The discovery of gut microbial metabolites as modulators of host susceptibility to acetaminophen-induced hepatotoxicity

Hyunwoo Lee¹,³, Xiaotong Yang¹, Pei-Ru Jin¹, Kyoung-Jae Won¹, Chang H. Kim⁴,⁵, and Hyunyoung Jeong¹,²

¹Department of Industrial and Molecular Pharmaceutics, College of Pharmacy, Purdue University, West Lafayette, IN 47907, USA
²Department of Pharmacy Practice, College of Pharmacy, Purdue University, West Lafayette, IN 47907, USA
³Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA
⁴Department of Pathology, University of Michigan School of Medicine, Ann Arbor, MI 48109, USA
⁵Mary H. Weiser Food Allergy Center and Rogel Center for Cancer Research, University of Michigan School of Medicine, Ann Arbor, MI 48109, USA

Correspondence: Hyunyoung Jeong, PharmD, PhD
Departments of Industrial and Molecular Pharmaceutics and Pharmacy Practice
Purdue University
E-mail: youngjeong@purdue.edu
Running title: Gut microbiota and APAP-induced hepatotoxicity

Summary:
Number of Table 2
Number of Figure 1
Number of References 98
Number of Words in Abstract 161
Number of Words in Manuscript 6,787

Abbreviations: ALT, alanine transaminase; APAP, acetaminophen; CYP2E1, cytochrome P450 2E1; GPR, G-protein coupled receptor; 2-HB, hydroxybutyric acid; I3C, indole-3-carboxylic acid; LPS, lipopolysaccharide; MMP12, matrix metallopeptidase 12; 5-MIAA, 5-methoxy indole acetic acid; NAPQI, N-acetyl-p-benzoquinone imine; NLRP6, NACHT, LRR and PYD domain-containing protein 6; NRF2, nuclear factor erythroid 2-related factor 2; ox-LDL, oxidatively modified low-density lipoprotein; 3-PPA, 3-phenylpropionic acid; PPD, 1-phenylpropane-1,2-dione; TMA, trimethylamine; TMAO, trimethylamine oxide
ABSTRACT

The mammalian gut microbiota plays diverse and essential roles in modulating host physiology. Key mediators determining the outcome of the microbiota-host interactions are the small molecule metabolites produced by the gut microbiota. The liver is the organ exposed to gut microbial metabolites, and it serves as the nexus for maintaining healthy interactions between the gut microbiota and the host. At the same time, the liver is the primary target of potentially harmful gut microbial metabolites. In this review, we provide an up-to-date list of gut microbial metabolites that have been identified to either increase or decrease host susceptibility to APAP-induced liver injury. The signaling pathways and molecular factors involved in the progression of APAP-induced hepatotoxicity are well-established, and we propose that the mouse model of APAP-induced hepatotoxicity serves as a model system for uncovering gut microbial metabolites with previously unknown functions. Furthermore, we envision that gut microbial metabolites identified to alter APAP-induced hepatotoxicity likely have broader implications in other liver diseases.

Significance statement

This review provides an overview of recent discoveries from investigating whether and how the gut microbiota modulates the host susceptibility to APAP-induced liver injury. It focuses on the roles of gut bacterial small molecule metabolites as mediators of the interaction between the gut microbiota and the liver. It also illustrates the utility of APAP-induced liver injury as a model to identify gut microbial metabolites with biological function.
1. Introduction

The mammalian gut contains a community of microorganisms (termed “gut microbiota”) that includes bacteria, fungi, and protozoans, as well as viruses. Numerous studies have established that the changes in the composition and abundance of gut bacteria affect host health and disease states. One key contribution of the gut microbiota to host physiology is the production of diverse small molecule metabolites (e.g., short-chain fatty acids, bile acids, and amino-acid metabolites). These metabolites play critical roles in mediating the beneficial or detrimental interaction between the gut microbiota and the host, consequently contributing to setting baseline health states (Dorrestein et al., 2014; Sharon et al., 2014; Nicolas and Chang, 2019; McCarville et al., 2020; Shine and Crawford, 2021).

The liver is positioned in a sequence with the intestine such that it readily receives signals from changes in the gut microbiota. It is the extraintestinal organ exposed to the gut microbial metabolites at the highest concentrations, and not surprisingly, the biological activities of many gut microbial metabolites manifest in the liver. As a result, the liver serves as the nexus in maintaining healthy interactions between the host and the gut microbiota, while it is also the primary target of unhealthy interactions (Macpherson et al., 2016).

Drugs are a significant cause of liver injury, accounting for 20-40% of all fulminant hepatic failure requiring liver transplantation (Lee, 2013; Sarges et al., 2016). Also, drug-induced liver injury is the most frequent reason for the withdrawal of drugs from the market (Bleibel et al., 2007; Senior, 2007). Acetaminophen (APAP; \(N\)-acetyl-\(p\)-aminophenol) is generally considered to be a safe drug; however, certain individuals are susceptible to APAP-induced hepatotoxicity even at therapeutic doses (Harrill et al., 2009). APAP is one of a few drugs whose hepatotoxicity in humans and primary human hepatocytes can be reproduced in mice and primary mouse hepatocytes (Xie et al., 2014; McGill and Jaeschke, 2019). Using mouse models, biochemical events and signal transduction pathways involved in APAP
hepatotoxicity have been well established (Han et al., 2010; Win et al., 2018; Jaeschke and Ramachandran, 2020b).

APAP-induced hepatotoxicity is caused by its reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Fig 1). NAPQI formation is catalyzed by phase I enzymes, mainly cytochrome P450 (CYP) 2E1. NAPQI is a strong electrophile and oxidant, covalently binding to proteins (forming APAP-protein adducts) and rapidly depleting cytoplasmic and mitochondrial glutathione in the liver. Glutathione depletion (particularly in mitochondria) causes oxidative stress and mitochondrial dysfunction, triggering a cascade of events leading to cell necrosis (Win et al., 2018; Jaeschke et al., 2019). Oxidative stress also activates the host defense system, such as nuclear factor erythroid 2-related factor 2 (NRF2) - the master regulator of the antioxidant defense system by upregulating the genes involved in neutralizing oxidative stress (Chan et al., 2001). Necrotic hepatocytes release damage-associated molecules, attracting immune cells, including monocyte-derived macrophages. During the early stages of APAP hepatotoxicity (during the first 24 h after APAP administration), the macrophages are of a highly inflammatory phenotype, potentially contributing to APAP-induced hepatotoxicity. However, at the later stage (48-72 h), these macrophages are critical in tissue repair and regeneration by promoting the resolution and elimination of necrotic lesions (Jaeschke and Ramachandran, 2020a; Feng et al., 2023).

APAP-induced liver injury has served as a model for identifying gut microbial metabolites exerting previously unknown biological functions, especially in the liver, based on its well-characterized pathogenesis and biochemical sequela. APAP-induced liver injury is a multifaceted event involving xenobiotic bioactivation, oxidative stress, antioxidant defense, and tissue repair and regeneration. As a result, it serves as a model system to examine changes in xenobiotic elimination and bioactivation, as well as the antioxidant defense mechanisms of the host. The same biological processes are involved in other hepatic diseases, such as acute liver injury (caused by other drugs) and alcoholic and non-alcoholic fatty liver disease. Therefore, the
findings obtained using APAP-induced liver injury as a model can also be applied to understanding the role of the gut microbiota and their metabolites in other liver diseases.

