INTRODUCTION

The gut microbiota plays a key role in maintaining host health through many aspects.1–3 Drug metabolism is also regarded to be influenced by the gut microbiota.4,5 Ample studies demonstrated the link between the gut microbiota and the hepatic activity of cytochrome P450 (Cyp).6–8 A major drug-metabolizing enzyme. In particular, previous papers comparing germ-free (GF) and specific pathogen-free

**Abstract**

Several studies revealed that substantial artificial changes in the gut microbiota resulted in modification of hepatic cytochrome P450 3a (Cyp3a) in mice. Consequently, we hypothesized that "normal" variation of the gut microbiota might also alter hepatic Cyp activity and lead to individual differences in drug metabolism. Therefore, this study investigated the effects of normal gut microbiota variation on hepatic Cyp activity under the same genetic and environmental conditions using ex-germ-free mice. Using the feces of three breeder BALB/c mice (Jcl, Slc, and Crj), germ-free BALB/cYit mice were conventionalized (Yit-Jcl, Yit-Slc, and Yit-Crj). The gut microbiota composition and hepatic Cyp activity of these donors and recipients were evaluated. 16S rRNA sequencing revealed clear differences of the gut microbiota among donors and among recipients. Cyp3a activity was significantly higher in Slc mice than in Jcl and Crj mice. Notably, among recipients, Cyp3a activity was significantly higher in Yit-Slc and Yit-Crj mice than in Yit-Jcl mice. Cyp2b activity was significantly higher in Slc mice than in Jcl and Crj mice. Cyp2b activity was significantly higher in Yit-Slc mice than in Yit-Jcl mice. Additionally, in correlation analysis, some genera displayed significant positive or negative correlations with Cyp activity, particular the strong positive correlation between *Clostridium sensu stricto 1* with Cyp3a activity. In conclusion, this study demonstrated that normal variation of the gut microbiota affected hepatic Cyp3a and Cyp2b activity, which might result in individual differences of drug metabolism.

**KEYWORDS**

16S rRNA gene, cytochrome P450, drug metabolism, intestinal flora, normalization
obtained from the breeding colony of Yakult Central Institute for environmental condition by conventionalizing GF mice using different individual variation of the gut microbiota could contribute to CYP activity under the same genetic and environmental factors that can directly affect CYP activity, no reports have demonstrated the impact of “normal” variation such as interindividual variation of the gut microbiota on hepatic CYP activity. Likely because of difficulties of excluding genetic and environmental factors that must also be considered. These findings led us to hypothesize that normal variation of the gut microbiota could contribute to CYP activity and result in individual differences of drug metabolism.

Thus, this study investigated the effects of normal gut microbiota variation on hepatic CYP activity under the same genetic and environmental condition by conventionalizing GF mice using different gut microbiota samples obtained from three mouse breeders.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Isopropanol, ethanol, sucrose, glycerol, Tris, EDTA-2K, potassium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from Fujifilm Wako Pure Chemical Co.. Meanwhile, 1 M Tris-HCl (pH 9.0), 0.5 M EDTA (pH 8.0), 10% SDS, TE buffer, TE-saturated phenol, phenol/chloroform/isoamyl alcohol (25:24:1), and 3 M sodium acetate (pH 5.2) were purchased from Nippon Gene Co., Ltd.

2.2 | Animals

As feces donors, male 8-week-old SPF BALB/cAJcl, BALB/cCrSlc, and BALB/cAnNCrCrj mice (Jcl, Slc, and Crj groups, respectively; n = 6/group) were purchased from CLEA Japan, Inc., Japan SLC, Inc., and Charles River Laboratories Japan, Inc., respectively. As feces recipients, male 4-week-old GF BALB/cYit mice (n = 18) were obtained from the breeding colony of Yakult Central Institute for conventionalization using Jcl (Yit-Jcl), Slc (Yit-Slc), and Crj (Yit-Crj) feces (n = 6/group). In addition, male GF BALB/cYit (Yit-Cont) mice were used as an untreated group (n = 6).

All mice were housed in cages in flexible film isolators. The breeding room was controlled under a 12-h/12-h light/dark cycle. Room temperature and humidity were maintained at 23 ± 3°C and 50% ±20%, respectively. Radiation-sterilized chow (FR-2 50 kGy, Funabashi Farm Co., Ltd.) and sterilized drinking water were available ad libitum. The sterility of GF mice was confirmed via microscopic and culture examination.

