

# Effect of gut microbiota on host whole metabolome

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## Abstract

**Introduction** Recent advances in microbiome research have revealed the diverse participation of gut microbiota in a number of diseases. Bacteria-specific endogenous small molecules are produced in the gut, are transported throughout the whole body by circulation, and play key roles in disease establishment. However, the factors and mechanisms underlying these microbial influences largely remain unknown.

**Objectives** The purpose of this study was to use metabolomics to better understand the influence of microbiota on host physiology.

**Methods** Germ-free mice (GF) were orally administered with the feces of specific pathogen-free (SPF) mice and were maintained in a vinyl isolator for 4 weeks for establishing the so-called ExGF mice. Comparative metabolomics was performed on luminal contents, feces, urine, plasma, and tissues of GF and ExGF mice.

**Results** The metabolomics profile of 1716 compounds showed marked difference between GF and ExGF for each matrix. Intestinal differences clearly showed the contribution of microbiota to host digestive activities. In addition, colonic metabolomics revealed the efficient conversion of primary to secondary metabolites by microbiota.

Furthermore, metabolomics of tissues and excrements demonstrated the effect of microbiota on the accumulation of metabolites in tissues and during excretion. These effects included known bacterial effects (such as bile acids and amino acids) as well as novel ones, including a drastic decrease of sphingolipids in the host.

**Conclusion** The diverse effects of microbiota on different sites of the host metabolome were revealed and novel influences on host physiology were demonstrated. These findings should contribute to a deeper understanding of the influence of gut microbiota on disease states and aid in the development of effective intervention strategies.

**Keywords** Fecal inoculation · Germ-free mice · Gut microbiota · Metabolomics · 16S rRNA analysis

## Abbreviations

5HL	5-Hydroxylysine
3MH	3-Methylhistidine
BCAA	Branched-chain amino acid
FA	Formic acid
FFA	Free fatty acid
GF	Germ-free
IACUC	Institutional animal care and use committees
MS	Mass spectrometry
NASH	Non-alcoholic steatohepatitis
NCI	National Cancer Institute
NGS	Next generation sequencer
OTU	Operational taxonomic unit
QIIME	Quantitative insights into microbial ecology
SPF	Specific pathogen-free
UPLC	Ultra-performance liquid chromatography
HESI-II	Heated electrospray ionization-II
PFPA	Perfluoropentanoic acid
HILIC	Hydrophilic interaction chromatography

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FDR False discovery rate  
SPF Specific pathogen-free

## 1 Introduction

Taxonomical analysis of human gut microflora has recently revealed many associations with host health and disease status. In addition to gastrointestinal dysfunction (Nagao-Kitamoto et al. 2016), metabolic (Arora and Bäckhed 2016), vascular (Yamashita et al. 2016; Felizardo et al. 2016), autoimmune (Kim et al. 2016), and even central nervous disorders (Sharon et al. 2016; Winek et al. 2016) have been demonstrated to be associated with microbiota composition. While next generation sequencer (NGS)-based metagenomics approaches contribute to the identification of responsible bacterial strains for disease phenotypes, underlying molecular mechanisms have been more efficiently explained by mass spectrometry (MS)-based metabolomics. Certainly, secreted molecules from bacteria have been proved to affect host physiology in both proximal and distal organs from the gut. Therefore, combined microbiota-based omics technologies expand the opportunity for drug discovery; typically, the bacterial and molecular candidates responsible for disease are efficiently selected using statistics and bioinformatics for further validation with cultured bacteria and germ-free/gnotobiotic models.