In this review, we will provide a summary of recent discoveries suggesting the role of the gut microbiota in modulating APAP-induced liver injury. We focus on small molecule metabolites derived from gut microbes (primarily bacteria) and their effects on APAP-induced hepatotoxicity.

2. Evidence for the gut microbiota modulating APAP-induced hepatotoxicity

Mice have been a frequently used animal model in studying APAP-induced hepatotoxicity, providing the mechanistic insights that we know today. Although the progression of APAP-induced liver injury is faster in mice than in humans, fundamental discoveries made in mice have been translatable to humans, enabling the development of treatment modalities against APAP-induced liver injury (Jaeschke et al., 2014). Mice have also served as a pivotal model in investigating microbiota-host interaction. While humans and mice differ in the composition of the gut microbiota, a high degree of overlap (>80-90%) exists between humans and mice in both the gut microbial metabolomic profiles and the annotated functions of the gut microbial metagenomes (Ley et al., 2005; Marcobal et al., 2013; Hugenholtz and de Vos, 2018), indicating functional similarities between human and mouse gut microbiotas. Consistent with this notion, many studies have demonstrated that the physiological effects of gut microbial metabolites identified in mouse models can be translated to humans and vice versa (Wang et al., 2011b; Maini Rekdal et al., 2019).

Studies using germ-free mice (i.e., with no gut microbiota), antibiotic-treated mice (i.e., pseudo-germ-free), or transgenic mice (with altered gut microbiota) identified important roles of the gut microbiota in modulating APAP-induced hepatotoxicity. In a study examining the host susceptibility to APAP-induced hepatotoxicity, the baseline hepatic glutathione level was found to be decreased in germ-free mice compared with conventional mice, but no significant differences were observed between the two mouse groups in hepatic alanine transaminase
ALT) levels and liver necrosis at 8 h post-APAP dosing (200 mg/kg intraperitoneal injection) (Possamai et al., 2015) (Table 1). These findings might indicate that the gut microbiota does not play a significant role in APAP-induced liver injury, despite its significant role in maintaining glutathione levels. Alternatively, the gut microbiota may simultaneously produce both beneficial and harmful gut microbial products, leading to failed detection of any changes in APAP-induced liver injury. In the latter scenario, a balance between beneficial and harmful gut microbial products would play a critical role in determining the magnitude of APAP-induced hepatotoxicity.

While germ-free animals serve as a model in addressing how the presence (or absence) of the gut microbiota impacts host physiology, direct comparisons between germ-free and conventional mice need caution because germ-free mice exhibit significant physiological differences from conventional mice (Al-Asmakh and Zadjali, 2015). Several studies employed a short-term (a few days to weeks) treatment of rodents with a cocktail of antibiotics to deplete the gut microbiota and examined the potential contribution of the gut microbiota to mouse susceptibility to APAP-induced hepatotoxicity (Table 1). In these studies, APAP was administered in the morning (ZT0), and mice were sacrificed at 24 h post-APAP treatment. The antibiotic cocktail treatment had minimal effects on APAP-induced liver injury in two studies (Thaiss et al., 2016; Gong et al., 2018), whereas it significantly increased APAP-induced hepatotoxicity in one study (Li et al., 2023b). The different susceptibility phenotypes arising from the depletion of gut microbiota by treating an antibiotic cocktail may signify variations among animal facilities in the gut microbiotas, producing distinct profiles of gut microbial products with beneficial or harmful activity against APAP-induced hepatotoxicity. Interestingly, individual antibiotics were shown to have differential effects on APAP-induced hepatotoxicity in mice (Li et al., 2023b). Ampicillin alone increased APAP-induced liver injury in mice, while other antibiotics such as neomycin, metronidazole, or vancomycin did not. On the other hand, vancomycin treatment decreased APAP-induced liver injury in rats (Zheng et al., 2020). The effects of individual antibiotics on APAP-induced liver injury appear to depend on the baseline gut
microbial community and be mediated by specific gut microbial taxa, resulting in contrasting outcomes in different animal facilities and rodent models.

While germ-free or antibiotic-treated mouse models drastic changes in the gut microbiota and host physiology, transgenic mouse models can provide relatively subtle but specific information about gut microbial dysbiosis. NLRP6 (NACHT, LRR, and PYD domain-containing protein 6) is an inflammasome sensor molecule that detects cytosolic pathogen-associated molecular patterns and is involved in the regulation of host defense against microbial infection (Levy et al., 2017). As compared with wild-type C57BL/6J mice, an isogenic mouse deficient in Nlrp6 (i.e., Nlrp6<sup>−/−</sup>) has altered gut microbial composition and abundance (termed intestinal dysbiosis). Nlrp6<sup>−/−</sup> mice were more susceptible to APAP-induced liver injury than wild-type mice (Schneider et al., 2021). Strikingly, the susceptibility difference between wild-type and Nlrp6<sup>−/−</sup> mice disappeared upon pretreatment with an antibiotic cocktail. Also, the increased APAP-induced hepatotoxicity in Nlrp6<sup>−/−</sup> mice was transferable by transplanting its gut microbiota into wild-type mice, demonstrating that the observed phenotype was driven by the Nlrp6<sup>−/−</sup> gut microbiota. The enhanced APAP-induced liver injury in Nlrp6<sup>−/−</sup> mice was ascribed at least in part to impaired gut barrier function and monocyte polarization towards the inflammatory phenotype. While the detailed mechanism (e.g., responsible gut microbial taxa and their roles) remains to be determined, this study using Nlrp6<sup>−/−</sup> mice demonstrates that gut dysbiosis can increase the basal susceptibility of the host to APAP-induced hepatotoxicity.

In humans, dysbiotic changes in the gut microbiota have also been associated with poor prognosis of developing acute liver failures. Analysis of clinical data on the 502,511 participants from the UK BioBank population-based study suggests that subjects who underwent long-term treatment with proton pump inhibitors or antibiotics had increased risks of developing acute liver failure after correction for other variables including age, sex, body mass index, and aspartate aminotransferase to platelet ratio index (Schneider et al., 2021). Not to mention antibiotics, proton pump inhibitors have been reported to cause unhealthy perturbations in the gut.
microbiota in humans (Imhann et al., 2016; Jackson et al., 2016; Paroni Sterbini et al., 2016). Many other medications are known to influence the gut microbiota (Imhann et al., 2017; Maier et al., 2018); however, whether their effects on the gut microbiota alter susceptibility to APAP-induced hepatotoxicity remains to be investigated.

The above studies illustrate how changes in the gut microbiota could alter the susceptibility to and progression of APAP-induced liver injury. In the next section, we will discuss gut microbial signals potentially responsible for the effects of the gut microbiota on APAP-induced hepatotoxicity by focusing on gut bacterial metabolites.