All experiments using animals were conducted under the supervision of the Institutional Animal Care and Use Committee of Yakult Central Institute and approved by the director of the Yakult Central Institute (approval number: 19-0070). All animals were cared for and used under a program accredited by AAALAC International.

2.3 | Treatments

Mice from each group were housed in separate isolators and permitted to acclimatize for 2 weeks. At 10 weeks of age, donor mice were removed from the isolators after the collection of feces. Then, 4-week-old recipient mice were moved from the GF isolator to the corresponding donor isolator. Nine pieces of donor feces were suspended in 3 ml of saline solution and 0.15 ml of fecal homogenates were orally administered to each recipient mouse. The recipient mice were then raised until reaching 10 weeks old. Additionally, Yit-Cont mice were raised until 10 weeks of age in separate isolators.

At 10 weeks of age, all mice were removed from their isolators after feces collection. Then, the animals were weighed and exsanguinated from the posterior vena cava and abdominal aorta under isoflurane anesthesia, and the liver of each animal was harvested at the same time on different days. After weighing, livers and feces were frozen in liquid nitrogen and then stored at −80°C until use.

2.4 | Gut microbiota analysis

DNA was extracted from feces using glass beads and phenol as described previously. The total counts of bacteria in feces were measured by quantitative PCR using a previously described method with slight modifications. Briefly, each reaction mixture (20 µl) was composed of 10 µl of 2× TB Green® Premix Ex Taq™ II (Takara Bio), 0.04 µl each of 100 µM forward primer (UniF: 5′-GTGSGACGGTGGTAAG-3′) and 100 µM reverse primer (UniR: 5′-TCCAGTCGTTGTCGTTT-3′), 0.4 µl of 50x ROX Reference Dye II, 4.52 µL of nuclease-free water (QIAGEN N.V.), and 5 µl of DNA. PCR was performed using the AB7500 system (Applied Biosystems) using the following conditions: 94°C for 5 min followed by 40 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 50 s. The fluorescent products were detected at the last step of each cycle. The total number of bacteria per gram of feces was calculated by substituting the Ct value obtained from the amplification curve of each sample into.
the standard curve, which was generated with 10-fold serial dilutions of DNA extracted from Faecalibacterium prausnitzii YIT 12316. The cell counts of the standard bacterial strain were determined microscopically using 4′,6-diamidino-2-phenylindole staining as described previously.19

2.5 | 16S rRNA sequencing

The V4 region of the bacterial 16S rRNA gene was amplified and sequenced using the primers 515F (5′-GTGCCAGCMGCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) according to a previously described method20 with small modifications. Briefly, bar-coded amplicons were generated using TB Green Premix Ex Taq II (Takara Bio) with 10 ng of template fecal DNA. The PCR program was as follows: 95°C for 30 s followed by 30 cycles of 95°C for 5 s, 50°C for 30 s, and 72°C for 40 s. The reaction was stopped immediately before DNA amplification reached a plateau. The amplicons were purified using an AMPure XP Kit (Beckman Coulter) and their concentrations were quantified using a Quant-iT™ PicoGreen™ dsDNA Assay (Invitrogen). The amplicons were pooled in equimolar amounts and then sequenced on a MiSeq system (Illumina) with a MiSeq Reagent Kit v2 500 cycle (Illumina).

The obtained sequence data were processed using QIIME221 (version 2020.8) with Silva (version 138) as a reference database to obtain the operational taxonomic unit (OTU) table (File S1) and the relative abundances of bacteria at the phylum and genus levels. Differences in occupancy among groups were determined via linear discriminant analysis (LDA) effect size (LEfSe) analysis22 with the condition of LDA score >4.0 using the Galaxy application (http://huttenhower.sph.harvard.edu/galaxy/). Principal coordinate analysis was used to visualize the difference of composition based on the unweighted and weighted UniFrac distances. The count of each constituent bacterium was calculated by multiplying the total number of bacteria by the occupancy. In addition, as alpha diversity indices, observed OTUs, Faith's phylogenetic diversity (Faith's PD), and the Shannon index were calculated using 20,000 reads per sample.

