Gut-microbiome mediated modulation of hepatic cytochrome P450 and P-glycoprotein: impact of butyrate and FOS-inulin

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Running title: *The gut microbiota influences hepatic gene expression*

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Nonstandard Abbreviations

Conv., conventional; Cyp, Cytochrome P450 superfamily of enzymes; FOS, Fructo-oligosaccharide; GF, germ-free; MDR1, multi-drug resistance protein 1; P-gp, P-glycoprotein; RT-qPCR, reverse-transcriptase quantitative polymerase chain reaction; SCFA, short-chain fatty acids

Conflict of interest

JFC & TGD have research funding from Dupont Nutrition Biosciences APS, Cremo SA, Alkermes Inc, 4D Pharma PLC, Mead Johnson Nutrition, Nutricia Danone, Suntory Wellness. JFC, TGD & GC have spoken at meetings sponsored by food and pharmaceutical companies. All other authors report no financial interests or potential conflicts of interest

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Abstract

Objectives: Our objective was to demonstrate microbial regulation of hepatic genes implicated in drug metabolism and transport using germ-free (GF) mice and to explore the impact of a microbial metabolite, butyrate, and a prebiotic dietary intervention on hepatic gene expression in mice.

Methods: Using reverse-transcriptase PCR, we investigated cytochrome P450 (CYP) and multidrug-resistance protein 1 (MDR1) expression in conventional, GF, and colonised GF mice. To investigate the effects of butyrate, sodium butyrate (3 g/L) was administered for 21 days to conventional or GF mice. In the prebiotic study, young-adult and middle-aged mice received diet-enriched with 10% fructo-oligosaccharide (FOS)-inulin for 14 weeks.

Key findings: Colonisation of GF animals normalised expression of Cyp3a11 and Mdr1b to conventional levels. Butyrate upregulated Cyp2b10 in conventional mice ($p<0.05$) but overall did not induce widespread changes in hepatic genes. FOS-inulin increased Cyp3a13 expression and had the opposite effect on Mdr1a expression in young-adult mice ($p<0.05$). Age, on the other hand, influenced the prebiotic effect on Cyp2a4 expression ($p<0.01$).

Conclusion: The expression of hepatic genes implicated in drug metabolism and transport displays sensitivity to the microbiome, microbiome-derived metabolites, and a microbial-targeted intervention. Our study may provide the impetus to explore microbiota-targeted interventions in normalising host metabolic activity and reducing inter-individual variability in drug pharmacokinetics.

Keywords: Microbiome, Cytochrome, Transporter, Hepatic, Drug, Metabolism

Introduction

The metabolic fate and toxicity of drugs are determined, in part, by the expression of drug-metabolising enzymes and drug transporters (1). In particular, the cytochrome P450 (CYP) enzyme superfamily and drug-efflux transporters are key drivers of oral drug bioavailability (2). Drug-efflux transporters, including multidrug-resistance protein 1 (MDR1), expel conjugated drugs from the liver into the bile ducts and thus make an essential contribution to drug pharmacokinetics (3). Significantly, CYP1-3 family members are implicated in the metabolism of 70-80% of all drugs in clinical use (4) and MDR1, also known as P-glycoprotein (P-gp), is an efflux pump with broad substrate specificity (2). While humans express a single MDR1 gene, rodents share the function of hepatic MDR1 between two highly homologous MDR1-type genes, Mdr1a and Mdr1b (5, 6).

Inter-individual variability in the expression of CYP genes is generally linked to age, race, genetics, concomitant disease, or co-administered drugs (4). However, the importance of the gut microbiota, the trillions of micro-organisms residing along the gastrointestinal tract (7), has recently come to the fore as an additional variable adding to this complexity. Evidence from germ-free (GF) mice, mice devoid of microbes, demonstrate altered expression of hepatic genes implicated in drug metabolism (8-10). The drug-metabolising capacity of an individual may vary, therefore, not only because of polymorphisms in genes encoding host drug-metabolising enzymes and the concomitant intake of drugs but also due to individual differences in the composition of the gut microbiota. This interconnectivity between the intestinal tract and the liver makes it essential to view drug metabolic processes as co-metabolism by the host and the gut microbiota (11).

The liver receives approximately 70% of its blood supply from the intestine and is thus continually exposed to microbial metabolites, including short-chain fatty acids (SCFA) (12).

One such SCFA, butyrate, is efficiently metabolised by the intestinal epithelial cells, but a proportion is absorbed and transported into the liver by the portal vein (13). Evidence suggests that butyrate can induce Cyp1a2 expression possibly linked to the modification of histones (14, 15). However, whether this effect is dependent on an intact gut microbiome or affects the expression of other CYP genes is unknown.

The impact of microbiota-targeted therapies, including antibiotics, probiotics (i.e., "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (16)), and prebiotics (i.e., "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (17)) on CYP and MDR1, and their potential knock-on effects on the response to co-administered medication (18), is a significant but underexplored area of drug metabolism. While there are several reports that a change in nutritional status affects hepatic levels of drug-metabolising enzymes (19), a commercially available probiotic mix, VSL#3, exerted a limited effect on CYP gene expression (8). Modulation of intestinal microbes by prebiotics may also, however, alter the drug-metabolising capacity of the host. Foods such as onions, leeks, and garlic are dietary sources of the prebiotic inulin (20), which protects against high-fat diet-induced alterations in both the expression and activity of Cyp1a1, Cyp1a2, and Cyp2e1 (19).

