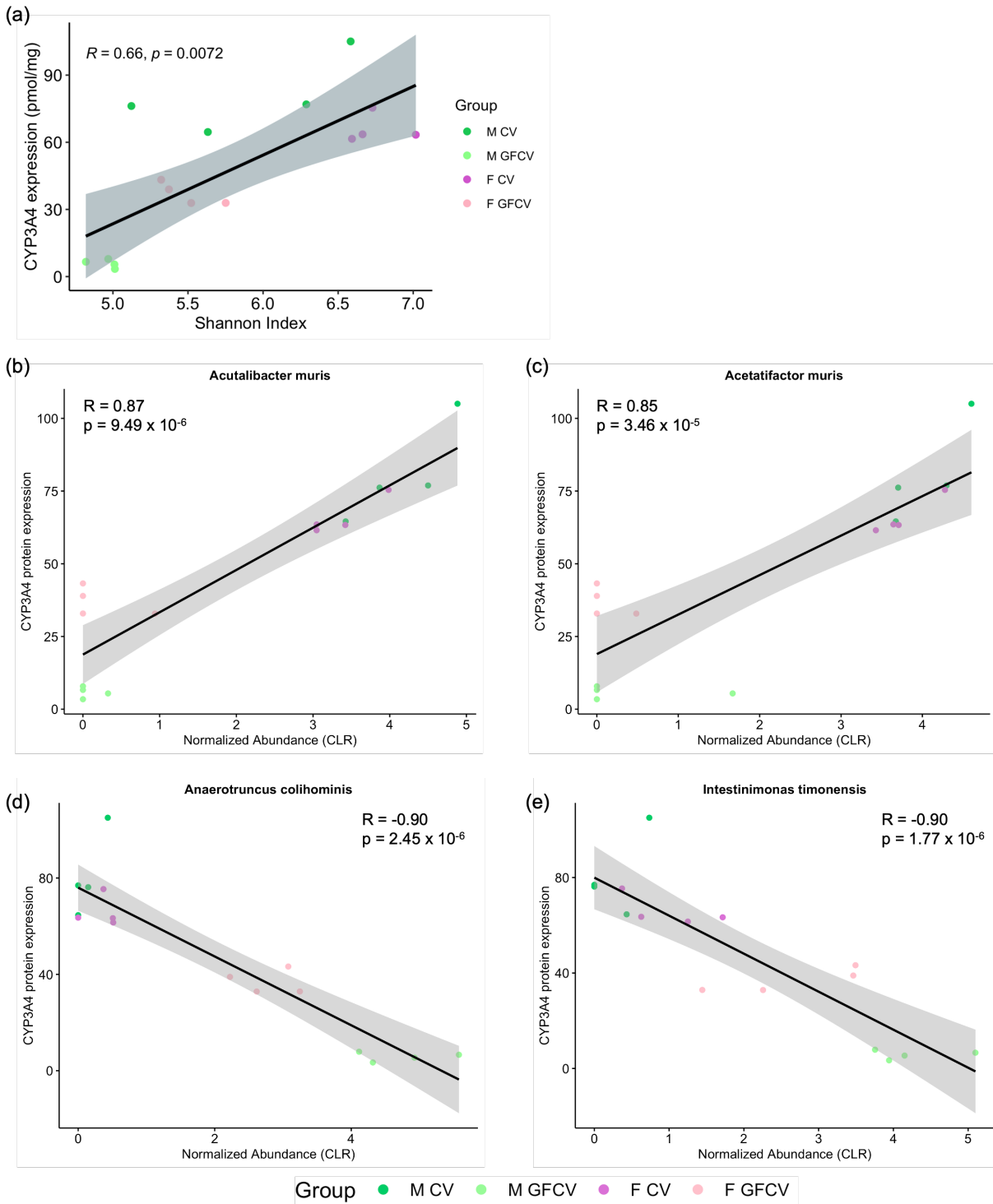


Figure 2.9. Correlation of CYP3A4 protein expression with bacterial species. (a) Spearman correlation between CYP3A4 protein expression and alpha diversity (Spearman's $R = 0.66$, $p = 0.0072$) where shaded area represents 95% confidence interval. (b) Representative bacterial

species with the highest positive and negative correlations with CYP3A4 protein expression where shaded area represents 95% confidence interval of the Spearman correlation and p-values were adjusted by the False Discovery Rate.

Chapter 3

The Gut-Liver Axis:

**A Multi-Omics Approach to Explore Gut Microbiome and Sex Effects
on Hepatic DMETs in PXR-CAR-CYP3A4/3A7 Mice**

3.1. Introduction

The gut microbiome is popularly known as the ‘second genome’ and its composition and diversity can vary due to intrinsic and environmental factors (e.g., age, sex, diet, lifestyle, etc.) (Zhang, 2022; Zhu, Wang, Li, 2010). Manipulation of the microbiome is being studied for several therapeutic purposes as gut dysbiosis has been associated with a range of diseases (Sorbara and Parmer, 2022). Another emerging field, pharmacomicrobiomics, aims to investigate the relationship between the gut microbiome and drugs. The relationship between the gut microbiome and drugs is bidirectional and ranges from direct metabolism of drugs to indirect regulation of the expression of drug metabolizing enzymes and transporters (DMETs) by microbes (Spanogiannopoulos et al., 2016). Understanding the impact of the microbiome on DMET is essential as it can influence drug pharmacokinetics (PK) and ultimately affect drug efficacy.

The liver is one of the major xenobiotic biotransformation organs and hepatic DMETs play a critical role in drug disposition (Almazroo, Miah, Venkataramanan, 2017). The gut-liver axis conceptualizes the interactions between the gut microbiome and the liver. Specifically, the gut microbiome is thought to be the core driver that shape intestinal and liver pathophysiology (Pabst et al., 2023). Some microbial metabolites are likely absorbed and transported to the liver where they can interact with transcription factors, inducing the expression of their respective DMETs (Dempsey and Cui, 2019). Several studies showed differing expression among major DMETs following alteration of microbial conditions in mice (Selwyn et al., 2015a; Selwyn et al., 2016; Togao et al., 2023). Yet, the mechanisms behind microbial DMET regulation are still unknown. A broader understanding of how differences in the gut microbiome affect the overall hepatic -omics is an important first step in explaining why changes in DMET expression are

seen.

Another limitation in the current literature is the paucity of microbiome studies in female mice. Conventional (CV) mice display significant sex differences in hepatic Cyp3a11 expression and Cyp3a11 is generally higher in male CV mice compared to female CV mice from 3 to 27 months of age (Fu et al., 2012). However, another study saw higher hepatic Cyp3a11 expression in female CV mice compared to male CV mice but no sex differences in germ-free (GF) mice (Jourová et al., 2020). The sex-specific regulation of the microbiome on DMETs has not yet been fully elucidated.

The objectives of this study were to: 1) identify microbial differences between sexes in CV mice to provide insight into possible microbes that are associated with differences in the hepatic DMETs; 2) use a global -omics approach to explore microbial and sex differences in the hepatic transcriptome and metabolome to gain insight into altered biological processes; and 3) investigate mRNA and protein expression of hepatic DMETs that differ depending on microbiome and sex to establish specific regulatory patterns. We used a humanized PXR-CAR-CYP3A4/3A7 mouse model to attempt to find microbial species and/or metabolites that impact DMET expression with the eventual goal of translating findings to humans. Overall, this study aims to lay a foundation for elucidating the mechanisms behind the microbial and sex-specific effects in the regulation of DMETs.

3.2. Materials and Methods

3.2.1. Materials

Ammonium bicarbonate (ABC; 98% purity), dithiothreitol (DTT), iodoacetamide (IAA), Pierce trypsin protease (MS-grade), stable isotope-labeled (SIL) peptides, bovine serum albumin

(BSA), hydrochloric acid (HCl), protease inhibitor cocktail, potassium phosphate dibasic and monobasic anhydrous were obtained from Thermo Fisher Scientific (Waltham, MA). Acetone, sucrose, and Trizma[®] base were purchased from Sigma Aldrich (St. Louis, MO), and Optima MS-grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ).

3.2.2. Animals and Procedures

This study expands on the study of humanized CV and GF PXR-CAR-CYP3A4/3A7 mice that were originally presented in Liem et al. (2026). All animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington (PROTO202000114 / 4035-07).

Conventional (CV) humanized PXR-CAR-CYP3A4/3A7 mice (C57BL/6-*Nr1i3*^{tm1(NR1I3)Arte} Is(5CYP3A4-CYP3A7;Del5Cyp3a57-Cyp3a59)2Arte *Nr1i2*^{tm1(NR1I2)Arte}) (Taconic stock #11585) were purchased from Taconic Bioscience (Germantown, NY). The germ-free (GF) colony was generated by rederivation of the conventional (CV) humanized PXR-CAR-CYP3A4/3A7 mice by the University of Washington Gnotobiotic Core. GF mice were housed in the Gnotobiotic Animal Core in isolators. All animals were maintained on 14-hr light/10-hr dark cycles with autoclaved Enrich-N'Pure bedding (The Andersons Inc., Maumee, OH). Autoclaved Breeder Chow #5021 (LabDiet, St. Louis, MO) and autoclaved non-acidified water were provided *ad libitum*. All mice were housed until the day of tissue collection when mice were 12-14 weeks of age.

Male and female CV and GF humanized PXR-CAR-CYP3A4/3A7 mice were studied

(n=4 per sex per group). On the day of tissue collection, mice were anesthetized with isoflurane, and blood was collected via cardiac puncture and immediately centrifuged ($1,500 \times g$ for 10 min at 4°C) to isolate plasma. Liver and colon were removed and washed in cold PBS. The colon contents were collected for microbial sequencing. Plasma, liver, and colon contents were snap-frozen on dry ice and stored at -80°C until analysis.

3.2.3. Bacterial DNA Extraction and Metagenomic Shotgun Sequencing

Details of bacterial DNA extraction and sequencing are provided in Liem et al. (2026). In brief, total genomic bacterial DNA was extracted from the colonic contents of male and female CV mice (n = 4 per sex per group) and sequencing was performed by Diversigen (New Brighton, MN). Sequences were aligned to a curated database with additional metagenomically assembled genomes (MAGs; completeness >90%, contamination <5%) and cell-cultured genomes. Alpha diversity was calculated by Shannon and Chao1 index and beta diversity was determined by Bray-Curtis index. All plots were created using ggplot2 in R version 2024.04.1+748.

3.2.4. Hepatic RNA Isolation and RNA Sequencing

Total RNA was isolated from livers of male and female GF and CV mice (n = 4 per sex per group) using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) following the manufacturer's protocol. Concentration of the RNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at 260 nm. RNA quality was assessed by formaldehyde-agarose gel electrophoresis with visualization of 18S and 28S rRNA bands under UV light and confirmed by an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA). Samples were sent to Novogene (Beijing, China) for RNA sequencing.

FASTQ files with paired-end sequence reads were mapped to the mouse and human genomes (UCSC mm10 and hg38, respectively) using HISAT2 (Kim et al., 2019). Sequence alignment/map (SAM) files were converted to their binary form and sorted using SAMtools version 1.2 (Li et al., 2009). Transcript abundances were estimated with featureCounts (Subread package, version 1.5.3; Liao, Smyth, and Shi, 2013) using the Gencode mouse and human gene transfer formats (GTF) vM25 and v44, respectively. Transcript abundance was normalized to transcripts per million (TPM). Differential expression analysis was performed using DESeq2 (Love, Huber, and Anders, 2014) and filtered for significance of $p < 0.05$. Gene ontology (GO) analysis was performed in R with a created function that conducts enrichment analysis for GO terms. Data were visualized using ggplot2 in R version 2024.04.1+748.

3.2.5. Untargeted Metabolomics with LC-MS

Male and female CV and GF mouse livers ($n = 4$ per group) were sent to Dr. Haiwei Gu at Arizona State University for untargeted metabolomics as described in previous studies (Gu et al., 2015; Wei et al., 2021). Briefly, the untargeted metabolomics was conducted with an Agilent 1200 SL LC-6520 Quadrupole Time of Flight (QTOF) MS system (Agilent Technologies, Inc., Santa Clara, CA). Samples were injected on a Waters Xbridge BEH Amide column ($2.5\ \mu\text{m}$, $2.1 \times 150\ \text{mm}$) at 40°C . The Q-TOF data were extracted with Agilent MassHunter Qualitative Analysis (version B.07.00), Quantitative Analysis (version B.07.01), and Mass Profiler Professional (MPP, version B.13.00) software. Features were matched against the commercial Agilent databases available in MPP (version B.13.00) and METLIN. The absolute intensity threshold for the LC-Q-TOF data extraction was 1000, and the mass accuracy limit was set to 10 ppm. Heatmap was created with pheatmap package and volcano plots were created using ggplot2

in R version 2024.04.1+748.

3.2.6. Targeted DMET Analyses

DMET Gene Expression. To explore differences in DMET targets based on microbial or sex differences, selected targets were abstracted from the RNAseq transcriptomics data and classified as Phase I enzymes, Phase II enzymes, drug transporters or transcription factors. A heatmap of all genes were created with pheatmap and patterns were manually identified. Individual plots of genes were created using ggplot2 in R version 2024.04.1+748.

Targeted DMET Proteomic Analysis. Using the Pierce Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, Waltham, MA), liver membrane proteins were isolated from male and female CV and GF mice (n = 4 per sex per group). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Biotechnology, Waltham, MA). Samples were sent to Precision Quantomics (Spokane, WA) for digestion and proteomic quantification as described in Liem et al (2026).

Twenty DMET proteins were selected for proteomics based on RNA sequencing results and four specific criteria: 1) transcripts per million (TPM) > 20 (chosen to represent genes that were relatively highly expressed); 2) genes that were differentially expressed based on RNA sequencing data and/or literature; 3) likely presence in hepatic membrane fractions; and 4) highly expressed in the liver (Lim et al., 2025). The selected proteins were: CYP3A4, Cyp1a2, Cyp2b10, Cyp2c38, Cyp2d22, Cyp2e1, Cyp3a13, Cyp4a14, Cyp7a1, Fmo5, Ces1g, Ugt2b1, Ugt2b35, Abcb11, Abcg5, Abcg8, Abcb4, Abcc2, Abcc3, and Slco1b2. CYP3A7, the other humanized protein, was not selected due to low abundance. PXR and CAR were not selected because they were unlikely to be isolated in the membrane fraction. Protein quantification was

performed using total protein abundance (TPA) method described previously by Precision Quantomics (Sharma et al., 2023). Global proteomics analysis was acquired on the EASY-Spray 1200 series nanoLC coupled with Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA) using an Acclaim PepMap™ trap column (75 µm x 150 mm, 3 µm) (Thermo Fisher Scientific, part number 164535) connected with analytical PepMap™ RSLC C18 column (75 µm x 250 mm, 2 µm) (Thermo Fisher Scientific, part number ES902). The mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in 80% acetonitrile (B), was run in a gradient mode (% B) of 2-6% (0-10 min), 6-30% (10-110 min), 30-100% (110-115 min), and 100% (115-131 min) with a flow rate of 300 nL/min. Data was acquired in the positive ion mode using data-independent acquisition (DIA) feature of Xcalibur software (Thermo Fisher Scientific, Version 4.3.73.11). Global proteomics data was processed using DIA-NN software and the quantitative values for target enzyme and transporters were determined using TPA method (Sharma et al., 2023).

3.2.7. Statistical Analysis

Significance for alpha diversity was assessed using Wilcoxon rank-sum test. Beta diversity differences were evaluated using PERMANOVA. All p-values calculated for RNA sequencing were adjusted by false discovery rate. Spearman's correlation was used for correlation analyses. A p-value < 0.05 was considered significant.

3.3. Results

3.3.1. Microbial composition and diversity between sexes in CV mice

Metagenomics shotgun sequencing was performed on the colonic contents of male and

female CV mice to compare microbial composition and diversity. GF mice had undetectable bacterial genomic DNA and were not sequenced (Liem et al., 2026). There were subtle sex differences in the microbial composition and diversity of CV mice (Figure 3.1). Overall, 44 families were identified, and there was variability within and between groups but roughly similar patterns in the composition (Figure 3.1a).

The alpha diversity of microbial species was compared using two indices: the Shannon Index (number of species and evenness) and the Chao1 Index (total number of species, accounting for rare species) in Figures 3.1b and 3.1c, respectively. The Shannon index was higher in female CV mice compared to male CV mice ($p < 0.05$) and although female CV mice trended higher than male CV mice for Chao1, the difference was not significant. The beta diversity between male and female CV mice differed ($p = 0.03$; Figure 3.1d) and were not tightly clustered for either sex.

3.3.2. Hepatic Transcriptomics

To investigate the changes in the hepatic transcriptome of male and female CV and GF mice, RNA sequencing was performed. We identified a total of 22,098 genes and visualized the overall variance and grouping using principal components analysis (PCA; Figure 3.2). The first two principal components, PC1 and PC2, accounted for 44.8% of the total variance. Though mice were generally clustered in each group, the male CV and male GF mice were more separated than the female CV and female GF mice.

3.3.2.1. Differential Hepatic Transcriptomics

The impact of the intestinal microbiome and sex on hepatic transcriptomics was

compared in GF vs. CV mice. In male GF vs. male CV mice, a total of 4656 differentially expressed genes (DEGs) were found (21% of total transcripts) of which 2185 were upregulated (47% of DEGs) and 2471 were downregulated (53% of DEGs). In female GF vs. female CV mice, a total of 939 DEGs were found (4.25% of total transcripts) of which 529 were upregulated (56% of DEGs) and 410 were downregulated (44% of DEGs). In female CV vs. male CV mice, a total of 2091 DEGs were found (9.5% of total transcripts) of which 1022 were upregulated (49% of DEGs) and 1069 were downregulated (51% of DEGs). In female GF vs. male GF mice, a total of 3477 DEGs (16% of total transcripts) were found of which 1737 were upregulated (50% of DEGs) and 1740 were downregulated (50% of DEGs).

3.3.2.2. Differential Hepatic Transcriptomics in CV and GF mice

Gene ontology (GO) analysis was conducted to classify gene products and reveal processes that were altered between CV and GF mice. The top ten differential gene ontology terms for each set of comparisons are shown in Figures 3.3. Amide metabolic processes and responses to oxidative stress were downregulated in male GF mice compared to male CV mice (Figure 3.3a). In contrast, oxidative phosphorylation, aerobic respiration, and ATP synthesis were upregulated in male GF mice compared to male CV mice (Figure 3.3b). Sterol biosynthetic processes, cholesterol biosynthetic processes, and secondary alcohol biosynthetic processes were downregulated in female GF mice compared to female CV mice, but unlike male mice, there were no changes in responses to stress (Figure 3.3c). No processes were upregulated in female GF vs. female CV mice (i.e., $-\log_{10}(\text{FDR}) < 3$; Figure 3.3d).

3.3.2.3. Differential Hepatic Transcriptomics in Male and Female Mice

Gene ontology (GO) analysis was conducted to classify gene products and reveal processes that were altered between sexes. The top ten differential gene ontology terms for each set of comparisons are shown in Figures 3.4. Responses to endoplasmic reticulum stress, cytoplasmic translation, xenobiotic metabolic process, and glutathione metabolic process were downregulated in female CV mice compared to male CV mice (Figure 3.4a). Small molecule catabolic process, organic acid catabolic process and carboxylic acid catabolic process were upregulated in female CV mice compared to male CV mice (Figure 3.4b). Cytoplasmic translation, oxidative phosphorylation, and aerobic respiration were downregulated pathways, whereas fatty acid metabolic process was upregulated in female GF mice compared to male GF mice (Figures 3.4c and 3.4d). In both GF and CV mice, cytoplasmic translation was downregulated in female mice compared to male mice (Figures 3.4a and 3.4c).

3.3.3. Hepatic Metabolomics

The impact of the intestinal microbiome and sex on hepatic metabolomics was compared in GF vs. CV mice. A total of 5419 metabolites were detected in the liver samples and 3222 metabolites passed quality control (those with coefficient of variation of less than 20%). From the 3222 metabolites, variance was visualized using PCA. PC1 and PC2 accounted for 41.1% of the total variance (Figure 3.5a). Similar to the transcriptome, the male CV and male GF mice were more separated than the female CV and female GF mice. A heatmap was used to visualize overall patterns of expression in the metabolome. Overall, there were 4 patterns of expression. The first pattern is where male CV had a high expression of metabolites, and the other three groups had lower expression. The second and third patterns were sex-specific where expression was high in one sex and low in the other. The last pattern is where metabolite expression was

low in male CV mice but high in the other three groups. Overall, the male CV mice had the most different expression pattern compared to the other three groups (Figure 3.5b).

3.3.3.1. Differential Metabolomics

A differential analysis was conducted and metabolites with a fold change greater than 2-fold and with $FDR < 0.05$ were considered significant. Comparing male GF to male CV mice, a total of 307 metabolites (9.5% of metabolites) differed by more than 2-fold of which 185 were upregulated (60%) and 122 were downregulated (40%; Figure 3.6a). Comparing female GF to female CV mice, only 2 unidentified metabolites differed (Figure 3.6b). Comparing female CV to male CV mice, 84 metabolites differed (2.6% of total) of which 28 were upregulated (33%) and 56 were downregulated (67%; Figure 3.6c). Comparing female GF to male GF mice, 8 metabolites differed (0.2%) of which 2 were upregulated (25%) and 6 were downregulated (75%; Figure 3.6d). Many of these metabolites that differed more than 2-fold were unidentified metabolites.

3.3.4. Targeted DMET Analyses

3.3.4.1. DMET Gene Expression

RNA sequencing genes were categorized into four groups of genes involved in drug disposition: Phase I enzymes, Phase II enzymes, transporters, and transcription factors. These genes were plotted as a heatmap to visualize patterns of expression. We identified six major patterns (Figure 3.7a, Table 3.1). We selected a representative gene to visualize from each pattern. In pattern 1, *Cyp1a2* was selected as an example of high expression in male CV mice but lower and comparable expression in the other three groups (Figure 3.7b). For pattern 2, *Cyp2d9*

was selected as an example where male CV and male GF mice have slightly higher expression compared to the female CV and female GF mice (Figure 3.7c). For pattern 3, *Nr1d1* was selected as an example where male GF and female GF mice had a higher expression compared to male CV and female CV mice (Figure 3.7d). For pattern 4, *Ces3a* was selected as an example where male GF had the highest expression while the other three groups were lower and comparable to each other (Figure 3.7e). For pattern 5, *Cyp2b9* was selected as an example where expression was lower in male CV and male GF mice and higher in female CV and female GF mice (Figure 3.7f). Pattern 6 was the most common pattern as 51.9% of genes fell into this category. *Ugt1a1* was selected as an example where expression was highest in male CV mice, lowest in male GF mice, but similar for female CV and female GF mice (Figure 3.7g).

Based on a differential expression analysis, the number of upregulated and downregulated genes for each comparison (GF vs. CV; female vs. male) are summarized in Figure 3.8 and Table 3.2. There was a higher percentage of upregulated or downregulated genes for male GF vs. male CV mice compared to female GF vs. female CV mice. For example, male GF mice had a total of 18% upregulated and 27% downregulated Phase I genes compared to male CV mice. In contrast, only 6% and 3% of Phase I genes were upregulated and downregulated, respectively for female GF compared to female CV mice. For Phase II genes comparing GF vs. CV mice, 17% of genes were upregulated and 29% were downregulated in male mice and 3% were upregulated and 8% were downregulated in female mice. For transporters comparing GF vs. CV mice, 23% of genes were upregulated and 18% were downregulated in male mice and 10% were upregulated and 3% were downregulated in female mice. For transcription factors comparing GF vs. CV mice, 17% of genes were upregulated and 15% were downregulated in male mice and 8% were upregulated and 7% were downregulated in

female mice.

The percentage of differentially expressed genes were similar when comparing female CV vs. male CV mice and female GF vs. male GF mice. In female CV vs. male CV mice, there were 17% upregulated and 15% downregulated Phase I genes, 21% upregulated and 22% downregulated Phase II genes, 20% upregulated and 18% downregulated transporter genes, and 10% upregulated and 5% downregulated transcription factor genes. For female GF vs. male GF mice, there were 20% upregulated and 10% downregulated Phase I genes, 22% upregulated and 16% downregulated Phase II genes, 15% upregulated and 18% downregulated transporter genes, and 10% upregulated and 14% downregulated transcription factor genes.

To further investigate potential regulatory patterns, five major transcription factors (*Ahr*, *hCAR*, *hPXR*, *Hnf4a*, and *Ppara*) and their downstream genes (i.e., Phase I, Phase II, and transporters) were visualized (Figure 3.9). The downstream genes were selected using TRRUST (Han et al., 2017) and GTRD database (Kolmykov et al., 2021). For most of the downstream genes, with the exception of *Cyp4a12b*, male GF mice either had lower or similar expression to male CV mice. Expression of *Cyp4a12b* was higher in male GF mice compared to male CV mice. For female mice, the selected downstream gene expression either was higher or similar for female GF mice compared to female CV mice. Most selected downstream genes had similar or lower expression in female CV mice compared to male CV mice, except for *Slc29a1* and *Slc10a1*. In contrast, for GF mice, female GF mice had similar or higher expression of the selected downstream genes compared to male GF mice, except for *Gstp2*.

3.3.4.2. Targeted DMET Protein Expression

Twenty DMET targets were selected for quantification using proteomics. For Cyp2c38,

values were below the level of quantification (BLQ; denoted as 0) for four male CV mice and three male GF mice. The protein expression of the quantified DMETs were correlated to mRNA expression (Table 3.3). Cyp2b10, Cyp2c38, Cyp4a14, CYP3A4, and Ugt2b35 protein expression was positively correlated with mRNA expression. Cyp1a2, Cyp2d22, Cyp2e1, Cyp3a13, Cyp7a1, Ces1, Fmo5, and Ugt2b1 protein expression was not correlated with mRNA expression. For most Abc transporters and the quantified Slc transporter (Slco1b2), no correlation was observed between protein and mRNA expression, except for Abcb4 and Abcg8 where protein and mRNA expression were positively correlated.

3.4. Discussion

This study utilized a multi-omics approach to identify the impact of microbiome and sex on the hepatic environment in a humanized PXR-CAR-CYP3A4/3A7 mice. The goal of this current work is to uncover microbial and sex-specific effects that may suggest potential mechanistic pathways in the regulation of DMETs.

First, the microbial composition and diversity of male and female CV mice were compared. The overall composition at the bacterial family level showed similar patterns between male and female CV mice (Figure 3.1a). Overall, female CV mice had a higher alpha diversity, describing richness and evenness (~1.1 fold for Shannon index, $p < 0.05$) compared to male CV mice, and beta diversity ($p = 0.033$) differed between groups (Figures 3.1b - d). This finding is consistent with what was seen in other mouse studies. For example, a study in two mouse strains (i.e., BALB/c and C57BL/6) showed that females have a higher alpha diversity than males (Elderman et al., 2018). However, there have been conflicting reports on the impact of sex on the gut microbiome in humans. For example, in two studies conducted in European individuals, one

study reported no differences in microbial diversity between sexes (Lay et al., 2005) while the other study found a higher alpha diversity in females compared to males (Falony et al., 2016).