2.6 | Cyp activities

Hepatic microsomes were prepared using previously described methods.10 The total protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Cyp3a, Cyp2b, and Cyp2c activities in hepatic microsomes were measured using the P450-Glo™ Assay (Promega) according to the manufacturer's instructions and a previous report.10 In detail, a luminogenic substrate, i.e., luciferin IPA for Cyp3a, luciferin 2B6, for Cyp2b, and luciferin H for Cyp2c, and hepatic microsomes in 0.2 M potassium phosphate buffer (pH 7.4) were preincubated for 10 min at 37°C. After adding NADPH regeneration systems (Promega), the reaction mixtures were incubated for 10, 20, or 30 min to measure Cyp3a, Cyp2b, or Cyp2c activity, respectively, at 37°C. Then, luciferin detection reagent was added and stabilized for 20 min at room temperature. Luminescence was measured using a LUMItstar OPTIMA (BMG LABTECH). As positive controls, Supersomes of CYP3A4, CYP2B6, and CYP2C9 (Corning) for luciferin IPA, luciferin 2B6, and luciferin H, respectively, were used. Similarly, Supersomes-CYP minus (Corning) served as a negative control.

2.7 | Gene expression levels in the liver

mRNA was extracted from small pieces of liver tissue using the ReliaPrep™ Miniprep System (Promega). First-strand cDNA was generated from approximately 1000 ng of total RNA using Rever Tra Ace qPCR RT Master Mix (Toyobo). cDNA was examined by real-time PCR using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) and the AB7500 system. As target genes, in addition to Cyp3a11, Cyp2b10, and Cyp2c29, which are the major mouse isofoms of Cyp3a, Cyp2b, and Cyp2c, respectively,23 pregnane X receptor (Pxr), a major transcriptional regulator of Cyp3a11 that is involved in the regulation of Cyp2b and Cyp2c,24 organic anion transport polypeptide C (Oatpc), a downstream gene of Pxr,25 and constitutive androstane receptor (Car), a major transcriptional regulator of Cyp2b10,24 were evaluated. The sequence of each primer is presented in Table S1. The delta-delta Ct method was used to calculate the relative levels of mRNA. β-actin was used for normalization.

2.8 | Statistical analysis

The Steel–Dwass method was used to compare the total counts of bacteria and alpha diversity indices among donors and among recipients. PERMANOVA was used to evaluate the difference of composition based on the unweighted and weighted UniFrac distances. The Bonferroni-corrected t-test method was used to compare hepatic Cyp activity and gene expression among donors and among recipients. Spearman's rank correlation analysis was used to evaluate the association between Cyp activity and the counts of constituent bacteria at the phylum and genus levels by pooling donor and recipient samples (the analysis was performed using the bacterial groups in which more than half of the samples were detected). A p value less than .05 was considered statistically significant. Two Yit-Crj individuals died after conventionalization, and they were therefore excluded from the analysis. All statistical analyses were performed using Bell Curve for Excel (Social Survey Research Information Co., Ltd.).

3 | RESULTS

3.1 | Gut microbiota

The total bacterial counts in donor and recipient mouse feces are presented in Figure 1A,B. Crj and Yit-Crj mice had the lowest total bacterial counts among donor and recipient mice, respectively.
To visualize the variation of gut microbiota in donors and recipients, the results of principal coordinate analysis based on unweighted and weighted UniFrac distances were presented in Figure 1D and Figure S3. Based on unweighted UniFrac distances, there were significant differences in the gut microbiota composition among donors and recipients \((p < .01)\). Conversely, the gut microbiota composition was similar between donors and their corresponding recipients. Likewise, based on weighted UniFrac distances, the gut microbiota of Yit-Crj mice was significantly different from those of Yit-Jcl and Yit-Slc mice \((p < .01)\). As indicators of alpha diversity, the observed OTUs (Figure 1E,F), Faith's PD, and Shannon's index (Figure S4) were measured. The observed OTUs were significantly higher in Crj mice than in Jcl and Slc mice \((p = .011)\). Additionally, the observed OTUs were significantly higher in Yit-Slc and Yit-Crj mice than in Yit-Jcl mice \((p = .011\) and \(p = .028\), respectively). Faith's PD was significantly larger in Crj mice than in Jcl and Slc mice \((p = .011\) and \(p = .018\), respectively), and the index was significantly larger in Slc mice than in Jcl mice \((p = .011)\). Among recipient mice, Faith's PD

### Figure 1

**Characteristics of the gut microbiome in donor and recipient mice.**

(A) Total counts of bacteria in feces from donor mice. (B) Total counts of bacteria in feces from recipient mice. (C) Fecal bacterial composition of donor and recipient mice at the genus level. (D) Principal coordinate analysis plot based on the unweighted UniFrac distances of fecal bacteria from donor and recipient mice. (E) Observed operational taxonomic units (OTUs) in feces from donor mice. (F) Observed OTUs in feces from recipient mice. The Steel–Dwass method was used to compare the total counts of bacteria and alpha diversity indices among donors and among recipients. Different letters (a and b) indicate significant differences among donors and among recipients.