In addition to conventional probiotics and prebiotics (Sánchez et al. 2016; Collins and Reid 2016), various intervention and drug modalities are currently being developed in preclinical and clinical stages, including fecal microbiota transplantation (Choi and Cho 2016), bacterial cocktails (Narushima et al. 2014), isolated bacteria (Woo et al. 2011), recombinant bacteria (Piraner et al. 2016), bacterial metabolites (Donia and Fischbach 2015), and inhibitors of bacterial enzymes (Wang et al. 2015). Regardless of therapeutic modality, modes of actions are primarily based on the alteration of molecules secreted from microorganisms. It is generally recognized that short chain fatty acids have significant effects on host physiology both in immunity and metabolism (Ríos-Covián et al. 2016; Kasubuchi et al. 2015). Trimethylamine-oxide has been demonstrated to be a prognosis marker for cardiovascular diseases (Tang et al. 2013). Moreover, the level of serotonin controlling gut motility is largely affected by gut microbiota through bile acids and aromatic amino acid derivatives (Yano et al. 2015). Studies on the metabolomics of inflammasome-deficient animals have resulted in the discovery of the curing effects of taurine through the combination of metagenomics and metabolomics approaches (Levy et al. 2015).

The accumulation of evidence underscoring the importance of bacteria-derived molecules in host physiology has encouraged scientists in metabolomics to include

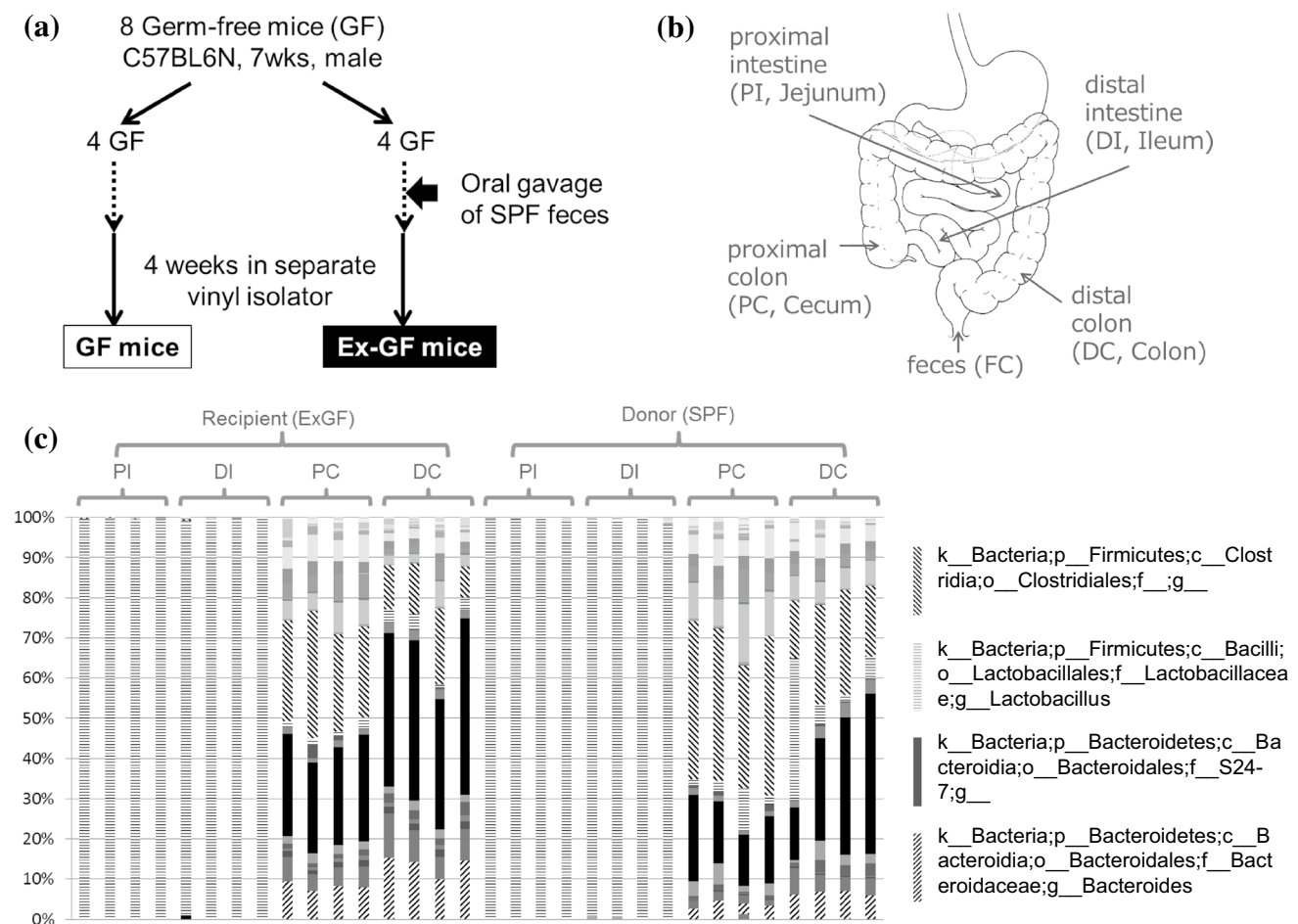
bacteria-specific metabolites in their analytical platforms. Furthermore, to clarify microbiota-related mechanisms of disease and drug efficacy, it is necessary to estimate the bacterial contribution to the tissue levels of each metabolite derived specifically from bacteria, as well as that commonly produced by both host and microbes.

