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

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30 Nonstandard Abbreviations

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

35 *Conflict of interest*

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Wellness. JFC, TGD & GC have spoken at meetings sponsored by food and pharmaceutical
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42 Abstract

43 *Objectives:* Our objective was to demonstrate microbial regulation of hepatic genes 44 implicated in drug metabolism and transport using germ-free (GF) mice and to explore the 45 impact of a microbial metabolite, butyrate, and a prebiotic dietary intervention on hepatic 46 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).</p>

57 *Conclusion:* The expression of hepatic genes implicated in drug metabolism and transport 58 displays sensitivity to the microbiome, microbiome-derived metabolites, and a microbial-59 targeted intervention. Our study may provide the impetus to explore microbiota-targeted 60 interventions in normalising host metabolic activity and reducing inter-individual variability 61 in drug pharmacokinetics.

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

63 Introduction

64 The metabolic fate and toxicity of drugs are determined, in part, by the expression of drug-65 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 66 67 (2). Drug-efflux transporters, including multidrug-resistance protein 1 (MDR1), expel 68 conjugated drugs from the liver into the bile ducts and thus make an essential contribution to 69 drug pharmacokinetics (3). Significantly, CYP1-3 family members are implicated in the 70 metabolism of 70-80% of all drugs in clinical use (4) and MDR1, also known as P-71 glycoprotein (P-gp), is an efflux pump with broad substrate specificity (2). While humans 72 express a single MDR1 gene, rodents share the function of hepatic MDR1 between two highly homologous MDR1-type genes, Mdr1a and Mdr1b (5, 6). 73

74 Inter-individual variability in the expression of CYP genes is generally linked to age, race, 75 genetics, concomitant disease, or co-administered drugs (4). However, the importance of the 76 gut microbiota, the trillions of micro-organisms residing along the gastrointestinal tract (7), 77 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 78 79 hepatic genes implicated in drug metabolism (8-10). The drug-metabolising capacity of an 80 individual may vary, therefore, not only because of polymorphisms in genes encoding host 81 drug-metabolising enzymes and the concomitant intake of drugs but also due to individual 82 differences in the composition of the gut microbiota. This interconnectivity between the 83 intestinal tract and the liver makes it essential to view drug metabolic processes as co-84 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.

92 The impact of microbiota-targeted therapies, including antibiotics, probiotics (i.e., "live 93 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 94 95 microorganisms conferring a health benefit" (17)) on CYP and MDR1, and their potential 96 knock-on effects on the response to co-administered medication (18), is a significant but 97 underexplored area of drug metabolism. While there are several reports that a change in 98 nutritional status affects hepatic levels of drug-metabolising enzymes (19), a commercially 99 available probiotic mix, VSL#3, exerted a limited effect on CYP gene expression (8). 100 Modulation of intestinal microbes by prebiotics may also, however, alter the drug-101 metabolising capacity of the host. Foods such as onions, leeks, and garlic are dietary sources 102 of the prebiotic inulin (20), which protects against high-fat diet-induced alterations in both 103 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.

110 Materials and Methods

111

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.

119

120 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 $55 \pm 10\%$. 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.

128

129 GF/Colonisation Study

At postnatal day 21, a subset of GF mice were transferred to the conventional animal facilityand 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 °C until further analysis.

134 Butyrate Study

135 Sodium butyrate (3 g/L; Sigma-Aldrich), or sodium chloride for sodium-matched controls, 136 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 137 138 research group and others investigating the impact of butyrate (600 mg/kg) on behaviour in 139 mice, combined with an estimated drinking water consumption of 5 ml/day (23-25). Drinking 140 water was filtered through a 0.2-micron syringe filter (Sarstedt) and refreshed twice per week. 141 As diet can contribute to the gastrointestinal and systemic levels of butyrate in vivo, food 142 intake was closely monitored across all experimental groups and no significant differences in 143 food consumption were observed. Mice were euthanised by decapitation, and liver samples 144 were immediately snap-frozen and stored at -80 °C until further analysis.