| Genus Level | Jcl | Slc | Crj | Yit-Jcl | Yit-Slc | Yit-Crj |
|-------------|-----|-----|-----|---------|---------|---------|
| Others      |     |     |     |         |         |         |
| Roseburia   |     |     |     |         |         |         |
| Parabacteroides |   |     |     |         |         |         |
| Lachnospiraceae |   |     |     |         |         |         |
| Alloprevotella |   |     |     |         |         |         |
| Clostridia UCG-014 |   |     |     |         |         |         |
| Faecalibaculum |   |     |     |         |         |         |
| Lachnospiraceae NK4A136 group |   |     |     |         |         |         |
| Others      |     |     |     |         |         |         |
| Bacteroides |     |     |     |         |         |         |
| Muribaculaceae |   |     |     |         |         |         |
| Lactobacillus |   |     |     |         |         |         |

**Figure 1A**

| Jcl | Slc | Crj | Yit-Jcl | Yit-Slc | Yit-Crj |
|-----|-----|-----|---------|---------|---------|
|     |     |     |         |         |         |

| Donor | Recipient |
|-------|-----------|
|       |           |

To visualize the variation of gut microbiota in donors and recipients, the results of principal coordinate analysis based on unweighted and weighted UniFrac distances were presented in Figure 1D and Figure S3. Based on unweighted UniFrac distances, there were significant differences in the gut microbiota composition among donors and recipients \((p < .01)\). Conversely, the gut microbiota composition was similar between donors and their corresponding recipients. Likewise, based on weighted UniFrac distances, the gut microbiota of Yit-Crj mice was significantly different from those of Yit-Jcl and Yit-Slc mice \((p < .01)\). As indicators of alpha diversity, the observed OTUs (Figure 1E,F), Faith's PD, and Shannon's index (Figure S4) were measured. The observed OTUs were significantly higher in Crj mice than in Jcl and Slc mice \((p = .011)\). Additionally, the observed OTUs were significantly higher in Yit-Slc and Yit-Crj mice than in Yit-Jcl mice \((p = .011\) and \(p = .028\), respectively). Faith's PD was significantly larger in Crj mice than in Jcl and Slc mice \((p = .011\) and \(p = .018\), respectively), and the index was significantly larger in Slc mice than in Jcl mice \((p = .011)\). Among recipient mice, Faith's PD

### Table 1

| Genus Level | Jcl | Slc | Crj | Yit-Jcl | Yit-Slc | Yit-Crj |
|-------------|-----|-----|-----|---------|---------|---------|
| Others      |     |     |     |         |         |         |
| Roseburia   |     |     |     |         |         |         |
| Parabacteroides |   |     |     |         |         |         |
| Lachnospiraceae |   |     |     |         |         |         |
| Alloprevotella |   |     |     |         |         |         |
| Clostridia UCG-014 |   |     |     |         |         |         |
| Faecalibaculum |   |     |     |         |         |         |
| Lachnospiraceae NK4A136 group |   |     |     |         |         |         |
| Others      |     |     |     |         |         |         |
| Bacteroides |     |     |     |         |         |         |
| Muribaculaceae |   |     |     |         |         |         |
| Lactobacillus |   |     |     |         |         |         |
was significantly larger in Yit-Slc and Yit-Crj mice than in Yit-Jcl mice ($p = .011$ and $p = .028$, respectively). Concerning Shannon’s index, no significant difference was found among donor mice, whereas the value was significantly higher in Yit-Slc mice than in Yit-Crj mice ($p = .028$).

### 3.2 | Hepatic Cyp activities

Hepatic Cyp activities in donors and recipients are presented in Figure 2. In donors, Cyp3a activity was significantly higher in Slc mice than in Jcl and Crj mice ($p < .001$ and $p = .002$, respectively). Among recipients, Cyp3a activity was significantly higher in all recipient groups than in the Yit-Cont group ($p < .001$). Furthermore, Cyp3a activity was significantly higher in Yit-Slc and Yit-Crj mice than in Yit-Jcl mice ($p < .001$). Cyp2b activity was significantly higher in Slc mice than in Jcl ($p < .001$) and Crj mice ($p = .010$). Cyp2b activity was significantly higher in Yit-Slc mice than in Yit-Cont mice ($p = .009$).