Here, we aimed to further validate the role of the gut microbiota in the regulation of CYP drug-metabolising enzymes and the drug-efflux transporter, MDR1. The microbial metabolite, butyrate, was investigated as a potential influencer of these host-microbe interactions. We further examined the impact of fructo-oligosaccharide-inulin (FOS-inulin), a dietary prebiotic known to alter the composition and function of the gut microbiome (21), on hepatic gene expression at different life-stages.

Materials and Methods

All experiments were conducted in accordance with the European Directive 86/609/EEC and the Recommendation 2007/526/65/EC. Ethical approval for each study was obtained from the Animal Experimentation Ethics Committee of University College Cork before the commencement of all animal-related experiments. For the impact of the GF/colonisation study, ethical approval (AE19130/P047) was granted on 16/02/2017. For the butyrate supplementation study, ethical approval (AE19130/P023) was granted on 13/01/2016. For the FOS-inulin intervention study, ethical approval (B100/3774) was issued on 18/12/2012.

Animals

Male F1-generation offspring from conventionally raised and GF C57BL/6J breeding pairs (Taconic, Germantown, New York, USA) were used as previously described (22). GF mice were housed in specific isolators. Animals were kept under a 12-h light/dark cycle, with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Food and water were given *ad libitum*. Conventional, GF, colonised GF, and butyrate-treated mice were fed an autoclaved diet (Special Diets Services, UK). See *FOS-inulin study* for corresponding diet and animal information.

GF/Colonisation Study

At postnatal day 21, a subset of GF mice were transferred to the conventional animal facility and were colonised by exposure to used cage bedding of age-, vendor- and sex-matched

conventional mice for 7-8 weeks. Mice were euthanised by decapitation, and liver samples were immediately snap-frozen and stored at -80 ºC until further analysis.

Butyrate Study

Sodium butyrate (3 g/L; Sigma-Aldrich), or sodium chloride for sodium-matched controls, was dissolved in sterile drinking water and administered for 21 days to conventional or GF male C57BL/6 mice (n=13-15/group). This dosage was based on previous studies by our research group and others investigating the impact of butyrate (600 mg/kg) on behaviour in mice, combined with an estimated drinking water consumption of 5 ml/day (23-25). Drinking water was filtered through a 0.2-micron syringe filter (Sarstedt) and refreshed twice per week. As diet can contribute to the gastrointestinal and systemic levels of butyrate in vivo, food intake was closely monitored across all experimental groups and no significant differences in food consumption were observed. Mice were euthanised by decapitation, and liver samples were immediately snap-frozen and stored at -80 ºC until further analysis.

FOS-Inulin Study

Previous work by our laboratory investigated prebiotic supplementation (FOS-Inulin) on the peripheral immune response and neuroinflammation in middle age (21). Here, we sought to examine the effects of prebiotic supplementation on hepatic gene expression from tissues collected from the same animals. In brief, young adult (approx. 2 months at start of treatment) and middle-aged (approx. 10 months at start of treatment) conventional male C57BL/6 mice (obtained from Harlan, Cambridgeshire) received a standard diet (ssniff-Spezialdiäten GmbH, Soest, Germany) or the diet enriched with 10% Oligofructose-enriched inulin (FOS-Inulin: mixture of 92±2% Inulin and 8±2% Fructo-oligosaccharide, Orafti®Synergy1; BENEO-Orafti N.V., Belgium) for 14weeks (n=9-10/group). Mice were euthanised by

156 decapitation, and liver samples were immediately snap-frozen and stored at -80 ºC until
157 further analysis.

158

159 **RNA extraction, Reverse transcription and RT-qPCR**

160 Total RNA was isolated from harvested liver tissue using the High Pure RNA Tissue Kit
161 (Sigma Aldrich) following the manufacturer's protocol or using the mirVana^{TM} miRNA
162 Isolation Kit (Thermo Scientific/Invitrogen; GF/Colonisation study). Tissue from the
163 GF/Colonisation study required the use of an RNA extraction kit well-suited for total and
164 miRNA isolation suitable for future downstream miRNA analyses. Both kits allowed for the
165 comparable high-quality, pure, intact collection of RNA used in the present study. Following
166 RNA extraction, RNA concentration and quality were determined using the standard
167 OD260/280 method using a Nanodrop spectrophotometer (Thermo Scientific). The
168 OD260/OD280 ratio for each RNA sample used in subsequent experiments was in the range
169 1.9-2.1. RNA was reversed transcribed to cDNA using the Exiqon cDNA Universal Synthesis
170 kit (Exiqon A/Q) or High Capacity cDNA Reverse Transcription kit (Thermo
171 Scientific/Applied Biosystems) in a G-storm thermocycler (G-storm, Surrey, UK).

172 Reverse-transcriptase PCR was employed to compare the mRNA expression of CYP drug-
173 metabolising enzymes and the two mouse isoforms of hMDR1, Mdr1a, and Mdr1b. The most
174 commonly studied CYP and MDR murine isoforms equivalent to humans are described in
175 *Table.1*. [**see Table 1**]

176 While the murine isoforms of hMDR1 show differential distribution in other physiological
177 areas, both Mdr1a and Mdr1b are widely distributed in the liver (26). There are, however,
178 some inter-species differences in CYP and MDR genes in mice and humans, in terms of
179 sequence homology and substrate specificity (27).