The effects of bacteria on the metabolome have been estimated by comparing GF mice with those orally gavaged with SPF feces (i.e., ExGF mice) or with certain bacterial strains (Matsumoto et al. 2012; Walker et al. 2014; Marcobal et al. 2015). Although these studies have clearly revealed bacterial effects, evaluations were conducted with limited sample types such as cecum or plasma. As greater correlations between disease and the microbiome are revealed, the more important it is to estimate what metabolites are affected by the bacteria. In this study, we have compared the metabolomes of GF and Ex-GF mice using nine different matrices, including four luminal contents, feces, plasma, liver, kidney, and urine. The spatial difference of the bacterial effect on the whole metabolome has provided evidence concerning where the metabolites are produced and how they are absorbed, accumulated, and excreted. This in-depth evaluation of the metabolome difference among these matrices has provided a better understanding of the contribution of bacteria to host physiology including digestive activities.

## 2 Materials and methods

### 2.1 Animals

All of the animal experiments using GF, ExGF and SPF mice were conducted at Sankyo-labo Service Co., Ltd. Mice were fed a CMF diet sterilized with 50 Gy-gamma radiation (Oriental Yeast Co., Ltd). Eight-week-old GF C57BL/6 N mice were divided into two groups (four mice per group). Mice in the first group were orally administered feces freshly prepared from SPF individual mice of the same C57BL/6N background and maintained in a vinyl isolator for an additional 4 weeks in order to stabilize gut microflora (Fig. 1a). GF mice in the second group were orally administered saline and bred for 4 weeks under germ-free conditions in a separate vinyl isolator. The day before sacrifice, both feces (FC) and urine (UR) samples were taken and the mice were fasted overnight. After refeeding for 5 h, mice were sacrificed and dissected. Luminal contents, plasma, liver and kidney (KD) were obtained. The luminal contents were taken from the gut and separated into four sections, namely, the proximal intestine (PI, mainly jejunum), distal intestine (DI, ileum), proximal colon (PC, cecum), and distal colon (colon) (Fig. 1b). After plasma samples (PL) were taken from the inferior



**Fig. 1** Establishment of ExGF mice and the resultant successful transfer of gut microflora. **a** Schematic view of the procedure for establishing ExGF mice, **b** position of luminal contents obtained in

this study, **c** bacterial taxonomy of the luminal contents of ExGF and SPF mice; the main 4 OTUs were indicated in the *right panel*

vena cava, the liver (LV) and kidney (KD) were isolated. All animal studies were performed in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the Institutional Animal Care and Use Committees (IACUC) of Sankyo-labo Service Co.

## 2.2 16S rRNA genome sequencing analysis

Genomic DNA was prepared from the luminal contents or feces using an ISOFEAL DNA Isolation Kit (Nippon-Gene). The variable region ranging from V3 to V4 was amplified by specific primers according to the Illumina instructions. The respective sequences of the forward and reverse primers were as follows:

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TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
CCTACGGGNGGCWGCAG
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA
GGACTACHVGGGTATCTAATCC
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After the index sequences were added using the Illumina Nextera XT Index Kit v2, the amplicons were mixed and applied to MiSeq. The obtained sequence reads were trimmed and quality-checked. All failed sequence reads, low-quality sequence ends, tags, and primers were removed. Sequence collections depleted homopolymers >5 bp in length, reads <200 bp, and chimeras. Operational taxonomic units (OTUs) were picked using an open reference OTU and the QIIME software (Caporaso et al. 2010) at 97% similarity against the Greengenes database (DeSantis et al. 2006).