### 3.3 | Hepatic Cyp and related gene expression

The hepatic expression of Cyp and related genes in donors and recipients is presented in Figure 3. Cyp3a11 expression was similar among the donor groups, whereas its expression was significantly higher in all recipient groups than in the Yit-Cont group ($p < .001$). However, Cyp3a11 expression did not differ among the recipient groups. The expression of both Cyp2b10 and Cyp2c29 was equivalent among the donor and among recipient groups.
Regarding nuclear factor expression, Pxr, Oatpc, and Car expression did not differ among the donor groups. Conversely, Pxr and Oatpc expression was significantly higher in all recipient groups than in the Yit-Cont group (\(p < .001\) and \(p < .05\), respectively). Car expression was significantly higher in Yit-Jcl mice than in Yit-Slc and Yit-Cont mice (\(p = .008\) and \(p = .001\), respectively).

### 3.4 Association between Cyp activity and gut microbiota

Correlation analysis was performed to examine the relationship between Cyp activity and gut bacterial counts (Figure 4). Positive correlations with Cyp activity were observed for several phyla and genera. Specifically, Actinobacteriota, Deferribacterota, and Proteobacteria displayed significant positive correlations with Cyp3a activity, and at the genus level, *Parabacteroides*, *Mucispirillum*, *Lactococcus*, *Bacilli RF39*, *Clostridium UCG-014*, *Clostridium vadinBB60* group, *Clostridium sensu stricto 1*, and *Intestinimonas* exhibited significant positive correlations with Cyp3a activity. In particular, *Clostridium sensu stricto 1* displayed a strong positive correlation with Cyp3a activity (rs = 0.734, \(p < .001\)).

Concerning Cyp2b activity, Actinobacteriota and Deferrribacterota exhibited significant positive correlations at the phylum level, and *Mucispirillum*, *Lactococcus*, *Bacilli RF39*, *Clostridium UCG-014*, *Clostridium vadinBB60* group, *Clostridium sensu stricto 1*, *Dorea*, *Intestinimonas*, and *Oscillospirales UCG-010* had significant positive correlations at the genus level.

No phylum displayed a positive correlation with Cyp2c activity, whereas the genera *Bacilli RF39*, *Clostridium sensu stricto 1*, and *Oscillospirales UCG-010* exhibited significant positive correlations.

Meanwhile, the total bacterial count had a significant negative correlation with Cyp2c activity. At the phylum level, *Bacteroidota* displayed significant negative correlations with Cyp2b and Cyp2c activity, and *Firmicutes* exhibited a significant negative correlation with Cyp2c activity. Furthermore, several genera displayed significant negative correlations with Cyp activity.

### 4 DISCUSSION

Using ex-GF mice conventionalized with feces from mice obtained from three breeders, we revealed that normal variation of the gut microbiota could affect hepatic Cyp activity, and correlation analysis illustrated that several bacterial groups might modulate Cyp activity.
from each breeder were used to measure the hepatic Cyp activity in our preliminary study in which commercially available mice livers in Cyp2c activity was observed. The same result was also obtained were higher than in the other donors and no significant difference was found in the other recipients, if any, are considered attributable to the difference in the donor. These results illustrated that recipients maintained the gut microbiota composition of the donor. These results were consistent with previous conventionalization studies. These differences could be attributable to, for example, the variation of the donor. These results illustrated that recipients maintained the gut microbiota composition of the donor. These results were consistent with previous conventionalization studies. These differences could be attributable to, for example, the variation of the donor. These results illustrated that recipients maintained the gut microbiota composition of the donor. These results were consistent with previous conventionalization studies.

FIGURE 4 Spearman's rank correlation heat map between bacterial counts and cytochrome P450 (Cyp) activity. Only phyla and genera that were significantly correlated with any of the Cyp isoforms are listed. *Means genus unidentified. **Means family and genus unidentified.

Regarding the gut microbiota, principal coordinate analysis based on unweighted UniFrac distances revealed clear differences in the gut microbiota composition among donors and among recipients. The result in donors was consistent with previous findings revealing breeder-based differences in the gut microbiota composition in C57BL/6 mice. The present study also identified similar microorganisms that were differentially abundant between donors and their corresponding recipients, indicating that the recipient generally inherited the microbiota of the donor. These results illustrated that recipients maintained the normal variation of the gut microbiota under the same genetic and environmental backgrounds. Therefore, the differences among recipients, if any, are considered attributable to the difference in the gut microbiota composition in the present study.