For the GF/colonisation study, RT-qPCR was performed using TaqMan Universal Master Mix II (Thermo Fisher Scientific/Applied Biosystems), and genes of interest were amplified using TaqMan probes (Integrated DNA Technologies). For the RT-qPCRs from the butyrate- or FOS-inulin study liver samples, SYBR Green detection chemistry was employed, utilising the ExiLENT SYBR^{R} GREEN Master Mix (Exiqon A/Q) or SensiFAST SYBR Lo-ROX kit (Bioline) respectively. SYBR Green compatible primers were obtained from Eurofins Genomics, and the primer oligosaccharide sequences are detailed in the supplementary material (*Table S1*). Reactions were run in GeneAMP PCR System 9700 (Applied Biosystems). Each transcript value was calculated as the average of at least duplicate samples across experimental conditions. Values were normalised to β-actin as the housekeeping gene whose expression was stable under these experimental conditions. Data were analysed with the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) (28) and presented as a fold change vs. conventional control group, or in the case of the FOS-inulin study, fold change vs. the middle-aged control mice.

Statistical analysis

Data were analysed using one-way ANOVA followed by Bonferroni's test. A two-way ANOVA, with Bonferroni post hoc test for further analysis, was used to compare the effects of age and FOS-inulin on hepatic gene expression. The Grubbs method was employed to identify any outliers (29). The threshold for statistical significance was set at $p<0.05$. Data are expressed as mean +SEM. All statistical procedures were performed using GraphPad Prism Software 6.0 (GraphPad Prism, USA).

Results

Microbial colonisation significantly alters hepatic CYP and MDR expression in GF mice

The expression of murine CYP drug-metabolising enzymes, Cyp2b10 and Cyp3a11, was markedly downregulated under GF conditions relative to conventional mice ($p<0.001$; Figure 1 (A)). [**see Figure 1**]

We further investigated whether colonisation could restore the expression of these two CYP drug-metabolising enzymes in GF mice. At the transcript level, Cyp2b10 expression in GF mice did not recover after exposure to a microbial environment while the expression of Cyp3a11 was normalised to conventional levels. Colonisation exerted a similar influence on Cyp2a4 expression, but the effect was not significant.

Neither GF status nor colonisation altered the mRNA expression of Mdr1a (Figure 1(B)). The Mdr1b isoform was, however, upregulated in GF mice relative to conventional mice ($p<0.01$). Notably, colonisation of GF mice normalised Mdr1b expression to conventional levels. The direction and magnitude of the effect of the gut microbiota on host metabolism and transport are likely, therefore, to be specific not only to the hepatic gene but also to the isoform of that gene.

Butyrate alters Cyp2b10 expression only in the presence of a complex microbiota

Butyrate supplementation did not induce widespread changes in hepatic genes. In the presence of a complex microbiota, butyrate only had a significant effect on the hepatic expression of Cyp2b10 (2.85-fold higher relative to conventional mice; $p<0.05$). No significant differences were observed in the other CYP or MDR1 genes in conventional mice.

A secondary objective of the butyrate intervention study was to see if this microbial metabolite could restore the gene expression of the enzymes altered in GF mice. The mRNA expression of Cyp2b10 in GF mice, however, remained perturbed after butyrate supplementation relative to conventional mice (Figure 2(A)). Moreover, the expression of Cyp3a11 in GF mice also remained extensively downregulated after butyrate supplementation relative to the corresponding conventional group ($p<0.01$; $p<0.001$, respectively). Butyrate, however, exerted an inhibitory effect on the expression of MDR1 (Figure 2(B)). Butyrate decreased the expression of Mdr1a in GF mice ($p<0.05$) relative to the butyrate-treated conventional group, despite no evident changes in this isoform under GF conditions or by colonisation. Mdr1b expression remained marginally elevated, but not significantly so, after butyrate supplementation relative to conventional counterparts. [**see Figure 2**]

To assess if butyrate had a broader impact on the CYP superfamily of enzymes, the mRNA expression of members of the Cyp-2c, -2d, and -2e families was further investigated. Notably, the expression of these enzymes was not affected by butyrate supplementation, regardless of the microbial status of the mice (Table S2).

The impact of FOS-inulin on hepatic CYP and MDR expression is gene-specific and age-dependant

Subsequently, we assessed whether the hepatic expression of CYP and MDR1 genes could be manipulated by modulating the gut microbiota with a prebiotic mix in young adult versus middle-aged mice.

A significant interaction was identified between age and prebiotic in dictating the expression of Cyp2a4 ($p<0.05$; $F(1,34) = 4.216$) (Figure 3(A)). In Cyp2a4, age affected the response to

249 FOS-inulin; Cyp2a4 gene expression was significantly upregulated in young-adult treated
250 relative to middle-aged treated mice ($p<0.01$).

251 Age and FOS-inulin did not alter Cyp2b10 expression. As no significant difference was
252 evident in Cyp3a11 expression, the impact of diet-enriched with 10% FOS-inulin on the other
253 CYP3A4/5 equivalent mouse isoform, Cyp3a13, was also investigated. For both Cyp3a13
254 and Mdr1a, a significant interaction between age and prebiotic was observed [($p<0.05$; F
255 $(1,35 = 5.159)$, ($p<0.01$; $F(1,32) = 11.00$) respectively]. Bonferroni's multiple comparisons
256 test revealed a significant downregulation of Cyp3a13 in young adult mice ($p<0.05$) and the
257 prebiotic mix upregulated hepatic Mdr1a expression in young adults ($p<0.05$). As evident in
258 Figure 3(B), the prebiotic mix did not elicit a significant effect on Mdr1a in middle-aged
259 mice. Interestingly, the age-related impact on Mdr1a was opposite to the FOS-inulin induced
260 upregulation in young mice ($p<0.05$). Conversely, increasing age was coupled with
261 decreased Mdr1b expression ($p<0.05$). [**see Figure 3**]