145

146 FOS-Inulin Study

147 Previous work by our laboratory investigated prebiotic supplementation (FOS-Inulin) on the 148 peripheral immune response and neuroinflammation in middle age (21). Here, we sought to 149 examine the effects of prebiotic supplementation on hepatic gene expression from tissues 150 collected from the same animals. In brief, young adult (approx. 2 months at start of treatment) 151 and middle-aged (approx. 10 months at start of treatment) conventional male C57BL/6 mice 152 (obtained from Harlan, Cambridgeshire) received a standard diet (ssniff-Spezialdiäten 153 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; 154 155 BENEO-Orafti N.V., Belgium) for 14weeks (n=9-10/group). Mice were euthanised by

decapitation, and liver samples were immediately snap-frozen and stored at -80 °C until
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 (Sigma Aldrich) following the manufacturer's protocol or using the mirVanaTM miRNA 161 Isolation Kit (Thermo Scientific/Invitrogen; GF/Colonisation study). Tissue from the 162 163 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 OD260/280 method using a Nanodrop spectrophotometer (Thermo Scientific). The 167 168 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 Exigon cDNA Universal Synthesis kit (Exiqon A/Q) or High Capacity cDNA Reverse Transcription kit (Thermo 170 171 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 drug173 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]

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

180 For the GF/colonisation study, RT-qPCR was performed using TaqMan Universal Master 181 Mix II (Thermo Fisher Scientific/Applied Biosystems), and genes of interest were amplified 182 using TaqMan probes (Integrated DNA Technologies). For the RT-qPCRs from the butyrate-183 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 184 185 (Bioline) respectively. SYBR Green compatible primers were obtained from Eurofins 186 Genomics, and the primer oligosaccharide sequences are detailed in the supplementary 187 material (Table SI). Reactions were run in GeneAMP PCR System 9700 (Applied 188 Biosystems). Each transcript value was calculated as the average of at least duplicate samples 189 across experimental conditions. Values were normalised to β -actin as the housekeeping gene 190 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. 191 192 conventional control group, or in the case of the FOS-inulin study, fold change vs. the 193 middle-aged control mice.

194

195 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). 202 **Results**

203

204 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.

219

220 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. 225 A secondary objective of the butyrate intervention study was to see if this microbial 226 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 227 228 supplementation relative to conventional mice (Figure 2(A)). Moreover, the expression of Cyp3a11 in GF mice also remained extensively downregulated after butyrate 229 230 supplementation relative to the corresponding conventional group (p<0.01; p<0.001, 231 respectively). Butyrate, however, exerted an inhibitory effect on the expression of MDR1 232 (Figure 2(B)). Butyrate decreased the expression of Mdr1a in GF mice (p<0.05) relative to 233 the butyrate-treated conventional group, despite no evident changes in this isoform under GF 234 conditions or by colonisation. Mdr1b expression remained marginally elevated, but not 235 significantly so, after butyrate supplementation relative to conventional counterparts. [see 236 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).

241

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

FOS-inulin; Cyp2a4 gene expression was significantly upregulated in young-adult treated 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]

262 **Discussion**

263 The implications of microbiome research for therapeutic interventions requires, in part, a mechanistic and predictive understanding of clinically-relevant microbiome-drug interactions 264 265 (30, 31). Whilst most research to date on microbial-mediated metabolism of drugs largely 266 centred around direct interactions between the drug substance and a microbe within the 267 bacterial-dense colon (32), the research presented herein highlights the underappreciated 268 indirect mechanisms by which the microbiota can dictate host metabolism in the liver. Here 269 we further validated the modulation of CYP enzymes and MDR1 by the gut microbiome and 270 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 271 272 gene expression cannot be generalised. Butyrate and FOS-inulin only modify the hepatic 273 expression of certain enzymes in a context and time-dependent manner. Neither intervention 274 exerted a consistent effect across all enzymes and transporters investigated in this study. 275 Given the gut microbiome is a complex ecosystem regularly exposed to a continually 276 changing cocktail of small and large molecules (33), it is unlikely that a single metabolite, or 277 prebiotic, could have a universal effect overall. There are likely to be a variety of pathways or 278 metabolites involved in microbiome-host interactions that will contribute to inter-individual 279 variability in drug metabolism and disposition. Our results may, however, provide the 280 impetus to explore the potential of prebiotic supplementation to modify CYP and MDR1 281 expression in a clinical setting