An overview of the transcriptome revealed that male CV mice had the most different expression compared to the other groups (Figure 3.2). Specifically, male CV mice and male GF mice had very different transcriptomes while female CV and female GF mice were more similar for their transcriptomes. Additionally, CV but not GF mice had sex differences in the transcriptome. A gene ontology analysis was performed to assess differences in biological processes. In both male and female mice, the lack of a microbiome led to downregulation in fatty acid metabolic process (Figure 3.3a and 3.3c). Changes in metabolic processes could be explained due to absence of a regular gut microbiome and the crosstalk between the gut and the liver. In female mice, the lack of a microbiome led to downregulation of several biosynthetic and metabolic processes but there were no significant upregulated processes, suggesting that there were not many upregulated processes or that the comparator group had a high baseline and hence significance of upregulated process is harder to achieve (Figure 3.3c and 3.3d). Interestingly, most of the downregulated processes in male GF mice were related to stress and the upregulated processes are related to boosting ATP synthesis in response to need for energy (Figure 3.3a and 3.3b). For example, upregulation in oxidative phosphorylation could be due to response to stress such as DNA damage or increased demand in energy (Brace et al., 2016).

Cytoplasmic regulation was downregulated in both female CV and female GF mice compared to their male counterparts (Figure 3.4a and 3.4c). Reduction in cytoplasmic translation is related to protein synthesis (Chen et al., 2023; Shyu et al., 2008). On the other hand, small molecule catabolic and fatty acid metabolic processes were upregulated in both female CV and female GF mice compared to male CV and male GF mice (Figure 3.4b and 3.4d). Higher fatty

acid metabolism was also reported to be higher in females in primary mouse hepatocytes (Hochmuth et al., 2021).

Similar to the transcriptomic results, the metabolome of male CV and male GF mice differed while the metabolome for female CV and female GF mice clustered more tightly (Figure 3.5a). Male CV mice had a metabolite pattern that differed from the other three groups (Figure 3.5b) and had the greatest number of differentially expressed metabolites with 122 upregulated and 185 downregulated metabolites (Figure 3.6a) when compared to male GF mice. In contrast, there were only two metabolites that differed between female GF vs. female CV mice (Figure 3.6b). The sex difference was more prominent in CV mice compared to GF mice (Figure 3.6c and 3.6d), which is consistent with what was seen in the PCA plot. Overall, global -omics analyses revealed that male CV mice had the most different hepatic environment and that the lack of microbiome had a bigger impact on male compared to female mice.

Focusing specifically on DMETs, a total of six patterns were identified and a representative gene was selected to showcase each pattern (Figure 3.7, Table 3.1). Almost half of the total DMET genes fall into the pattern 6 where expression is the lowest in male GF mice, highest in male CV mice but comparable for female CV and female GF mice (Figure 3.7g). Similar to the global transcriptome and metabolome, the greatest differences in DMET genes were between male GF and male CV mice (Figure 3.8, Table 3.2). These patterns provide a general idea of how the microbiome and sex may impact DMET expression and highlight that the lack of a microbiome influences male mice more than female mice. Major drug-related transcription factors with examples of downstream Phase I, Phase II, and transporter genes followed the general pattern where expression was highest in male CV mice, intermediate in female GF and female CV mice, and lowest in male GF mice (Figure 3.9). The differences in

transcription factor expression between male and female CV and GF mice did not always translate to their downstream genes.

Consistent with other studies in male mice, most DMETs were expressed at higher levels in male CV mice compared to male GF mice (Selwyn et al., 2015a). However, we noted an opposite pattern in female mice, where female CV mice had a lower expression of most DMETs compared to female GF mice. Selwyn et al. reported that many hepatic DMETs were lower or not different in female CV mice compared to female GF mice, but that the effect was age-dependent (Selwyn et al., 2015b). For example, *Aldh3a2*, *Cyp4a10*, *Cyp4a31*, and *Cyp4a32* were lower in 30-day old female CV mice but not different in 90-day old female mice compared with age-matched female GF mice. Overall, consistent with our findings, Selwyn et al. also saw a greater microbial impact on DMETs in male mice compared to female mice. This sex-specific regulation of DMETs may be mediated by sex hormones that have been shown to impact growth hormone secretion resulting in sexually dimorphic expression of DMETs (Buckley and Klaassen, 2009; Waxman and O'Connor, 2006). Further studies need to be done to elucidate the mechanism behind this sex-specific effect on the microbial regulation of DMETs.

In our data with the humanized PXR-CAR-CYP3A4/3A7 mice, female CV mice generally had lower expression of DMETs (e.g., *CYP3A4*) compared to male CV mice. In contrast, most DMETs were more highly expressed in female GF mice compared to male GF mice. Other studies with the PXR-CAR-CYP3A4/3A7 mouse model found higher CYP3A4 expression and activity in female mice compared to male mice (Crosby and Riddick, 2019; Fashe et al., 2025). In human studies, women have higher hepatic CYP3A4 expression (mRNA and protein) and activity (through studies on CYP3A4 substrates) compared to males (Greenblatt and von Moltke, 2013; Tanaka, 1999; Wolbold et al., 2003; Yang et al., 2012). It is unknown why

there is a discrepancy in CYP3A4 expression in our study of male and female CV mice.

Protein and mRNA expression were not always correlated for the quantified DMETs (Table 3.3). This is expected as mRNA expression is typically not able to predict protein abundance due to several intermediate processes (Samih et al., 2026). Therefore, changes in DMETs mRNA expression do not necessarily translate to changes in DMET activity and hence drug PK. Future studies should incorporate global proteomics to identify whether the same mRNA patterns are observed at the protein level.

There are several limitations to our study. First is the small sample size ($n = 4$ per sex per group). This is the first study to explore the hepatic environment of PXR-CAR-CYP3A4/3A7 mice using a multi-omics approach, and despite the limited number of mice, these data will inform the design of future studies. In particular, to verify whether female CV mice have lower CYP3A4 expression compared to male CV mice, CV and GF mice of different ages or wild-type C57BL/6 mice could be studied to determine if humanization of the mouse with hPXR, hCAR, CYP3A4 and CYP3A7 (and deletion of associated mouse genes), results in unpredictable changes in the hepatic environment and regulation of DMETs by the microbiome and sex. It is important to see whether the influence of the microbiome on human PXR and CAR differ. Male CV mice demonstrated substantial interindividual variability in their microbiome, transcriptome, and metabolome. This variability was not as prominent for the other three groups and future studies should confirm whether this variability persists in a larger group of mice. Lastly, cage-specific effects (not recorded in the presented study) should be considered as a cause of variability in male CV mice and potential contributor to differences seen between groups.

3.5. Conclusion

In summary, utilizing this multi-omics approach allowed for a deeper understanding of how the gut microbiome and sex influenced the hepatic environment and DMETs in the humanized PXR-CAR-CYP3A4/3A7 mouse model. First, we showed that the microbial diversity of female CV mice was higher than male CV mice. Additionally, male CV mice displayed a much higher variability in their bacterial diversity while female mice had minimal interindividual variability. This variability was also reflected in the transcriptome and metabolome of male CV mice where PCA plots revealed that the male CV mice did not cluster as tightly as the mice in other groups. Lastly, the lack of a microbiome had a bigger impact on the transcriptomic and metabolomic environment as well as DMET expression in male mice compared to female mice.

3.6. References

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3.7. Tables and Figures

Table 3.1. List of drug processing genes that fall into each pattern as determined in the heatmap.

Pattern	Category			
	Phase I	Phase II	Transporters	Transcription Factors
1	Ces1a, Ces1b, Cyp1a1, Cyp1a2, Cyp2a5, Cyp4f17, Cyp4f39, Cyp7a1, Cyp51, Dhrr4, Dhrr7c, Dhrr11, Akr1c19, Akr7a5, Gpx2, Gpx4, Sdr9c7, Aox1, Gclc, Txnrd1, Ephx1, Aldh1a1, Aldh112, Aldh18a1, Cbr3, Nqo1	Gsta1, Gsta2, Gsta4, Gstm1, Gstm2, Gstm3, Gstm4, Gclm, Gsr, Glyat, Comt, Ugdh, Ugt2b5, Ugt2b35, Ugt2b36	Slc51a, Abcc4, Abcg2	Nr1h4, Nfe212, Pparg, Rorc, Esrra
2	Cbr2, Cyp2c67, Cyp2d9, Cyp4a12a, Cyp4a12b, Cyp4a32, Cyp7b1, Ephx3	Gstp1, Gstb2, Matg, Nat2, Nat8, Ugt2b1	Slc22a4, Slco1b2, Abcb1b	Nr2f2, Nr3c2
3	Cyp2d12, Cyp2t4, Gpx5, Gpx6, Gpx7, Gpx8, Aldh3b2, Akr1c18	Ugt1a2, Ugt1a6a, Gstm6, Nat9, Mgst2	Slc28a2, Abcg5	Nr1d1
4	Ces2b, Ces2h, Ces3a, Ces3b, Ces4a, Ephx4, Gpx3, Cyp2j8, Cyp2j9, Cyp4f13, Cyp2u1, Cyp21a1	Gstt2, Tpm1, Mgst1	Slc22a21, Slco1a1	Nr4a1, Nr4a2, Thra, Rara
5	Cyp2a4, Cyp2a12, Cyp2b9, Cyp2b13, Cyp2c23, Cyp2c40, Cyp2c50, Cyp2c54, Cyp2c55, Cyp2c69, Cyp2c70, Cyp2d22, Cyp2e1, Cyp2g1, Cyp2j6, CYP3A7, Cyp4f14, Cyp4f15, Cyp4f16, Cyp4v3, Cyp17a1, Cyp46a1, Emo3, Emo4, Pon1, Gpx1, Akr1c6, Akr1c21, Aldh1a2, Adh7, Adhfe1, Prdx4, Amacr	Gstm7, Gstk1, Gstt1, Sult1a1, Sult1b1, Sult1c2, Sult1d1, Sult1e1, Sult2a1, Sult2a2, Sult2a3, Sult2a4, Sult2a5, Sult2a6, Sult2a7, Sult3a1, Sult4a1, Ugt1a10, Ugt2a3, Ugt2b37, Nat1	Slc10a2, Slc27a2, Slc29a1, Slc47a1, Slco1a4, Abcc6, Abcg8	Nr1d2, Esr1, Esrrg, Hnf4g
6	Cyp1b1, Cyp2a22, Cyp2b10, Cyp2c29, Cyp2c37, Cyp2c38, Cyp2c39, Cyp2c65, Cyp2c68, Cyp2d10, Cyp2d11, Cyp2d26, Cyp2f2, Cyp2r1, Cyp4a10, Cyp4a14, Cyp4a31, Cyp3a13, CYP3A4, Cyp4b1, Cypf418, Cyp8b1, Cyp20a1, Cyp26a1, Cyp26b1, Cyp27a1, Cyp27b1, Cyp26c1, Cyp2d40, Cyp2j5, Cyp39a1, Prdx2, Prdx5, Prdx6, Hsd3b7, Hsd17b4, Ces1c, Ces1d, Ces1e, Ces1f, Ces1g, Ces2a, Ces2c,	Gsta3, Gstd, Gstm5, Gsto1, Gsto2, Gss, Gstt3, Gstz1, Nat10, Nat14, Mat1a, Mat2a, Mat2b, Sult5a1, Ugt1a1, Ugt1a5, Ugt1a6b, Ugt1a7c, Ugt1a9, Ugt2b34, Ugt2b38, Ugt3a1, Ugt3a2, Mgst3,	Slc4a2, Slc10a1, Slc22a3, Slc22a1, Slc22a5, Slc22a7, Slc27a5, Slc28a1, Slc29a2, Slc29a3, Slc51b, Slco3a1, Abca1, Abcb1a, Abcb4, Abcb11,	Nr0b2, NR112, NR113, Nr6a1, Nr1h2, Nr1h3, Nr2c1, Nr2c2, Nr2f1, Nr2f6, Nr3c1, Nr5a2, Ar, Esrrb, Ppara, Ppard,

	Ces2e, Ces2g, Adh1, Adh4, Adh5, Aldh1i1, Aldh1a3, Adh1a7, Aldh1b1, Aldh2, Aldh3a2, Aldh3b1, Aldh4a1, Aldh5a1, Aldh6a1, Aldh7a1, Aldh8a1, Aldh9a1, Aldh16a1, Hmgcr, Akr1a1, Akr1b8, Akr1b10, Akr1b3, Akr1b7, Akr1c12, Akr1c13, Akr1c14, Akr1c20, Akr1d1, Akr1e1, Cbr1, Cbr4, Dhrs3, Dhrs1, Dhrs7, Dhrs7b, Dhrs9, Dhrs13, Zfp385a, Fmo1, Fmo2, Fmo5, Ache, Bche, Sdr39u1, Sdr42e1, Maoa, Maob, Nqo2, Por, Txnrd2, Txnrd3, Prdx1, Prdx3, Ephx2, Atp8b1, Moccos, Baat, Pon2, Pon3, Acox2, Xdh	Papss1, Papss2, As3mt, Ugp2, Gusb	Abcc1, Abcc2, Abcc3	Rxra, Rxrb, Rxrg, Rarb, Rarg, Hnfla, Hnf4a, Ahr, Thrb, Rora
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Table 3.2. List of differentially expressed genes in each comparison group for each category.

	Impact of Microbiome		Sex Differences	
	Male GF vs. Male CV	Female GF vs. Female CV	Female CV vs. Male CV	Female GF vs. Male GF
Phase I Enzymes				
Upregulated	Adh7, Adhfe1, Aldh4a1, Akr1c6, Akr7a5, Cbr4, Ces1e, Ces3a, Ces3b, Ces4a, Cyp2a12, Cyp2c23, Cyp2c40, Cyp2c50, Cyp2c54, Cyp2c67, Cyp2c70, Cyp2d9, Cyp2d12, Cyp2d22, Cyp2d40, Cyp2e1, Cyp2g1, Cyp2j6, Cyp2j9, Cyp2u1, Cyp4a12a, Cyp4a12b, Cyp4f13, Cyp4f14, Cyp4f15, Cyp7b1, Cyp21a1, Gpx1, Gpx6, Gpx7, Nqo2, Prdx2, Prdx4, Prdx5	Akr1b7, Cyp2a5, Cyp2a12, Cyp2c23, Cyp2c29, Cyp2c38, Cyp2c67, Cyp2e1, CYP3A4, Fmo2, Fmo3, Gpx6, Por, Xdh,	Aldh1b1, Aldh4a1, Aldh9a1, Ces2e, Ces3a, Ces3b, Ces4a, Cyp2a4, Cyp2a12, Cyp2b9, Cyp2b13, Cyp2c23, Cyp2c37, Cyp2c40, Cyp2c50, Cyp2c54, Cyp2c68, Cyp2c69, Cyp2c70, Cyp2d22, Cyp2d26, Cyp2d40, Cyp2g1, Cyp4f14, Cyp4f15, Cyp4f16, Cyp17a1, Cyp26a1, Cyp46a1, CYP3A7, Fmo3, Fmo4, Gpx1, Gpx6, Gpx7, Nqo2, Pon1, Prdx4	Adh1, Aldh1b1, Aldh3a2, Akr1b7, Akr1c20, Akr1d1, Bche, Ces1c, Ces1d, Ces1g, Ces2g, Cyp2a4, Cyp2b9, Cyp2b10, Cyp2b13, Cyp2c29, Cyp2c37, Cyp2c38, Cyp2c39, Cyp2c40, Cyp2c55, Cyp2c68, Cyp2c69, Cyp2g1, Cyp3a13, Cyp3a57, Cyp3a59, CYP3A4, Cyp4f14, Cyp4f16, Cyp17a1, Cyp39a1, Dhrr7, Dhrr9, Fmo1, Fmo2, Fmo3, Fmo4, Fmo5, Gusb, Maob, Maoa, Pon3, Por, Txnrd2, Xdh

	Impact of Microbiome		Sex Differences	
	Male GF vs. Male CV	Female GF vs. Female CV	Female CV vs. Male CV	Female GF vs. Male GF
Downregulated	Adh1, Aldh1a1, Aldh1a7, Aldh1l2, Aldh18a1, Aldh3a2, Aldh3b1, Aldh5a1, Akr1a1, Akr1b7, Akr1b8, Akr1c13, Akr1c14, Akr1c19, Aox1, Ces1b, Ces1c, Ces1d, Ces1g, Cbr1, Cbr3, Cyp1a1, Cyp2a4, Cyp2a5, Cyp2b10, Cyp2c29, Cyp2c38, Cyp2c39, Cyp3a13, Cyp3a57, Cyp3a59, CYP3A4, Cyp4a14, Cyp4b1, Cyp4f39, Cyp7a1, Cyp17a1, Cyp26b1, Dhrrs1, Dhrrs7, Dhrrs9, Ephx1, Fmo1, Fmo2, Fmo3, Fmo5, Gsr, Gusb, Maoa, Mocos, Nqo1, Pon2, Pon3, Por, Sdr9c7, Txnrd1, Txnrd2, Txnrd3, Xdh	Ces2e, Cyp2d40, Cyp26c1, Cyp46a1, Cyp51, Maoa, Sdr9c7	Aldh1a1, Aldh1l2, Aldh18a1, Akr1a1, Akr1c13, Akr1c14, Akr1c19, Aox1, Cbr1, Cbr3, Ces1b, Cyp1a1, Cyp1a2, Cyp1b1, Cyp2a5, Cyp2d9, CYP3A4, Cyp3a59, Cyp4a12a, Cyp4a12b, Cyp4f39, Cyp7a1, Cyp39a1, Dhrrs9, Ephx1, Fmo5, Gpx2, Gsr, Nqo1, Pon3, Sdr9c7, Txnrd1, Xdh	Akr7a5, Aox1, Ces1e, Ces3a, Ces3b, Ces4a, Cyp2c67, Cyp2d9, Cyp2j9, Cyp2u1, Cyp4a12a, Cyp4f13, Cyp4f17, Cyp7b1, Cyp8b1, Cyp21a1, Cyp51, Dhrrs11, Gpx4, Gsr, Prdx2, Prdx5, Sdr9c7
Phase II Enzymes				
Upregulated	Comt, Gstk1, Gstm6, Gstm7, Gstz1, Mgst1, Nat2, Nat9, Sult1b1, Sult1e1, Sult5a1, Tpm1, Ugt1a6a, Ugt2a3, Ugt2b1	Sult1d1, Sult1e1, Ugp2	Gstm6, Gstm7, Gstk1, Gstt1, Gstt3, Gstz1, Papss2, Sult1b1, Sult2a1, Sult2a2, Sult2a3, Sult2a5, Sult2a6, Sult2a7, Sult3a1,	As3mt, Gstt3, Gsto1, Mat1a, Papss2, Sult1a1, Sult1c2, Sult1d1, Sult1e1, Sult2a1, Sult2a2, Sult2a3, Sult2a5, Sult2a6, Sult2a7,

	Impact of Microbiome		Sex Differences	
	Male GF vs. Male CV	Female GF vs. Female CV	Female CV vs. Male CV	Female GF vs. Male GF
			Sult5a1, Ugt2a3, Ugt2b37, Ugt3a1	Sult3a1, Ugt1a1, Ugt1a5, Ugt1a9, Ugt2b37
Downregulated	As3mt, Gclc, Gclm, Gsta1, Gsta2, Gsta3, Gsta4, Gss, Gsted, Gstp1, Gstp2, Gsto1, Gstm1, Gstm2, Gstm3, Gstm4, Gstm5, Mgst3, Mat1a, Ugdh, Ugt1a7c, Ugt1a9, Ugt2b5, Ugt2b34, Ugt2b35, Ugt2b36	Gstm2, Gstm4, Gstm5, Mgst3, Nat10, Sult3a1, Sult5a1	Gclc, Gclm, Gsta1, Gsta2, Gsta3, Gsta4, Gstp1, Gstp2, Gstm1, Gstm2, Gstm3, Gstm4, Nat8, Ugdh, Ugp2, Ugt1a9, Ugt2b5, Ugt2b34, Ugt2b35, Ugt2b36	Comt, Glyat, Gstm1, Gstp1, Gstp2, Mgst1, Nat8, Nat9, Sult5a1, Tpm1, Ugdh, Ugt2b1, Ugt2b5, Ugt2b35
Transporters				
Upregulated	Abcc6, Abcg5, Abcg8, Slc10a2, Slc22a7, Slc22a21, Slc47a1, Slco1a1, Slco1a4	Abcc2, Abcg5, Abcg8, Slco1a4	Abcc6, Slc10a1, Slc10a2, Slc22a7, Slc29a1, Slc47a1, Slco1a1, Slco1a4	Abcb1a, Abcb11, Abcc1, Abcc3, Abcc4, Slc29a1, Slco1a4
Downregulated	Abcb1a, Abcb4, Abcb11, Abcc1, Abcc3, Abcc4, Slc22a5	Slc22a7	Abcb4, Abcc2, Abcc3, Abcc4, Abcg2, Slc22a5, Slc51a	Abcb1b, Abcg2, Slc22a4, Slc22a7, Slc22a21, Slco1a1
Transcription Factors				
Upregulated	Esrrg, Nr0b2, Nr1d1, Nr1d2, Nr1h3, Nr2f2, Nr4a1, Nr4a2, Rara, Thra	Ahr, Nr1d1, Nr1d2, Nr5a2, Ppara	Ar, Esr1, Esrrg, Nr0b2, Nr1d1, Nr1h3	Ahr, Esr1, Hnf4a, Nr1i2, Nr2c2, Nr5a2, Ppara, Rarb
Downregulated	Ahr, Esr1, Hnf4a, Nr1i2, Nr2c2, Pparg, Ppard, Rora, Rxrg	Ar, Nr0b2, Ppard, Rxrg	Ahr, Pparg, Rora	Nr0b2, Nr1h4, Nr4a1, Nr4a2, Rara, Thra

Table 3.3. Spearman correlation between mRNA and protein expression of selected genes.

Gene	Spearman's rank correlation	p-value ^a
Cyp1a2	0.39	-- ^b
Cyp2b10	0.53	0.037
Cyp2c38	0.61	0.012
Cyp2d22	0.43	--
Cyp2e1	0.18	--
CYP3A4	0.73	0.0019
Cyp3a13	-0.44	--
Cyp4a14	0.63	0.011
Cyp7a1	0.48	--
Ces1	0.065	--
Fmo5	0.17	--
Ugt2b1	0.45	--
Ugt2b35	0.78	0.00053
Abcb4	0.75	0.0011
Abcb11	0.05	--
Abcc2	0.26	--
Abcc3	0.32	--
Abcg5	0.23	--
Abcg8	0.65	0.0079
Slco1b2	-0.021	--

^a Corrected by false discovery rate

^b -- not significant

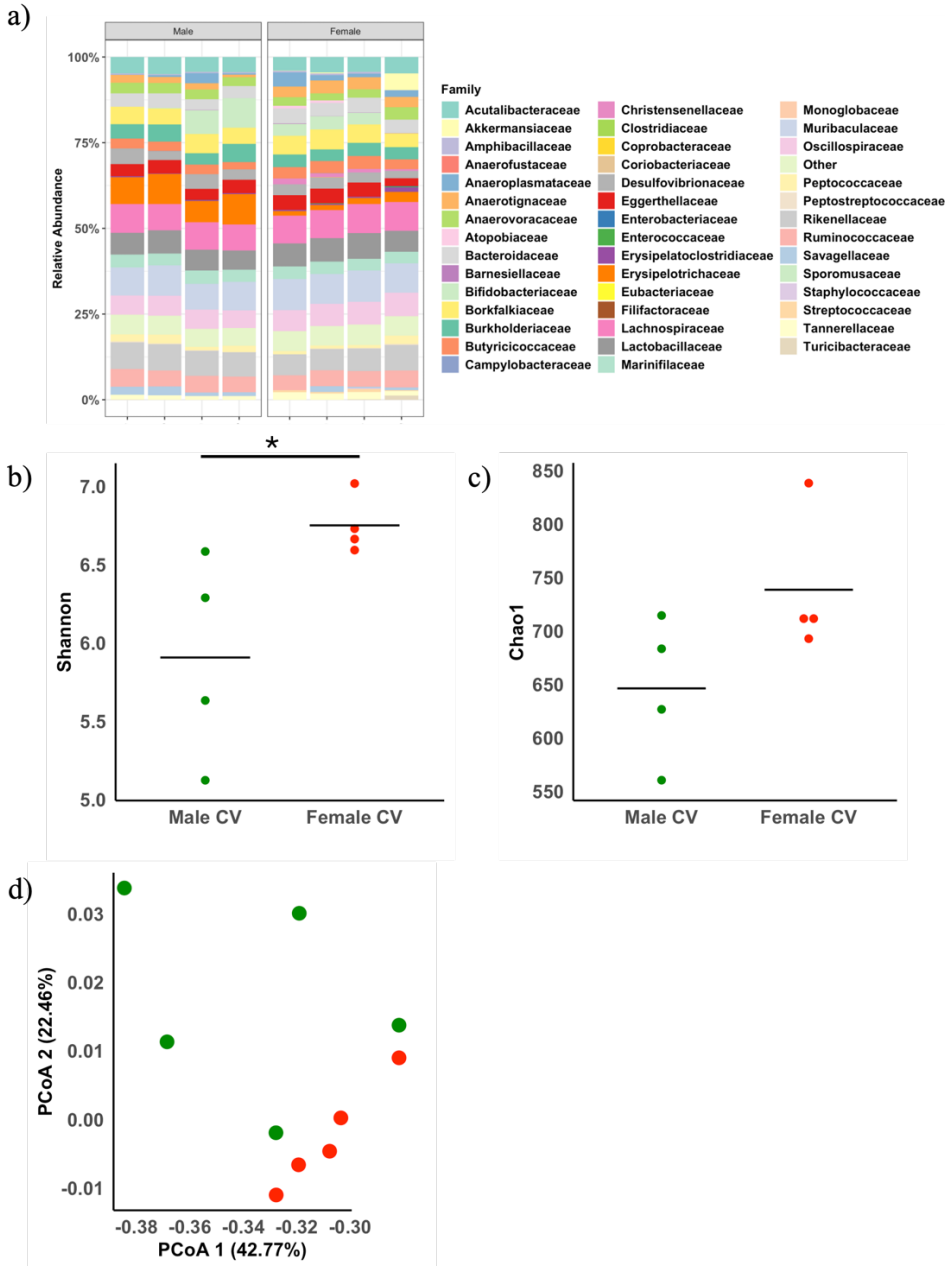


Figure 3.1. Microbial composition and diversity in male and female CV PXR-CAR-CYP3A4/3A7 mice (n = 4 per sex per group). (a) Microbial families identified in colon contents of each CV mouse. Alpha diversity as calculated with (b) Shannon index and (c) Chao1 index with significance determined by Wilcoxon rank-sum test (* for p-value < 0.05). (d) Beta diversity was calculated by the Bray-Curtis Distance Index and significance was determined by PERMANOVA (p = 0.033). Male CV mice are in green and female CV mice are in red.