Regarding donor Cyp activity, Cyp3a and Cyp2b activities in Slc were higher than in the other donors and no significant difference in Cyp2c activity was observed. The same result was also obtained in our preliminary study in which commercially available mice livers from each breeder were used to measure the hepatic Cyp activity (data not shown). Therefore, the difference is not by chance but owing to specific differences among these breeder mice.

Regarding Cyp3a activity in recipients, all recipient mouse groups had higher activity than the Yit-Cont group. This result is consistent with those of previous conventionalization studies. It must be emphasized that Yit-Slc and Yit-Crj mice had higher Cyp3a activity than Yit-Jcl mice. These results indicated that the reduction efficiency of hepatic Cyp3a activity following conventionalization depends on the colonizing bacteria. Additionally, as Yit-Crj mice had lower total bacterial counts in the gut microbiota and high hepatic Cyp3a activity, it is suggested that the bacterial composition has a stronger impact on Cyp3a activity than total bacterial counts. Taken together, these results suggested that the gut microbiota greatly enhanced hepatic Cyp3a activity, and even within the normal range, variation of the gut microbiota can lead to differences in Cyp3a activity.

Cyp2b activity was higher in Yit-Slc mice than in Yit-Jcl and Yit-Cont mice. However, Yit-Jcl and Yit-Crj mice did not have significantly different Cyp2b activity than Yit-Cont mice. Concerning the effects of the microbiota on Cyp2b activity, the results obtained to date are controversial. Kuno et al. reported higher Cyp2b activity in SPF C57BL/6NCrSlc mice obtained from Japan SLC, Inc. than in GF mice. Conversely, Li et al. found that Cyp2b activity was lower in conventional C57BL/6J mice obtained from Jackson Laboratory than in GF mice. In this regard, the breeder in the former study was the same as that used in the present study. It follows that bacteria that enhance Cyp2b activity might be present in the gut microbiota of Slc mice. Further study is required to determine the precise effects of the gut microbiota on hepatic Cyp2b activity.

Cyp2c activity was lower in Yit-Slc mice than in Yit-Jcl and Yit-Cont mice. An earlier study reported that gut microbiomes enhanced Cyp2c activity, however, we previously reported lower Cyp2c activity in SPF mice than in GF mice in a study using BALB/cAJcl mice which is the same strain with Jcl in this study. Thus, bacteria that attenuate Cyp2c activity could be present in the gut microbiota of Jcl mice. Taken together, these results suggested that even under normal conditions, the composition of the gut microbiota could attenuate the activity of certain Cyp isoforms including Cyp2c.

Regarding Cyp gene expression, Cyp3a11 expression was higher in all recipient groups than in the Yit-Cont group, consistent with previous conventionalization studies. These findings suggested the existence of gut microbiomes enhanced Cyp3a activity by modulating gene expression. Contrarily, there were no differences in Cyp2b10 and Cyp2c29 among all recipient groups compared with the Yit-Cont group. The results of Cyp2b10 were consistent with previous conventionalized mice studies. Furthermore, there were no differences among donors or recipients regarding Cyp3a11, Cyp2b10, and Cyp2c29 expression, and thus, the results for Cyp gene expression were not consistent with those for Cyp activity. In this regard, discrepancies between mRNA and activity of Cyp are common and have also been reported in previous reports using conventional and GF mice. These differences could be attributable to, for example,
post-transcriptional regulation. Some reports indicated that CYPs including CYP3A, CYP2B, and CYP2C isoforms are significantly controlled by microRNA. These effects might explain the difference between gene expression and Cyp activity.

The expression of the nuclear receptor Pxr and its downstream gene Oatpc was higher in all recipient groups than in the Yit-Cont group. This was also recorded in previous reports, which identified significant upregulation of Pxr in conventionalized mice. Taken together, it is possible that the gut microbiota modulates Cyp gene expression via Pxr, which influences Cyp activity.

The expression of the nuclear receptor Car was higher in Yit-Jcl mice than in Yit-Slc and Yit-Cont mice. However, Cyp2b10 gene expression did not differ among the groups, whereas Cyp2b activity was lower in Yit-Jcl mice than in Yit-Slc mice but similar to that in Yit-Cont mice. Thus, the present study could not support the contribution of Car to the effects of the gut microbiota on Cyp activity.