Discussion

The implications of microbiome research for therapeutic interventions requires, in part, a mechanistic and predictive understanding of clinically-relevant microbiome-drug interactions (30, 31). Whilst most research to date on microbial-mediated metabolism of drugs largely centred around direct interactions between the drug substance and a microbe within the bacterial-dense colon (32), the research presented herein highlights the underappreciated indirect mechanisms by which the microbiota can dictate host metabolism in the liver. Here we further validated the modulation of CYP enzymes and MDR1 by the gut microbiome and illustrated the altered expression of hepatic genes in GF animals that can be rescued, in some cases, by colonisation. The overall impact of butyrate and prebiotic supplementation on host gene expression cannot be generalised. Butyrate and FOS-inulin only modify the hepatic expression of certain enzymes in a context and time-dependent manner. Neither intervention exerted a consistent effect across all enzymes and transporters investigated in this study. Given the gut microbiome is a complex ecosystem regularly exposed to a continually changing cocktail of small and large molecules (33), it is unlikely that a single metabolite, or prebiotic, could have a universal effect overall. There are likely to be a variety of pathways or metabolites involved in microbiome-host interactions that will contribute to inter-individual variability in drug metabolism and disposition. Our results may, however, provide the impetus to explore the potential of prebiotic supplementation to modify CYP and MDR1 expression in a clinical setting

Consistent with previous findings, GF conditions resulted in the most prominent changes in hepatic genes, most notably a downregulation in mRNAs of Cyp2b10 and Cyp3a11, and a substantial upregulation of Mdr1b. The colonisation of GF mice restored Cyp3a11 expression to conventional levels illustrating that Cyp3a11 may be particularly susceptible to changes in

286 the composition of the gut microbiota. This finding may have important clinical implications
287 as Cyp3a11 is the murine equivalent gene of hCYP3A4/5. In particular, the hCYP3A gene
288 family is responsible for the oxidation of approximately 50% of drugs (34). The normalized
289 Cyp3a11 gene expression in the livers of colonised GF mice is consistent with previous
290 studies using colonisation or secondary bile acid replacement approaches (8, 35, 36). In
291 contrast to others (8), however, GF status substantially reduced Cyp2b10 in our study.
292 Cyp2b10 is the murine equivalent gene of hCYP2B6, which is linked to the metabolism of
293 anaesthetics and analgesics (37). However, a more recent study, using RNA-sequencing, by
294 the same research group supported our finding of reduced Cyp2b10 in GF mice (38).

295 Our study is the first to demonstrate a clear role of the gut microbiome on drug transporters.
296 P-gp works in tandem with drug-metabolising enzymes, specifically CYP3A4/5, to reduce
297 the oral bioavailability of certain drug molecules, which are substrates of both genes (39).
298 Intestinal and hepatic drug transporters can dictate the amount of drug in the systemic
299 circulation by influencing drug absorption from the gut lumen or by facilitating the evasion of
300 drug metabolism on the first pass through the gut and liver. Factors affecting transporter
301 function or expression may, therefore, be important determinants of drug pharmacokinetics
302 (40). Our results illustrate that both murine isoforms of MDR1 are susceptible to microbiota-
303 related changes as evidenced by the induction of Mdr1b by GF conditions, or by the
304 inhibitory effect of butyrate on Mdr1a and Mdr1b. Previously, colonisation with *Bacteroides*
305 *thetaiotaomicron* downregulated Mdr1a expression in GF mice (41). Earlier research has also
306 indicated a sex-related food effect on the protein level of intestinal P-gp in rats (42). The
307 induction of Mdr1a expression by diet-enriched FOS-inulin in our study may provide further
308 insights into the dietary impact on host P-gp expression levels.

309 Overall, butyrate supplementation did not induce widespread changes in hepatic gene
310 expression. Butyrate supplementation did not cause extensive changes in hepatic genes of

conventional mice except for Cyp2b210. In the case of GF mice, transcript levels of Cyp2b10 remained downregulated even after butyrate supplementation, but this microbial metabolite had a significant inhibitory effect on Mdr1a expression in butyrate-treated GF mice relative to conventional counterparts. Butyrate-induced effects on hepatic genes, therefore, may depend on the microbial status of the host, highlighting the complexity of microbe-liver interactions, and the difficulty in extrapolating from GF animals to those with a conventional microbiota. Future studies employing a longer duration of butyrate supplementation or investigating the effect of alternative SCFAs (e.g., acetate, propionate) or a combination of SCFAs, may provide further mechanistic insight into the role SCFAs play in microbiome-influenced host gene expression. Indeed, investigating the impact of different microbial metabolites, such as tryptophan and bile acids, on hepatic CYP expression may help to further delineate the molecular underpinnings of this host-microbe interaction. Moreover, the microbial regulation of the hepatic transcriptome has been linked to the circadian oscillations of serum metabolites which can affect the detoxification pattern in the liver (43), therefore, the impact of microbial metabolites at different times of the day also merits consideration.