## 2.3 Metabolomics

The non-targeted metabolomics platform was a combination of four technological platforms of Metabolon, Inc. All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer

interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. Sample preparation was carried out as described previously (Ohta et al. 2009; Evans et al. 2009, 2014). An automated liquid handler (Hamilton LabStar, Salt Lake City, UT) was used to add methanol containing recovery standards to the experimental samples to facilitate protein precipitation. Following centrifugation, sample extracts were dried and reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic-positive ion conditions and chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1×100 mm, 1.7 μm) using water and methanol containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic-positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA, and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic-negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however, with 6.5 mM ammonium bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1×150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate (pH 10.8). The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 70–1000 m/z.

## 2.4 Bioinformatics and statistics

For data visualization and statistical analysis, the amounts of each metabolite were rescaled and normalized relative to measurements made in a matrix sample. A pooled matrix sample was generated for each matrix by taking a small volume of each experimental sample. The matrix sample was run in parallel with experimental samples on all run days. Any missing values were assumed to be below the limits of detection and designated as compound minimum (minimum value imputation). However, if the metabolite was only detected in one group (GF or ExGF), the metabolite was considered a metabolite that microbiota had completely degraded or that microbiota had specifically produced, respectively. Following scaling and imputation of missing values, statistical analysis of log-transformed

data was performed using “R” (<http://cran.r-project.org/>). Metabolites that differed significantly between experimental groups were determined using Welch’s two-sample *t* test. *p* values <0.05 were considered statistically significant and *p* values <0.10 showing important trends. In order to profile each sample metabolome, principal component analysis (PCA) using MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/faces/home.xhtml>) was conducted (Xia and Wishart 2016).

## 3 Results

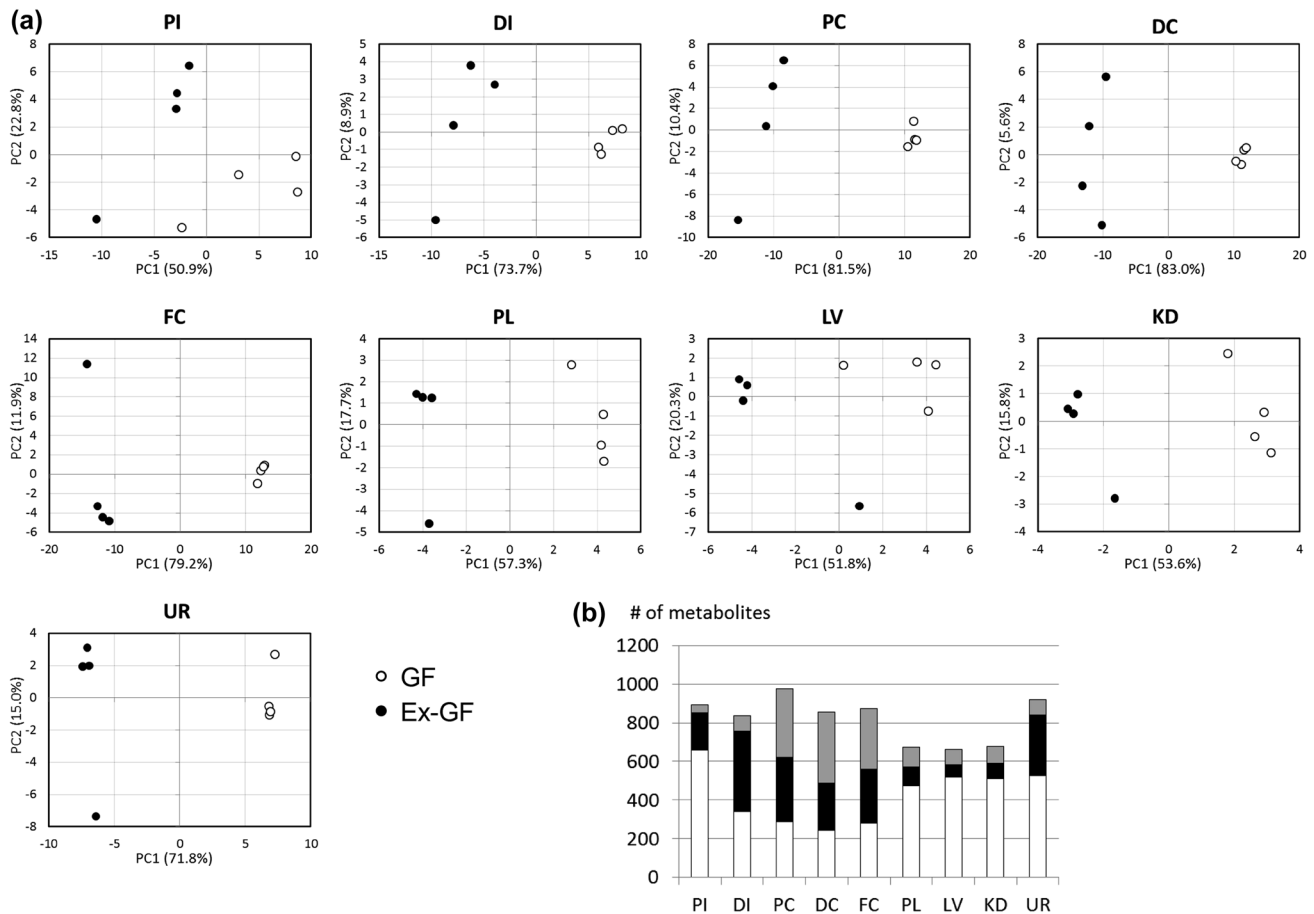
### 3.1 Confirmation of bacterial transfer to different luminal contents