282 Consistent with previous findings, GF conditions resulted in the most prominent changes in 283 hepatic genes, most notably a downregulation in mRNAs of Cyp2b10 and Cyp3a11, and a 284 substantial upregulation of Mdr1b. The colonisation of GF mice restored Cyp3a11 expression 285 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. P-gp works in tandem with drug-metabolising enzymes, specifically CYP3A4/5, to reduce 296 297 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 gene310 expression. Butyrate supplementation did not cause extensive changes in hepatic genes of

311 conventional mice except for Cyp2b210. In the case of GF mice, transcript levels of Cyp2b10 312 remained downregulated even after butyrate supplementation, but this microbial metabolite 313 had a significant inhibitory effect on Mdr1a expression in butyrate-treated GF mice relative 314 to conventional counterparts. Butyrate-induced effects on hepatic genes, therefore, may 315 depend on the microbial status of the host, highlighting the complexity of microbe-liver 316 interactions, and the difficulty in extrapolating from GF animals to those with a conventional 317 microbiota. Future studies employing a longer duration of butyrate supplementation or 318 investigating the effect of alternative SCFAs (e.g., acetate, propionate) or a combination of 319 SCFAs, may provide further mechanistic insight into the role SCFAs play in microbiome-320 influenced host gene expression. Indeed, investigating the impact of different microbial 321 metabolites, such as tryptophan and bile acids, on hepatic CYP expression may help to 322 further delineate the molecular underpinnings of this host-microbe interaction. Moreover, the 323 microbial regulation of the hepatic transcriptome has been linked to the circadian oscillations 324 of serum metabolites which can affect the detoxification pattern in the liver (43), therefore, 325 the impact of microbial metabolites at different times of the day also merits consideration.

326 Fermentation of fibre is one of the primary sources of SCFAs. Diet-derived butyrate must 327 also be considered in terms of experimental design as it may have implications for butyrate-328 mediated physiological functions (44), albeit dietary sources may, however, be more 329 important in small intestine where bacterial fermentation is lowest (45). Through regular 330 monitoring of food intake across the butyrate-supplemented and non-treated groups, we 331 confirmed no differences in the potential dietary sources of butyrate across all groups. 332 Previous research has illustrated that the majority of SCFAs in the gut come from bacterial 333 fermentation as has been reported previously with levels of 1020 mumol/kg in caecum of 334 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, 335

336 altered propionate, and valerate levels in the caecum (21), further substantiating previous 337 links between SCFAs and prebiotics (46-48). Our results suggest FOS-inulin-induced effects 338 on hepatic gene expression are specific to the gene isoform. This prebiotic mix significantly 339 altered Cyp3a13 and Mdr1a expression in the liver of young adult mice but exerted no 340 influence on the Cyp3a11 or Mdr1b gene isoforms. Overall, FOS-inulin supplementation for 341 14 weeks did not translate to marked differences in the expression of hepatic genes in 342 conventional animals. Previously, a one-month treatment with a cocktail of probiotics, 343 VSL#3, was also found insufficient to alter the hepatic expression of many drug-metabolising 344 genes (8). It is plausible that microbiota-targeted interventions, including prebiotics and 345 probiotics, may require extended chronic treatment to elicit more extensive changes in 346 metabolic pathways under healthy or naïve conditions or that the effects may be contingent 347 on the host, such as age or gender.