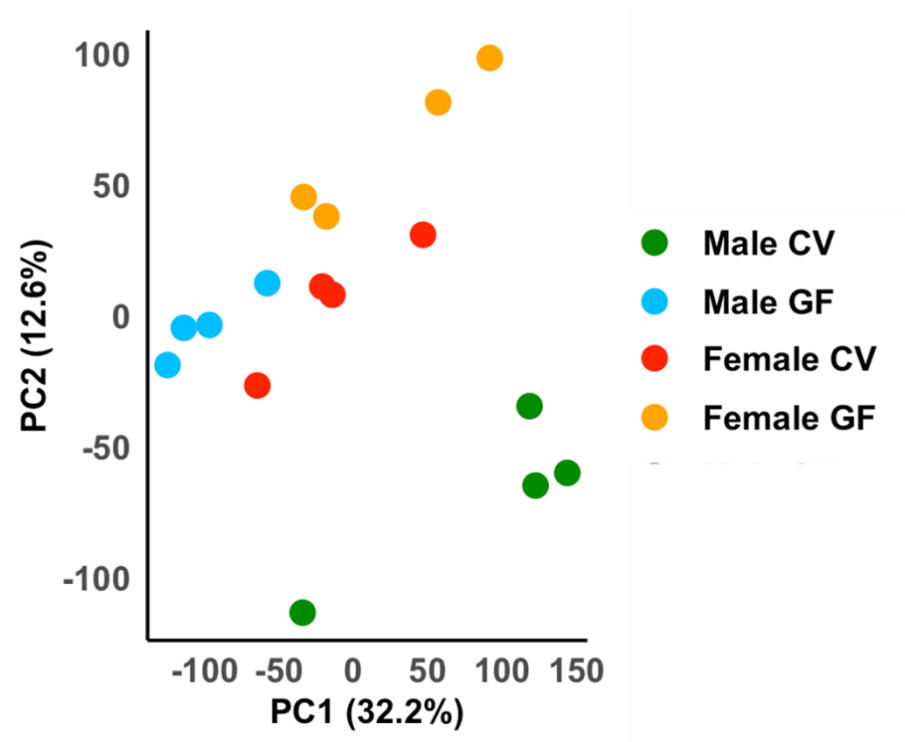


Figure 3.2. Group differences as shown by PCA in the liver transcriptome of male and female CV and GF PXR-CAR-CYP3A4/3A7 mice (n = 4 per sex per group).

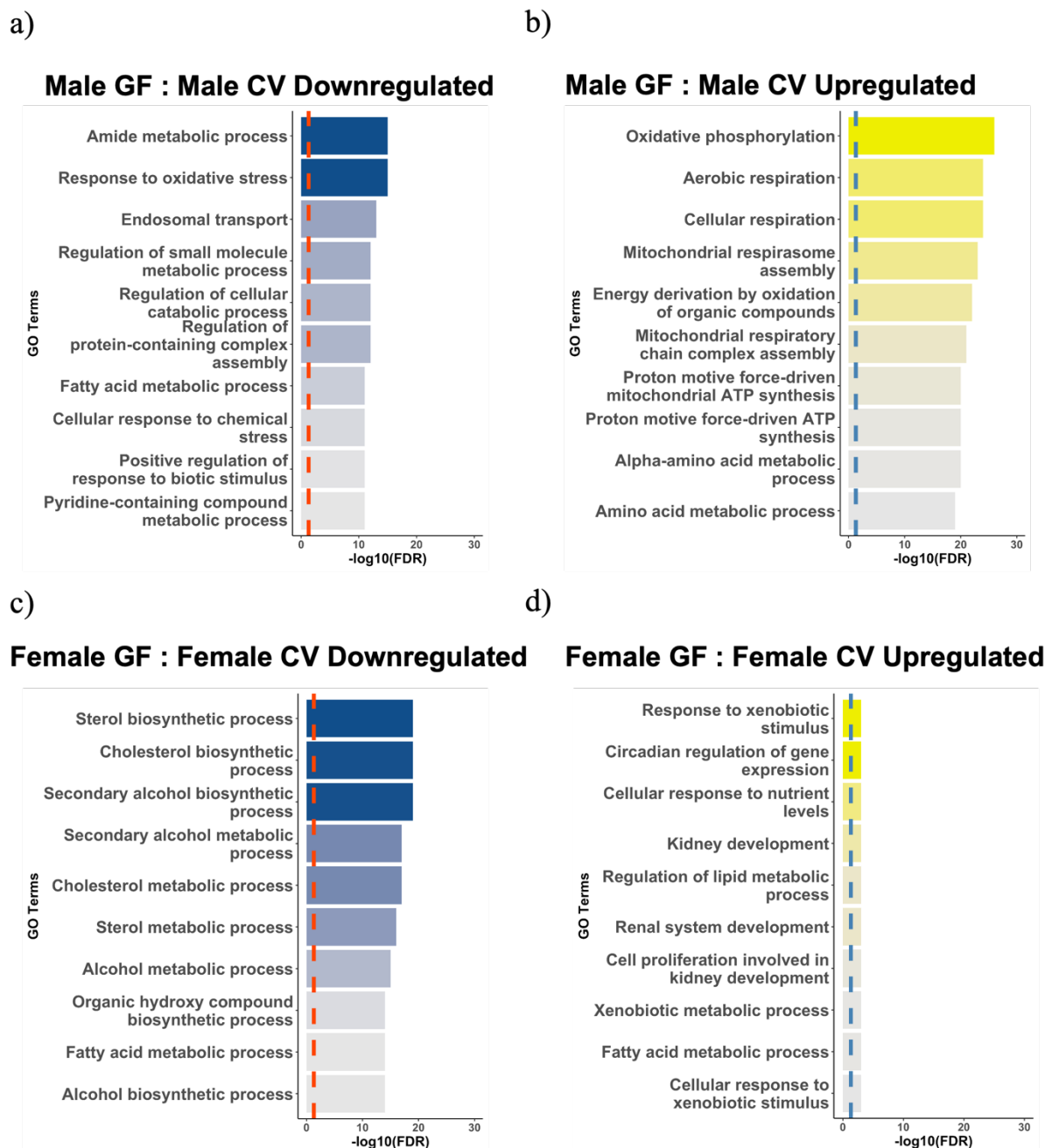
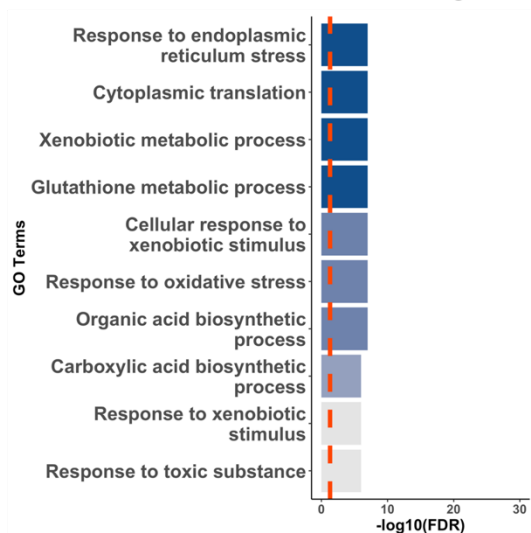
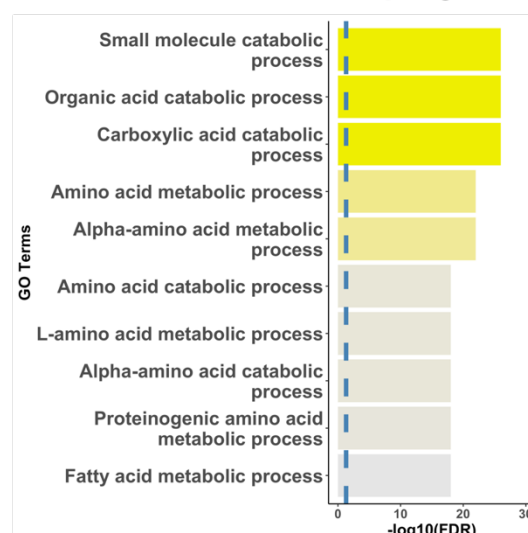


Figure 3.3. Top ten results from transcriptomic gene ontology analysis comparing GF to CV PXR-CAR-CYP3A4/3A7 mice for male mice (a, b) and female mice (c, d).

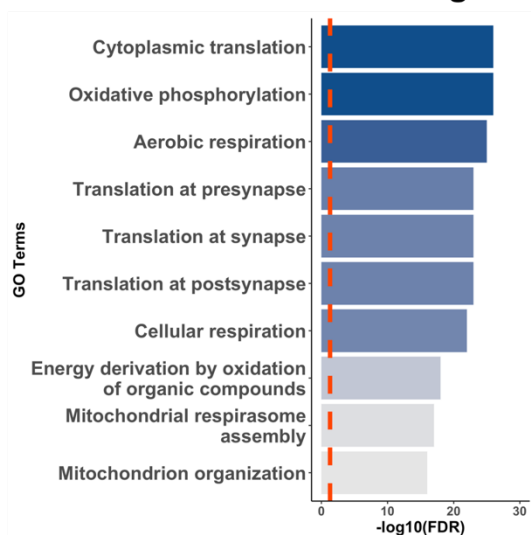
a)

Female CV : Male CV Downregulated

b)

Female CV : Male CV Upregulated

c)

Female GF : Male GF Downregulated

d)

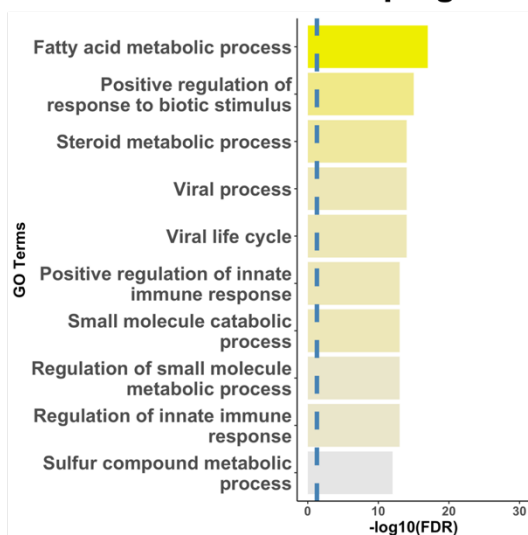
Female GF : Male GF Upregulated

Figure 3.4. Top ten results from transcriptomic gene ontology analysis comparing female to male PXR-CAR-CYP3A4/3A7 mice for CV mice (a, b) and GF mice (c, d).

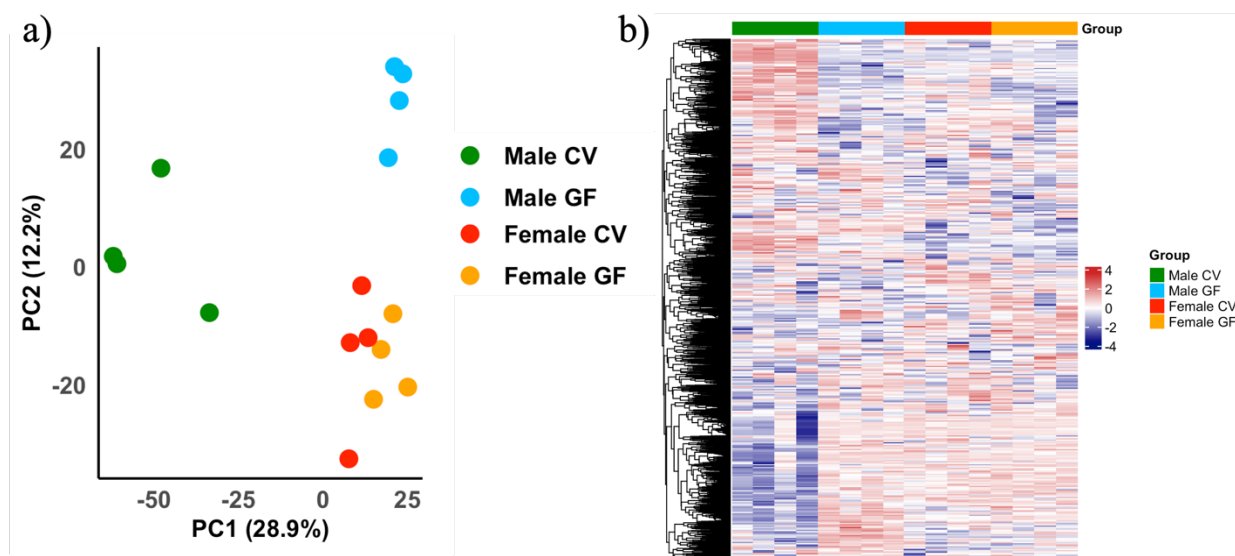


Figure 3.5. Differential metabolite analysis of hepatic metabolomics in male and female CV and GF PXR-CAR-CYP3A4/3A7 mice (n = 4 per sex per group). (a) Group differences as shown by PCA in the liver metabolome and (b) heatmap of untargeted metabolomics data.

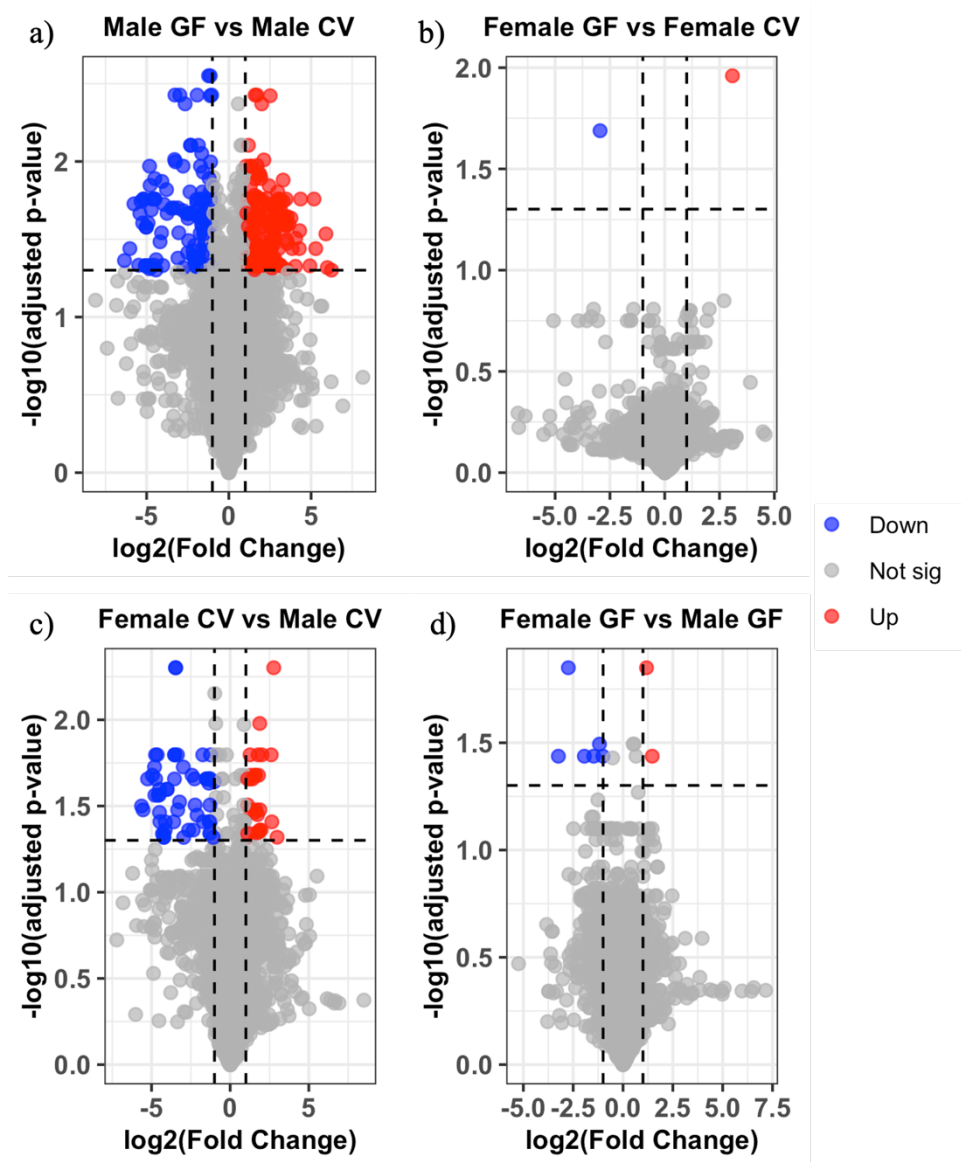


Figure 3.6. Volcano plot highlighting metabolites that were significantly decreased (blue) or increased (red) in male and female PXR-CAR-CYP3A4/3A7 mice ($n = 4$ per sex per group). The impact of microbiome is shown in comparison of (a) male GF vs. male CV and (b) female GF vs. female CV. The impact of sex is shown in the comparison of (c) female CV vs. male CV and (d) female GF vs. male GF. The thresholds were set at $|\text{fold-change}| > 2$ and $p\text{-value} < 0.05$.

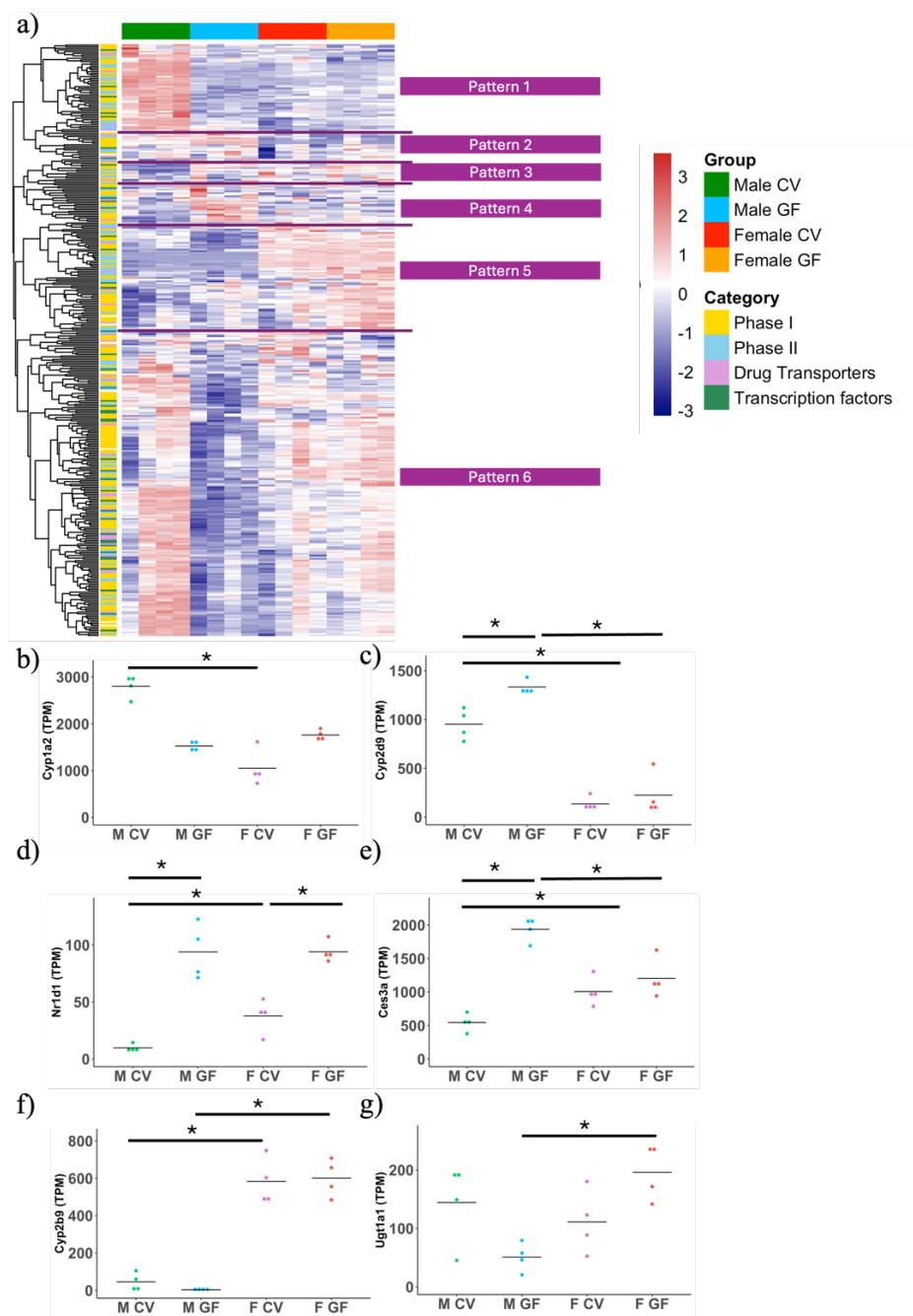


Figure 3.7. (a) Heatmap showing 6 major patterns of quantified drug processing genes in male and female CV and GF PXR-CAR-CYP3A4/3A7 mice (n = 4 per sex per group). Representative genes were selected for each pattern: (b) Pattern 1: *Cyp1a2*, (c) Pattern 2: *Cyp2d9*, (d) Pattern 3: *Nr1d1*, (e) Pattern 4: *Ces3a*, (f) Pattern 5: *Cyp2b9*, and (g) Pattern 6: *Ugt1a1* where * indicates FDR-adjusted p-value < 0.05).

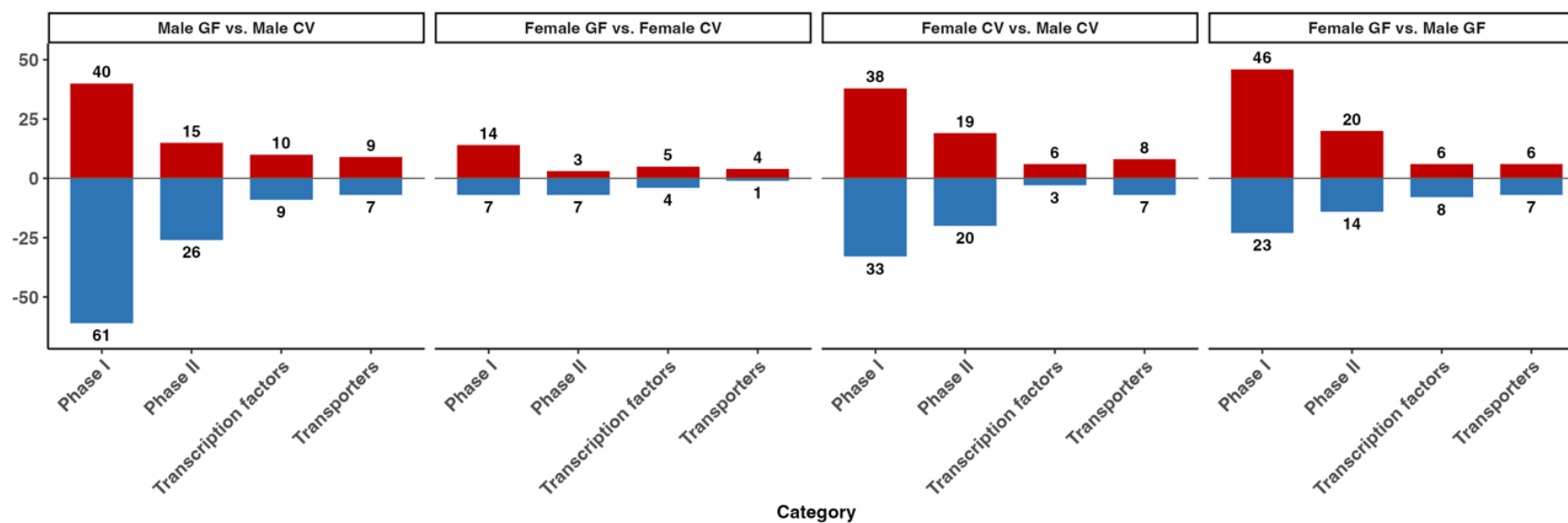


Figure 3.8. Number of upregulated (red) and downregulated (blue) drug processing genes (Phase I, Phase II, transporters, and transcription factors) in male and female CV and GF PXR-CAR-CYP3A4/3A7 mice (n = 4 per sex per group).

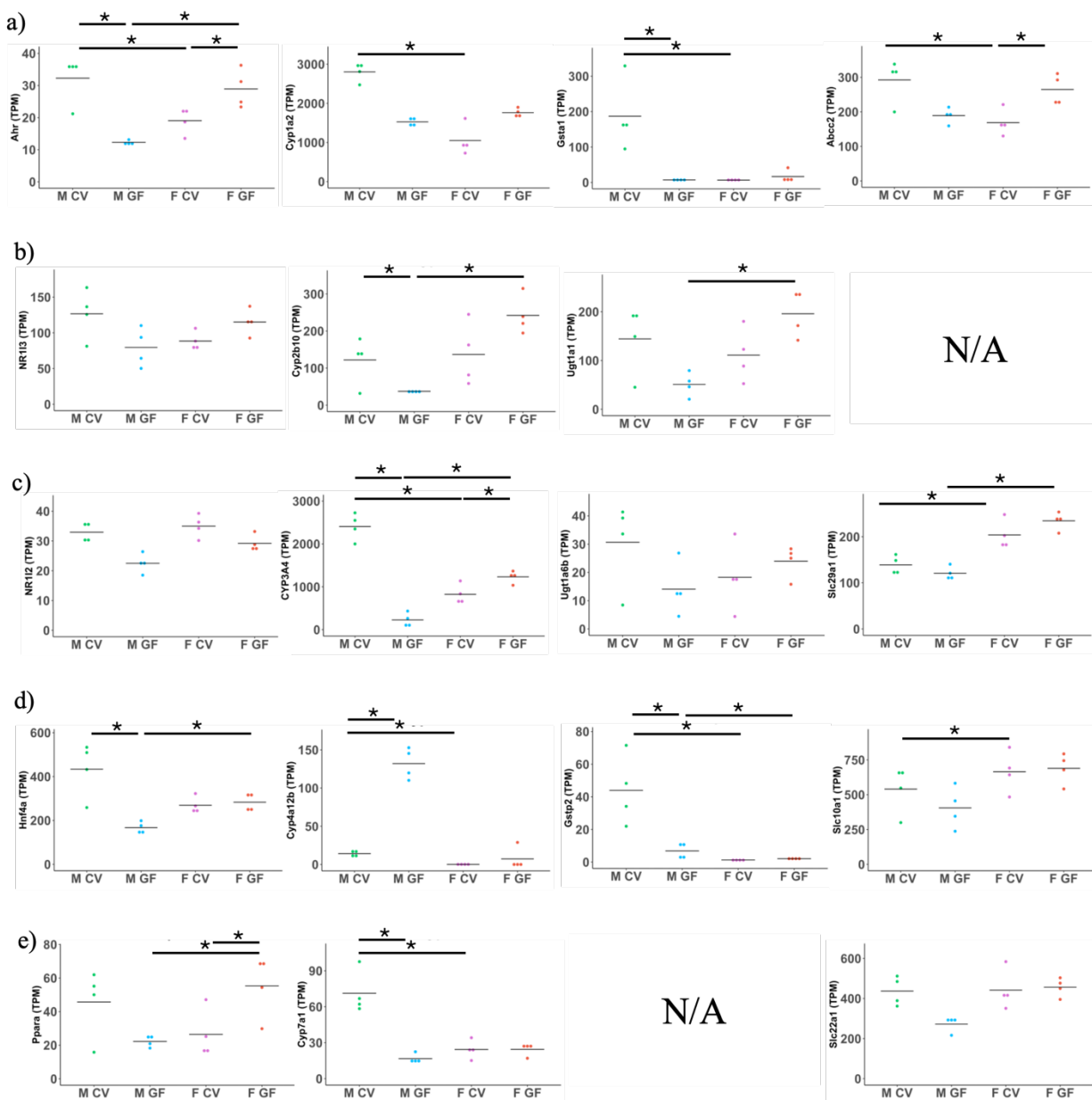


Figure 3.9. mRNA expression of major drug-related transcription factors: (a) *Ahr*, (b) *Nr1i3* (*CAR*), (c) *Nr1i2* (*PXR*), (d) *Hnf4a*, (e) *Ppara* and their representative downstream Phase I (2nd column), Phase II (3rd column), and transporter (4th column) genes in male and female CV and GF PXR-CAR-CYP3A4/3A7 mice (n = 4 per sex per group). * false discovery rate-corrected p-value < 0.05. Transcription factors that do not have a representative gene of that group is denoted as N/A.