3. Gut microbial metabolites altering host susceptibility to APAP-induced hepatotoxicity

Multiple factors affect the composition and/or abundance of the gut microbiota (Gacesa et al., 2022). Accordingly, different approaches can be employed to cause or capture distinct perturbations in the gut microbiota when studying the effects of gut microbiota on host physiology. Depending on the approaches used, the extent of changes in the gut microbial composition and/or abundance vary widely, ranging from almost complete or partial depletion of the gut microbiota (i.e., antibiotic treatment) to subtle or significant variations originating from differences in animal husbandry. Therefore, it is not surprising that studies performed in different institutes and/or employing different approaches have identified distinct gut microbial metabolites differentially affecting APAP-induced hepatotoxicity. We have categorized these metabolites based on the directional changes in APAP-induced hepatotoxicity upon increased metabolite exposure, as the biological consequence may be of utmost interest to most readers (summarized in Table 2).

3.1. Aggravating APAP-induced hepatotoxicity

3.1.1. p-Cresol

Interindividual variability in drug efficacy and toxicity has been a major challenge in drug therapy. Pharmacometabolomics is one of the approaches used to identify the sources of
variability and predict drug response in an individual based on the analysis of metabolites produced in the body. Clayton et al. examined the utility of NMR-based metabolomic profiling of biological samples in identifying potential biomarkers of drug disposition in individuals (Clayton et al., 2009). The authors determined metabolite profiles in urine samples collected before and after a single therapeutic oral dose (1 gram) of APAP in 99 healthy subjects. As expected, significant urinary excretion of major APAP metabolites, i.e., APAP-sulfate and APAP-glucuronide, was noted, accounting for ~85% of the total amounts of APAP metabolites in the samples. Of the metabolites (produced by the host and the gut microbes) detected in predose urine samples, \( p \)-cresol sulfate exhibited a significant negative correlation with the ratio of APAP-sulfate/APAP-glucuronide. \( p \)-Cresol sulfate is a metabolite resulting from co-metabolism by host and gut microbiota; \( p \)-Cresol is produced by the gut bacterial metabolism of L-tyrosine (Gryp et al., 2017) and is sulfated to \( p \)-cresol sulfate by the host. Both \( p \)-cresol and APAP are substrates of the hepatic SULT1A1 enzyme. Considering that APAP sulfation is a low-capacity saturable process (Klaassen and Boles, 1997) and the urinary excretion of \( p \)-cresol sulfate is of the same magnitude as that of APAP sulfate (Clayton et al., 2009), the authors proposed the following working model: the gut bacterial metabolite \( p \)-cresol competes against APAP sulfation, potentially diverting APAP to the bioactivation pathway and increasing hepatotoxicity. While this study has raised an exciting possibility of a gut bacterial metabolite modulating the hepatic disposition of APAP, the proposed model remains to be verified experimentally, especially under the condition of APAP overdose. When a massive amount of APAP is introduced to the liver in the case of overdose, the amount of \( p \)-cresol produced by gut bacteria may not reach a high enough level to compete for sulfation. In mice, the hepatic amount of \( p \)-cresol sulfate at baseline was lower than the hepatic APAP-sulfate levels (measured at 15 min post-APAP dosing) by three orders of magnitude (Cho et al., 2023). Competition between APAP and \( p \)-cresol over hepatic sulfotransferase enzymes likely has a minor effect on hepatotoxicity caused by APAP overdose. Despite this potential limitation, it is important to note the possibility that gut bacterial
metabolite(s) may alter host susceptibility to APAP-induced hepatotoxicity at therapeutic APAP doses.

3.1.2. 1-Phenylpropane-1,2-dione (PPD)

The mammalian circadian clock regulates various physiological processes, adjusting the body to daily environmental changes such as light and food intake. Host susceptibility to APAP-induced hepatotoxicity also exhibits diurnal variation: mice receiving APAP at night (ZT12) show more severe hepatotoxicity than mice receiving APAP (500 mg/kg via intraperitoneal injection) in the morning (ZT0) (Thaiss et al., 2016). Interestingly, such diurnal variation in susceptibility disappears in germ-free mice or in mice pretreated with an antibiotic cocktail, suggesting that the gut microbiota controls diurnal variation in susceptibility to APAP-induced hepatotoxicity. Interestingly, the gut microbiota also undergoes diurnal oscillations in gut bacterial composition, gut bacterial localization (termed biogeography), and metabolic outputs (metabolites that define the function of the gut microbiota) (Thaiss et al., 2016). These diurnal fluctuations in the gut microbiota were found to be associated with changes in the intestinal and hepatic transcriptomes.

Following up on the findings above, Gong et al. embarked on a study to identify gut microbial metabolites potentially responsible for the diurnal variation in the APAP-induced hepatotoxicity (Gong et al., 2018). The transplantation of ZT0 or ZT12 gut microbiota into the gut microbiota-depleted mice (by antibiotic treatment) reproduced the differences in APAP-induced hepatotoxicity when all mice received APAP (300 mg/kg via oral gavage) at ZT0, indicating the causative role of the gut microbiota in the diurnal susceptibility variation. Untargeted metabolomic analysis of mouse cecal contents at ZT0 and ZT12 led to the identification of three metabolites more abundant in ZT12 samples: propionate, isovalerate, and PPD. PPD exhibited the largest difference (~2-fold) between the groups. PPD, when administered orally at 1 μg/kg at ZT0, significantly increased APAP-induced liver injury in mice,
with minimal changes in APAP pharmacokinetic profiles and hepatic CYP2E1 expression. The PPD treatment led to a small (~5%) but statistically significant decrease in the baseline glutathione levels in liver tissue. A similar reduction in glutathione levels was also observed in primary mouse hepatocytes treated with PPD at 100 nM, although the underlying mechanism was unknown. These results led to the conclusion that the gut bacterial metabolite PPD causes the diurnal variation in the susceptibility to APAP-induced hepatotoxicity by reducing the level of hepatic glutathione that defends cells against APAP-induced oxidative stress.

Concerning the effect of PPD on APAP-induced hepatotoxicity, the molecular target(s) of PPD remains to be identified. The basal PPD levels detected in the mouse cecal content and liver appear to be very low (0.7-10 pmol/g cecum content and 0.34-0.61 pmol/g mouse liver). It is unlikely that PPD lowers hepatic glutathione levels by directly reacting with glutathione (1-10 mM in mouse liver) (Vairetti et al., 2021). It is more likely that PPD interferes with step(s) in the glutathione biosynthetic pathway or other molecular factors involved in controlling hepatic glutathione levels.