Fermentation of fibre is one of the primary sources of SCFAs. Diet-derived butyrate must also be considered in terms of experimental design as it may have implications for butyrate-mediated physiological functions (44), albeit dietary sources may, however, be more important in small intestine where bacterial fermentation is lowest (45). Through regular monitoring of food intake across the butyrate-supplemented and non-treated groups, we confirmed no differences in the potential dietary sources of butyrate across all groups. Previous research has illustrated that the majority of SCFAs in the gut come from bacterial fermentation as has been reported previously with levels of 1020 mumol/kg in caecum of Norwegian GF mice vs levels of 124,600 mumol/kg in the caecum of conventional mice (45). Recently, our group illustrated that supplementation with the prebiotic mix, FOS-inulin,

altered propionate, and valerate levels in the caecum (21), further substantiating previous links between SCFAs and prebiotics (46-48). Our results suggest FOS-inulin-induced effects on hepatic gene expression are specific to the gene isoform. This prebiotic mix significantly altered Cyp3a13 and Mdr1a expression in the liver of young adult mice but exerted no influence on the Cyp3a11 or Mdr1b gene isoforms. Overall, FOS-inulin supplementation for 14 weeks did not translate to marked differences in the expression of hepatic genes in conventional animals. Previously, a one-month treatment with a cocktail of probiotics, VSL#3, was also found insufficient to alter the hepatic expression of many drug-metabolising genes (8). It is plausible that microbiota-targeted interventions, including prebiotics and probiotics, may require extended chronic treatment to elicit more extensive changes in metabolic pathways under healthy or naïve conditions or that the effects may be contingent on the host, such as age or gender.

As age is a well-established influential factor for drug metabolism capacity (4, 49-51), we, therefore, sought to explore whether the response to prebiotics was age-dependant. Increasing age is associated with an approximate 40-45% downregulation of detoxification enzymes (34). In this study, the specific life-stages of young adult and middle-aged were chosen to examine if the response to FOS-inulin depended on the age of the host while avoiding the confounding effect of old age-related decline in hepatic function (52). Like the prebiotic-induced effects, age significantly modified the expression of CYP and MDR1 isoforms in an isoform-specific manner. Moreover, age dictated the impact of prebiotics on Cyp2a4, suggesting that age-related changes in hepatic CYP isoforms may influence the efficacy and safety of drugs. However, the effects of ageing on the expression and activity of CYP enzymes in humans remains controversial due to the many confounding factors, including concomitant diseases and personal medical history.

Overall, these results lend further support to the role the gut microbiota plays on host drug metabolism. To our knowledge, this study provides the first evidence on the influence the gut microbiota exerts on a drug-efflux transporter gene, MDR1. Having identified current gaps in our understanding of the mechanistic basis for these microbiome-liver interactions, the impact of butyrate supplementation on a much broader range of host drug-metabolising enzymes and transporters was investigated, extending to previous work on butyrate-induced changes specific to the Cyp1a family (14, 15). A limitation of the study herein is that data obtained on the mRNA level only hints on a general pattern of expression, and future studies should now focus on protein levels and enzyme activity to confirm the microbial regulation of these hepatic genes implicated in drug metabolism and transport. Herein, butyrate did not exert an extensive impact on a range of hepatic genes and research efforts may need to be shifted towards alternative microbial metabolites. Nonetheless, the study herein represents an important stepping stone for further studies exploring the microbiome-liver crosstalk. Furthermore, there is still uncertainty concerning the existence of species differences in genes implicated in drug metabolism and transport (27), and thus, there is a requirement for more studies in this area to establish a sound basis for correlation of preclinical studies to clinical research (5).

Conclusion

This data further strengthens the increasing body of evidence linking the gut microbiota as a modulator of host gene expression, specifically in influencing hepatic enzymes involved in drug metabolism and disposition. Not only may the gut microbiota alter how the host metabolises drugs but may, through the modified efflux process from the liver to the bile duct, also influence the distribution and elimination process of drugs. On a mechanistic level, it appears the microbial metabolite butyrate is not singularly involved in mediating these effects on host metabolism and transport. Butyrate-induced effects on CYP and P-gp expression are gene-specific and, even in some cases, dependent on the specific isoform of the gene, as evidenced by its impact on Cyp2b10 and MDR1 isoforms, respectively. Further studies are required to elucidate microbiota-induced changes in host gene expression at the protein level and to unravel the mechanistic basis for this crosstalk between the gut microbiome and the liver, including the impact of other SCFAs or different microbial metabolites such as tryptophan. Furthermore, prebiotic supplementation modulates host gene expression and may play a role in normalising metabolic activity or reducing inter-individual variability in drug pharmacokinetics.

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References

1. Bleasby K, Castle JC, Roberts CJ, Cheng C, Bailey WJ, Sina JF, et al. Expression profiles of 50 xenobiotic transporter genes in humans and pre-clinical species: a resource for investigations into drug disposition. Xenobiotica. 2006;36(10-11):963-88.
2. Miller DS, Bauer B, Hartz AMS. Modulation of P-glycoprotein at the blood-brain barrier: opportunities to improve central nervous system pharmacotherapy. Pharmacological reviews. 2008;60(2):196-209.
3. Marquez B, Van Bambeke F. ABC multidrug transporters: target for modulation of drug pharmacokinetics and drug-drug interactions. Curr Drug Targets. 2011;12(5):600-20.
4. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & Therapeutics. 2013;138(1):103-41.
5. Sadiq MW, Uchida Y, Hoshi Y, Tachikawa M, Terasaki T, Hammarlund-Udenaes M. Validation of a P-Glycoprotein (P-gp) Humanized Mouse Model by Integrating Selective Absolute Quantification of Human MDR1, Mouse Mdr1a and Mdr1b Protein Expressions with In Vivo Functional Analysis for Blood-Brain Barrier Transport. 2015;10(5):e0118638.
6. Brinkmann U, Eichelbaum M. Polymorphisms in the ABC drug transporter gene MDR1. The Pharmacogenomics Journal. 2001;1(1):59-64.
7. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
8. Selwyn FP, Cheng SL, Klaassen CD, Cui JY. Regulation of Hepatic Drug-Metabolizing Enzymes in Germ-Free Mice by Conventionalization and Probiotics. Drug Metab Dispos. 2016;44(2):262-74.
9. Selwyn FP, Cui JY, Klaassen CD. RNA-Seq Quantification of Hepatic Drug Processing Genes in Germ-Free Mice. Drug Metab Dispos. 2015;43(10):1572-80.
10. Björkholm B, Bok CM, Lundin A, Rafter J, Hibberd ML, Pettersson S. Intestinal microbiota regulate xenobiotic metabolism in the liver. PloS one. 2009;4(9):e6958-e.
11. Swanson HI. Drug Metabolism by the Host and Gut Microbiota: A Partnership or Rivalry? Drug Metabolism and Disposition. 2015;43(10):1499-504.
12. Son G, Kremer M, Hines IN. Contribution of Gut Bacteria to Liver Pathobiology. Gastroenterology Research and Practice. 2010;2010:453563.
13. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 1987;28(10):1221-7.
14. Matis G, Neogrady Z, Csiko G, Kulcsar A, Kenez A, Huber K. Effects of orally applied butyrate bolus on histone acetylation and cytochrome P450 enzyme activity in the liver of chicken - a randomized controlled trial. Nutr Metab (Lond). 2013;10(1):12.
15. Matis G, Gyorgý C, Katalí J, Zsuzsanna V, Fébel H, Anna K, et al. Investigation of the effect of butyrate supplementation of the diet on hepatic cytochrome P450 enzymes in rats. Magyar Allatorvosok Lapja. 2013;135:109-16.
16. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology &Amp; Hepatology. 2014;11:506.
17. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nature Reviews Gastroenterology & Hepatology. 2017;14(8):491-502.