Chapter 4

Exploration of Microbial Metabolites as Regulators of CYP3A4

4.1. Introduction

The gut microbiome is a complex and diverse ecosystem, encompassing a large collection of microbes that can perform several key functions to maintain host health (Van Hul et al., 2024). Imbalance in the microbiome (i.e., dysbiosis) has been associated with several types of disease states such as colorectal cancer (Sobhani et al., 2011) and Parkinson's disease (Huang et al., 2023). In the past couple of years, the gut microbiome has gained popularity for its impact on drug disposition and action (Zhao et al., 2023). There are several types of interactions that the gut microbiome and drugs are thought to have, ranging from direct metabolism (e.g., digoxin metabolism by *Eggerthella Lenta*) (Dobkin et al., 1983) to competition (e.g., acetaminophen and *p*-cresol) (Clayton et al., 2009). Notably, one way that the gut microbiome is hypothesized to influence drug disposition is through microbial metabolites. Specifically, certain microbial metabolites can activate nuclear receptors like pregnane X receptor (PXR), leading to downstream induction of drug metabolizing enzymes and transporters (DMETs) (Dempsey and Cui, 2019).

One key DMET that has been identified to be altered with microbial manipulation in animal models is cytochrome P450 3a (Cyp3a), with its expression and activity decreased by the absence or reduction of gut microbes (Selwyn et al., 2016; Togao et al., 2021). Although these studies have exposed the presence of an interaction, the mechanism behind the regulation of Cyp3a by the gut microbiome remains largely unknown. We hypothesize the interaction between the gut microbiome and Cyp3a is mediated by microbial metabolites. The three major microbial metabolite groups: bile acids, short chain fatty acids (SCFA), and tryptophan metabolites have been shown to induce Cyp3a expression. Notably, lithocholic acid, a secondary bile acid, has been repeatedly shown to be an inducer of PXR and the downstream gene, Cyp3a in mouse

models (Staundinger et al., 2001; Toda et al., 2009). *In vitro*, a mixture of SCFA treatment (1:1:1 of acetate:butyrate:propionate) led to an increase in CYP3A4 expression in human pluripotent stem cell-derived liver organoids but individual SCFA did not cause induction (Mun et al., 2021). More recently, Togao et al. showed that in DPX-2 and HepaRG cells, lithocholic acid and butyric acid (a SCFA) did not induce CYP3A4 activity. However, they found that indoxyl sulfate, a downstream metabolite of tryptophan could induce and inhibit CYP3A4 activity in human hepatocytes, but the effect was hepatocyte donor-specific (Togao et al., 2025). Apart from these groups of microbial metabolites, other microbial metabolites have not been identified as regulators of CYP3A4.

The goals of this study were to identify microbial metabolites that are associated with CYP3A4 expression in a humanized mouse model, determine whether the identified microbial metabolites can regulate CYP3A4 expression *in vitro*, and help further elucidate the relationship between the gut microbiome and CYP3A4.

4.2. Materials and Methods

4.2.1. Materials

Human liver microsomes (pooled from 50 donors), cryopreserved HepaRGTM cells, media components (HepaRGTM Thaw, Plate, & General Purpose Medium supplement, HepaRGTM Serum-free Induction Medium Supplement, William's E medium, GlutaMAXTM Supplement, and Penicillin-Streptomycin), 1-deoxynojirimycin (DNJ), dimethyl sulfoxide (DMSO), rifampin, ketoconazole, PureLinkTM RNA Mini Kit, TaqMan Fast Advanced Master Mix, and TaqMan probes (GAPDH: Hs99999905_m1; CYP3A4: Hs00604506_m1; P-gp: Hs00184500_m1; CYP2C9: Hs04260376_m1) were purchased from ThermoFisher Scientific

(Waltham, MA). Collagen I-coated 24-well plates were purchased from Corning Inc. (Corning, NY). The High-Capacity cDNA Reverse Transcription Kit was obtained from Applied Biosystems (Waltham, MA). Ethanol, betaine BioUltra, and L-pyroglutamic acid BioXtra (LPGA) were purchased from Sigma-Aldrich (St. Louis, MO). The P450-Glo CYP3A4 Luciferin-IPA Assay and NADPH regeneration system were purchased from Promega (Madison, WI).

4.2.2. Animal Procedures

The animal studies have been previously published in Liem et al. (2026). In brief, three groups of PXR-CAR-CYP3A4/3A7 mice (Taconic stock #11585) were studied: conventional (CV), germ-free (GF), and conventionalized (GFCV) which were generated by inoculation of GF mice at 8 weeks old with sex-matched pooled human feces purchased from BioIVT (Westbury, NY). Mice were sacrificed at 12-14 weeks. Liver tissues were collected, snap frozen, and stored at -80°C until analysis.

4.2.3. Untargeted Metabolomics with LC-MS

Mouse livers (n = 4 per sex per group, 24 total) were sent to Dr. Haiwei Gu at Arizona State University for untargeted metabolomics. The methodology was similar to previous published studies (Gu et al., 2015, Wei et al., 2021). Briefly, untargeted metabolomics was conducted on Agilent 1200 SL LC-6520 Quadrupole Time of Flight (QTOF) MS system. Extracted features were matched against commercial Agilent databases and METLIN.

To assess associations between hepatic metabolites and CYP3A4 expression, identified metabolites were correlated with CYP3A4 protein expression using Spearman's correlation with

additional filtering criteria. Significantly correlated metabolites were cross-referenced against a publicly available database Microbial Metabolome Database 2.0 (Wishart et al., 2023) to identify microbial-derived metabolites. All analyses were done on Rstudio (Pang et al., 2024).

4.2.4. Induction Experiments using HepaRG cells

Terminally differentiated HepaRG cells were plated in 24-well collagen I-coated plates at a density of 0.96×10^6 cells/mL. Cells were initially seeded with William's Medium E supplemented with GlutaMax (1x), HepaRG™ Thaw, Plate, & General Purpose Medium supplement, and penicillin-streptomycin (1%) and incubated for 6 hours and replenished with fresh plating media. After incubation for 72 hours, the plating media was replaced with William's Medium E supplemented with GlutaMax (1x), HepaRG™ Serum-free Induction Medium supplement, and penicillin-streptomycin (1%) containing either: vehicle control (DMSO), rifampin (10 μ M), deoxynojirimycin (DNJ), betaine, or L-pyroglutamic acid (LPGA) at selected concentrations of 15 and 150 μ M. For an LPGA dose response experiment, selected concentrations were 1, 10, 50, 100, 200, and 500 μ M. Rifampin, DNJ, and LPGA were dissolved in DMSO, whereas betaine was dissolved in water. For betaine, media was spiked with DMSO. The final concentration of DMSO was 0.1%. Media containing these treatments were refreshed every 24 hours for 72 hours. All *in vitro* experiments were done in triplicate.

4.2.5. Target mRNA expression by RT-qPCR

After treatment, total RNA was extracted from HepaRG cells after lysis using the PureLink RNA Mini Kit, per manufacturer's protocol. Total RNA concentration and purity were determined with a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). Isolated RNA

was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit per the manufacturer's protocol. With this cDNA, quantitative real-time polymerase chain reaction (qPCR) was performed to measure mRNA expression of PXR-target genes (i.e., *CYP3A4*, *P-glycoprotein* and *CYP2C9*) using *GAPDH* as the reference gene. Relative expression was determined by the $2^{-\Delta\Delta C_t}$ method.

4.2.6. *CYP3A4* Inhibition

CYP3A4 inhibition was assessed with the P450-Glo™ assay with human liver microsomes (pooled from 50 donors), per the manufacturer's instructions. Briefly, microsomes, luciferin IPA substrate, and potassium phosphate buffer (pH 7.4) were combined with selected concentrations of DNJ (10 and 100 μ M), betaine (100 and 1000 μ M), or LPGA (100 and 1000 μ M). The mixture was pre-incubated at 37°C for 10 min, followed by the addition of 25 μ L of NADPH regenerating system to initiate the reaction. Samples were incubated for 10 min and 50 μ L of the luciferin detection reagent was added and the plate was gently tapped to mix. The plate was equilibrated at room temperature for 20 min and luminescence was measured with a plate reader. Ketoconazole (1 μ M) was used as a positive control and 0.25% DMSO in water was used as a vehicle control. DNJ and ketoconazole were dissolved in DMSO while LPGA and betaine were dissolved in water and spiked with DMSO (0.25% final concentration). All experiments were done in triplicate.

4.2.7. Statistical Analysis

Associations between metabolites and CYP3A4 expression were assessed with Spearman's correlation and corrected for multiple comparisons with false discovery rate. For

induction and inhibition experiments, significance was determined by ANOVA followed by Dunnett's test compared to vehicle control. A p-value < 0.05 was considered significant.

4.3. Results

4.3.1. Selection of microbial metabolites

The relationship between hepatic CYP3A4 and the different microbial conditions tested in our humanized mice was previously published (Liem et al., 2026). This study focuses on identifying and testing microbial metabolites as potential regulators of CYP3A4 expression. A total of 1092 metabolites were identified in the liver samples of humanized mice using global metabolomics (Figure 4.1). We performed Spearman's correlations to identify metabolites that were significantly correlated with hepatic CYP3A4 protein expression ($n = 170$). To filter for more robust associations, only metabolites with $\rho \geq 0.6$ were selected ($n = 33$). The publicly available database Microbial Metabolome Database 2.0 (Wishart et al., 2023) was used to identify microbial metabolites that are products of microbiome mediated metabolism and/or are found in the human microbiome. The filtered list of 33 metabolites were compared to the Microbial Metabolome Database and only six metabolites were identified as microbial metabolites (Table 4.1). To test our hypothesis that microbial metabolites induce hepatic CYP3A4 expression and/or activity, only metabolites with positive correlations were selected ($n = 4$). Finally, we prioritized metabolites that are commercially available as a nutritional supplement (i.e., more likely to be consumed with the potential for a drug-natural product interaction). Three metabolites were selected: 1-deoxynojirimycin (DNJ), betaine, and L-pyroglutamic acid (LPGA). Figure 4.2 shows the correlation between hepatic CYP3A4 protein expression and liver abundance of 1-deoxynojirimycin ($\rho = 0.72, p = 1 \times 10^{-4}$), betaine ($\rho = 0.66,$

$p = 0.00056$), and L-pyroglutamic acid ($p = 0.62$, $p = 0.0015$). The chemical structure of each metabolite is depicted in Figure 4.3. Overall, these selected microbial metabolites were strongly and positively correlated with hepatic CYP3A4 protein expression in humanized mice.

4.3.2. Induction Screen with HepaRG cells

To identify whether the metabolites induce hepatic *CYP3A4* mRNA, HepaRG cells were treated with 15 and 150 μM of each metabolite. DMSO and rifampin served as a vehicle and positive control, respectively. *CYP3A4* mRNA expression was quantified with RT-qPCR. Although not significant, the initial screen suggested LPGA might be a moderate inducer of *CYP3A4* (2.05-fold, $p = 0.07$; Figure 4.4a). DNJ and betaine treatment had no effect. We did not observe any changes in the expression of *P-gp* and *CYP2C9*, other downstream genes of PXR (Figure 4.4b and 4.4c, respectively). We tested increasing doses of LPGA ranging from 1 to 500 μM to determine whether LPGA exhibits dose-dependent induction of *CYP3A4* in the HepaRG cells. In this experiment, *CYP3A4* mRNA induction was not seen at any concentration of LPGA tested (Figure 4.5).

4.3.3. Inhibition screen with pooled liver microsomes

To ascertain whether these microbial metabolites could have a dual effect (i.e., induction and inhibition), liver microsomes were incubated with a luciferin substrate and a low and high concentration of each microbial metabolite. DMSO and ketoconazole served as a vehicle and positive control, respectively. As DNJ is only soluble in DMSO and a maximum of 0.25% DMSO was allowable in each 50 μL well, only 10 and 100 μM concentrations of DNJ were tested. As LPGA and betaine are soluble in water, concentrations of 100 and 1000 μM were tested with DMSO spiked in to reach 0.25%. Across all metabolites and concentrations, no

CYP3A4 inhibition was observed (Figure 4.6).

4.4. Discussion

In our previous study (Liem et al., 2026), we saw that different microbial conditions led to changes in hepatic CYP3A4 expression in the humanized PXR-CAR-CYP3A4/3A7 mouse model. In this study, we conducted untargeted metabolomics in the mouse liver samples (n = 4 per sex per group, 24 total) and used a correlation analysis with hepatic CYP3A4 expression to identify microbial metabolites that may be regulating CYP3A4 (Table 4.1). From 1092 identified metabolites, three microbial metabolites were selected for subsequent screening (Figure 4.1). These metabolites were: DNJ, betaine, and LPGA. All three metabolites demonstrated moderate positive correlations with hepatic CYP3A4 expression (Figure 4.2). The male CV group was an outlier for all three metabolites. Regardless, these three microbial metabolites had the strongest correlations, and we explored whether they are potential inducers of CYP3A4.

DNJ (Figure 4.3a) is an iminopyranose alkaloid. It was first found in mulberry leaves and is the most abundant of the mulberry iminosugars (Asano et al., 2001). Mulberry leaves have been used as a traditional medicine in China due to their potential anti-diabetic properties (Cui et al., 2023). DNJ is currently being studied for its ability to inhibit activity of glucosidase in glucose, lipid, and amino acid metabolism (Hu et al., 2017). Aside from mulberry leaves, DNJ has been isolated from other plants (e.g., *H. orientalis*), insects (e.g., silkworm) and microbial strains (e.g., *B. subtilis*), classifying it as a microbial metabolite (Zhang et al., 2019). Mulberry extracts with DNJ as a constituent are available commercially and are purported to be potent and effective for various health conditions.

Betaine (Figure 4.3b), also known as trimethylglycine, is a non-essential amino acid and

is commonly found in food products. Aside from exogenous sources like beets, betaine can also be produced endogenously via choline metabolism (Dobrijević et al., 2023). Betaine is being studied for its antioxidant properties (Alirezaei et al., 2015), in liver disease (Chen et al., 2021), kidney disease (Sharma et al., 2021), and cardiovascular disease (Rosas-Rodríguez and Valenzuela-Soto, 2021), as an anti-cancer agent (Van Puyvelde et al., 2023), for neuroprotective effects (Li et al., 2022), and to increase overall athletic performance (Ashtary-Larky et al., 2021). Betaine is sold as a supplement but is also available as an FDA-approved prescription (Cystadane) for treatment of homocystinuria (Valayannopoulos et al., 2019).

LPGA (Figure 4.3c) is an endogenous amino acid and is being studied for several different neurological properties, specifically for neurochemical effects (de Mello et al., 1995), as an anxiolytic (Beni et al., 1988), and for brain energy production (Silva et al., 2001). More recently, LPGA was identified as a potential diagnostic biomarker for systemic lupus erythematosus (Zhang et al., 2021). LPGA is sold commercially with claims of beneficial neurological effects.

We aimed to test physiologically relevant concentrations of the microbial metabolites as reported in literature. We based DNJ concentrations on estimates from a clinical study where subjects consumed 1.2 g of mulberry extract that contained 6.3 mg of DNJ, and a maximum plasma concentration of 520 ng/mL ($\sim 3.18 \mu\text{M}$) was observed at 1.5 hr (Nakagawa et al., 2008). This concentration is lower than the low concentration tested in our screening. For betaine, plasma concentrations are typically 20 – 70 μM (Day and Kempson, 2016) and when subjects were dosed with 1, 3, or 6 g of betaine, plasma concentrations reached a maximum of 239, 551, and 966 μM , respectively (Ursula et al., 2006). Lastly, physiological plasma concentrations of LPGA are reported to be $39 \pm 6 \mu\text{M}$, with highest concentrations up to 100 μM (Ghezzi et al.,

1983). However under disease conditions, LPGA plasma concentrations can be as high as 300 μM in patients with hepatic dysfunction (Mullins et al., 2020) or up to 16 mM in patients with metabolic acidosis (Liss et al., 2013).

To identify whether these microbial metabolites were inducers of CYP3A4, an *in vitro* screen was performed in HepaRG cells. Although LPGA at 150 μM had a trend for moderate *CYP3A4* induction (2.05-fold change), DNJ and betaine did not induce *CYP3A4* mRNA expression at 15 and 150 μM , nor did any of these microbial metabolites induce *P-gp* or *CYP2C9* mRNA expression (Figure 4.4a-4.4c). None of the treatments (including rifampin) induced *CYP2C9* mRNA (Figure 4.4c). The lack of *CYP2C9* induction may be due to the nature of HepaRG cells. Induction of CYP2C9 by rifampin in HepaRG cells has been shown to be at a lesser magnitude than for CYP3A4 (Berger et al., 2016; Kanebratt and Andersson, 2008). Berger et al. (2016) observed up to a 30-fold induction in *CYP3A4* mRNA expression but only a 3-fold induction in *CYP2C9* mRNA in HepaRG cells with treated with 20 μM rifampin. Furthermore, when they tested CYP3A4 activity, rifampin induced CYP3A4 activity by 8-fold but did not induce CYP2C9 activity in HepaRG cells. Kanebratt and Andersson (2008) found with increasing rifampin concentrations in HepaRG cells, the induction of *CYP3A4* expression peaked at 24 ± 12 -fold while *CYP2C9* induction only reached 2.3 ± 1.4 -fold at the highest rifampin concentration (4 μM). Overall, these studies may explain why no *CYP2C9* induction was observed in our experiments, even in our positive control. Furthermore, CYP3A4 inducers tend to induce other PXR genes such as *P-gp* and *CYP2C9* at a lower magnitude (Lutz et al., 2018). Based on area under the curve (AUC) ratios of probe drugs in healthy volunteers comparing induced vs. baseline conditions, rifampin was a strong inducer of CYP3A4 activity (probe drug: midazolam), but only a weak to moderate inducer of P-gp (probe drug: dabigatran) and CYP2C9

(probe: tolbutamide). This may explain why we observed only 2-fold induction of *P-gp* mRNA expression by rifampin and no induction of *P-gp* with 150 μ M LPGA even though *CYP3A4* expression had a slight increase. With this in mind, we decided to further explore LPGA as a *CYP3A4* inducer and treated HepaRG cells with increasing concentrations of LPGA (i.e., 1, 10, 50, 100, 200, 500 μ M). No induction was seen across all concentrations (Figure 4.5). Overall, these results indicate that DNJ, betaine and LPGA are not *CYP3A4* inducers.

As Togao et al. (2025) saw a dual effect (i.e., induction and inhibition) with indoxyl sulfate, we wanted to see whether our selected microbial metabolites could potentially inhibit *CYP3A4*. To test the *CYP3A4* inhibition potential by the microbial metabolites, pooled human liver microsomes were incubated with a *CYP3A4*-specific luciferin substrate and a low and high concentration of each microbial metabolite. Ketoconazole at 1 μ M served as a positive control. Aside from the positive control, no inhibition was observed across all concentrations and treatments (Figure 4.6). Therefore, the selected microbial metabolites are unlikely to be inhibitors of *CYP3A4* under the conditions tested.

Overall, although the selected microbial metabolites demonstrated strong correlations with hepatic *CYP3A4* protein expression in humanized mice, none of the metabolites were shown to regulate *CYP3A4 in vitro*. There can be multiple reasons for this discrepancy. First, *in vitro* models do not perfectly replicate the mouse model's environment (e.g., gut-liver axis). For example, the regulation of *CYP3A4* by these metabolites may require other components or metabolites to trigger an effect. Our selected *in vitro* model for induction screening, the HepaRG cells, may not have been adequate to capture this regulation. Another hypothesis is that the microbial metabolites may be products of *CYP3A4* metabolism and thus the strong correlation reflects their ability to reflect *CYP3A4* expression. Therefore, we did not see regulation of

CYP3A4 *in vitro*.

There are several limitations with our study. First, as mentioned previously, HepaRG cells may not be the most physiologically relevant model as they are derived from a hepatocarcinoma patient. Primary human hepatocytes may be preferable as they more closely reflect CYP inducibility *in vivo*. However, HepaRG cells have been shown to be a suitable surrogate for primary human hepatocytes (Berger et al., 2016) and are extensively used in drug development screening. Second, as untargeted metabolomics does not report absolute concentrations, we do not know what concentrations were present in the mouse liver samples. Determining microbial metabolite concentrations with specific and sensitive assays may provide better insight as to whether we tested appropriate concentrations *in vitro*. Similarly, we did not confirm whether the microbial metabolites were successfully getting into the cells. As DNJ is hydrophobic, there is a possibility for non-specific binding and that intracellular concentrations were lower than expected. Next, although the untargeted metabolomics method was able to identify over 1000 metabolites, there are likely many metabolites that were not captured by the method. Future studies could perform more extensive metabolomics or lipidomics to identify microbial metabolites that were missed and in a larger number of animals. Lastly, the lack of inhibition by these microbial metabolites may depend on the substrate being studied. Detection of CYP3A4 inhibition can depend on substrate being used and follow-up studies with standard CYP3A4 probes (i.e., midazolam and testosterone) may be necessary to confirm that metabolites are not CYP3A4 inhibitors.

4.5. Conclusion

In summary, three microbial metabolites, DNJ, betaine, and LPGA, were identified to be strongly correlated to hepatic CYP3A4 protein expression in humanized mice. These three metabolites were screened for CYP3A4 induction and inhibition *in vitro*. However, none of the selected metabolites influenced CYP3A4 mRNA expression or activity, indicating that these microbial metabolites are unlikely to contribute to the regulation of CYP3A4. Although these microbial metabolites had no effect on CYP3A4 *in vitro*, with the increased popularity of natural products and the availability of some microbial metabolites as supplements, it will be important to ascertain potential interactions with CYP3A4 to avoid harmful drug-natural product interactions.

4.6. References

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4.7. Tables and Figures

Table 4.1. List of microbial metabolites that were significantly correlated with CYP3A4 protein expression.

Metabolite	Spearman's rank correlation	p-value
1-Deoxynojirimycin	0.72	1×10^{-4}
Betaine	0.72	0.00013
L-Pyroglutamic acid	0.62	0.0015
Sphinganine	0.63	0.0013
N1-Acetylspermidine	-0.67	0.00052
Kaempferol 3- (6"-p-coumarylgalactoside)	-0.65	0.00077

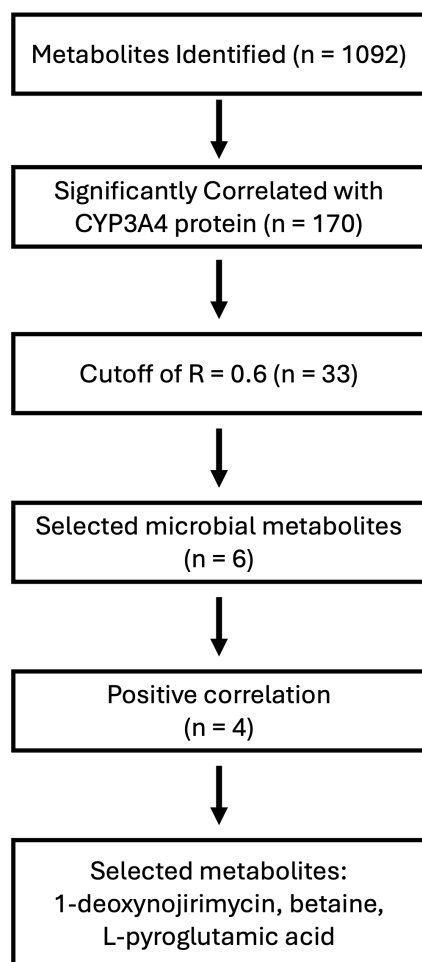


Figure 4.1. Workflow for selection of microbial metabolites from untargeted metabolomics data of PXR-CAR-CYP3A4/3A7 mouse livers (n = 4 per sex per group, 24 total).

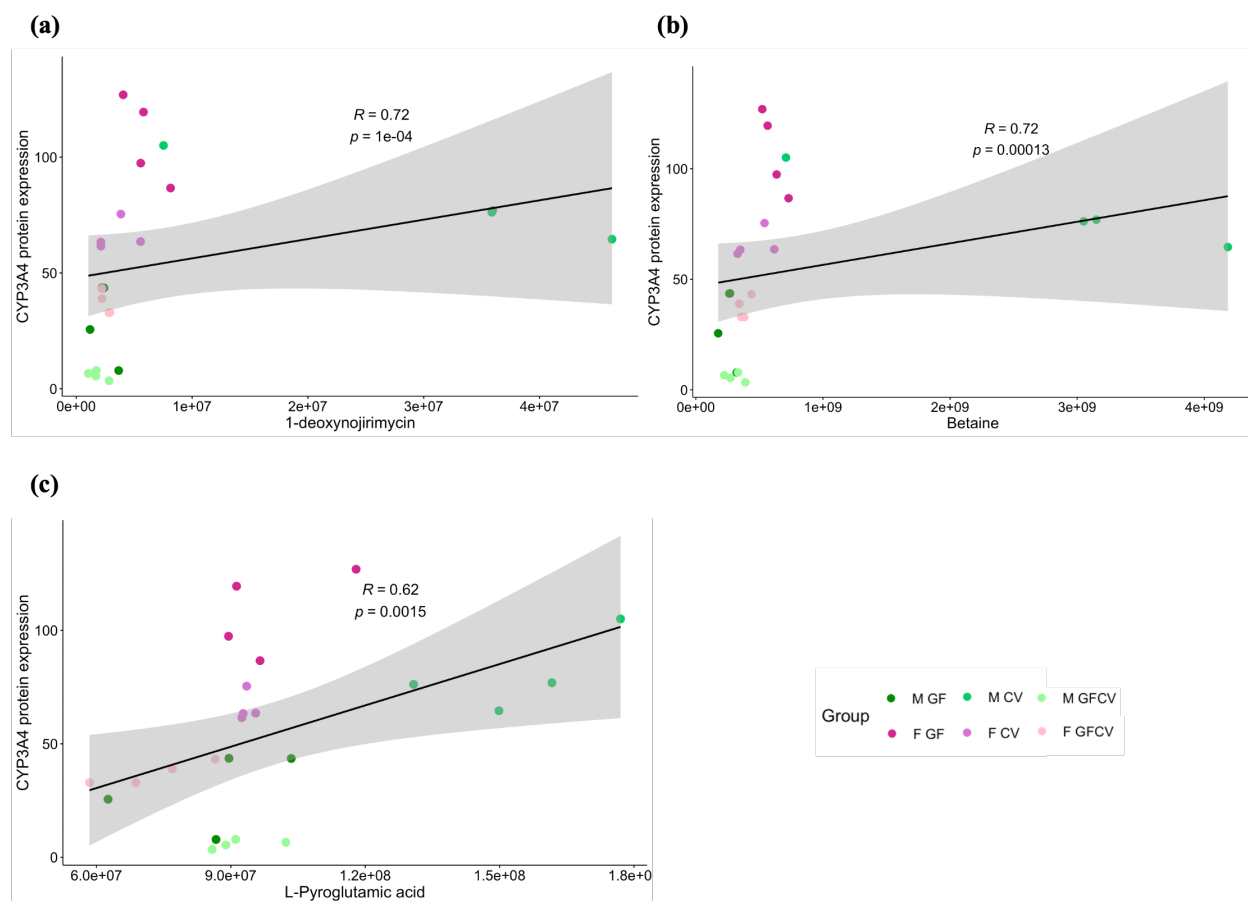


Figure 4.2. Spearman's correlation between CYP3A4 expression and (a) 1-deoxynojirimycin, (b) betaine, and (c) L-pyroglutamic acid as determined by global metabolomics in humanized PXR-CAR-CYP3A4/3A7 mice. Groups: Male and female conventional (CV), germ-free (GF) and conventionalized (GFCV) were analyzed ($n = 4$ per sex per group).