PPD is a buttery, honey, and pepper-tasting compound found in plants (e.g., coffee) and used as a flavoring agent (Burdock, 1997; Dötterl and Schäffler, 2007). PPD was detected in the culture of gut bacteria such as *Escherichia coli*, *Citrobacter freundii*, *Clostridioides difficile*, and *Enterococcus faecalis* (but not *Lactobacillus casei* and *Bacteroides thetaiotaomicron*), although the extent of PPD production by gut bacteria is unknown (Gong et al., 2018). Oral administration of a cocktail of four different antibiotics led to a two-fold decrease in cecal PPD levels in mice, suggesting that at least half of PPD detected in the mouse cecum is attributable to non-bacterial origins such as diet. Regardless of the source of PPD, the intestinal PPD levels could be manipulated by the administration of a microbe capable of degrading PPD. *Saccharomyces cerevisiae*, a yeast known to metabolize PPD, decreased the cecal PPD level by ~90%. This was accompanied by ~3-fold lower liver injury by APAP administered at ZT12 (Gong et al., 2018), demonstrating that reducing the intestinal levels of PPD lowers the host susceptibility to
APAP-induced hepatotoxicity. It is currently unclear which fungi other than *S. cerevisiae* can also metabolize PPD. However, *Saccharomyces* is one of the commensal fungi in mammals, including mice and humans (Chin et al., 2020; Mims et al., 2021). Therefore, the presence and abundance of gut fungi metabolizing PPD, in parallel with gut bacteria producing PPD, is likely to determine host exposure to PPD and modulate host susceptibility to APAP-induced hepatotoxicity.

3.1.3. Trimethylamine (TMA)

Carnitine and choline are abundant in animal-derived diets such as red meat, eggs, and dairy products. These nutrients are metabolized by multiple gut bacteria (e.g., *Streptococcus sanguis*, *Desulfovibrio desulfuricans*, and *Escherichia coli*, to name a few) to TMA. Once absorbed, TMA is oxidized by hepatic flavin monooxygenase 3 to TMA oxide (TMAO), the biologically active co-metabolite. The extent of gut microbial TMA production governs host exposure to TMAO, and chronic TMAO exposure has been implicated in various pathological conditions, including atherosclerosis (Wang et al., 2011b; Koeth et al., 2013). TMAO significantly enhances the formation of foam cells and atherogenic plaque, in part by stimulating macrophage migration towards oxidatively modified low-density lipoprotein (ox-LDL) and promoting the expression of inflammatory cytokines (Wang et al., 2011b; Geng et al., 2018).

Macrophages are known to mediate the inflammation during the early stage of APAP-induced liver injury, while they are involved in tissue repair and regeneration in the later stage (Jaeschke and Ramachandran, 2020a). Yan et al. investigated whether TMAO affects APAP-induced liver injuries. TMAO pretreatment (109 mg/kg intraperitoneal injection 2 h before 300 mg/kg of APAP intraperitoneal injection; attaining the maximum plasma concentration of ~200 μM) exacerbated APAP-induced hepatotoxicity in mice (Yan et al., 2022). While the levels of CYP2E1 protein or oxidative stress markers were similar between control and TMAO-treated mice, more pronounced hepatic centrilobular sinusoidal hemorrhage and congestion at 12 h
post-APAP were noted in TMAO-treated mice. This was accompanied by decreased levels of hepatic proliferating cell nuclear antigen (PCNA), the marker of liver regeneration after injuries, suggesting that TMAO delays liver regeneration and repair during the progression of APAP-induced hepatotoxicity. Considering the well-established role of the macrophages in mediating hepatocyte regeneration after APAP dosing (Jaeschke and Ramachandran, 2020a), the effects of TMAO on macrophage function were further investigated. TMAO pretreatment led to lower recruitment of CD68(+) cells (i.e., macrophage and monocytes) after APAP dosing in mice, despite increased expression of CCL2 (a chemokine attracting monocytes and stimulating their inflammatory phenotypes). This was attributed to reduced expression of matrix metalloproteinase 12 (MMP12), a protease critical for macrophage migration (Shipley et al., 1996). MMP12 also cleaves interferon γ (IFN-γ), turning off pro-inflammatory IFN-γ signaling. Therefore, the decreased MMP12 expression/activity by TMAO would exacerbate inflammation to potentially increase host susceptibility to APAP (Dufour et al., 2018).

In RAW264.7 mouse macrophage cells, TMAO reduced MMP12 expression and suppressed cell migration across the transwell when stimulated by serum chemokines or lipopolysaccharide (LPS) (Yan et al., 2022). This finding contradicts the previous report where TMAO promoted RAW264.7 migration across the transwell upon stimulation by ox-LDL (Geng et al., 2018). Further studies are needed to parse TMAO’s effects on macrophage functions in APAP-induced acute liver injury. Also, it remains to be determined whether gut microbiota changes impacting TMA production (and subsequently systemic TMAO exposure) will indeed modulate APAP-induced hepatotoxicity.

3.2. Alleviating APAP-induced hepatotoxicity

3.2.1. Butyric acid

_Akkermansia muciniphila_ is a mucin-degrading Gram-negative bacteria isolated from human stools (Derrien et al., 2004). It is highly prevalent and abundant, representing 1-3% of
the total fecal bacterial cells in humans (Derrien et al., 2008). Numerous studies have reported a negative correlation between \textit{A. muciniphila} abundance and metabolic parameters linked to diabetes and obesity (reviewed in (Karamzin et al., 2021; Yan et al., 2021)). For example, a high-fat diet or overeating-induced obesity in different rodent models led to significant decreases in \textit{A. muciniphila} abundance in the fecal or cecal samples. Similarly, fecal \textit{A. muciniphila} abundance was lower in subjects with type 2 diabetes (than in healthy controls) and negatively correlated with BMI and fasting blood glucose. Importantly, \textit{A. muciniphila} supplementation corrected the metabolic phenotypes in mice and humans, establishing a cause-and-effect relationship between reduced intestinal \textit{A. muciniphila} abundance and the progression of metabolic diseases.

The beneficial effects of \textit{A. muciniphila} against metabolic diseases have been partly attributed to its production of short-chain fatty acids in the proximity of intestinal epithelial cells. \textit{A. muciniphila} degrades and ferments mucin into metabolites such as oligosaccharides, acetate, and propionate (Derrien et al., 2004). Acetate and propionate are known to activate G-protein coupled receptor (GPR) 41 and GPR43, which are short-chain fatty acid-sensing receptors expressed in enteroendocrine cells and immune cells, and show anti-obesity and immunomodulatory effects upon activation (Offermanns, 2014; Tomioka et al., 2022). Additionally, \textit{A. muciniphila} metabolically interacts with butyrogenic gut bacteria, stimulating butyrate production (Belzer et al., 2017). The protective effects of butyrate against hepatic diseases (e.g., non-alcoholic fatty liver disease) and impaired gut barrier function have been reported in numerous studies (reviewed in (Parada Venegas et al., 2019; Pant et al., 2023). Butyrate is also known to reduce oxidative stress by activating NRF2, the master regulator of the antioxidant defense system in the host (Guo et al., 2020). Xia et al. investigated the potential benefits of \textit{A. muciniphila} against acute liver injury (Xia et al., 2022). In mice given \textit{A. muciniphila} ($3\times10^9$ cfu/day, oral gavage for 14 days) followed by an APAP overdose (300 mg/kg intraperitoneal injection), APAP-induced liver injury was
significantly alleviated. The beneficial effects of *A. muciniphila* supplementation were associated with various changes in the liver, including decreased oxidative stress and inflammatory response, activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling, and reduced apoptosis. Interestingly, *A. muciniphila* supplementation also alleviated APAP-induced intestinal toxicity. APAP overdose is known to induce apoptosis of intestinal stem cells and cause a breach in the intestinal permeability barrier in mice (Chopyk et al., 2019), which can lead to increased translocation of gut microbial components such as LPS and may worsen the progression of APAP-induced hepatotoxicity (Chen, 2019). *A. muciniphila* supplementation in mice restored the fecal levels of acetate and butyrate, reversed APAP-induced gut barrier impairment, and normalized the amount of LPS reaching the systemic circulation (Xia, Lv et al. 2022).