It has been reported that the stabilization of mammalian gut microflora is completed 2–4 weeks after oral administration of fresh feces (Turnbaugh et al. 2009). Therefore, we dissected mice 4 weeks after fecal inoculation and the four parts of luminal contents and feces of both donors (SPF) and recipients (ExGF) were taxonomically analyzed by 16S rRNA amplicon sequencing. As shown in Fig. 1c, the microflora of each luminal region in ExGF mice was quite similar to those identified in the corresponding sites of SPF mice. Both the proximal and distal parts of the intestinal contents were occupied with *Lactobacillus*, whereas colonic contents and feces contained a diversity of bacterial species. These results demonstrate that SPF gut microflora for each region was successfully transferred to the corresponding luminal region of ExGF mice by oral administration of fresh feces.

### 3.2 Metabolomics overview

Global metabolomics of the nine matrices detected a total of 1716 metabolites. PCA plots of the analyzed nine matrices are shown in Fig. 2a. The metabolome profiling between GF and ExGF mice was clearly separated in all matrices, showing the significant effect of gut microbiota not only in regions where they exist, but also distant tissues from the gut. Figure 2b represents the numbers of detected metabolites in each matrix and those of significantly increased and decreased ones by bacterial colonization were colored with black and gray, respectively. The observed gradual increase in metabolic changes from the upstream to the downstream inside of the gut correlated with the diversity and abundance of gut microbiota (Figs. 1c, 2b). For example, in the DC, 71.7% of the detected metabolites were significantly changed.

Compared with luminal contents and feces, metabolic changes in plasma, liver, and kidney were relatively small; however, the percentages of significantly changed



**Fig. 2** Overview of the metabolomes of nine matrices in GF and ExGF. **a** Principal component analysis of metabolome data obtained by the analysis of the nine matrices; *white* and *black dots* represent the profiles of GF and ExGF mice, respectively. **b** Number of detected

metabolites in metabolomics for each matrix; *white*, *black* and *gray bars* represent those of unchanged, increased, and decreased ones, respectively ( $p < 0.05$ )