18. Nichols RG, Peters JM, Patterson AD. Interplay Between the Host, the Human Microbiome, and Drug Metabolism. Human Genomics. 2019;13(1).
19. Sugatani J, Sadamitsu S, Wada T, Yamazaki Y, Ikari A, Miwa M. Effects of dietary inulin, statin, and their co-treatment on hyperlipidemia, hepatic steatosis and changes in drug-metabolizing enzymes in rats fed a high-fat and high-sucrose diet. Nutr Metab (Lond). 2012;9(1):23.
20. Carlson JL, Erickson JM, Lloyd BB, Slavin JL. Health Effects and Sources of Prebiotic Dietary Fiber. Current developments in nutrition. 2018;2(3):nzy005-nzy.
21. Boehme M, van de Wouw M, Bastiaanssen TFS, Olavarría-Ramírez L, Lyons K, Fouhy F, et al. Mid-life microbiota crises: middle age is associated with pervasive neuroimmune alterations that are reversed by targeting the gut microbiome. Molecular Psychiatry. 2019.
22. Hoban AE, Stilling RM, Moloney G, Shanahan F, Dinan TG, Clarke G, et al. The microbiome regulates amygdala-dependent fear recall. Molecular Psychiatry. 2018;23(5):1134-44.
23. Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci. 2015;18(7):965-77.
24. Stilling RM, van de Wouw M, Clarke G, Stanton C, Dinan TG, Cryan JF. The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? Neurochem Int. 2016;99:110-32.
25. Van De Wouw M, Boehme M, Lyte JM, Wiley N, Strain C, O'Sullivan O, et al. Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain-gut axis alterations. The Journal of Physiology. 2018;596(20):4923-44.
26. Cui YJ, Cheng X, Weaver YM, Klaassen CD. Tissue Distribution, Gender-Divergent Expression, Ontogeny, and Chemical Induction of Multidrug Resistance Transporter Genes (Mdr1a, Mdr1b, Mdr2) in Mice. Drug Metabolism and Disposition. 2009;37(1):203-10.
27. Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol. 2006;2(6):875-94.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
29. Grubbs FE. Sample criteria for testing outlying observations. Annals of Mathematical Statistics. 1950;21(1):27-58.
30. Walsh J, Griffin BT, Clarke G, Hyland NP. Drug–gut microbiota interactions: implications for neuropharmacology. British Journal of Pharmacology. 2018;175(24):4415-29.
31. Spanogiannopoulos P, Bess EN, Carmody RN, Turnbaugh PJ. The microbial pharmacists within us: a metagenomic view of xenobiotic metabolism. Nat Rev Microbiol. 2016;14(5):273-87.
32. Sousa T, Paterson R, Moore V, Carlsson A, Abrahamsson B, Basit AW. The gastrointestinal microbiota as a site for the biotransformation of drugs. International Journal of Pharmaceutics. 2008;363(1):1-25.
33. Pellock SJ, Redinbo MR. Glucuronides in the gut: Sugar-driven symbioses between microbe and host. J Biol Chem. 2017;292(21):8569-76.
34. Fu ZD, Selwyn FP, Cui JY, Klaassen CD. RNA Sequencing Quantification of Xenobiotic-Processing Genes in Various Sections of the Intestine in Comparison to the Liver of Male Mice. Drug metabolism and disposition: the biological fate of chemicals. 2016;44(6):842-56.