Previous studies have shown that supplementation of pasteurized (i.e., dead) *A. muciniphila* or Amuc_1100 (an outer membrane protein of *A. muciniphila*) improved the gut barrier and corrected liver dysfunction (Plovier et al., 2017; Depommier et al., 2019). While the study by Xia et al. attests to the potential of *A. muciniphila* as a probiotic that may enhance intrinsic resistance to APAP-induced intestinal and liver toxicity, the extent of the contribution of butyrate to the protective effects of *A. muciniphila* against APAP-induced toxicity remains to be further defined.

As mentioned above about the effects of antibiotic treatment (Section 2), in mice treated with a cocktail of antibiotics (i.e., ampicillin, metronidazole, neomycin, and vancomycin; oral gavage for 3 days), the susceptibility to liver injury from APAP overdose (500 mg/kg oral gavage) significantly increased (Li et al., 2023b). Transplantation of the fecal microbiota of antibiotic-cocktail-treated mice into pseudo-germ-free (i.e., antibiotics-treated) mice recapitulated the phenotype, suggesting that altered gut microbiota by antibiotics is responsible for the increased susceptibility to APAP-induced hepatotoxicity. Interestingly, the treatment with ampicillin but not other antibiotics phenocopied the enhanced APAP-induced hepatotoxicity caused by the
antibiotic cocktail, indicating that the antibiotic cocktail effect was driven by ampicillin. The analysis of the gut microbiota uncovered a reduced abundance of *Lactobacillus*, *Akkermansia*, and *Ruminococcaceae* in the mice treated either with the antibiotic cocktail or ampicillin alone. Oral supplementation of *Lacticaseibacillus rhamnosus* (previously known as *Lactobacillus rhamnosus*) (1x10^9 cfu/day for 2 weeks) corrected the enhanced APAP-induced hepatotoxicity by ampicillin. This was accompanied by significant increases in fecal levels of butyrate (but not acetate and propionate). Butyrate administration (500 mg/kg/day via oral gavage for 7 days) phenocopied the beneficial effects of *L. rhamnosus* against APAP-induced liver injury and also reversed ampicillin-enhanced oxidative stress. Notably, supplementation of either *L. rhamnosus* or butyrate promoted the expression of host genes regulated by NRF2, the master regulator of the antioxidant defense system, suggesting that NRF2 mediates, at least in part, the protective effects of *L. rhamnosus* or butyrate. The study by Li et al. illustrates how the intake of a specific antibiotic, such as ampicillin, can decrease the abundance of beneficial bacteria and metabolite (butyrate) levels, consequently leading to enhanced oxidative injury from APAP overdose.

Results from two studies by Xia et al. and Li et al. suggest that butyrate produced by gut bacteria is one of the gut microbial factors determining host susceptibility to APAP-induced hepatotoxicity. However, the mechanism for butyrate’s effects on improving liver function is rather unclear and merits further investigation.

3.2.2. 3-Phenylpropionic acid (3-PPA)

Substrains of C57BL/6 mice from different vendors, 6J from the Jackson Laboratory and 6N from Taconic Biosciences, carry distinct gut microbiota, and they have been utilized in identifying gut bacteria with biological functions (Sivan et al., 2015; Robertson et al., 2019). For example, *Bifidobacterium*, a commensal bacterium more abundant in 6J vs. 6N, was identified as a beneficial microbe in cancer therapy by enhancing antitumor immunity and response to anti-PD-L1 immunotherapy (Sivan et al., 2015). The mouse substrain difference has also been
implicated in altered susceptibility to APAP-induced liver injury (from 300 mg/kg intraperitoneal dose); 6N was more susceptible to APAP-induced hepatotoxicity than 6J (Bourdi et al., 2011; Duan et al., 2016). While it was attributed in part to genetic drifts between the substrains, e.g., loss-of-function mutation in nicotinamide nucleotide transhydrogenase (Nnt) in 6J mice (Almodovar et al., 2013), the underlying mechanisms remained unclear.

Cho et al. investigated the potential role of the differential gut microbiota between 6J and 6N in the altered APAP-induced liver injury (Cho et al., 2023). Co-housing of 6J and 6N, a process used to assimilate the gut microbiota of mice housed in the same cage, abolished the susceptibility difference between 6J and 6N to APAP-induced hepatotoxicity. Moreover, the susceptibility difference was transferable by gut microbiota transplantation into germ-free mice. These results indicate that apart from the genetic differences between 6J and 6N, the differential gut microbiota alters the host susceptibility to APAP-induced hepatotoxicity.

The levels of short-chain fatty acids in the cecum contents were similar between 6N and 6J mice, ruling out their involvement. Untargeted metabolomic analyses of the portal vein serum and liver tissue samples from these mice identified 19 metabolites with differential abundances between 6J or 6N gut microbiotas. Of interest, four metabolites more abundant in 6J samples were intermediates or final metabolic products derived from the metabolism of L-phenylalanine (L-Phe) by gut bacteria and subsequently by host: 3-PPA, cinnamoyl glycine, phenylpropionyl glycine, and hippuric acid. L-Phe is metabolized by gut bacteria to 3-PPA. 3-PPA is then absorbed from the intestine and undergoes hepatic β-oxidation, producing hippuric acid as the final product and cinnamoyl glycine as a potential intermediate (Badenhorst et al., 2014; Pruss et al., 2023). 3-PPA can also undergo hepatic glycine conjugation into phenylpropionyl glycine (Badenhorst et al., 2014; Pruss et al., 2023). Considering that 3-PPA production by the gut microbiota could govern the host exposure to the 3-PPA-related metabolites listed above, 3-PPA levels in cecal contents were measured, and significantly higher 3-PPA levels were found in 6J samples. 3-PPA was not detected in the cecal contents of germ-free mice, indicating that it
is derived from the gut microbiota. 3-PPA supplementation (0.4% w/v in drinking water for 4 weeks) to 6N mice (with low 3-PPA) led to significant increases in 3-PPA serum levels and alleviated APAP-induced hepatotoxicity.