Correlation analysis revealed that several phyla and genera had positive correlations with Cyp activity, suggesting that differences of the bacterial composition in the gut microbiota enhanced Cyp activity. It has been reported that secondary bile acids produced mainly by Lachnoclostridium spp., such as Clostridium scindens and Clostridium hylemonae can modify hepatic Cyp activity. In this study, no clear correlation between Lachnoclostridium counts and Cyp activity was observed. Contrarily, Clostridium sensu stricto 1 had positive correlations with Cyp3a, Cyp2b, and Cyp2c activities. In particular, a strong correlation of Clostridium sensu stricto 1 was observed with Cyp3a activity. The previous study reported that Clostridium butyricum, which belongs to Clostridium sensu stricto 1, enhanced the transcription of MicroRNA-200c in Caco-2 BBe cells, and evidence suggests that MicroRNA-200c affects human hepatic CYP3A4 activity. In addition, Clostridium butyricum produces high levels of butyrate, which is suggested to induce certain Cyp isoforms. Taken together, it is possible that Clostridium sensu stricto 1 spp. can enhance Cyp activity. Further studies are needed to evaluate the precise effects of individual bacteria on the modulation of Cyp activity and elucidate the responsible mechanisms.

At the same time, several phyla and genera exhibited negative correlations with Cyp activity. Although the mechanisms were not clarified in this study, it is possible that the bacteria in these phyla and genera are involved in the attenuation of Cyp activity, including that of Cyp2c, which was attenuated by the gut microbiota in the present study. Therefore, the gut microbiota may be involved in both the enhancement and attenuation of Cyp activity.

There are several limitations in this study. First, we only obtained data about Cyp activity; thus, whether normal variation of the gut microbiota affects in vivo drug metabolism or the clearance of clinically relevant drugs is uncertain. Furthermore, there could be a species difference in effects of microbiota on Cyp activity. Hence, further study is required to address these problems.

In conclusion, this study demonstrated that normal variation of the gut microbiota affected hepatic Cyp activity, including Cyp3a activity, in mice. Additionally, it was estimated that several bacterial groups can modulate Cyp activity. Our results suggest that normal variation of the gut microbiota could contribute to hepatic CYP activity and explain the individual differences in drug metabolism in humans.

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DISCLOSURE
The authors declare no conflicts of interest associated with this manuscript.

AUTHOR CONTRIBUTIONS
 Participated in research design: Togao, Tajima, Wagai, Otsuka, and Kawakami. Conducted experiments: Togao, Tajima, Kurakawa, and Kawakami. Performed data analysis: Togao, Kurakawa, and Kawakami. Wrote or contributed to the writing of the manuscript: Togao, Tajima, Kurakawa, Wagai, Otsuka, Kado, and Kawakami.

ETHICS APPROVAL STATEMENT
All experiments using animals were conducted under the supervision of the Institutional Animal Care and Use Committee of Yakult Central Institute and approved by the director of the Yakult Central Institute (approval number: 19-0070). All animals were cared for and used under a program accredited by AAALAC International.

DATA AVAILABILITY STATEMENT
Research data are not shared.

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REFERENCES
1. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. Cell. 2012;148:1258-1270.
2. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. N Engl J Med. 2016;375:2369-2379.
3. Valdes AM, Walter J, Segal E, Spector TD. Role of the gut microbiota in nutrition and health. BMJ. 2018;361:36-44.
4. Collins SL, Patterson AD. The gut microbiome: an orchestra of xenobiotic metabolism. Acta Pharm Sin B. 2020;10:19-32.
5. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature. 2019;570:462-467.
6. Kuno T, Hirayama-Kurogi M, Ito S, Ohutsuki S. Effect of intestinal flora on protein expression of drug-metabolizing enzymes and transporters in the liver and kidney of germ-free and antibiotics-treated mice. Mol Pharm. 2016;13:2691-2701.
7. Kuno T, Hirayama-Kurogi M, Ito S, Ohutsuki S. Reduction in hepatic secondary bile acids caused by short-term antibiotic-induced dysbiosis decreases mouse serum glucose and triglyceride levels. Sci Rep. 2018;8(1253):1-15.
8. Selwyn FP, Cheng SL, Claassen CD, Cui JY. Regulation of hepatic drug-metabolizing enzymes in germ-free mice by conventionalization and probiotics. Drug Metab Dispos. 2016;44:262-274.
9. Toda T, Saito N, Ikarashi N, et al. Intestinal flora induces the expression of Cyp3a in the mouse liver. *Xenobiotica*. 2009;39:323-334.