513 35. Toda T, Saito N, Ikarashi N, Ito K, Yamamoto M, Ishige A, et al. Intestinal flora
514 induces the expression of Cyp3a in the mouse liver. Xenobiotica. 2009;39(4):323-34.
515 36. Claus SP, Ellero SL, Berger B, Krause L, Bruttin A, Molina J, et al. Colonization-
516 induced host-gut microbial metabolic interaction. MBio. 2011;2(2):e00271-10.
517 37. Hedrich WD, Hassan HE, Wang H. Insights into CYP2B6-mediated drug-drug
518 interactions. Acta pharmaceutica Sinica B. 2016;6(5):413-25.
519 38. Fu ZD, Selwyn FP, Cui JY, Klaassen CD. RNA-Seq Profiling of Intestinal Expression
520 of Xenobiotic Processing Genes in Germ-Free Mice. Drug Metabolism and Disposition.
521 2017:dmd.117.077313.
522 39. Shugarts S, Benet LZ. The role of transporters in the pharmacokinetics of orally
523 administered drugs. Pharmaceutical research. 2009;26(9):2039-54.
524 40. Zhang Y, Benet LZ. The gut as a barrier to drug absorption: combined role of
525 cytochrome P450 3A and P-glycoprotein. Clin Pharmacokinet. 2001;40(3):159-68.
526 41. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular
527 Analysis of Commensal Host-Microbial Relationships in the Intestine. Science.
528 2001;291(5505):881.
529 42. Dou L, Mai Y, Madla CM, Orlu M, Basit AW. P-glycoprotein expression in the
530 gastrointestinal tract of male and female rats is influenced differently by food. European
531 Journal of Pharmaceutical Sciences. 2018;123:569-75.
532 43. Thaiss CA, Levy M, Korem T, Dohnalová L, Shapiro H, Jaitin DA, et al. Microbiota
533 Diurnal Rhythmicity Programs Host Transcriptome Oscillations. Cell. 2016;167(6):1495-
534 510.e12.
535 44. Sakata T. Pitfalls in short-chain fatty acid research: A methodological review. Animal
536 Science Journal. 2019;90(1):3-13.
537 45. Høverstad T, Midtvedt T. Short-Chain Fatty Acids in Germfree Mice and Rats. The
538 Journal of Nutrition. 1986;116(9):1772-6.
539 46. Mistry RH, Gu F, Schols HA, Verkade HJ, Tietge UJF. Effect of the prebiotic fiber
540 inulin on cholesterol metabolism in wildtype mice. Scientific Reports. 2018;8(1):13238.
541 47. Yang J, Martinez I, Walter J, Keshavarzian A, Rose DJ. In vitro characterization of
542 the impact of selected dietary fibers on fecal microbiota composition and short chain fatty
543 acid production. Anaerobe. 2013;23:74-81.
544 48. Baxter NT, Schmidt AW, Venkataraman A, Kim KS, Waldron C, Schmidt TM.
545 Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary
546 Interventions with Three Fermentable Fibers. MBio. 2019;10(1).
547 49. Lawrence SM, Corriden R, Nizet V. Age-Appropriate Functions and Dysfunctions of
548 the Neonatal Neutrophil. Frontiers in pediatrics. 2017;5:23-.
549 50. Xu S-F, Hu A-L, Xie L, Liu J-J, Wu Q, Liu J. Age-associated changes of cytochrome
550 P450 and related phase-2 gene/proteins in livers of rats. PeerJ. 2019;7:e7429-e.
551 51. Yun KU, Oh SJ, Oh JM, Kang KW, Myung CS, Song GY, et al. Age-related changes
552 in hepatic expression and activity of cytochrome P450 in male rats. Arch Toxicol.
553 2010;84(12):939-46.
554 52. Woodhouse K, Wynne HA. Age-related changes in hepatic function. Implications for
555 drug therapy. Drugs Aging. 1992;2(3):243-55.
556 53. Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW.
557 Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes,
558 including nomenclature recommendations for genes, pseudogenes and alternative-splice
559 variants. Pharmacogenetics. 2004;14(1):1-18.
560 54. Bandiera EGHaSM. Expression, Function and Regulation of Mouse Cytochrome
561 P450 Enzymes: Comparison With Human Cytochrome P450 Enzymes. Current Drug
562 Metabolism. 2009;10(10):1151-83.

Tables

Table 1. Overview of the human equivalent mouse CYP enzymes. The previously identified murine Cyps most similar or equivalent to human CYP enzymes, and examples of corresponding substrate drugs are illustrated. (a)www.drugbank.ca/drugs.

Gene (mouse)	**Gene (human)**	**Substrate Drugs**	**References**
Cyp1a2	CYP1A2	Chlorpromazine, Amitriptyline, Zolmitriptan	(4, 53, 54) (a)
Cyp2a4	CYP2A6	Letrozole, Nicotine, Nifedipine	
Cyp2b10	CYP2B6	Ketamine, Selegiline, Methadone	
Cyp3a11	CYP3A4/5	Clarithromycin, Citalopram, Alprazolam, Morphine	
Cyp3a13			
Mdr1a	MDR 1	Digoxin, Verapamil, Domperidone, Ranitidine (Strong overlap with CYP3A4/5 substrates)	