Time profiles of APAP-induced liver injury in 3-PPA-supplemented mice revealed that 3-PPA reduced the liver damage at the early stage of pathogenesis (i.e., before 6 h of APAP dosing). Decreases in the APAP-protein adduct formation, as well as in the hepatic microsomal CYP2E1 protein expression, were noted in the 3-PPA-supplemented mouse liver. This occurred without significant changes in Cyp2e1 mRNA levels, indicating the involvement of posttranscriptional regulation. CYP2E1 protein is known to undergo degradation by slow lysosomal and fast ubiquitin-proteasome pathways (protein half-life being 32 and 7 h, respectively) (Roberts et al., 1995). These processes are regulated by the availability of (endogenous) CYP2E1 substrates and the activation of multiple signaling pathways, including protein kinases A and C (PKA and PKC) (Wang et al., 2011a). While 3-PPA is not a CYP2E1 substrate (Cho et al., 2023), it remains unknown whether 3-PPA supplementation affects the levels of other endogenous CYP2E1 substrates and/or alters the activities of PKA and PKC. Various endogenous fatty acids (e.g., arachidonic acid) are known to undergo (ω-1) hydroxylation by CYP2E1 (Laethem et al., 1993; Roy et al., 2005), and it is unknown whether 3-PPA affects hepatic lipid metabolism. The detailed mechanisms of CYP2E1 regulation in 3-PPA-supplemented mice remain to be determined.

Decreased CYP2E1 expression by 3-PPA may have broader implications beyond APAP-induced liver injury. Higher CYP2E1 expression was associated with the development of metabolic phenotype linked to obesity (Abdelmegeed et al., 2012; Zong et al., 2012). Also, CYP2E1 catalyzes the bioactivation of other hepatotoxic chemicals, such as carbon tetrachloride (CCl₄) and alcohol. Indeed, 3-PPA supplementation also significantly decreased CCl₄-induced acute liver injury (Cho et al., 2023). Whether 3-PPA supplementation provides benefits for other CYP2E1-related diseases remains to be examined.
Importantly, the study by Cho et al. demonstrates how seemingly healthy mice raised in different environments exhibit different phenotypes, i.e., altered susceptibility to APAP-induced liver injury. This result indicates that the baseline liver physiology of “conventional” mice raised in different facilities may differ from each other due to differential gut microbiota.

3.2.3. 5-Methoxyindoleacetic acid (5-MIAA)

Saeedi et al. investigated the gut microbiota-liver axis by focusing on the host signaling pathways potentially mediating the interaction with the gut microbiota. The authors first compared the hepatic transcriptomes between germ-free and conventionalized (i.e., germ-free mice transferred to cages with bedding from conventional mice) mice (Saeedi et al., 2020). The upregulated genes in the conventionalized mouse liver samples were the ones associated with antioxidant and xenobiotic responses (e.g., \textit{Gstm1}, \textit{Gclc}, and \textit{Nqo1}), for which \textit{NRF2} is the master regulator of expression. Using the transgenic \textit{Nrf2} reporter system in \textit{Drosophila melanogaster}, four bacterial species were examined for their abilities to activate \textit{NRF2}, and two bacterial species, i.e., \textit{Lactobacillus plantarum} and \textit{Lacticaseibacillus rhamnosus} GG (LGG; a commonly used probiotic strain), were identified as \textit{NRF2} activators. LGG pretreatment (2x10^8 cfu/day, oral gavage for 14 days) significantly increased hepatic \textit{NRF2} signals in mice and conferred protection against oxidative toxicants, such as paraquat and APAP, in fruit flies and mice. Such protection was abolished when the \textit{Nrf2} gene was deleted in a liver-specific manner, indicating that hepatic \textit{NRF2} is essential for the LGG effect. Untargeted metabolomic analysis of portal vein serum from the mice administered with LGG led to the identification of six compounds more abundant in the LGG group. Among these, only one compound, 5-MIAA at 5 \textmu M, could activate \textit{NRF2} in a luciferase reporter assay in HepG2 cells. While these results suggest a potential role of 5-MIAA in mediating the beneficial effects of LGG against oxidative stress in the liver, the \textit{in vivo} effect of 5-MIAA on the \textit{NRF2} system and its contribution to the anti-oxidative effects of LGG in the liver requires further investigation.
5-MIAA was first described as a metabolite of melatonin found in the bovine pineal gland (Lerner et al., 1960). It was detected in the urine of healthy individuals (excretion rate being 4.8 µg/day) (Higa and Markey, 1985). The serum concentration of 5-MIAA was reported to be ~1 nM in rats (Ho et al., 2001), but it could reach as high as 2 µM in the mouse portal vein samples after the oral administration of LGG (Saeedi et al., 2020). The vast (i.e., >3 orders of magnitude) differences in 5-MIAA concentrations may reflect inter-species differences in melatonin metabolism (Kennaway et al., 2002). While gut bacteria such as LGG may significantly increase hepatic exposure to this metabolite by producing large amounts of 5-MIAA, the baseline exposure to 5-MIAA is expected to be very low. Supporting the idea, while 5-MIAA is readily detectable in the culture supernatants of many *Lactobacillus* bacteria (Saeedi et al., 2020), it was not detected in human stool samples (Higa and Markey, 1985). By comparing the wild-type LGG with an LGG mutant that cannot produce 5-MIAA, the function of 5-MIAA in mediating the probiotic activities of LGG, including those on APAP-induced hepatotoxicity, can be further defined.

3.2.4. Indole-3-carboxylic acid (I3C)

Magnesium (Mg) serves as a cofactor in various enzymatic reactions in the cells. A recent study has demonstrated the important role of Mg in drug-induced liver injury; the disturbance in intracellular Mg homeostasis caused by the induced expression of a mitochondrial Mg efflux transporter has been implicated in the propagation of APAP-induced hepatotoxicity in mice (Gonzalez-Recio et al., 2022). Li et al. have further shown that MgCl₂ supplementation alleviates APAP-induced liver injury in mice when administered orally but not intraperitoneally (Li et al., 2023a). The phenotype was transferable by gut microbiota transplantation from MgCl₂-administered mice or humans into (antibiotic-pretreated) pseudogerm-free mice, suggesting an important role for the gut microbiota in mediating the beneficial
effect of MgCl$_2$. Gut microbiome analysis of the mice administered with MgCl$_2$ revealed increased abundances of *Bifidobacterium* genera, and oral administration of different strains in *Bifidobacterium* genera (*B. longum, B. bifidum, B. breve, and B. animalis*; 2x10$^8$ cfu/day oral gavage for 3 days) decreased APAP-induced liver injury in mice. Of note, the culture supernatant of *Bifidobacterium* also decreased APAP-induced hepatotoxicity in mice, suggesting that the bacterial metabolites mediate the benefits of *Bifidobacterium*. Untargeted metabolomic analyses of the culture supernatants of four *Bifidobacterium* bacteria and the feces of MgCl$_2$-administered mice, led to the identification of I3C as a metabolite enriched in the samples. In mice, oral administration of *E. coli* overexpressing the bacterial enzyme responsible for I3C production increased serum I3C concentrations from ~1.3 to ~3.2 nM and significantly decreased APAP-induced liver injury (300 mg/kg oral gavage), suggesting that I3C may be the bacterial metabolite mediating the beneficial effects of *Bifidobacterium*. Indeed, I3C (100 mg/kg, oral gavage) administered 1 h before the APAP challenge significantly alleviated APAP-induced liver injury, and this was accompanied by decreased hepatic levels of APAP-adduct formation, suggesting reduced APAP bioactivation by I3C. I3C or MgCl$_2$ treatment did not affect hepatic CYP2E1 protein levels in mice; however, in the homogenates of mouse liver collected 1 h after an oral dose of I3C (100 mg/kg, oral gavage), CYP2E1 enzyme activity was significantly lower as compared to those from the control group. These results suggest the I3C may inhibit CYP2E1 activity without affecting its expression. Direct binding between I3C and CYP2E1 was shown by multiple in vitro binding assays including surface plasmon resonance analysis. However, the estimated binding affinity of I3C to CYP2E1 was weak (e.g., $K_d = 16$ µM). Therefore, it is uncertain whether I3C inhibits CYP2E1 at physiologically relevant concentrations, which range only at a few nanomolar levels in the blood. It remains to be determined whether other gut bacterial metabolites, in addition to I3C, mediate the beneficial effects of *Bifidobacterium* against acute liver injury. Regardless, this study presents I3C as a potentially
beneficial gut bacterial metabolite conferring protection against APAP-induced liver injury via CYP2E1 inhibition.