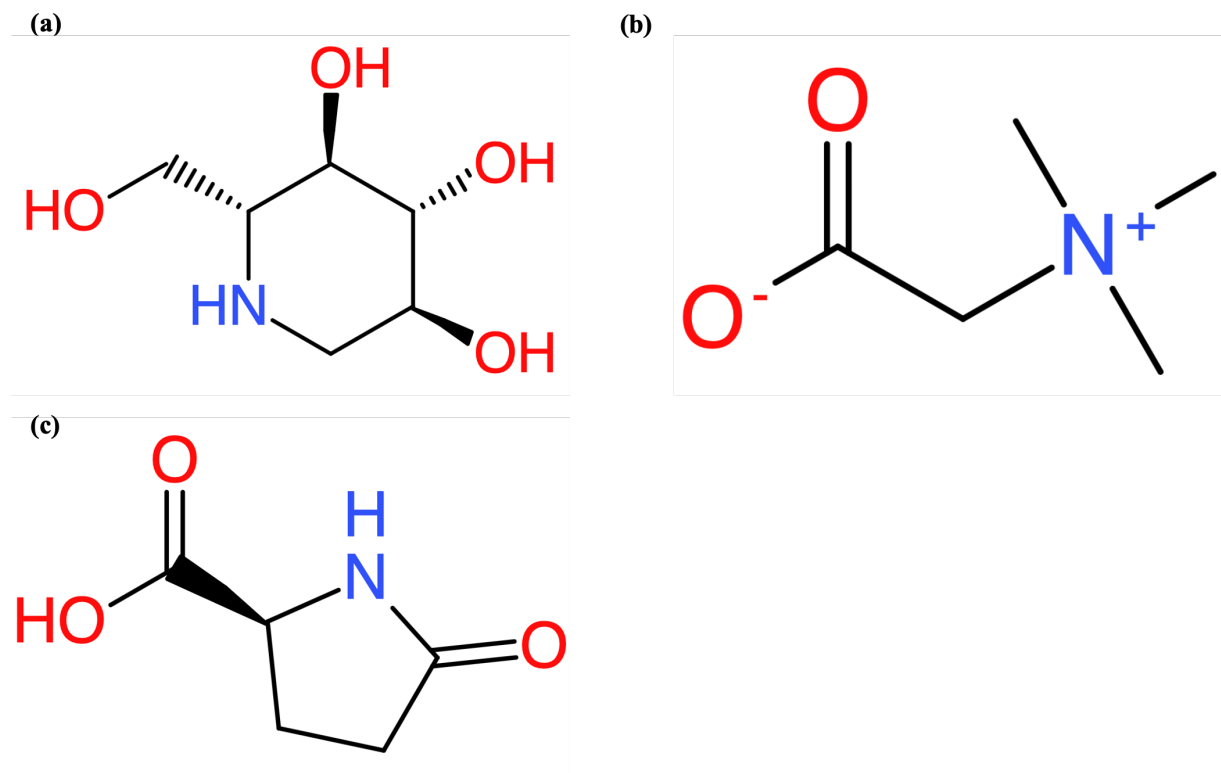


Figure 4.3. Chemical structures of selected metabolites: (a) 1-deoxynojirimycin, (b) betaine, and (c) L-pyroglutamic acid.

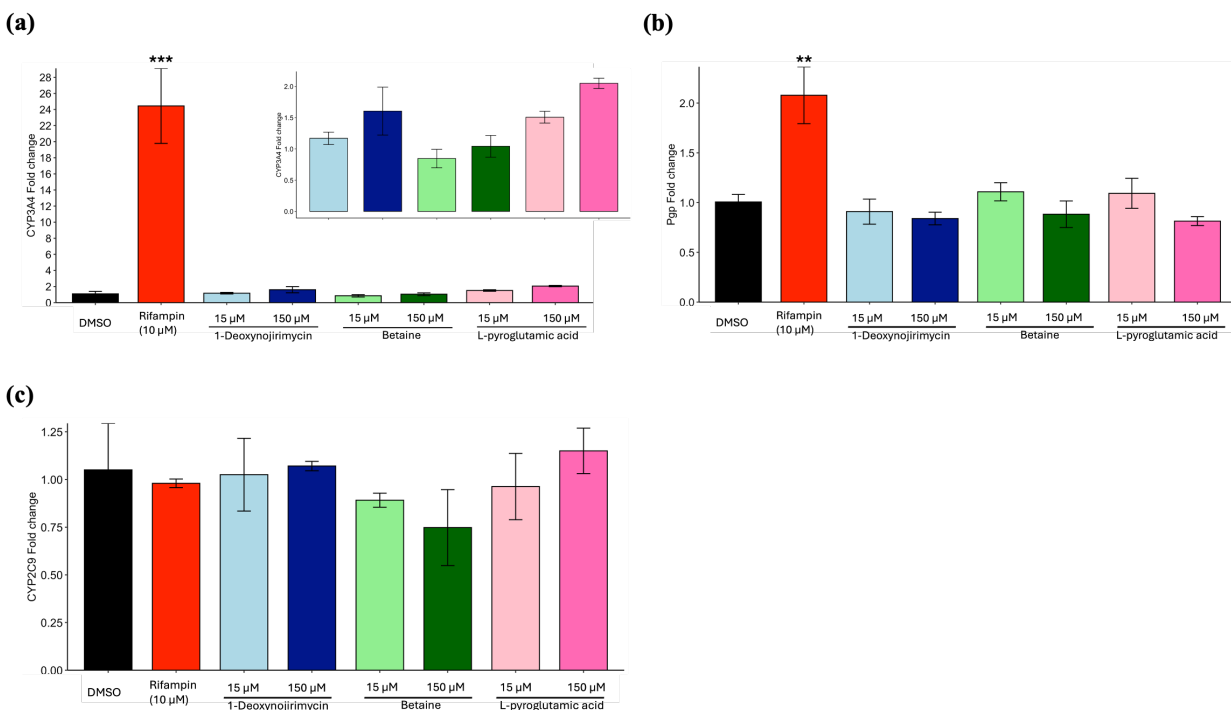


Figure 4.4. Target gene mRNA expression in HepaRG cells treated with 15 and 150 μ M concentrations of 1-deoxyneojirimycin, betaine, L-pyroglutamic acid. Fold-change in mRNA expression of: (a) *CYP3A4*, (b) *P-gp*, and (c) *CYP2C9*. Experiments were conducted in technical triplicate and significance was determined by ANOVA followed by Dunnett's test (p-value < 0.05 (*), < 0.01 (**), < 0.001 (***) compared to vehicle control)

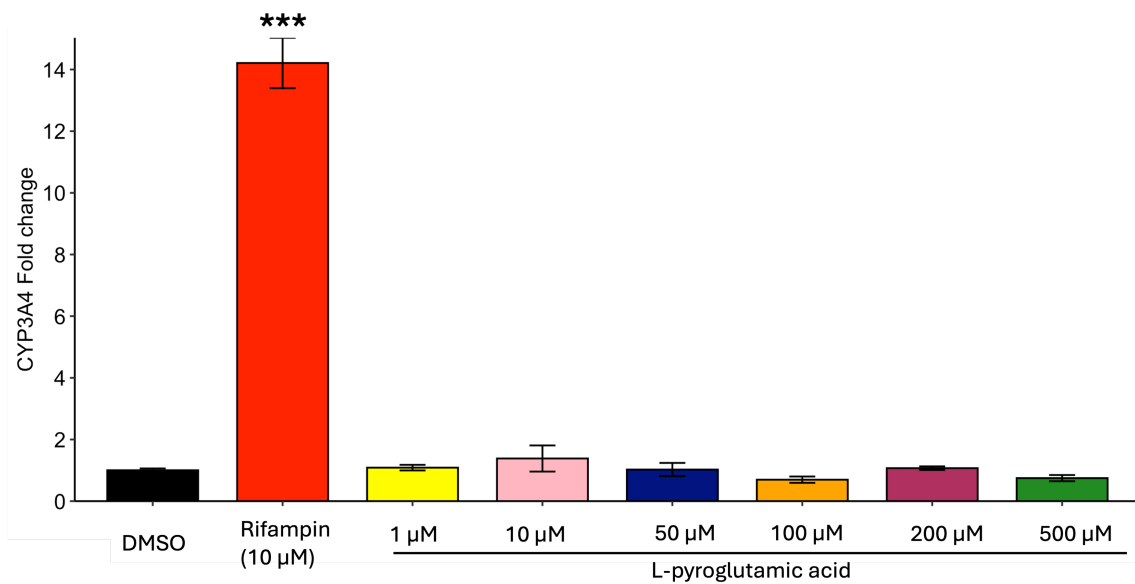


Figure 4.5. CYP3A4 mRNA expression following treatment with increasing doses of LPGA in HepaRG cells. Experiment was conducted in technical triplicate and significance was determined by ANOVA followed by Dunnett's test (p-value < 0.05 (*), < 0.01 (**), < 0.001 (***)).

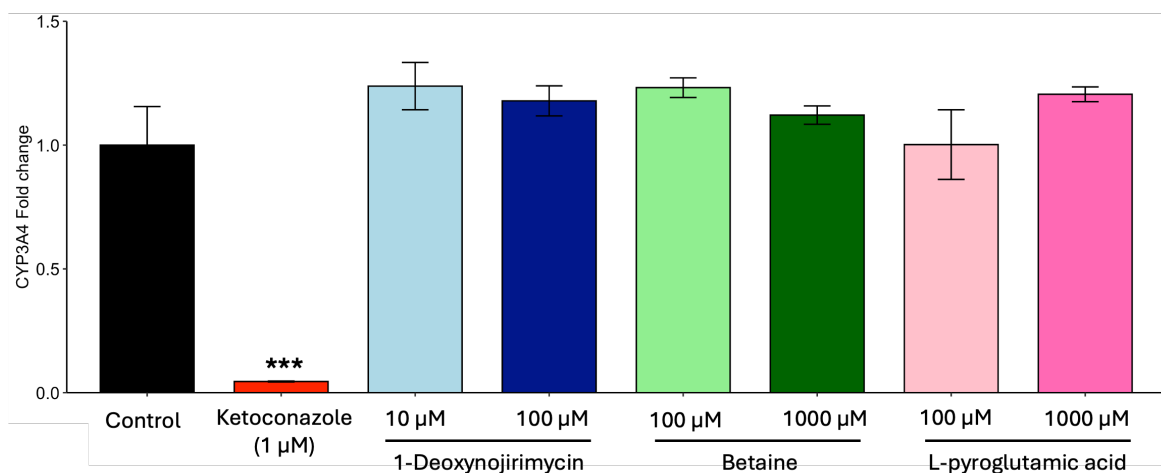


Figure 4.6. Inhibition of CYP3A4 activity by selected microbial metabolites in pooled human liver microsomes as quantified by luminescence. Experiment was conducted in technical triplicate and significance was determined by ANOVA followed by Dunnett's test (p-value < 0.05 (*), < 0.01 (**), < 0.001 (***)).

Chapter 5

Associations between the Gut Microbiome and CYP3A Activity in a Small Cohort of Healthy Male Volunteers

5.1. Introduction

The CYP3A subfamily is highly expressed both in the intestine and liver and is responsible for the metabolism of a large percentage of drugs on the market (Paine et al., 2006; Shimada et al., 1994; Zanger and Schwab, 2013). The CYP3A subfamily consists of 4 different isoforms, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, with CYP3A4 being the most abundant (Williams et al., 2002; Zanger and Schwab, 2013). Although many substrates, such as erythromycin, dextromethorphan, and verapamil, have been studied as probes of CYP3A activity (Krecic-Shepard et al., 1999), their use is limited by issues with administration (i.e., intravenous vs. oral), involvement of transporters or metabolism by other enzymes. Midazolam is considered the ‘gold standard’ probe for CYP3A as it is primarily metabolized by CYP3A into 1’-hydroxymidazolam and 4-hydroxymidazolam, can be administered both intravenously and orally, and has little unchanged drug excreted into the urine (Hyland et al., 2009; Thummel et al., 1994; Wandel et al., 1994). Additionally, midazolam is highly sensitive to CYP3A inducers and inhibitors, making it a favorable substrate (Backman et al., 1996; Krishna et al., 2009; Olkkola et al., 1994).

Using midazolam as a probe, CYP3A activity can be quantified using various metrics. First, midazolam clearance (CL) after IV dosing is highly correlated with hepatic CYP3A content (Thummel et al., 1994). With oral and IV dosing of midazolam, CYP3A activity is reflected by the gut and hepatic extraction ratios (ER_G and ER_H , respectively) that can be derived from the absolute oral bioavailability determined as a dose-normalized ratio of drug exposure following oral and IV dosing ($F = AUC_{PO}/AUC_{IV} \cdot dose_{IV}/dose_{PO}$). F can be separated into subcomponents of F_A , F_G , and F_H ($F = F_A \times F_G \times F_H$) (Gibaldi and Perrier, 1982). F_A represents the initial fraction absorbed, F_G is the fraction that escapes GI metabolism, and F_H is the fraction that escapes liver metabolism but is also related to $1 - CL/Q$ (where Q is hepatic blood flow).

Through defined relationships between bioavailability and extraction ratio (i.e., $ER_G = 1 - F_G$), the extraction ratios, ER_G and ER_H , can be calculated and will allow understanding of the CYP3A activity in the gut and liver, respectively. Lastly, the metabolite-to-parent AUC ratio (e.g., 1'-hydroxymidazolam AUC/midazolam AUC) reflects the formation of metabolite by CYP3A with the caveat that the ratio also incorporates the clearance of the metabolite through glucuronidation (Seo et al., 2010).

Traditionally, hepatic and intestinal CYP3A-mediated metabolism are assessed by determination of midazolam pharmacokinetics (PK) following separate instances of IV and oral dosing of midazolam. However, dosing on separate days can introduce error into the determination of certain PK parameters. For example, some individuals display considerable intraindividual variability in F (coefficient of variation of $26 \pm 15\%$) upon repeated midazolam dosing over 8 weeks (Kvitne et al., 2023). Interestingly, the authors did not see the same variability in clearance of midazolam. Additionally, separate dosing also leads to added cost and time which may burden both the participants and study team. A semi-simultaneous dosing approach has been utilized as a more efficient way to assess both hepatic and intestinal metabolism (Lee et al., 2002; van Rongen et al., 2015; Brill et al., 2014; Fetzner et al., 2011; Hafner et al., 2010; Kvitne et al., 2022a-2022b, 2023; Mikus et al., 2017). With this method, subjects receive oral and IV doses of midazolam separated by a fixed time interval on the same day. The semi-simultaneous dosing method allows for the sampling to be completed within one visit, eliminating sources of intraindividual variability associated with separate sampling visits.

In surgically obtained liver and small intestine tissues, CYP3A expression between individuals varied as much as 40-fold, indicating markedly large interindividual variability (Lin et al., 2002). The variability in hepatic and intestinal CYP3A expression can arise from several

different factors such as age, genetics, inflammation, and pregnancy (Subash and Prasad, 2024; Christensen and Hermann, 2012; Dresser, Spence, and Bailey, 2000; Tracy et al., 2005). For example, with CYP3A5, the largest cause of variability is genetic variation, especially between racial groups (Kuehl et al., 2001). On the other hand, hepatic CYP3A4 can differ by age with maximal CYP3A4 expression at around 2 years of age and then a continual decline until adulthood (Subash and Prasad, 2024). Variability in CYP3A expression or activity results in differences in drug clearance that may impact the efficacy and/or toxicity of drugs, especially drugs with a narrow therapeutic index (Kuehl et al., 2001; Lin et al., 2002; Thummel et al., 1996). Therefore, identifying factors that can influence intestinal and hepatic CYP3A expression will allow for a better understanding of factors that can impact basal hepatic and intestinal CYP3A variability.

Based on data from mouse studies, the gut microbiome has been proposed to be one of the contributing factors in interindividual variability of CYP3A (Fu et al., 2017; Selwyn et al., 2016; Togao et al., 2021). These studies found that the absence of a gut microbiome significantly altered the expression and activity of Cyp3a in the liver and intestine, suggesting that the gut microbiome can play a role in the regulation of Cyp3a. Togao et al. (2021) found that when different donor microbiomes were transplanted into germ-free mice, hepatic Cyp3a activity was restored to varying levels. Thus, it is possible that normal variation in the gut microbiome can influence CYP3A activity. However, no studies have been done to identify whether normal variation in the gut microbiome influences CYP3A activity in humans, and whether certain microbes and/or microbial metabolites might influence regulation of hepatic and intestinal CYP3A.

The aim of this current work is to identify whether normal variation in the gut microbiome and microbial metabolite levels is associated with intestinal or hepatic CYP3A activity as assessed by various metrics determined following semi-simultaneous dosing of midazolam. We then used an *in vitro* system to determine whether identified microbial metabolites impacted *CYP3A4* expression and induce CYP3A4 as PXR ligands.

5.2. Materials and Methods

5.2.1. Materials

Midazolam, 1'-hydroxymidazolam (1'-OH MDZ), 4-hydroxymidazolam (4-OH MDZ), midazolam-d4, 1'-hydroxymidazolam-d4, and 4-hydroxymidazolam-d5 were purchased from Cerilliant Corporation (Sigma, Round Rock, TX, USA). Methanol and formic acid were purchased from Fisher Scientific (Hampton, NH, USA). ZymoBIOMICS DNA Kits were purchased from Zymo Research (Irvine, CA, USA). Media components (HepaRG™ Thaw, Plate, & General Purpose Medium supplement, HepaRG™ Serum-free Induction Medium Supplement, William's EMmedium, GlutaMAX™ Supplement, and penicillin-streptomycin), dimethyl sulfoxide (DMSO), rifampin, PureLink™ RNA Mini Kit, TaqMan Fast Advanced Master Mix, and TaqMan probes (GAPDH: Hs99999905_m1; CYP3A4: Hs00604506_m1) were purchased from ThermoFisher Scientific (Waltham, MA). Collagen I coated 24-well plates were purchased from Corning Inc. (Corning, NY). The High-Capacity cDNA Reverse Transcription Kit was obtained from Applied Biosystems (Waltham, MA). Ethanol, serotonin, and xanthurenic acid were purchased from Sigma-Aldrich (St. Louis, MO). The Human Pregnane X Receptor Reporter Assay was purchased from Indigo Biosciences (State College, PA).

5.2.2. Clinical Study Design

Study Participants. A pilot study was conducted in healthy male volunteers to ascertain the impact of probiotic supplementation on the pharmacokinetics of acetaminophen and midazolam. Due to the Covid-19 pandemic, only baseline samples were collected from seven healthy participants. This study was approved by the Institutional Review Board at the University of Washington, Seattle, WA, and was registered as Study NCT04281407 at Clinical Trials.gov. Only the results of baseline midazolam administration are reported herein.

The inclusion criteria included male participants in good health between the ages of 18 and 40 years old and a body mass index between 25 to 32 kg/m². Participants could not take any known substrates, inhibitors, inducers, or activators of CYP3A4 at least 2 weeks before the study, and refrain from those CYP3A4 modulating drugs and any antibiotics, retinoids, corticosteroids, or immunosuppressant medications for the entire duration of the study. They must also be willing to fast overnight before study days and abstain from alcohol-containing beverages 24 hours before and during the study visits.

The exclusion criteria included milk allergy or lactose intolerance, current use of prescription medications and herbal products, systemic disorders affecting the immune system (e.g., human immunodeficiency virus, connective tissue disorders, cancers, etc.), current cigarette smokers, and any history of ulcerative colitis or Crohn's disease or documented intestinal stricture, stenosis, obstruction, fistula, abscess, or ileostomy. Individuals with self-reported history of liver, kidney, gastrointestinal or heart disease, abnormal liver or kidney function tests based on the Comprehensive and Hepatic Panel (below the lower end or greater than 15% of the upper end of the reference range), or a known or suspected history of alcohol or drug abuse were also excluded. Subjects allergic to midazolam, triazolam, diazepam, or

lorazepam and/or taking CYP3A4 inducers and inhibitors upon recruitment were also ineligible. Informed consent was obtained prior to study procedures.

Clinical Study Procedures. Subjects fasted overnight before the baseline visit. Pre-dose blood and pre-visit fecal samples were collected before the administration of midazolam. Midazolam (MDZ; 2 mg oral syrup) and acetaminophen (500 mg) were administered orally, and blood samples (7 mL each) were collected at 15 min, 30 min, 45 min, 1, 1.5, 2, and 3 hr post-oral administration. After the 3 hr timepoint, MDZ (1 mg) was given intravenously and blood samples were collected at 5 min, 15 min, 30 min, 45 min, 1, 2, 3, 6 and 9 hr post-IV dosing. Plasma and fecal samples were stored at -80°C until analysis.

5.2.3. Quantification of Midazolam in Plasma

Participant plasma samples or calibration curve samples (0.25 mL) were combined with 1 mL of methanol and 20 µL of internal standard (MDZ-d4, 1'-MDZ-d4, and 4-MDZ-d5) and vortexed for 2 minutes, then centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant was transferred to a clean tube, evaporated to dryness under nitrogen, and reconstituted in 75 µL of 60:40 of 0.1% formic acid and methanol. The samples were centrifuged at 20,000 x g for 10 minutes and transferred to a 96-well LC-MS plate.

5.2.4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

An Agilent 1290 series HPLC coupled to an Agilent Technologies 6460 tandem mass spectrometer (Agilent Technologies, Palo Alto, CA) was used for the analysis. For analysis, 5 µL of each sample was injected onto an Agilent Zorbax C-18 column (2.1 x 150 mm, 5.0 µm) held

at 35°C. The mobile phases were 0.1% formic acid (A) and methanol (B) with a flow rate of 0.250 mL/min for a run time of 12 minutes. The elution gradient was set as follows (% B): starting at 40%, ramping to 50% from 3 to 7 minutes and up to 90% from 7.5 to 9 minutes and back down to 40% at 9.10 to 12 minutes. The mass transitions used for quantification were: MDZ m/z 326 $\rightarrow$ 291.2, 1'-OH MDZ m/z 342 $\rightarrow$ 168.1, 4-OH MDZ m/z 342.1 $\rightarrow$ 297, MDZ-d4 m/z 330 $\rightarrow$ 291.2, 1'-OH MDZ-d4 m/z 346 $\rightarrow$ 168, and 4-OH-MDZ-d5 m/z 347.1 $\rightarrow$ 300. Analytes were monitored in the positive electrospray ionization mode. The drying gas temperature was fixed at 350°C and nitrogen drying gas flow was set at 10 L/min at 35 psig. Capillary voltage was set at 4000 V.

5.2.5. Pharmacokinetic Analysis

The midazolam concentration vs. time data were fit using a semi-simultaneous model that was developed using PKAnalix (MonolixSuite 2024R1) from SimulationPlus (Lancaster, CA, USA). Absolute oral bioavailability (F), rate constants (k_{12} , k_{21} , k_{10} , and k_a), and volume of distribution for the central compartment (V_c) were estimated. Hepatic clearance (CL_H) was calculated as $k_{10} \cdot V_c$ (Rowland and Tozer, 2011). Hepatic extraction ratio (ER_H) was calculated as CL_H/Q_H , where Q_H (L/h) is the hepatic blood flow that was estimated by $3.75 \times (\text{total body weight (kg)})^{0.75}$ (Brown et al., 1997). The intestinal extraction ratio (ER_G) was calculated as $1 - F/(1 - ER_H)$, assuming that intestinal absorption (F_A) of midazolam is complete ($F_A=1$) (Smith et al., 1981).

Area under the curve (AUC) values for MDZ, 1'-OH MDZ, and 4-OH MDZ were obtained by non-compartmental analysis (NCA) using PKAnalix. $AUC_{0-\infty}$ for oral administration was defined as the AUC after dose to infinity. $AUC_{0-\infty}$ for IV administration was calculated as

AUC from the start of IV administration (at 3 hours) to infinity ($AUC_{3-\infty}$) minus the extrapolated AUC from the oral administration ($AUC_{3-\infty,po}$). Total AUC was calculated as the addition of both oral and IV AUC.

5.2.6. Microbial DNA Extraction and Shotgun Metagenomics Sequencing

Microbial DNA was extracted from fecal samples ($n = 7$) with the ZymoBIOMICS DNA Kit per the manufacturer's protocol and stored at -20°C until analysis. DNA samples were shipped to NovoGene (Sacramento, CA, USA) for sequencing. Raw FASTQ files were trimmed and quality control was performed using fastp (Chen, 2025). The microbiome analysis was conducted as outlined in Lu et al. (2022). Briefly, host DNA was removed by aligning reads to host genome using bowtie2 (Langmead and Salzberg, 2012). Microbiome samples were classified using kraken2 (Wood, Lu, and Langmead, 2019). Lastly, an abundance estimation was generated with bracken (Lu et al., 2017). Downstream analysis (i.e., characterization and diversity measurements) were performed in Rstudio (version 2026.01.0+392).