10. Togao M, Kawakami K, Otsuka J, Wagai G, Ohta-Takada Y, Kado S. Effects of gut microbiota on in vivo metabolism and tissue accumulation of cytochrome P450 3A metabolized drug: midazolam. *Biopharm Drug Dispos*. 2020;41:275-282.

11. Zuber R, Anzenbacherová E, Anzenbacher P. Cytochromes P450 and experimental models of drug metabolism. *J Cell Mol Med*. 2002;6:189-198.

12. Ishii M, Toda T, Ikarashi N, et al. Gastrectomy increases the expression of hepatic cytochrome P450 3A by increasing lithocholic acid-producing enteric bacteria in mice. *Bioll Pharm Bull*. 2014;37:298-305.

13. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the human intestinal microbial flora. *Science*. 2005;308:1635-1638.

14. Naidoo P, Chetty VV, Chetty M. Impact of CYP polymorphisms, ethnicity and sex differences in metabolism on dosing strategies: the case of efavirenz. *Eur J Clin Pharmacol*. 2014;70:379-389.

15. Takagi S, Nakajima M, Mohri T, Yokoi T. Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem*. 2008;283:9674-9680.

16. Matsuki T. Procedure of DNA extraction from fecal sample for the analysis of intestinal microflora. *J Intest Microbiol*. 2006;20:259-262.

17. Shima T, Amamoto R, Kaga C, et al. Association of life habits and fermented milk intake with stool frequency, defecatory symptoms and intestinal microbiota in healthy Japanese adults. *Benef Microbes*. 2019;10:841-854.

18. Fuller Z, Louis P, Mihajlovski A, Rungapamestry V, Ratcliffe B, Duncan AJ. Influence of cabbage processing methods and prebiotic manipulation of colonic microflora on glucosinolate breakdown in man. *Br J Nutr*. 2007;98:364-372.

19. Jansen GJ, Wildeboer-Veloo ACM, Tonk RHJ, Franks AH, Welling GW. Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. *J Microbiol Methods*. 1999;39:215-221.

20. Caporaso JG, Lauber CL, Walters WA, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*. 2012;6:1621-1624.

21. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37:852-857.

22. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12:R60.

23. Martignoni M, Groothuis GMM, 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;2:875-894.

24. di Masi A, De Marinis E, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: molecular, functional, and biomedical aspects. *Mol Aspects Med*. 2009;30:297-343.

25. Li Y, Ross-Viola JS, Shay NF, Moore DD, Ricketts ML. Human CYP3A4 and murine Cyp3a11 are regulated by equal and genistein via the pregnane X receptor in a species-specific manner. *J Nutr*. 2009;139:898-904.

26. Ivanov II, Atarashi K, Manel N, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. 2009;139:485-498.

27. Sadler R, Singh V, Benakis C, et al. Microbiota differences between commercial breeders impacts the post-stroke immune response. *Brain Behav Immun*. 2017;66:23-30.

28. Li CY, Lee S, Cade S, et al. Novel interactions between gut microbiome and host drug-processing genes modify the hepatic metabolism of the environmental chemicals polybrominated diphenyl ethers. *Drug Metab Dispos*. 2017;45:1197-1214.

29. Claus SP, Ellero SL, Berger B, et al. Colonization-induced host-gut microbial metabolic interaction. *MBio*. 2011;2:e00271-10.

30. Walsh J, Gheorghe CE, Lyte JM, et al. Gut microbiome-mediated modulation of hepatic cytochrome P450 and P-glycoprotein: impact of butyrate and fructo-oligosaccharide-inulin. *J Pharm Pharmacol*. 2020;72:1072-1081.

31. Han LW, Wang L, Shi Y, et al. Impact of microbiome on hepatic metabolizing enzymes and transporters in mice during pregnancy. *Drug Metab Dispos*. 2020;48:708-722.

32. Nakajima M, Yokoi T. MicroRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. *Pharmacol Ther*. 2011;131:330-337.

33. Rieger JK, Klein K, Winter S, Zanger UM. Expression variability of absorption, distribution, metabolism, excretion-related microRNAs in human liver: influence of nongenetic factors and association with gene expression. *Drug Metab Dispos*. 2013;41:1752-1762.

34. Xiao Y, Dai X, Li K, Gui G, Liu J, Yang H. Clostridium butyricum partially regulates the development of colitis-associated cancer through miR-200c. *Cell Mol Biol*. 2017;63:59-66.

35. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther*. 2008;27:104-119.

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