Figure Legends

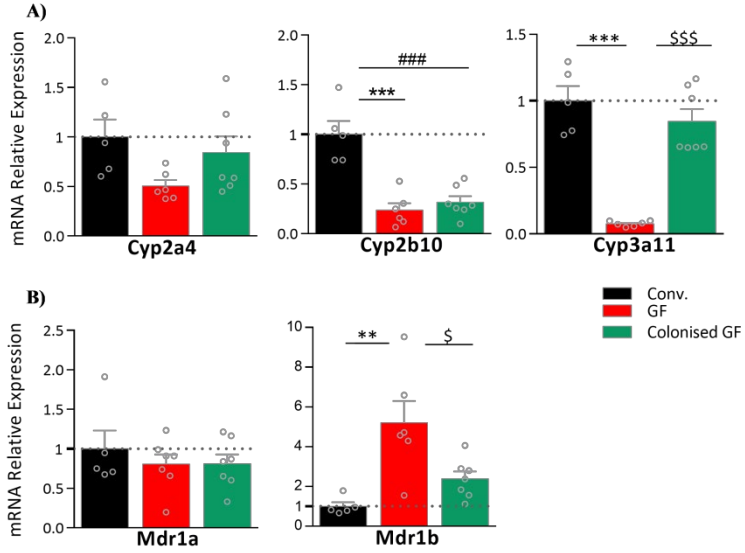


Figure 1. Microbial status alters mRNA expression of hepatic genes. (A) Relative mRNA expression of CYP450 drug-metabolising genes in the livers of germ-free (GF), colonised GF, and conventionally raised C57BL/6 mice. **(B)** Relative mRNA expression of two murine isoforms of hMDR1, Mdr1a, and Mdr1b, in the livers of GF, colonised GF, and conventionally raised C57BL/6 mice. Data analysed by one-way ANOVA with Bonferronis' multiple comparisons test and represented as mean + SEM (n=5-6). (* Conv. vs GF; # Conv. vs GF colonised; $ GF vs GF colonised; $ = p<0.05; **, p<0.01; ###, p<0.001; n=5-6/group).

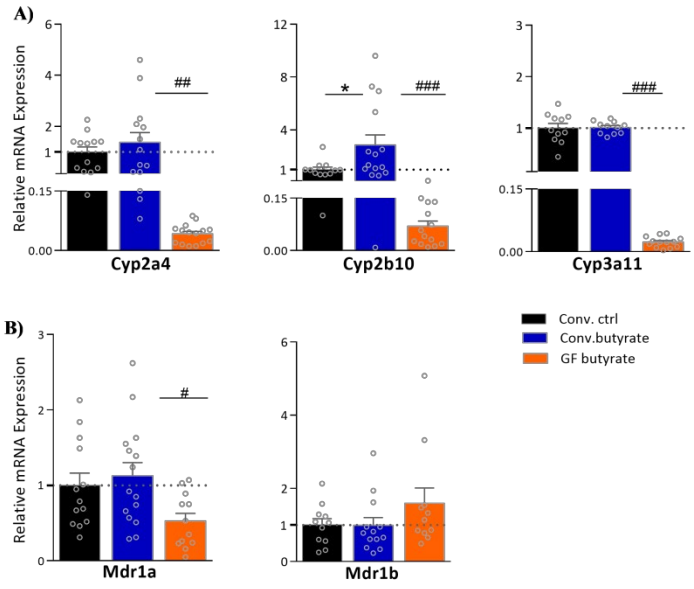
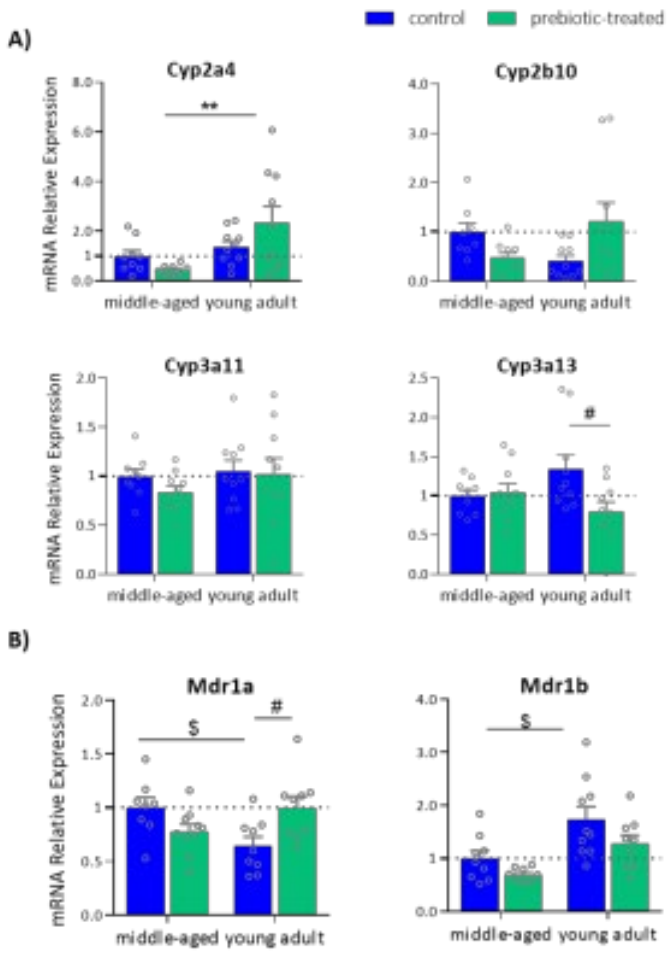


Figure 2. Impact of butyrate supplementation on hepatic genes. Relative mRNA expression of murine hepatic **(A)** CYP isoenzymes and **(B)** MDR1 transporter in conventionally raised and GF mice supplemented with sodium butyrate or sodium-matched saline (n=12-15/group). Data analysed by one-way ANOVA with Bonferronis' multiple comparisons test and represented as mean + SEM. * p<0.05; ##, p<0.01; ###, p<0.001; Conv, conventionally raised; GF, germ-free.



583

584 **Figure 3. FOS-inulin impact on hepatic gene expression**. Relative mRNA expression of
585 murine hepatic **(A)** CYP isoenzymes and **(B)** MDR1 transporter respectively in young and
586 middle-aged conventionally raised male mice receiving chow supplemented with FOS-inulin
587 or standard chow. Data analysed by two-way ANOVA and Bonferroni's multiple
588 comparisons test. Data represented as mean + SEM (n=9-10). (#or $, $p<0.05$; **, $p<0.01$).
589 n=9-10/group.