5.2.7. Targeted Metabolomics

Aqueous metabolites for targeted LC-MS profiling of seven plasma samples were extracted using a protein precipitation method as described previously (Kurup et al., 2021; Meador et al., 2020). Samples were thawed at 4°C for 60 mins, vortexed for 10 sec, and 50 μL of each sample was transferred to a 2 mL Eppendorf tube and 50 μL of 50:50 methanol:water containing 28 stable isotope-labeled internal standards (SILISs) was added. Afterwards, 250 μL of cold methanol containing two additional SILISs was added to each sample (124 μM 6C13-glucose and 25.9 μM 2C13-glutamate). The samples were vortexed, stored for 30 min at -20°C ,

and centrifuged for 15 min at 14,000 rpm at 4°C. Lastly, 250 µL of supernatant was collected from each sample and dried on a SpeedVac and reconstituted in 0.5 mL of LC-matching solvent containing 17.8 µM 2C13-tyrosine and 39.2 µM 3C13-lactate. Samples were transferred into LC vials and placed into a temperature-controlled autosampler for LC-MS analysis. 32 SILISs total were added to the samples in various sample preparation steps to monitor sample preparation, assay performance, and to determine absolute concentrations for metabolites that had corresponding SILISs.

Targeted LC-MS metabolite analysis was performed on a duplex-LC-MS system composed of two Shimadzu UPLC pumps, a CTC Analytics PAL HTC-xt temperature-controlled auto-sampler and AB Sciex 6500+ Triple Quadrupole MS equipped with an electrospray ionization (ESI) source (Kurup et al., 2021; Meador et al., 2020). UPLC pumps were connected to the autosampler in parallel and could perform two chromatography separations in parallel independently from each other. Each sample was injected twice on two identical analytical columns (Waters XBridge BEH Amide Premier 2.1 x 150 mm) performing separations in hydrophilic interaction liquid chromatography (HILIC) mode. While one column performed separation for MS data acquisition in ESI+ ionization mode, the other column was equilibrated for sample injection, chromatography separation and MS data acquisition in ESI- mode. Each chromatography separation was 16 min (total analysis time per sample was 32 min), and MS data acquisition was performed in multiple-reaction-monitoring (MRM) mode. The LC-MS system was controlled using AB Sciex Analyst 1.7.1 software. The LC-MS assay targeted 372 metabolic species and 32 SILISs (404 MRMs). In addition to the study samples, two sets of quality control (QC) samples were used to monitor the assay performance and data reproducibility. One in-house QC [QC(I)] was a pooled human serum sample used to monitor system performance, and

the other QC [QC(S)] consisted of pooled study samples, which was used to monitor data reproducibility. Each QC sample was injected before and after study samples, respectively. A total of 205 to 207 metabolites (plus 32 spiked standards) were measured across the serum set and the median CV was 5.3 % (based on measured peak areas without any signal normalization). Metabolites were quantified as relative abundance by area-under-the-curves of the MRM transitions using AB Sciex MultiQuant 3.0.3 software. Since the samples were spiked with 32 SILIS, the absolute concentrations of 30 metabolites with the corresponding SILISs were calculated as $[(\text{Peak Area of Metabolite})/(\text{Peak Area SILIS})] \times (\text{Known Concentration SILIS}, \mu\text{M})$. Metabolites were compared against Microbial Metabolome Database (MiMeDB) 2.0 (Kruger et al., 2025) to determine those that were microbially derived.

5.2.8. Treatment with HepaRG cells and mRNA expression by RT-qPCR

Terminally differentiated HepaRG cells were plated in a 24-well collagen-I coated plate. Cells were plated with William's Medium E, supplemented with Glutamax (1x), HepaRG™ Thaw, Plate, and General Purpose Medium supplement, and penicillin-streptomycin (1%) and incubated for 6 hours, and refreshed with the same media. After a 72-hour incubation, media was replaced with William's Medium E, supplemented with GlutaMax (1x), HepaRG™ Serum-free Induction Medium supplement, and penicillin-streptomycin (1%) containing either: vehicle control (DMSO), rifampin (10 μM), serotonin (ranging from 1 nM to 100 μM), and xanthurenic acid (1, nM to 100 μM). These treatments were replenished every 24 hours for 72 hours. Serotonin and xanthurenic acid were dissolved in DMSO. Cells were treated in triplicate.

RNA was extracted from lysed HepaRG cells using the PureLink RNA Mini Kit per manufacturer's protocol. RNA purity and concentration were determined with a NanoDrop 2000

spectrophotometer (Thermofisher Scientific). The isolated RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit per manufacturer's protocol. Quantitative real-time polymerase chain reaction (qPCR) was performed to measure mRNA expression with GAPDH as a housekeeping gene and relative expression was determined by the $2^{-\Delta\Delta C_t}$ method.

5.2.9. PXR Reporter Assay

The assay was done per manufacturer's protocol. Briefly, the reporter cells were pre-incubated with the provided compound screening media. After 4 hours of incubation, media was discarded and replaced with treatment media. Each treatment was dispensed in quadruplicate. Final incubation concentrations were: rifampin (positive control) at (0.0412, 0.123, 0.370, 1.111, 3.333, 10, and 30 μ M) and serotonin and xanthurenic acid at 0.001, 0.01, 0.1, 0.3, 1, 10, and 100 μ M. After a 24-hour incubation, media was discarded, and a luciferase detection reagent was added. Luminescence was quantified using the Agilent Biotek Synergy HTX microplate reader. Response data were calculated as signal quantified divided by the background signal.

5.2.10. Statistical Analysis

Due to the small sample size, statistical analysis was not conducted on microbial diversity determinations (i.e., alpha and beta diversity calculations). Correlation analyses were performed using Spearman's rank correlation and p-values were adjusted by false discovery rate. *In vitro* experiments significance was determined by one-way ANOVA followed by Dunnett's test compared to vehicle control. p-value of less than 0.05 was considered significant.

5.3. Results

5.3.1. Quantification of midazolam and its metabolites in plasma

Seven healthy volunteers participated in this study and their demographics are summarized in Table 5.1. The mean values of age, body weight, and height were 27.4 ± 4.7 years, 68.6 ± 8.0 kg, and 177.4 ± 6.1 cm, respectively. One individual's weight was not recorded and was assumed to be 70 kg for calculation of liver blood flow. The observed midazolam, 1'-OH MDZ, and 4-OH MDZ concentration vs. time profiles following semi-simultaneous administration are shown in Figure 5.1. The semi-simultaneous model was built on PKAnalix (Figure 5.2a) and F , V_c , k_a , k_{10} , k_{12} and k_{21} were estimated. Individual fits for each participant is demonstrated in Figure 5.2b, showcasing a relatively good fit for all participants. Table 5.2 summarizes the average and standard deviation of estimated parameters. Bioavailability ranged from 0.20 to 0.45 with a CV of 25.6% and volume ranged from 42.9 to 87.9 L with a CV of 24.9%. Rate constant k_{12} had the most variability (CV of 78.4%) while k_a , k_{10} , and k_{21} had similar variability (39.5%, 34.7%, and 37.7%, respectively). From these estimates, CL , ER_H , and ER_G were calculated, and each individual participant's values were summarized in Table 5.3. Non-compartmental analysis (NCA) was used to calculate AUC of midazolam, 1'-OH MDZ, and 4-OH MDZ (Table 5.2). From the estimated AUCs, the AUC metabolite-to-parent ratio was 0.32 ± 0.11 for 1'-OH MDZ/MDZ and 0.13 ± 0.02 for 4-OH MDZ/MDZ (Table 5.3). All calculated parameters were correlated to each other (Table 5.4). There were only two metrics that were positively correlated to each other (CL with ER_H and 1'-OH MDZ/MDZ AUC ratio with 4-OH MDZ/MDZ AUC ratio).

5.3.2. Microbiome composition and diversity

The microbial composition for each individual was characterized at the genus and family level (Figure 5.3). The dominant phyla for five of the participants was *Bacteroidota*, while the dominant phyla for the other two participants was *Bacillota* (Figure 5.3a). Three participants' dominant family was *Prevotellaceae* and *Oscillospiraceae* while the other four participants' most dominant family was *Lachnospiraceae* (Figure 5.3b). Alpha diversity (within-sample diversity) was quantified by four different indices (Figure 5.4). The Chao1 index quantifies species richness accounting for rare species. The richness index quantifies overall species richness. The Shannon index quantifies both species richness and evenness and gives weight to rare species. The Simpson index also emphasizes richness and evenness, focusing on more abundant species (Cassol et al., 2025). There were larger differences between the alpha diversity of individuals when only taking into account richness and minimal differences between individuals when taking into account species evenness. Beta diversity (between sample diversity) was quantified by Bray-Curtis weighted dissimilarity (Figure 5.5). Three samples were clustered together and the other four were each separated from the rest. As expected, there were basal differences in microbiome composition and diversity among the participants.

5.3.3. Associations between the microbiome and CYP3A activity

To identify whether normal variation in microbial diversity has an influence on CYP3A activity, a correlation analysis was done between the alpha diversity and CYP3A indices. Both richness-based indices (i.e., Chao1 and Richness) had a negative correlation with 1'-OH MDZ/MDZ AUC ratio ($\rho = -0.86$) and 4-OH MDZ/MDZ AUC ratio ($\rho = -0.89$) but not CL, ER_H, and ER_G (Figure 5.6). As Chao1 and Richness indices were identical, only Chao1

correlation plots are shown for simplicity. There were no correlations between richness and evenness-based indices (i.e., Simpson and Shannon) and any parameter (Table 5.5). Overall, the richness of the microbiome was not correlated with CYP3A activity but may influence glucuronides.

A list of all correlated microbial species with corresponding CYP3A metrics are listed in Table 5.6. Briefly, only one species was positively correlated with CL and no negative correlations were observed. Three species were positively correlated with ER_H and 20 species were negatively correlated with ER_H. Eight species were positively correlated with ER_G and 3 species were negatively correlated with ER_G. Only one species was positively correlated with both 1'-OH and 4-OH MDZ/MDZ AUC ratios, while 124 and 79 species were negatively correlated with 1'-OH and 4-OH MDZ/MDZ AUC ratios, respectively.

5.3.4. Correlation analysis between microbial metabolites and CYP3A activity

Out of 370 aqueous metabolites, 73 of these are involved in microbial metabolism. A Spearman's correlation was conducted to test the association of CL, ER_H, ER_G, 1'-OH MDZ/MDZ and 4-OH MDZ/MDZ AUC ratios with the microbial metabolites identified by targeted metabolomics (Table 5.7). All correlated metabolites were cross-referenced with the Microbial Metabolome Database (MiMeDB) 2.0 (Kruger et al., 2025) and only microbial metabolites were selected. A total of 3 microbial metabolites were correlated with CL, 8 metabolites were correlated with ER_H, 4 with ER_G, 7 metabolites were correlated with 1'-OH MDZ/MDZ AUC ratio, and 8 metabolites were correlated with 4-OH MDZ/MDZ AUC ratio. Taurine was the only microbial metabolite that correlated with both ER_G and ER_H. Sucrose and guanosine monophosphate were positively correlated with both 1'-OH MDZ/MDZ and 4-OH

MDZ/MDZ AUC ratio. Serotonin was positively correlated with ER_H and both AUC ratios.

Xanthurenic acid was positively correlated with ER_H and 4-OH MDZ/MDZ AUC ratio.

Serotonin and xanthurenic acid, which are both tryptophan metabolites, were selected for further in vitro testing as other tryptophan metabolites have demonstrated an influence on CYP3A4. Additionally, these metabolites are the only ones that were positively correlated with both ER_H and one or both AUC ratios. To determine whether these microbial metabolites could regulate CYP3A4, HepaRG induction studies were conducted with increasing concentrations of serotonin and xanthurenic acid. To ascertain if upregulation was caused by PXR, these metabolites were dosed to PXR reporter cells.

5.3.5. In vitro experiments with serotonin and xanthurenic acid

To identify whether the positively correlated tryptophan metabolites had a regulatory effect on hepatic CYP3A4, HepaRG cells were initially treated with 1, 10, and 100 μ M of serotonin and xanthurenic acid for 72 hours. With both metabolites, no induction was seen across all concentrations (Figure 5.7b). At 100 μ M, serotonin demonstrated a trend of 1.8-fold downregulation of *CYP3A4*, but this was not statistically significant ($p = 0.056$). Interestingly, at all concentrations of xanthurenic acid treatment, we observed cell death. To more closely mimic physiological concentrations and prevent cell toxicity, we tested 1, 10, and 100 nM concentrations of both metabolites (Figure 5.7a). At 100 nM of serotonin, CYP3A4 mRNA expression was induced 2.0-fold ($p < 0.01$). Xanthurenic acid induced *CYP3A4* mRNA expression nearly 2-fold at 10 and 100 nM concentrations (1.8- and 2-fold, respectively; $p < 0.01$ for both). To explore whether this induction was PXR mediated, serotonin and xanthurenic acid at concentrations ranging from 1 nM to 100 μ M were tested in a PXR reporter system. Neither

serotonin nor xanthurenic acid demonstrated PXR agonism at any concentration tested (Figure 5.8).

5.4. Discussion

This is the first study to explore whether normal variation in the gut microbiome can influence CYP3A activity in humans. We sought to answer this question by leveraging semi-simultaneous dosing of midazolam in healthy volunteers that allowed for the deconvolution of intestinal and hepatic CYP3A activity using ER_G and ER_H, respectively. Additionally, through the quantification of midazolam's metabolites, 1'-hydroxymidazolam and 4-hydroxymidazolam, the metabolite-to-parent AUC ratios were quantified to characterize overall CYP3A activity. Baseline (pre-dose) fecal and plasma samples were used to characterize the microbiome and microbial metabolites, respectively. Overall, we found that normal variation in microbial diversity do not influence CYP3A activity but may impact glucuronide activity. Two tryptophan metabolites: serotonin and xanthurenic acid were positively correlated to ER_H and induced *CYP3A4* expression by 2.0-fold *in vitro* at 100 nM concentrations.

Participant demographics were summarized in Table 5.1. The average plasma concentration vs. time curves for midazolam, 1'-OH MDZ, and 4-OH MDZ are demonstrated in Figure 5.1. The pharmacokinetic model for midazolam and individual fits of the model is illustrated in Figure 5.2 and estimated and calculated values are summarized in Table 5.2 and Table 5.3. We also conducted a correlation analysis and saw positive correlations between CL and ER_H as well as between 1'-OH and 4-OH MDZ/MDZ AUC ratio (Table 5.4). The estimated F was 0.36 ± 0.10 in our subjects, which is comparable to what was previously reported when dosed on different days (0.340 ± 0.100) and semi-simultaneous methods (0.343 ± 0.094) were compared (Lee et al., 2002), as well as previous findings of bioavailability in men through

traditional dosing (0.29 ± 0.11) (Thummel et al., 1996) and 0.24 ± 0.04 in healthy volunteers (Mandema et al., 1992). The calculated clearance of 697 ± 81.5 mL/min was slightly higher compared to previous values reported in men of 416 ± 134 mL/min (Thummel et al., 1996) or healthy volunteers of 517 ± 165 mL/min (Kvitne et al., 2023) and 523 ± 31 mL/min (Mandema et al., 1992). Calculated ER_H was 0.47 ± 0.07 (range: 0.37 – 0.55) and ER_G was 0.31 ± 0.22 (range: 0.08 – 0.62; Table 1) were comparable to reported values of 0.44 ± 0.14 and 0.43 ± 0.24 , respectively (Thummel et al., 1996). AUC ratio for 1'-OH MDZ was 0.34 ± 0.12 and for 4-OH MDZ was 0.13 ± 0.02 (Table 2), which is also comparable to previously reported values of 0.3 – 0.6 and 0.06 – 0.08, respectively (Eap et al., 2004; Kupferschmidt et al., 1995; Link et al., 2008; Mandema et al., 1992; Niemi et al., 2003). These values indicate that the variability in CYP3A activity metrics in our participants is consistent with previously reported values, suggesting that despite the small n, our samples reflect the variability typically observed in CYP3A activity.

There was variability in the microbiome composition at the phylum and family level in our participants (Figure 5.3). The two most dominant phyla in the human gut are *Bacteroidota* (or *Bacteroidetes*) and *Bacillota* (or *Firmicutes*) (Houtman et al., 2022; Turnbaugh et al., 2009). In our data, these two phyla were also dominant with participants' having a range of 10-50% *Bacteroidota* and 25-75% *Bacillota* (Figure 5.3a). Furthermore, microbial variability is also apparent at the family level, which is expected as many environmental factors, such as diet and built environment, influence the gut microbiome (Ahn et al., 2021). For example, the most dominant family was *Lachnospiraceae* for 4 participants, *Prevotellaceae* for 2 participants and *Oscillospiraceae* for 1 participant (Figure 5.3b).

The variability between participants was confirmed by alpha and beta diversity calculations (Figures 5.4 and 5.5, respectively). Alpha diversity was calculated by four different

metrics and the variability was more apparent when looking at Chao1 and Richness, presence-based metrics, with up to 7.3-fold differences between participants compared to Shannon and Simpson, presence- and evenness-based metrics, where the difference was 1.4-fold between participants. Differences in Chao1 and Richness but not Shannon and Simpson indicate that richness of the microbiome largely varies between participants and is the driving force of the variability. Beta diversity further confirmed that participants had differing microbiomes, with three participants clustered together and the other four participants were distinct from each other. We wanted to answer whether CYP3A activity is influenced by microbial diversity. Firstly, no parameters were significantly correlated with Shannon and Simpson metrics (Table 5.5). On the other hand, Chao1 and Richness have a strong negative correlation to both 1'-OH MDZ/MDZ and 4-OH MDZ/MDZ AUC ratios, but were not associated with CL, ER_H or ER_G (Figure 5.6). Based on these correlations, microbial diversity and richness do not influence CYP3A activity. Alternatively, increased glucuronidation of 1'-OH MDZ and 4-OH MDZ (i.e., metabolite clearance) would result in decreased metabolite-to-parent AUC ratios. Thus, patients with richer microbial compositions may have increased 1'-OH MDZ and 4-OH MDZ glucuronidation.

Although there was no correlation between microbial diversity and CYP3A, species abundance was correlated with CYP3A metrics to identify microbial species that may be associated with CYP3A activity (Table 5.6). We found that there were many more negatively correlated species than positively correlated for ER_H and both AUC ratios. There were 6 positively correlated and 3 negatively correlated species for ER_G. Interestingly, only one microbe was significantly correlated with CL and this was *Mediterraneibacter faecis*, which is an anaerobic bacteria found in stool (Togo et al., 2018).

A total of three microbial metabolites were correlated with CL, eight microbial metabolites were correlated with ER_H, four microbial metabolites were correlated with ER_G, seven microbial metabolites were correlated with the 1'-OH MDZ/MDZ AUC ratio, and eight microbial metabolites were correlated with the 4-OH MDZ/MDZ AUC ratio (Table 5.7). Overall, there were more positively correlated metabolites compared to negatively correlated metabolites (with the exception of equal numbers for ER_G), which was opposite to what was seen with microbial species. Note that these metabolites were measured in the plasma and not feces. Circulating levels of metabolites are not fully representative of metabolites present in the intestinal lumen and/or liver tissue.

There were only a few metabolites that were correlated to more than one CYP3A metric. For example, taurine was negatively correlated with ER_G but positively correlated with ER_H. Sucrose and guanosine monophosphate were positively correlated with 1'-OH MDZ/MDZ and 4-OH MDZ/MDZ AUC ratios. Serotonin, a tryptophan metabolite was positively correlated with ER_H and both AUC ratios. Another downstream metabolite of tryptophan, xanthurenic acid, was also found to be correlated with ER_H and 4-OH MDZ/MDZ AUC ratio. Tryptophan derived metabolites, specifically indole propionic acid, have been found to induce CYP3A4 (Ren et al., 2025). Therefore, we hypothesized the positive correlation of serotonin and xanthurenic acid seen in our data may indicate a potential regulatory role of other tryptophan metabolites on CYP3A.

To test whether serotonin and xanthurenic acid can upregulate CYP3A4 expression, we treated HepaRG cells with up to 100 μ M of serotonin and xanthurenic acid. No *CYP3A4* induction was observed across all concentrations for both metabolites (Figure 5.7b). Interestingly, there was a 1.8-fold downregulation of *CYP3A4* with 100 μ M of serotonin.

However, this effect was not statistically significant ($p = 0.056$) and therefore, may not be meaningful. Regardless, a previous study was done where rats that were fed a tryptophan-free diet had an increase in CYP3A protein expression and activity (quantified by testosterone 2 β - and 6 β -hydroxylation) for 3 weeks (Kot et al., 2012). Interestingly, plasma serotonin concentrations were increased after one week but decreased after three weeks after a tryptophan-free diet (Kot et al., 2012). This rat study highlights that there may be a complex interaction between serotonin and CYP3A that warrants further studies. On the other hand, although xanthurenic acid did not display any effect on *CYP3A4*, we did observe cell death as early as 24 hours post treatment across all concentrations. Previous studies have shown xanthurenic acid toxicity in primary smooth muscle, retinal, and lens epithelial cells (Malina et al., 2001; Malina et al., 2002; Malina and Hess, 2004). An observational study saw that an increase in xanthurenic acid correlated with hepatic encephalopathy in patients with liver cirrhosis (Reshetova et al., 2024). The physiological plasma concentrations of serotonin and xanthurenic acid are typically in the nanomolar range (Beck et al., 1993; Oxenkrug, 2015), and we conducted the induction experiment with lower concentrations (1 to 100 nM) (Figure 5.7a). Serotonin and xanthurenic acid induced *CYP3A4* mRNA expression by approximately 2-fold at 100 nM in HepaRG cells. When we tested whether the induction was PXR-mediated using reporter assay (Figure 5.8), we observed no PXR agonism with concentrations up to 100 μ M. Serotonin and xanthurenic acid-mediated induction of CYP3A4 does not appear to be through PXR. The CYP3A4 induction could be mediated through other nuclear receptors such as the constitutive androstane receptor (CAR) or aryl hydrocarbon receptor (AhR). Alternatively, a metabolite derived from serotonin or xanthurenic acid could be responsible for the upregulation of hepatic CYP3A4 observed in vivo, but the formation of that metabolite may have been limited in our in vitro systems.

There are limitations to this study. First, as this was a small cohort study ($n = 7$) and is not an interventional study, the correlations between microbial species and metabolites with CYP3A metrics are associations and may not be causation. Additionally, as this study was done only on male participants, we are unable to determine whether sex differences would exist. Women are shown to have a higher bacterial abundance, with a bacteria-to-human cell ratio of 2.2 as compared to 1.3 in men (Yoon et al., 2021). There may be more of an impact of species diversity on CYP3A metrics in women. We saw a strong negative correlation between microbial diversity and 1'-OH MDZ/MDZ and 4-OH MDZ/MDZ AUC ratios but did not quantify glucuronides (i.e., midazolam glucuronide, 1'-hydroxymidazolam glucuronide, and 4-hydroxymidazolam glucuronide) in our samples and therefore cannot conclude with certainty that the gut microbiome influences glucuronidation. Future directions should include this quantification of glucuronide metabolites to confirm this relationship. Although we identified that normal variation in the gut microbiome does not influence CYP3A activity, alterations to the microbiome may still impact CYP3A. Therefore, interventional studies in which the microbiome is manipulated (e.g., through biotic administration) can inform whether CYP3A activity is influenced by microbial changes. Lastly, we saw induction of *CYP3A4* by serotonin and xanthurenic acid *in vitro*. Future studies should explore this interaction either by testing it out in a more physiologically relevant system (e.g., primary human hepatocytes) or dosing of mice with these compounds.

5.5. Conclusions

We determined five metrics of CYP3A activity following semi-simultaneous dosing of midazolam that provided estimates of overall CYP3A activity as well as intestinal and hepatic

CYP3A. Overall, we saw no correlation between microbial diversity and CYP3A activity but a potential impact on glucuronide activity. However, we saw strong correlations between tryptophan metabolites (i.e., serotonin and xanthurenic acid) and hepatic CYP3A, and these metabolites moderately induced *CYP3A4* expression *in vitro*, but were not PXR agonists. Future studies are warranted to further elucidate the underlying mechanism behind the microbial influence on hepatic CYP3A.

5.6. References

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5.7. Tables and Figures

Table 5.1. Demographic Characteristics of Study Subjects.

	Value
Number of subjects	7
Age (yr) ^a	27.4 ± 4.7
Weight (kg) ^a	68.6 ± 8.0
Height (cm) ^a	177.4 ± 6.1
Male (%)	100
Race	
Caucasian	4
African American	1
Asian American	1
Other	1

^a Mean ± Standard Deviation

Table 5.2. Estimated pharmacokinetic parameters for midazolam following semi-simultaneous dosing of 2 mg oral midazolam (at 0 hours) and 1 mg IV midazolam (at 3 hours) in healthy male participants (n=7). Model parameters were determined by compartmental modeling. AUC parameters were determined using non-compartmental modeling.

Pharmacokinetic Parameter	Estimate^a	Range
F	0.36 ± 0.10	0.20 – 0.45
Vc (L)	65.9 ± 16.4	42.9 – 87.9
ka (h ⁻¹)	3.42 ± 1.35	1.26 – 4.68
k ₁₂ (h ⁻¹)	0.36 ± 0.28	0.08 – 0.84
k ₂₁ (h ⁻¹)	0.41 ± 0.16	0.19 – 0.58
k ₁₀ (h ⁻¹)	0.68 ± 0.24	0.47 – 1.08
AUC _{0-∞}		
Midazolam (ng/mL/min)	2513 ± 400	1934 – 3198
1'-OH Midazolam (ng/mL/min)	843 ± 288	385 – 1058
4-OH Midazolam (ng/mL/min)	331 ± 58.0	250 – 397

^a Mean ± Standard Deviation

Table 5.3. CYP3A activity metrics for each participant following semi-simultaneous dosing of 2 mg oral midazolam (at 0 hours) and 1 mg IV midazolam (at 3 hours) in healthy male participants (n=7).

Participant	CL (L/h)	ER_H	ER_G	1'-OH MDZ/MDZ AUC Ratio	4-OH MDZ/MDZ AUC Ratio
1	33.5	0.37	0.41	0.18	0.11
2	42.2	0.53	0.11	0.50	0.15
3	42.1	0.51	0.08	0.31	0.13
4	45.8	0.45	0.22	0.28	0.10
5	46.3	0.55	0.16	0.43	0.14
6	45.5	0.48	0.62	0.23	0.13
7	37.0	0.40	0.55	0.34	0.13
Average ± SD	41.8 ± 4.89	0.47 ± 0.07	0.31 ± 0.22	0.32 ± 0.11	0.13 ± 0.02

Table 5.4. Correlation of CYP3A metrics.

	CL	ER _H	ER _G	1'-OH MDZ/MDZ AUC Ratio	4-OH MDZ/MDZ AUC Ratio
CL	1	0.77*	-0.32	0.34	0.23
ER _H		1	-0.64	0.71	0.71
ER _G			1	-0.56	-0.28
1'-OH MDZ/MDZ AUC Ratio				1	0.77*
4-OH MDZ/MDZ AUC Ratio					1

* = significant correlation with p-value < 0.05

Table 5.5. Correlation between alpha diversity indices Shannon and Simpson and CYP3A activity metrics.

CYP3A Activity Metric	Correlation (p-value)	
	Shannon Diversity	Simpson Diversity
CL	-0.39 (0.4)	-0.36 (0.44)
ER _H	-0.071 (0.91)	0.14 (0.78)
ER _G	-0.18 (0.71)	-0.5 (0.27)
1'-OH MDZ/MDZ AUC Ratio	-0.5 (0.27)	-0.21 (0.66)
4-OH MDZ/MDZ AUC Ratio	-0.036 (0.96)	-0.21 (0.66)

Table 5.6. Correlation between microbial species and CYP3A activity metrics.