3.2.5. 2-Hydroxybutyric acid (2-HB)

Vancomycin is a tricyclic glycopeptide antibiotic used to treat Gram-positive bacterial infections (Rubinstein and Keynan, 2014). When administered orally, vancomycin is not appreciably absorbed from the intestine due to its large molecular weight (1,485 Da) and significantly alters gut microbiota composition and abundance. Zheng et al. showed that vancomycin pretreatment (200 mg/kg/day, oral gavage for 4 days) attenuated APAP-induced liver injury (from 400 mg/kg oral gavage) in Sprague-Dawley rats (Zheng et al., 2020). Untargeted metabolomic analysis of serum samples from vancomycin-pretreated and APAP-challenged rats led to the identification of 13 metabolites whose abundances differed between the mouse groups. Specifically, vancomycin pretreatment was associated with high cecum levels of 2-HB. Pretreatment of 2-HB (250 mg/kg/day, ip for 3 days) significantly reduced APAP-induced hepatotoxicity in mice, although the underlying mechanisms were unclear.

2-HB is a metabolic product of amino acids (methionine and threonine) by the action of lactate dehydrogenase (LDH), the enzyme expressed in both gut bacteria and host. In the host, 2-HB is the byproduct of the methionine transsulfuration pathway linked to the synthesis of glutathione (the major antioxidant molecule). Serum 2-HB level was proposed as an early biomarker of insulin resistance in a non-diabetic population and considered to reflect the extent of the liver’s glutathione need (e.g., oxidative stress) (Gall et al., 2010).

The capability to produce 2-HB appears prevalent in the gut microbiota. Bioinformatic analysis of the human gut microbiome in search of the 2-HB biosynthetic pathway revealed a high prevalence of 2-HB-producing bacteria (Qin et al., 2023). Despite such prevalence, the gut microbiota appears not to be the major determinant of systemic 2-HB exposure; 2-HB levels in the systemic circulation did not correlate with those in the cecal contents in vancomycin-treated
rats (Zheng et al., 2020). Also, oral administration of an antibiotic cocktail had minimal effects on serum 2-HB concentrations in mice, indicating that the primary source of circulating 2-HB is likely the host metabolism (Qin et al., 2023). Regardless of the source, 2-HB supplementation alleviated APAP-induced liver injury (Zheng et al., 2020). A detailed mechanism for 2-HB reducing APAP-induced liver injury remains to be determined.

4. Conclusion and future perspective

While the origin and the mechanism of action of some metabolites listed in this review need to be clarified and better defined, it appears to be clear that the gut microbiota plays a significant role in determining basal susceptibility to APAP-induced hepatotoxicity. With advancements in analytical tools such as high-resolution mass spectrometry and NMR spectroscopy of improved sensitivity, the number of chemically defined small molecules derived from gut microbes is ever-increasing. The APAP-induced liver injury can serve as a model to identify those gut microbial metabolites and investigate their potential biological roles in other related diseases.

The mouse as an animal model has served as a gold standard for identifying the roles of gut microbial metabolites in APAP-induced hepatotoxicity. While different gut bacterial metabolites have been successfully identified using the mouse models, similarities in animal husbandry (inbred laboratory mice, standardized mouse diets, limited vendors, etc.) may have limited the metabolic capacity of the gut microbiota, even if the origins of mice are different. The human gut microbiome harbors ever-increasing numbers of biosynthetic gene clusters that potentially produce thousands of chemically diverse metabolites with unknown biological functions. Many of these metabolites are likely produced only under certain conditions (Donia et al., 2014; Sugimoto et al., 2019), and it is unclear whether the gut microbiome of laboratory mice fully reflects the chemical diversity of the human gut microbiome. Thousands of culturable gut bacterial isolates are available to date, and an in vitro system (e.g., primary mouse
hepatocytes) may be used to screen gut bacterial cultures for those altering APAP-induced cytotoxicity, followed by further verification in a mouse model of APAP-induced hepatotoxicity.

Despite certain limitations in mouse models, chemical-induced, diet-induced, or other perturbations altering gut microbial metabolic outputs likely lead to identifying as-yet-unknown gut microbial metabolites that modulate the susceptibility to APAP-induced liver injury. The knowledge gained from those efforts can potentially be applied to a better understanding of diseases with shared pathologies with APAP-induced liver injury.
Acknowledgments

None

Data Availability Statement

This review article contains no datasets generated or analyzed during the current study.

Authorship Contributions

H Lee, X Yang, P-R Jin, K-J Won, CH Kim, H Jeong: wrote or contributed to the writing and editing of the manuscript.
References


Bourdi M, Davies JS, and Pohl LR (2011) Mispairing C57BL/6 substrains of genetically engineered mice and wild-type controls can lead to confounding results as it did in studies of JNK2 in acetaminophen and concanavalin A liver injury. Chem Res Toxicol 24:794-796.