348 As age is a well-established influential factor for drug metabolism capacity (4, 49-51), we, 349 therefore, sought to explore whether the response to prebiotics was age-dependant. Increasing 350 age is associated with an approximate 40-45% downregulation of detoxification enzymes 351 (34). In this study, the specific life-stages of young adult and middle-aged were chosen to 352 examine if the response to FOS-inulin depended on the age of the host while avoiding the 353 confounding effect of old age-related decline in hepatic function (52). Like the prebiotic-354 induced effects, age significantly modified the expression of CYP and MDR1 isoforms in an 355 isoform-specific manner. Moreover, age dictated the impact of prebiotics on Cyp2a4, 356 suggesting that age-related changes in hepatic CYP isoforms may influence the efficacy and 357 safety of drugs. However, the effects of ageing on the expression and activity of CYP 358 enzymes in humans remains controversial due to the many confounding factors, including 359 concomitant diseases and personal medical history.

360 Overall, these results lend further support to the role the gut microbiota plays on host drug 361 metabolism. To our knowledge, this study provides the first evidence on the influence the gut 362 microbiota exerts on a drug-efflux transporter gene, MDR1. Having identified current gaps in 363 our understanding of the mechanistic basis for these microbiome-liver interactions, the 364 impact of butyrate supplementation on a much broader range of host drug-metabolising 365 enzymes and transporters was investigated, extending to previous work on butyrate-induced 366 changes specific to the Cyp1a family (14, 15). A limitation of the study herein is that data 367 obtained on the mRNA level only hints on a general pattern of expression, and future studies 368 should now focus on protein levels and enzyme activity to confirm the microbial regulation 369 of these hepatic genes implicated in drug metabolism and transport. Herein, butyrate did not 370 exert an extensive impact on a range of hepatic genes and research efforts may need to be 371 shifted towards alternative microbial metabolites. Nonetheless, the study herein represents an 372 important stepping stone for further studies exploring the microbiome-liver crosstalk. 373 Furthermore, there is still uncertainty concerning the existence of species differences in genes 374 implicated in drug metabolism and transport (27), and thus, there is a requirement for more 375 studies in this area to establish a sound basis for correlation of preclinical studies to clinical 376 research (5).

377 **Conclusion**

378

379 This data further strengthens the increasing body of evidence linking the gut microbiota as a 380 modulator of host gene expression, specifically in influencing hepatic enzymes involved in 381 drug metabolism and disposition. Not only may the gut microbiota alter how the host 382 metabolises drugs but may, through the modified efflux process from the liver to the bile 383 duct, also influence the distribution and elimination process of drugs. On a mechanistic level, 384 it appears the microbial metabolite butyrate is not singularly involved in mediating these 385 effects on host metabolism and transport. Butyrate-induced effects on CYP and P-gp 386 expression are gene-specific and, even in some cases, dependent on the specific isoform of 387 the gene, as evidenced by its impact on Cyp2b10 and MDR1 isoforms, respectively. Further 388 studies are required to elucidate microbiota-induced changes in host gene expression at the 389 protein level and to unravel the mechanistic basis for this crosstalk between the gut 390 microbiome and the liver, including the impact of other SCFAs or different microbial 391 metabolites such as tryptophan. Furthermore, prebiotic supplementation modulates host gene 392 expression and may play a role in normalising metabolic activity or reducing inter-individual 393 variability in drug pharmacokinetics.

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Tables

563 **Table 1. Overview of the human equivalent mouse CYP enzymes.** The previously 564 identified murine Cyps most similar or equivalent to human CYP enzymes, and examples of 565 corresponding substrate drugs are illustrated. (a)www.drugbank.ca/drugs.

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

Figure Legends



567

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).



575

576 Figure 2. Impact of butyrate supplementation on hepatic genes. Relative mRNA 577 expression of murine hepatic (A) CYP isoenzymes and (B) MDR1 transporter in 578 conventionally raised and GF mice supplemented with sodium butyrate or sodium-matched 579 saline (n=12-15/group). Data analysed by one-way ANOVA with Bonferronis' multiple 580 comparisons test and represented as mean + SEM. * p<0.05; ##, p<0.01; ###, p<0.001; Conv, 581 conventionally raised; GF, germ-free.



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