CYP3A Activity Metric	Positively Correlated	Negatively Correlated
CL	<i>Mediterraneibacter faecis</i> (0.79)	N/A
ER _H	<i>Eggerthella lenta</i> (0.79), <i>Mediterraneibacter glycyrrhizinilyticus</i> (0.82), <i>Paenibacillus sp. FSL K6-3182</i> (0.86)	<i>Clostridium sp. AWRP</i> , <i>Desulfitobacterium dichloroeliminans</i> , <i>Thermoanaerobacterium sp. RBIITD</i> , <i>Lactiplantibacillus plantarum</i> , <i>Alistipes onderdonkii</i> , <i>Alistipes senegalensis</i> , <i>uncultured Alistipes sp.</i> , <i>Barnesiella intestinihominis</i> (-0.79) <i>Ihubacter sp. rT4E-8</i> , <i>Bacteroides salyersiae</i> , <i>Butyricimonas faecalis</i> , <i>Prevotella sp. Rep29</i> (-0.82) <i>Coprococcus sp. ART55/1</i> , <i>Peptoclostridium acidaminophilum</i> , <i>Halalkalibacterium halodurans</i> , <i>Alistipes communis</i> , <i>Coprobacter secundus</i> (-0.85) <i>Bacillus pumilus</i> , <i>Alistipes finegoldii</i> , <i>Simiaoa sunii</i> (-0.89)
ER _G	<i>Streptococcus sp. Marseille-Q3533</i> (0.77) <i>Lachnospira eligens</i> , <i>Haemophilus parainfluenzae</i> (0.79) <i>Streptococcus sp. KHUD_014</i> , <i>Streptococcus equinus</i> (0.82) <i>Eubacterium hominis</i> (0.86) <i>Lactobacillus delbrueckii</i> (0.92) <i>Sutterella wadsworthensis</i> (0.93)	<i>Bacillus amyloliquefaciens</i> (-0.79), <i>Clostridium sp. C1</i> (-0.82), <i>Pelodicyton luteolum</i> (-0.86)
1'-OH MDZ/MDZ AUC Ratio	<i>Bifidobacterium dentium</i> (0.79)	<i>Paenibacillus sp. FSL W8-0194</i> (-0.76) <i>Paenibacillus tundrae</i> , <i>Streptococcus sp. Marseille-Q3533</i> (-0.77)

		<i>Acetivibrio thermocellus</i>, <i>Actinomyces</i> sp. oral taxon 414, <i>Alistipes</i> sp. i18-0019-D1, <i>Anaerostipes</i> sp. PC18, <i>Bacillus</i> <i>mycoides</i>, <i>Chakrabartyella piscis</i>, <i>Chitinophaga niabensis</i>, <i>Clostridium intestinale</i>, <i>Delftia</i> <i>lacustris</i>, <i>Desulfotobacterium</i> <i>dichloroeliminans</i>, <i>Enterocloster</i> <i>lavalensis</i>, <i>Filifactor alocis</i>, <i>Haloimpatiens lingqiaonensis</i>, <i>Kineothrix</i> sp. MB12-C1, <i>Lacrimispora sinapis</i>, <i>Mesobacillus foraminis</i>, <i>Microtetraspora malaysiensis</i>, <i>Paenibacillus algicola</i>, <i>Phascolarctobacterium</i> <i>succinatutens</i>, <i>Pyramidobacter</i> sp. YE332, <i>Reyranella</i> sp., <i>Rothia</i> <i>dentocariosa</i>, <i>Selenomonas</i> sp., <i>Streptococcus</i> sp. KHUD_014, <i>Succinivibrio dextrinosolvens</i>, <i>Thermoanaerobacterium</i> sp. RBIITD, <i>Treponema socranskii</i>, uncultured <i>Alistipes</i> sp., <i>Vallitalea</i> <i>guaymasensis</i>, <i>Aedoeadaptatus</i> <i>ivorii</i> (-0.79) <i>Acidilutibacter cellobiosedens</i>, <i>Anaerocolumna</i> sp. AGMB13020, <i>Bacteroides faecis</i>, <i>Bacteroides</i> <i>fragilis</i>, <i>Bacteroides salyersiae</i>, <i>Butyricimonas faecalis</i>, <i>Caloramator</i> sp. mosi_1, <i>Emergencia timonensis</i>, <i>Enterococcus faecalis</i>, <i>Gemella</i> <i>sanguinis</i>, <i>Lactobacillus</i> <i>amylovorus</i>, <i>Lactococcus</i> <i>cremoris</i>, <i>Mogibacterium</i> <i>neglectum</i>, <i>Paenibacillus</i> <i>albicereus</i>, <i>Parabacteroides</i> <i>chongii</i>, <i>Parabacteroides faecis</i>, <i>Schaalia meyeri</i>, <i>Simiaoa sunii</i>, <i>Variovorax paradoxus</i> (-0.82)
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		<i>Paenibacillus</i> sp. H1-7 (-0.83) <i>Bifidobacterium subtilis</i> (-0.85) <i>Acidaminococcus timonensis</i>, <i>Acidocella</i> sp. MX-AZ03, <i>Alistipes</i> sp. dk3624, <i>Aminipila terrae</i>, <i>Anaerocolumna sedimenticola</i>, <i>Anaerocolumna</i> sp. MB42-C2, <i>Bengtsoniella intestinalis</i>, <i>Bifidobacterium eulemuris</i>, <i>Brevibacillus brevis</i>, <i>Butyricimonas paravirosa</i>, <i>Butyrivibrio proteoclasticus</i>, <i>Butyrivibrio</i> sp. JL13D10, <i>Candidatus Formimonas</i> <i>warabiya</i>, <i>Chordicoccus</i> <i>furentiruminis</i>, <i>Christensenella</i> sp. MSJ-20, <i>Clostridium</i> <i>fermenticellae</i>, <i>Clostridium</i> sp. SY8519, <i>Delftia acidovorans</i>, <i>Delftia</i> sp. Cs1-4, <i>Delftia</i> sp. HK171, <i>Desulfitobacterium</i> <i>hafniense</i>, <i>Enterococcus avium</i>, <i>Faecalibacterium</i> sp. 7, <i>Fodinisporobacter ferrooxydans</i>, <i>Fusobacterium</i> sp. SB021, <i>Helicovermis profundus</i>, <i>Lachnoanaerobaculum umeaense</i>, <i>Megamonas funiformis</i>, <i>Mogibacterium diversum</i>, <i>Olsenella timonensis</i>, <i>Oscillibacter</i> <i>hominis</i>, <i>Paenibacillus</i> sp. MMS20-IR301, <i>Paenibacillus</i> <i>thiaminolyticus</i>, <i>Pectinatus</i> <i>frisingensis</i>, <i>Peptoniphilus</i> <i>equinus</i>, <i>Proteiniborus</i> sp. MB09- C3, <i>Ruminiclostridium</i> <i>herbifermentans</i>, <i>Salinicoccus</i> <i>halodurans</i>, <i>Streptococcus</i> <i>acidominimus</i>, <i>Streptococcus</i> <i>dysgalactiae</i>, <i>Streptococcus</i> <i>lutetiensis</i>, <i>Streptococcus</i> <i>sanguinis</i>, <i>Streptomyces</i> sp. <i>enrichment culture</i>,
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		<i>Tepidanaerobacter acetatoxydans</i>, <i>Treponema ruminis</i> (-0.86) <i>Mycobacterium canetti</i> (-0.88) <i>Alistipes finegoldii</i>, <i>Anaerocolumna cellulolytica</i>, <i>Anaerocolumna chitinolytica</i>, <i>Anaeromicrophila herbolytica</i>, <i>Anaerostipes rhamnosivorans</i>, <i>Bacillus pumilus</i>, <i>Butyrivibrio</i> <i>hungatei</i>, <i>Clostridium</i> sp. OS1-26, <i>Geosporobacter ferrireducens</i>, <i>Lachnoclostridium</i> <i>phytofermentans</i>, <i>Paenibacillus</i> <i>bovis</i>, <i>Paenibacillus</i> <i>physcomitrellae</i>, <i>Saccharibacillus</i> <i>brassicae</i> (-0.89) <i>Agrobacterium vitis</i>, <i>Clostridium</i> sp. AWRP, <i>Copro bacter secundus</i>, <i>Faecalibacterium</i> sp. I3-3-89, <i>Fusibacter</i> sp. JL216-2, <i>Kineothrix</i> <i>sedimenti</i>, <i>Paenibacillus silvae</i>, <i>Peptoclostridium</i> <i>acidaminophilum</i>, <i>Solibaculum</i> <i>mannosilyticum</i>, <i>Stomatobaculum</i> sp. F0698, <i>Vagococcus fluvialis</i> (- 0.93)
4-OH MDZ/MDZ AUC Ratio	<i>Bifidobacterium dentium</i> (0.96)	<i>Paenibacillus</i> sp. FSL W8-0194 (- 0.76) <i>Clostridium caseinolyticum</i>, <i>Halobacillus litoralis</i>, <i>Sodaliphilus</i> <i>pleomorphus</i> (-0.77) <i>Aminipila terrae</i>, <i>Arachnia</i> <i>propionica</i>, <i>Bacteroides</i> <i>helcogenes</i>, <i>Bacteroides</i> <i>salyersiae</i>, <i>Bacteroides sedimenti</i>, <i>Bacteroides zooglyphiformans</i>, <i>Butyricimonas paravirosa</i>, <i>Christensenella</i> sp. MSJ-20, <i>Clostridium fermenticellae</i>, <i>Coprococcus eutactus</i>, <i>Faecalibacterium</i> sp. 7,

		<i>Oscillibacter hominis</i>, <i>Pectinatus frisingensis</i>, <i>Peptoniphilus equinus</i>, <i>Phascolarctobacterium succinatutens</i>, <i>Prevotella heparinolytica</i>, <i>Proteiniborus</i> sp. MB09-C3, <i>Streptococcus dysgalactiae</i>, <i>Streptomyces</i> sp. enrichment culture (-0.79) <i>Alistipes</i> sp. dk3624, <i>Anaerocolumna sedimenticola</i>, <i>Anaerocolumna</i> sp. MB42-C2, <i>Bengtsoniella intestinalis</i>, <i>Brevibacillus brevis</i>, <i>Butyrivibrio proteoclasticus</i>, <i>Butyrivibrio</i> sp. JL13D10, <i>Candidatus Formimonas warabiya</i>, <i>Chakrabartyella piscis</i>, <i>Chordicoccus furentiruminis</i>, <i>Clostridium intestinale</i>, <i>Desulfitobacterium dichloroeliminans</i>, <i>Enterococcus avium</i>, <i>Fusobacterium</i> sp. SB021, <i>Helicovermis profundus</i>, <i>Lachnoanaerobaculum umeaense</i>, <i>Paenibacillus thiaminolyticus</i>, <i>Prevotella</i> sp. E9-3, <i>Reyranella</i> sp., <i>Ruminiclostridium herbifermentans</i>, <i>Segatella hominis</i>, <i>Tepidanaerobacter acetatoxydans</i>, <i>Thermoanaerobacterium</i> sp. RBIITD, <i>Treponema ruminis</i>, uncultured <i>Alistipes</i> sp. (-0.82) <i>Mycobacterium canetti</i>, <i>Streptococcus</i> sp. Marseille-Q3533 (-0.83) <i>Acetivibrio thermocellus</i>, <i>Candidatus Desulforudis audaxviator</i>, <i>Clostridium</i> sp. OS1-26, <i>Copro bacter secundus</i>, <i>Fusibacter</i> sp. JL216-2, <i>Halalkalibacterium halodurans</i>, <i>Kineothrix sedimenti</i>, <i>Odoribacter</i>
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		<i>splanchnicus</i>, <i>Streptococcus</i> sp. KHUD_014, <i>Succinivibrio dextrinosolvens</i>, <i>Treponema socranskii</i>, <i>Variovorax paradoxus</i> (-0.86) <i>Bifidobacterium eulemuris</i>, <i>Clostridium</i> sp. AWRP, <i>Megamonas funiformis</i>, <i>Olsenella timonensis</i>, <i>Parabacteroides chongii</i>, <i>Prevotella</i> sp. Rep29, <i>Salinicoccus halodurans</i>, <i>Streptococcus acidominimus</i> (-0.89) <i>Alistipes finegoldii</i>, <i>Bacillus pumilus</i>, <i>Simiaoa sunii</i>, <i>Streptococcus sanguinis</i> (-0.93) <i>Alistipes communis</i>, <i>Butyricimonas faecalis</i>, <i>Parabacteroides faecis</i>, <i>Peptoclostridium acidaminophilum</i>, <i>Selenomonas</i> sp. (-0.96)
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Table 5.7. List of significantly correlated microbial metabolites and CYP3A activity metrics.

PK Parameter	Positively Correlated	Negatively Correlated
CL	Aspartic Acid (0.82), agmatine (0.82)	Glycerol 3-phosphate (-0.79)
ER _H	Nicotinamide adenine dinucleotide (0.79), cysteine-S-sulfate (0.79), taurine (0.82), agmatine (0.86), xanthurenic acid (0.86), serotonin (0.94), niacinamide (0.96), carnitine (0.96)	None
ER _G	Guanidinoacetate (0.79), cholic acid (0.79)	Urate (-0.82), taurine (-0.82)
1'-OH MDZ/MDZ AUC ratio	Serotonin (0.76), phosphorylcholine (0.79), O-phosphoethanolamine (0.79), sucrose (0.82), linoleic acid (0.86), erythrose-4-phosphate (0.87), Guanosine monophosphate (0.96)	None
4-OH MDZ/MDZ AUC ratio	Beta-alanine (0.79), Carnitine (0.82), Guanosine monophosphate (0.82), Nicotinamide adenine dinucleotide (0.82), serotonin (0.92), xanthurenic acid (0.96), sucrose (0.96)	Betaine (-0.79)

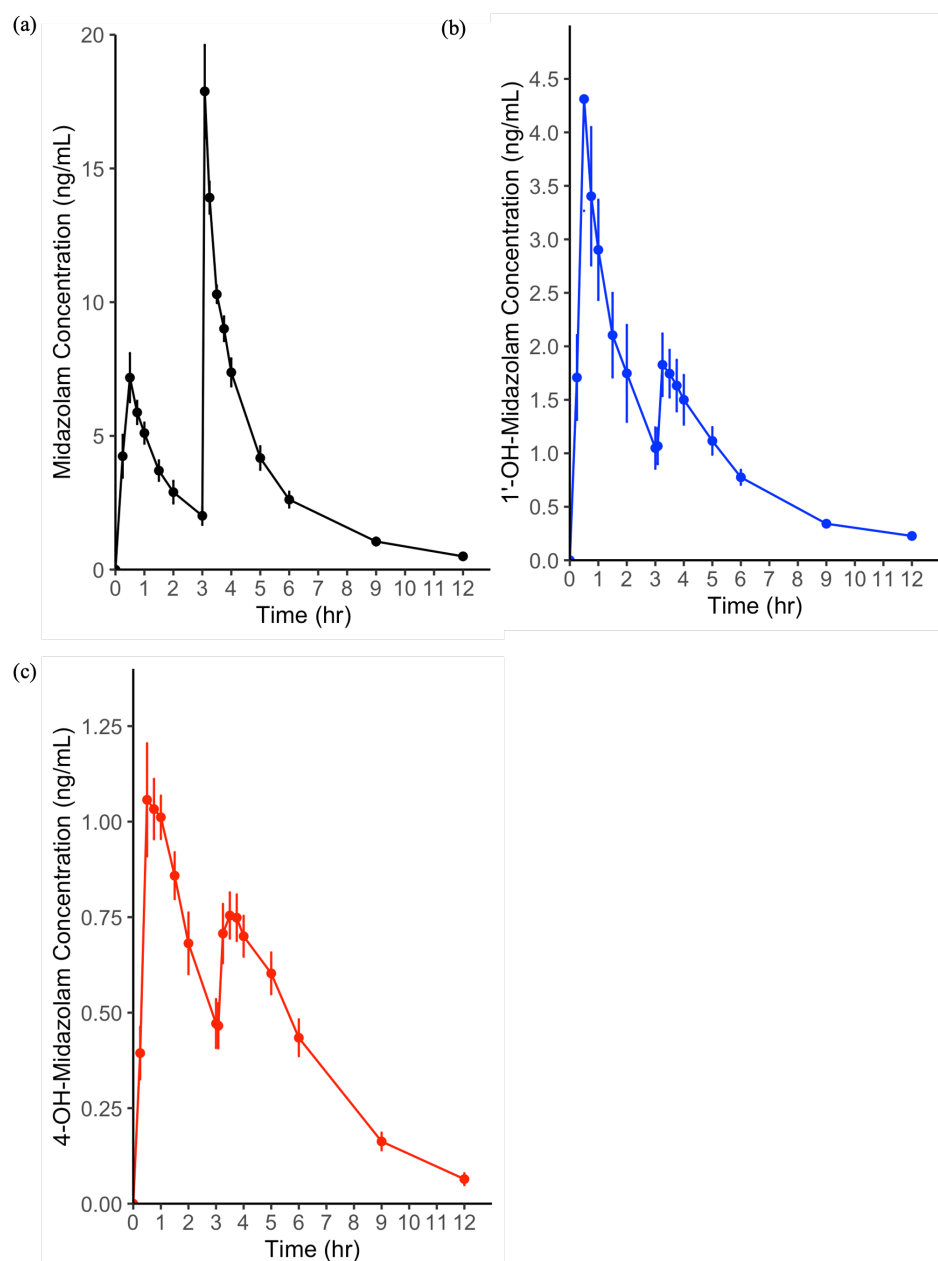


Figure 5.1. Average plasma concentration vs. time profiles for (a) midazolam, (b) 1'-hydroxymidazolam, and (c) 4-hydroxymidazolam following semi-simultaneous dosing of 2 mg oral midazolam (at 0 hours) and 1 mg IV midazolam (at 3 hours) in healthy male participants (n=7).

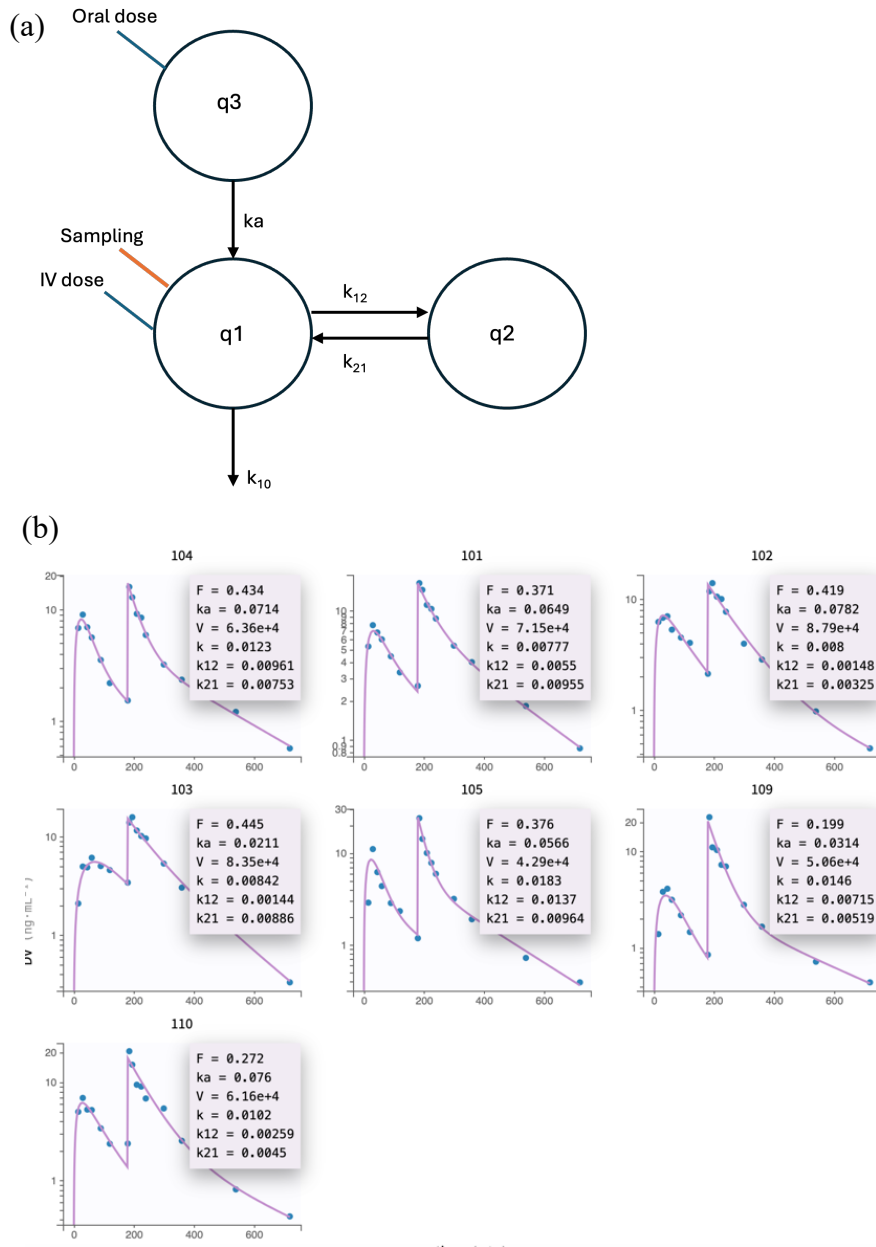


Figure 5.2. (a) Pharmacokinetic two-compartment model of midazolam where q1 is the central compartment, q2 is the peripheral compartment, and q3 is the depot compartment. The rate constants are: k_a (absorption rate constant); k_{10} (elimination rate constant); and k_{12} and k_{21} (intercompartmental transfer rate constants). Oral dosing occurs at time 0 min and IV dosing occurs at 180 min. (b) Individual fits of each participant using PKAnalix.

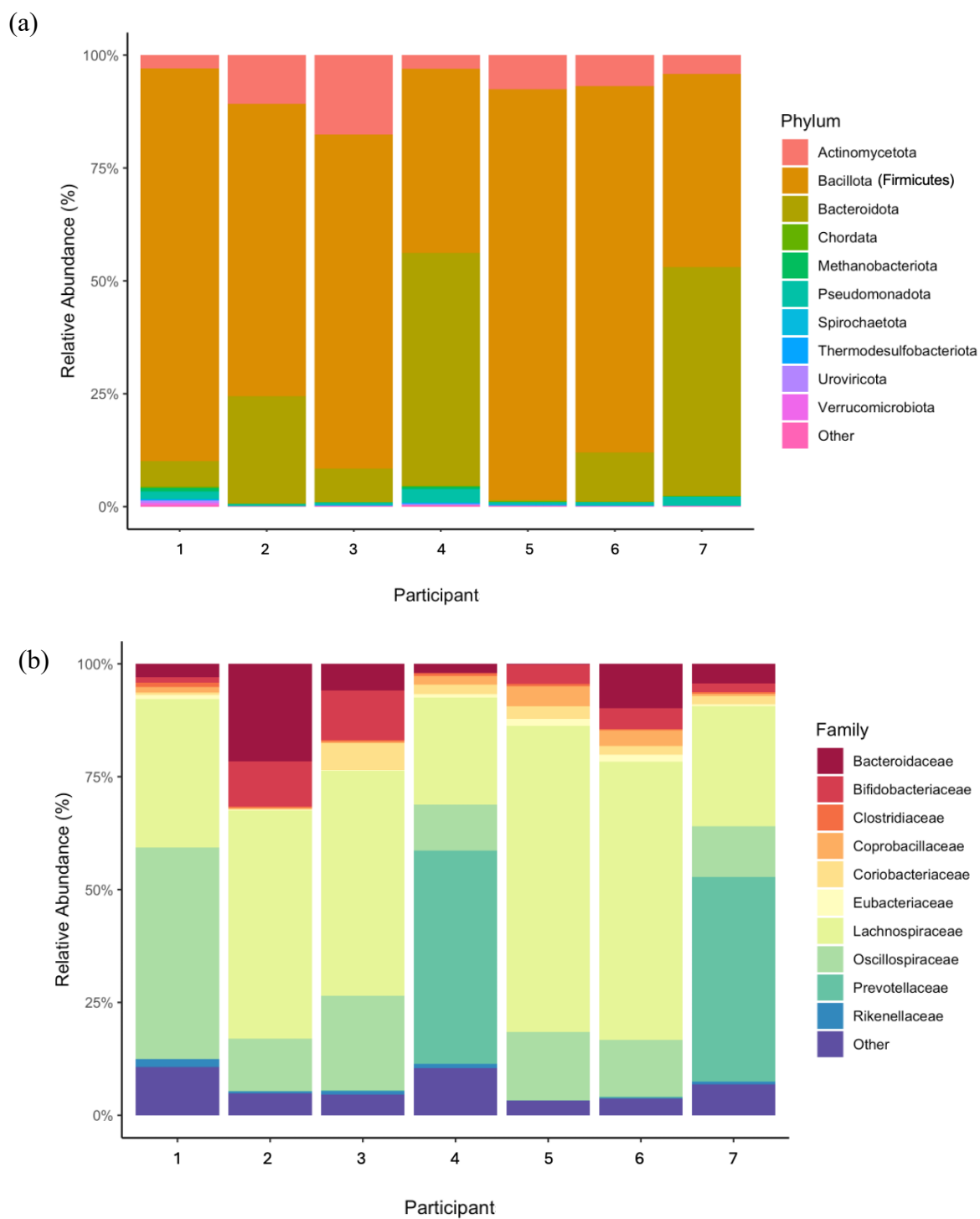


Figure 5.3. Microbiome composition at the (a) phylum and (b) family level for all participants ($n = 7$).