32


**Footnotes**

This work was supported by the National Institute of Health (R21AT011391 and R21AT011565). No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legend

Figure 1. Overview of gut microbial metabolites known to modulate APAP-induced liver injury. Metabolites in green and red are known to alleviate and worsen APAP-induced hepatotoxicity, respectively. I3C, indole-3-carboxylic acid; LPS, lipopolysaccharide; 5-MIAA, 5-methoxy indole acetic acid; PPD, 1-phenylpropane-1,2-dione; 3-PPA, 3-phenylpropionic acid; TMAO, trimethylamine oxide. Gut bacterial production of sedanolide is unknown. Please see the text for details. This figure was created with BioRender.com.
Table 1. Evidence of gut microbiota modulating APAP-induced liver injury in mice.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Method of microbiome analysis</th>
<th>APAP dosing</th>
<th>Effects on APAP-induced liver injury</th>
<th>Author, year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germ-free mice</td>
<td>Germ-free C3H/HeH mice</td>
<td>n/a</td>
<td>200 mg/kg ip after overnight fasting</td>
<td>No change in ALT and necrosis at 8 h post-APAP dosing as compared to the conventional mice</td>
</tr>
<tr>
<td>Genetic modification</td>
<td>Nlrp6−/− mice</td>
<td>Relative abundance in the hepatic microbiota was assessed by the 16S rRNA gene (V3-V4 regions) sequencing</td>
<td>500 mg/kg ip after overnight fasting</td>
<td>Increased ALT at 12 h post-APAP</td>
</tr>
<tr>
<td>Antibiotic treatment</td>
<td>Vancomycin (0.5 g/L), ampicillin (1 g/L), kanamycin (1 g/L), and metronidazole (1 g/L) in drinking water for 3 weeks in C57Bl/6 mice</td>
<td>Gut microbiota depletion was assessed by enumeration of bacteria attached to the colonic epithelium by the 16S rRNA gene (V6 region) qPCR; Relative abundance in the fecal microbiota was assessed by the 16S rRNA gene (V1-V2 regions) sequencing</td>
<td>500 mg/kg ip</td>
<td>No change in ALT, AST, and liver necrosis at 10 h post-APAP dosing when APAP was given at ZT0; decreased ALT, AST, and hepatic histology score with APAP dosing at ZT12</td>
</tr>
<tr>
<td></td>
<td>Vancomycin (100 mg/kg), ampicillin (200 mg/kg), neomycin (200 mg/kg), and metronidazole (200 mg/kg) oral gavage once daily for 3 days in Balb/C mice</td>
<td>Gut microbiota depletion was assessed in cecal contents by the 16S rRNA gene (V4 region) qPCR</td>
<td>300 mg/kg oral gavage</td>
<td>No change in ALT and liver necrosis at 24 h post-APAP dosing when APAP was given at ZT0; decreased plasma ALT and necrosis with APAP dosing at ZT12</td>
</tr>
<tr>
<td></td>
<td>Vancomycin (100 mg/kg), ampicillin (200 mg/kg), neomycin (200 mg/kg), metronidazole (200 mg/kg) via oral gavage once daily for 3 days in C57Bl/6 mice</td>
<td>Relative abundance in the fecal microbiota was assessed by the 16S rRNA gene (V3-V4 regions) sequencing</td>
<td>500 mg/kg oral gavage</td>
<td>Increased ALT at 24 h post-APAP</td>
</tr>
<tr>
<td></td>
<td>Ampicillin (200 mg/kg) via oral gavage once daily for 3 days in C57Bl/6 mice</td>
<td>Relative abundance in the fecal microbiota was assessed by the 16S rRNA gene (V3-V4 regions) sequencing</td>
<td>500 mg/kg oral gavage</td>
<td>Increased ALT at 24 h post-APAP dosing</td>
</tr>
<tr>
<td></td>
<td>Vancomycin (100 mg/kg) via oral gavage once daily for 3 days in C57Bl/6 mice</td>
<td>Relative abundance in the fecal microbiota was assessed by the 16S rRNA gene (V3-V4 regions) sequencing</td>
<td>500 mg/kg oral gavage</td>
<td>No changes in liver necrosis at 24 h post-APAP dosing</td>
</tr>
<tr>
<td></td>
<td>Vancomycin (200 mg/kg) via oral gavage once daily for 4 days in Sprague Dawley</td>
<td>Relative abundance in the cecal microbiota</td>
<td>2000 mg/kg oral gavage</td>
<td>Decreased ALT at 24 h post-APAP dosing</td>
</tr>
<tr>
<td>rats</td>
<td>was assessed by the 16S rRNA gene (V3 region) sequencing</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Gut microbial metabolites identified to modulate APAP-induced liver injury.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Intestinal concentration</th>
<th>Host concentration</th>
<th>Source</th>
<th>Effects on host physiology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolites alleviating APAP-induced hepatotoxicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyric acid</td>
<td>20-30 mM in human colon lumen</td>
<td>15-65 (\mu M) in human portal vein blood; 1-12 (\mu M) in human peripheral blood</td>
<td>Gut bacteria</td>
<td>Enhances NRF2 activity; improves impaired gut barrier impairment</td>
<td>(Cummings et al., 1987; Xia et al., 2022)</td>
</tr>
<tr>
<td>2-Hydroxybutyric acid (2-HB)</td>
<td>4.8-28.8 nmol/g mouse cecum content</td>
<td>4.8-14.4 (\mu M) in mouse serum</td>
<td>Host (liver) and gut bacteria</td>
<td>Unknown</td>
<td>(Zheng et al., 2020; Qin et al., 2023)</td>
</tr>
<tr>
<td>Indole-3-carboxylic acid (ICA)</td>
<td>0.2-1.3 nmol/g mouse cecum content</td>
<td>1-15 nM in mouse plasma</td>
<td>Diet and gut bacteria</td>
<td>Decreases CYP2E1 activities</td>
<td>(Dong et al., 2020; Tian et al., 2022; Li et al., 2023a)</td>
</tr>
<tr>
<td>5-Methoxy indole acetic acid (MIAA)</td>
<td>~2 (\mu M) in the portal vein of LGG-administered mice</td>
<td>~1 nM in rat serum</td>
<td>Gut bacteria and host</td>
<td>Activates basal hepatic NRF2 in mice</td>
<td>(Lerner et al., 1960; Ho et al., 2001; Saeedi et al., 2020)</td>
</tr>
<tr>
<td>3-Phenylpropionic acid (3-PPA)</td>
<td>10-400 nmol/g mouse cecum content</td>
<td>0.2-2.5 (\mu M) in mouse cardiac serum</td>
<td>Gut bacteria (e.g., Clostridium sporogenes)</td>
<td>Decreases basal hepatic CYP2E1 protein levels in mice</td>
<td>(Cho et al., 2023)</td>
</tr>
<tr>
<td><strong>Metabolites aggravating APAP-induced hepatotoxicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p)-Cresol</td>
<td></td>
<td>(p)-cresol sulfate: 15-36 (\mu M) human serum</td>
<td>Gut bacteria</td>
<td>May compete against APAP sulfation</td>
<td>(Clayton et al., 2009; Gryp et al., 2017)</td>
</tr>
<tr>
<td>1-Phenylpropane-1,2-dione (PPD)</td>
<td>0.7-10 pmol/g mouse cecum content</td>
<td>0.34-0.61 pmol/g mouse liver</td>
<td>Gut bacteria (Escherichia coli, Citrobacter freundii, Clostridioides difficile, Enterococcus faecalis)</td>
<td>Decreases basal glutathione levels in mice</td>
<td>(Gong et al., 2018)</td>
</tr>
<tr>
<td>Trimethylamine (TMA)</td>
<td></td>
<td>2.40 (\mu M) TMAO in healthy individual plasma; 200-300 (\mu M) TMAO in renally impaired patient plasma</td>
<td>Gut bacteria</td>
<td>TMAO Reduces MMP12 expression, macrophage migration, and liver regeneration after APAP</td>
<td>(Yan et al., 2022; Tacconi et al., 2023)</td>
</tr>
</tbody>
</table>