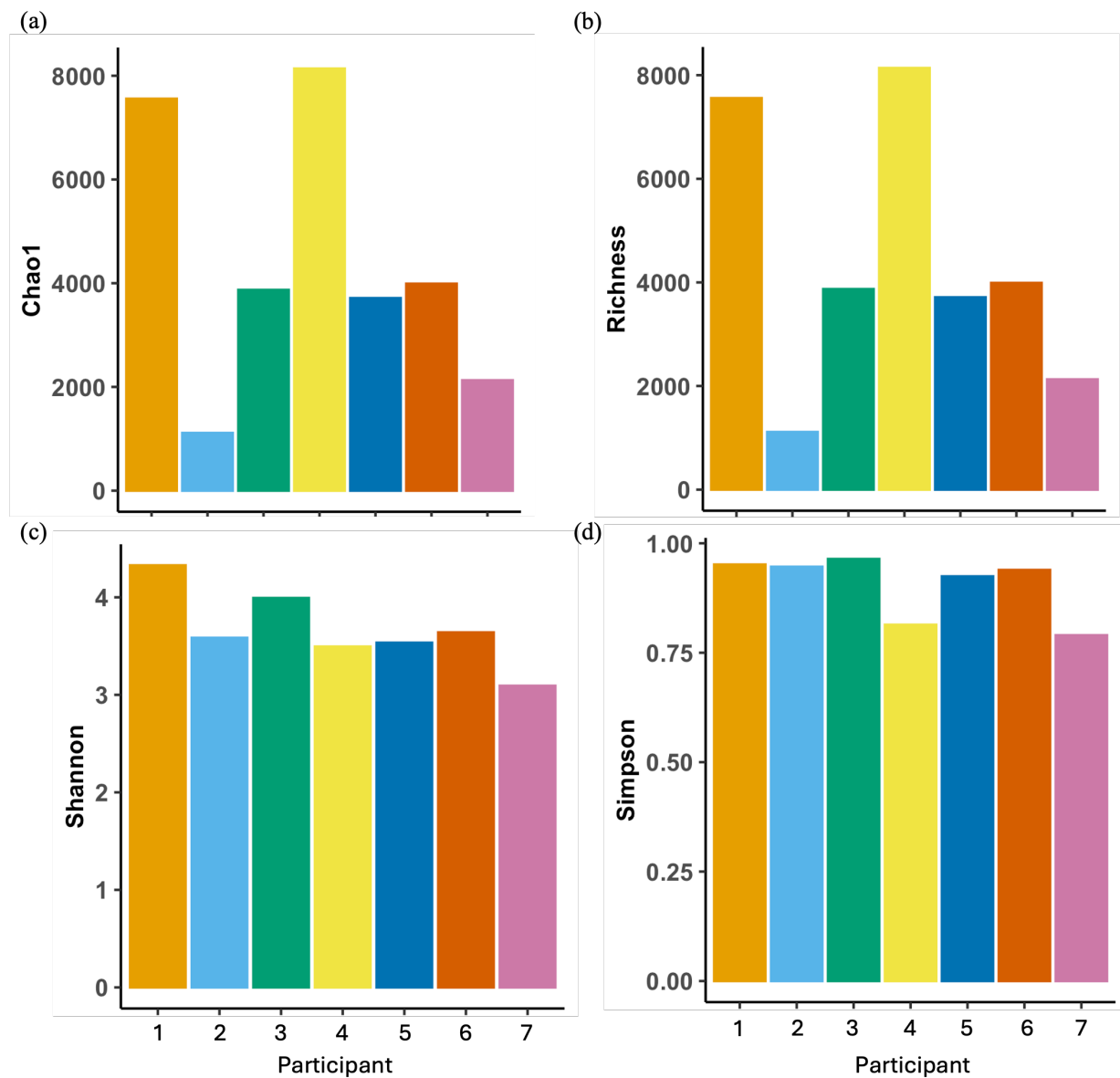


Figure 5.4. Microbial species alpha diversity determined from baseline fecal samples in healthy male participants as quantified by (a) Chao1, (b) Richness, (c) Shannon, and (d) Simpson indices.

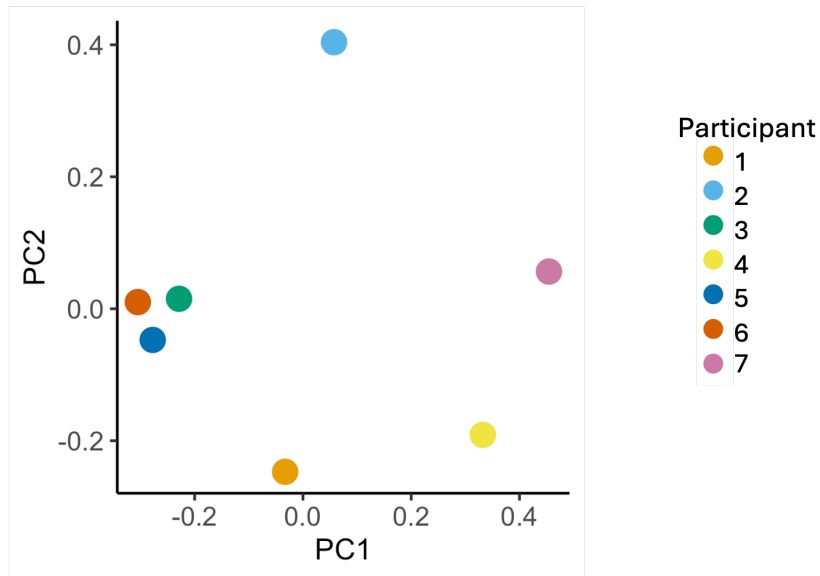


Figure 5.5. Microbial species beta diversity determined from baseline fecal samples in healthy male participants (n=7).

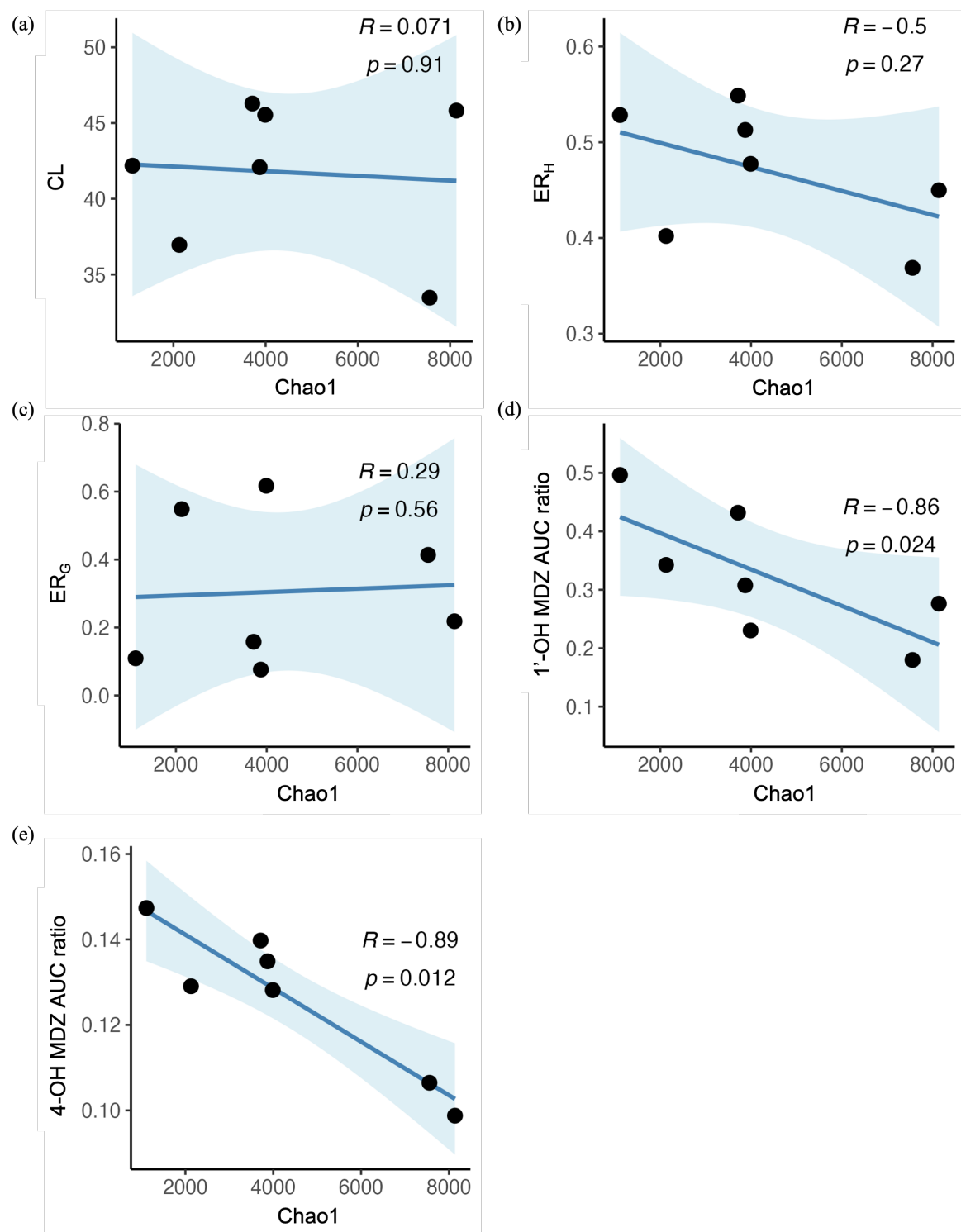


Figure 5.6. Correlation between Chao1 and (a) CL, (b) ER_H , (c) ER_G (d) 1'-OH MDZ/MDZ AUC ratio, (e) 4-OH MDZ/MDZ AUC ratio in healthy male participants (n=7).

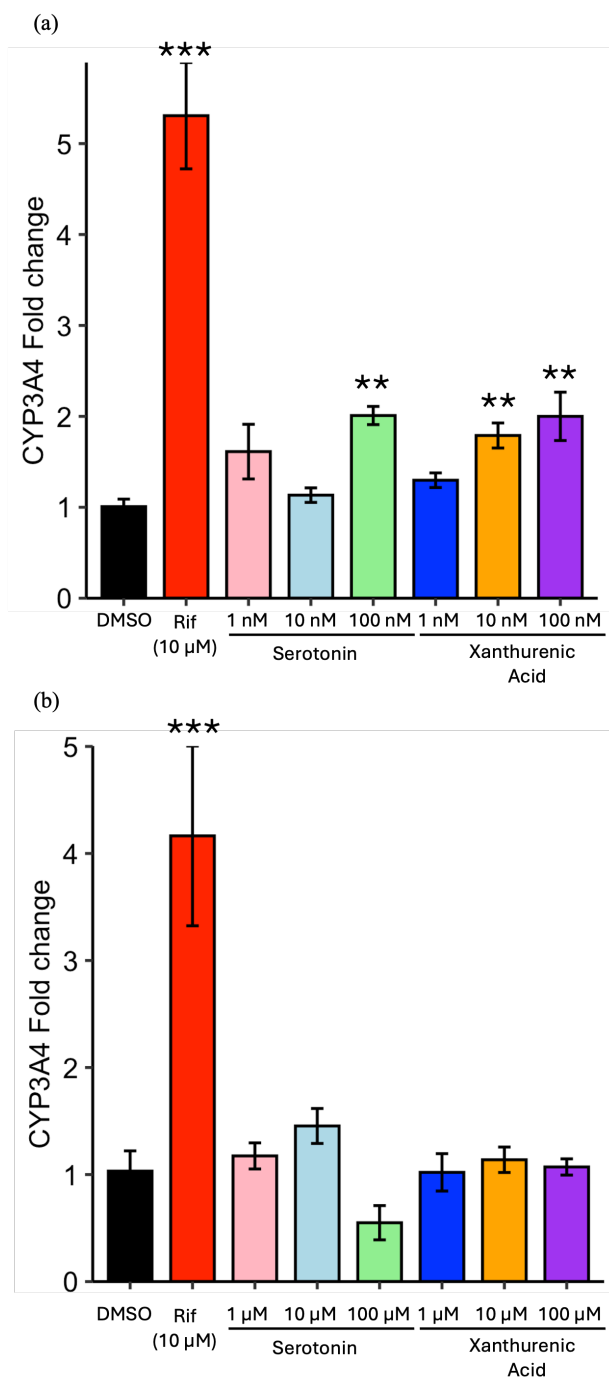


Figure 5.7. *CYP3A4* mRNA expression after treatment with serotonin and xanthurenic acid in HepaRG cells. (a) 1, 10, and 100 nM and (b) 1, 10, and 100 µM or treatment in HepaRG cells. Experiment was conducted in triplicate. Significance was determined by one-way ANOVA followed by Dunnett's test (p-value < 0.05 (*), < 0.01 (**), < 0.001 (***)).

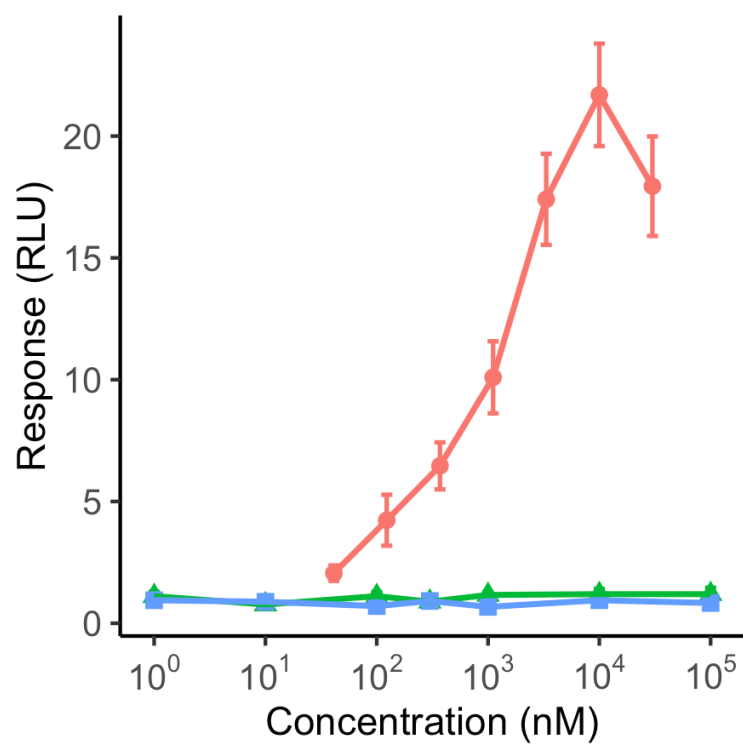


Figure 5.8. PXR response in relative luminescence unit (quantified signal / background signal) when reporter cells were treated with rifampin (red circle), serotonin (green triangle), or xanthurenic acid (blue square) in DMSO (n = 4 per concentration).

Chapter 6

Conclusions and Future Directions

6.1. Chapter Conclusions

The gut microbiome consists of a diverse community of microorganisms and is sensitive to change, specifically with disease states. For example, with inflammatory bowel disease (IBD), a study that analyzed nine metagenomic cohorts of IBD patients found two specific microbial species, *Asaccharobacter celatus* and *Gemmiger formicilis*, were depleted across six distinct IBD cohorts (Ning et al., 2023). Many groups have also looked at the impact of the gut microbiome on neurological diseases. For example, patients with Parkinson's disease were shown to have worse severity with *Helibacter pylori* infection (Tan et al., 2015). These are just two examples out of many studies that have been done to identify associations between the gut microbiome and disease states. Because of these findings, the microbiome is currently being studied as a therapeutic target to improve drug efficacy. For example, clinical studies suggest that the combination of standard therapy and microbiome manipulation (e.g., through probiotics or fecal microbiome transplantation) could improve clinical outcomes in patients with IBD (Lu et al., 2025; Shen et al., 2024).

In the last couple of decades, the field of pharmacomicrobiomics has emerged with the aim of further understanding the bidirectional relationship between the gut microbiome and drug action (Spanogiannopoulos et al., 2016). One way in which this relationship manifests is hypothesized to be through the influence of the microbiome on host receptors. We and others hypothesize that distinct microbial metabolites can activate nuclear receptors and lead to changes in the expression of drug metabolizing enzymes and transporters (DMETs). This dissertation aimed to investigate how the gut microbiome impacts CYP3A4 activity and drug metabolism enzymes and transporter expression using a combination of *in vitro* and *in vivo* mouse and human studies.

In Chapter 2, we used a humanized PXR-CAR-CYP3A4/3A7 mouse model to identify how different microbial conditions impacted CYP3A4 expression. Three groups of male and female humanized mice were studied: conventional (CV), germ-free (GF), and germ-free conventionalized using sex-matched pooled human fecal samples (GFCV). The presence of microbiome upregulated *CYP3A4* expression by 7.6-fold in male CV mice ($p < 0.001$) but downregulated *CYP3A4* expression by 1.69-fold in female CV mice ($p = 0.012$) compared to GF mice. The transplantation of a human fecal microbiome to sex-matched GF mice resulted in decreased microbial diversity ($p < 0.05$ for males and $p < 0.01$ for females) and was not effective in restoring *CYP3A4* expression, suggesting complex underlying microbe-CYP3A4 interactions. We found that the hepatic CYP3A4 mRNA and protein expression were strongly correlated ($R = 0.91$; $p = 2.6 \times 10^{-6}$). A total of 57 bacterial species from the mouse gut microbiome were identified to be significantly correlated with CYP3A4 protein expression ($FDR < 0.05$). Microbial diversity was strongly correlated with CYP3A4 protein expression ($R = 0.66$; $p = 0.0072$). Lastly, we found that five bile acids and no short chain fatty acids were correlated with CYP3A4 protein expression. Overall, we found that differences in microbial conditions do influence CYP3A4 expression in the humanized mouse model.

In Chapter 3, our goal was to use a multi-omics approach to better understand the effect of microbiome and sex on hepatic DMET expression in male and female humanized PXR-CAR-CYP3A4/3A7 mice. Metagenomics shotgun sequencing was performed on colonic contents and liver samples were analyzed using transcriptomics, proteomics, and metabolomics. In CV mice, female mice demonstrated a more diverse microbiome as quantified by Shannon Index ($p < 0.05$) and male and female mice showed differing microbial profiles as determined by beta diversity ($p = 0.03$). PCA analysis of the transcriptome and metabolome revealed that male CV mice were

the most variable and did not cluster unlike the male GF, female CV and female GF groups. Expression of a microbiome had a greater impact on male mice compared to female mice. For example, more metabolites were different when comparing male GF vs. male CV mice (307 metabolites) as opposed to female GF vs. female CV mice (2 metabolites). There was also a higher percentage of DMET genes that were differentially expressed in male GF vs. male CV mice compared to female GF vs. female CV mice. The overall impact of sex on CV and GF mice differed. Hepatic metabolomic changes were more frequent when comparing female CV to male CV mice (84 metabolites) versus female GF to male GF mice (8 metabolites). For the transcriptomics, the percentage of differentially expressed DMET genes was comparable when comparing sexes for CV and GF mice. Overall, we found that male CV mice demonstrated the most interindividual variability and had the most different transcriptome and metabolome compared to the other groups. The impact of the microbiome was also different between male and female mice.

In Chapter 4, our aim was to identify microbial metabolites that may be regulating hepatic CYP3A4 using untargeted metabolomics of liver samples of the humanized PXR-CAR-CYP3A4/3A7 mice and subsequent *in vitro* testing in HepaRG cells. Through a correlation analysis and subsequent filtering, three microbial metabolites were identified: 1-deoxynojirimycin (DNJ; $\rho = 0.72$, $p = 1 \times 10^{-4}$), betaine ($\rho = 0.66$, $p = 0.00056$), and L-pyroglutamic acid (LPGA; $\rho = 0.62$, $p = 0.0015$). In our initial *in vitro* induction screen in HepaRG cells, 150 μM LPGA had a trend towards induction of CYP3A4 mRNA expression (2.05-fold induction, $p = 0.07$), whereas no induction was seen with DNJ or betaine. However, subsequent testing with 1 to 500 μM LPGA did not show induction of CYP3A4 mRNA. In addition, we tested whether the microbial metabolites inhibited CYP3A4 activity in pooled liver

microsomes, but CYP3A4 activity was not inhibited by any of the metabolites. Overall, the three selected microbial metabolites demonstrated no induction or inhibition of CYP3A4, suggesting that these microbial metabolites are unlikely to contribute to the intraindividual variability in CYP3A4 under the conditions tested.

In Chapter 5, we determined whether the gut microbiome and microbial metabolite levels were associated with CYP3A activity metrics determined from semi-simultaneous dosing of midazolam. Seven healthy male volunteers were dosed with oral and intravenous midazolam separated by three hours. Blood samples were collected over the course of 12 hours and midazolam and its primary metabolites, 1'-hydroxymidazolam and 4-hydroxymidazolam, were measured using LC/MS. We developed a two-compartment model with first-order absorption that allowed estimation of PK parameters following semi-simultaneous administration of midazolam. Metrics related to CYP3A activity (clearance, hepatic and intestinal extraction ratios, and metabolite-to-parent AUC ratios) were estimated or calculated. Metagenomic shotgun sequencing was used to determine microbial species composition and diversity from a baseline fecal sample and targeted metabolomics was used to identify circulating microbial metabolites from plasma samples. In vitro experiments with HepaRG and PXR-ligand assays were used to confirm results. There was an inverse relationship between microbial species richness with AUC metabolite-to-parent ratios (1'-OH MDZ/MDZ AUC ratio: $R = -0.86$, $p = 0.024$ and 4-OH MDZ/MDZ AUC ratio: $R = -0.89$, $p = 0.012$) but no relationship with clearance, hepatic extraction ratio, or intestinal extraction ratio. The tryptophan metabolites, serotonin and xanthurenic acid demonstrated a strong positive correlation with hepatic CYP3A ($R = 1$, $p = 0.017$ and $R = 0.86$, $p = 0.024$, respectively). When HepaRG cells were treated with 100 nM of serotonin or xanthurenic acid, a 2.0-fold induction of *CYP3A4* mRNA expression was observed

($p < 0.01$). However, when tested in a reporter assay, these metabolites were not PXR agonists. Overall, normal variation in the gut microbiome does not influence CYP3A activity in this pilot study of 7 subjects but there may be specific microbial species or metabolites that can impact hepatic CYP3A activity.

Overall, we used a humanized mouse model, *in vitro* systems, and clinical samples to investigate the impact of the gut microbiome on CYP3A4. We found that microbial diversity was positively correlated to CYP3A4 expression in the humanized mouse model but does not correlate to CYP3A activity in our clinical samples. Although the identified metabolites from mouse liver samples were found to have no influence on CYP3A4 *in vitro*, the identified metabolites from the clinical samples, serotonin and xanthurenic acid, at 100 nM induced CYP3A4 by 2-fold ($p < 0.01$).

6.2. Future Directions

There are several future directions that can be taken to further investigate the relationship between the gut microbiome and drug disposition. First, as our mouse studies have focused on hepatic CYP3A4, next steps can include exploring the impact of different microbial conditions on intestinal CYP3A4 in the humanized model. Interestingly, our human data suggest fewer microbial metabolites were associated with the intestinal CYP3A (ER_G) than hepatic CYP3A activity (ER_H). This discrepancy may be due to nuclear receptor differences. Hepatic CYP3A4 is primarily regulated by PXR and constitutive androstane receptor (CAR) while intestinal CYP3A4 is regulated by vitamin D receptor (VDR). The metabolites responsible for the regulation of CYP3A may be PXR/CAR but not VDR agonists and therefore can influence hepatic but not intestinal CYP3A. One way to test this hypothesis is to test whether our identified

metabolites are agonists in PXR-, CAR-, or VDR-reporter cell systems.

In Chapter 2, we identified 57 bacterial species that were associated with hepatic CYP3A4 expression in humanized PXR-CAR-CYP3A4/3A7 mice. It is possible that the microbes identified are only of mouse origin and may not be applicable to humans. Nonetheless, experiments that co-culture the bacterial species with cells of hepatic origin can be conducted to determine whether specific microbial species and/or their metabolites can induce or repress CYP3A4 expression. Though co-culturing microbial species with hepatic models may be difficult, Yang et al. (2012) were able to infect HepG2 cells with a strain of *Klebsiella pneumoniae* to study how this infection affected liver abscesses. A major limitation of this method is that strictly anaerobic bacteria cannot be co-cultured as hepatic models need aerobic conditions.

Should our findings in the humanized PXR-CAR-CYP3A4/3A7 mice from Chapter 3 be specific to these humanized mice, we could conduct a multi-omics study in wild-type C57BL/6 mice under conventional (CV), germ-free (GF) and conventionalized (CVGF) conditions. Comparing the hepatic environment of wild-type mouse to the humanized PXR-CAR-CYP3A4/3A7 mouse can inform whether changes in the microbiome impact wild-type and humanized mice differently.

Although we did not see any induction of *CYP3A4* by the selected microbial metabolites in HepaRG cells, repeating this experiment with a more physiologically relevant model (e.g., primary human hepatocytes) can confirm whether this result was a true negative. Analytical procedures (i.e., LCMS) can be used to measure intracellular concentrations of the metabolites to ensure the metabolites are present at anticipated concentrations.

Finally, interventional studies can be conducted in mice or humans based on the results of

Chapter 5. Although normal variation in the gut microbiome did not influence CYP3A in our small sample size, an altered gut microbiome may still have an impact on CYP3A. This hypothesis can be tested by manipulating the gut microbiome of healthy volunteers through probiotic or postbiotic administration. Probiotic administration can alter the community of the gut microbiome but there are many different strains of probiotics, and a limitation will be that the impact on CYP3A may be strain specific. On the other hand, certain postbiotics (e.g., microbial metabolites or inactivated bacteria) may not reshape the gut microbiome in terms of colonization but influence the overall physiological effects (e.g., gut barrier function). A limitation of postbiotics is that there may not be direct changes in microbial diversity. Nonetheless, healthy volunteers can be dosed semi-simultaneously with midazolam at the start of the study and given a probiotic or postbiotic daily over the course of a month to affect a change in the microbiome and reach a new steady-state level for CYP3A expression. At the end of the month, volunteers will be dosed with midazolam again. Pre- and post-dosing CYP3A activity metrics can be compared to identify whether manipulation of the microbiome influences CYP3A. Additionally, the microbiome should be sequenced to assess changes in composition or diversity of the microbiome. Metabolomics should also be done to identify changes in microbial and host metabolites. Lastly, as we saw strong correlations between microbial diversity and metabolite-to-parent AUC for 1'-OH and 4-OH MDZ, glucuronides should also be measured. The strongest impact of gut microbiome alterations may be on intestinal or hepatic glucuronidation.

Since we saw an induction of *CYP3A4* by serotonin and xanthurenic acid, follow-up studies can be done to confirm this interaction. Humanized PXR-CAR-CYP3A4/3A7 mice can be dosed with serotonin and/or xanthurenic acid to confirm whether these metabolites can induce CYP3A4. An observational study can be done comparing healthy volunteers and patients with

serotonin syndrome. CYP3A activity can be measured through endogenous biomarkers (i.e., urinary 6 β -hydroxycortisol/cortisol ratio, plasma 1 β -hydroxydeoxycholic acid, and plasma 4 β -hydroxycholesterol) and compared between the two groups. A limitation to this is that endogenous biomarkers only reflect hepatic CYP3A activity, and an observational study can only reveal a correlation that may not be a true causal relationship.

6.3. Conclusion

The field of pharmacomicrobiomics aims to study the effect of variations in the microbiome on the disposition, action, and toxicity of drugs. This dissertation has contributed to the understanding of how variation in the microbiome can impact drug disposition in a humanized mouse model, *in vitro* model, and clinical samples. Specifically, we found that major differences in microbial conditions (i.e., GF vs. CV) does impact hepatic CYP3A4 in a humanized mouse model. Previous studies have only looked at the mouse orthologue, *Cyp3a11* and we confirmed this relationship also exists in human CYP3A4. We also identified two microbial metabolites, serotonin and xanthurenic acid, as inducers of hepatic *CYP3A4*. Previous studies have looked at tryptophan metabolites of the indole pathway but not the serotonin and kynurenine pathways. Lastly, through an observational clinical study, we found that normal variation in the gut microbiome does not influence CYP3A activity. This was the first study to date that looked at the influence of the gut microbiome on CYP3A in humans. Overall, understanding how the gut microbiome impacts drug disposition, in this case through CYP3A4, can lead to a better understanding of sources of variability in CYP3A4 and aid in better dosing strategies and treatment efficacy for drugs with a narrow therapeutic range.

6.4. References

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