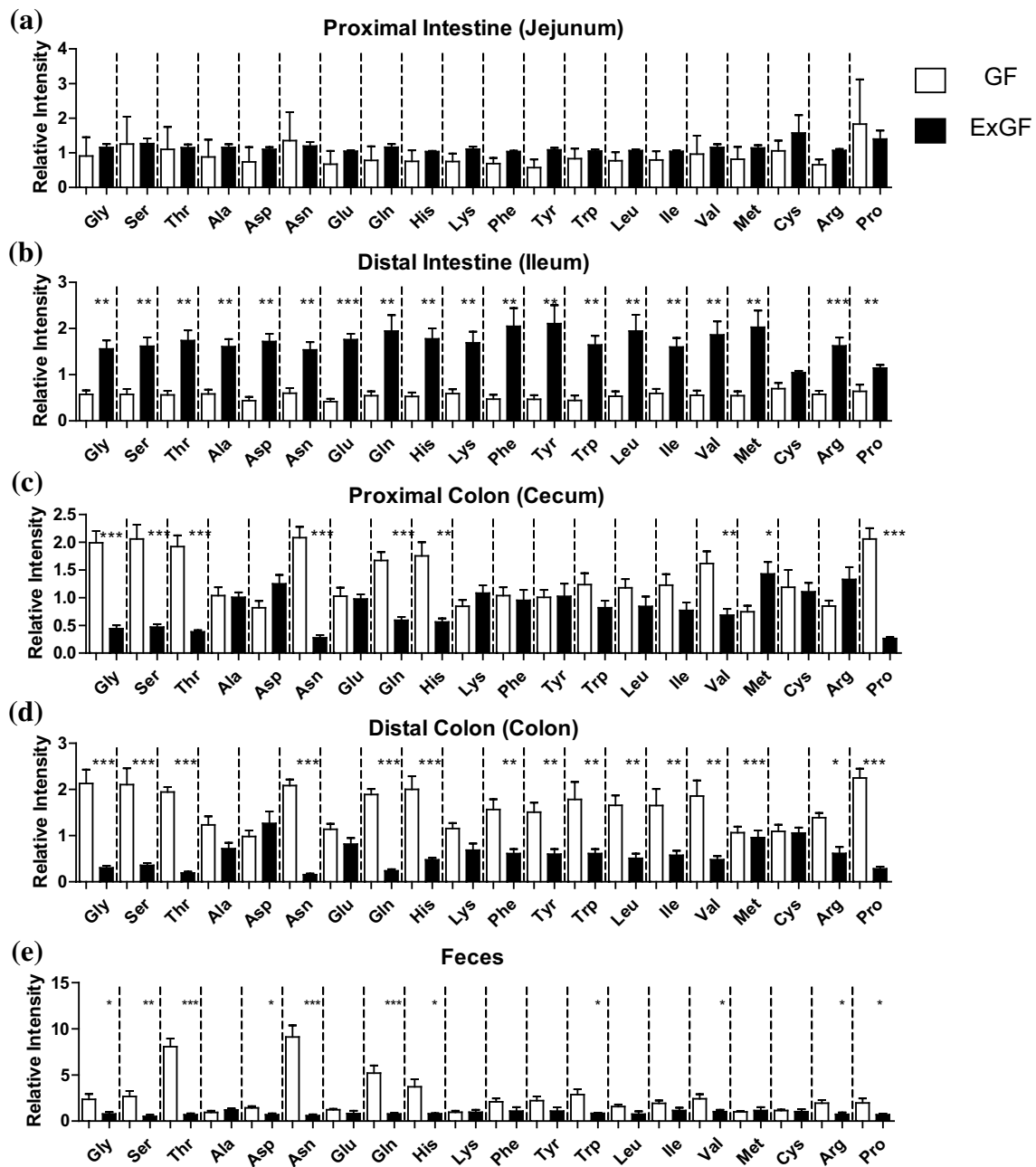
metabolites were still high (29.9, 21.5, and 24.9%, respectively). In contrast, the numbers of increased metabolites were much higher in urine than those in the above three host regions. Supplemental Fig. 1 shows the categorized numbers (amino acids, carbohydrates, lipids, nucleotides, vitamins, and xenobiotics). While the bacterial effects were relatively small in the liver, the number of significantly changed lipids was the same as other tissues. On the contrary, the bacterial effects on amino acids and xenobiotics were more dominant in urine. These results may be due to both the solubility of the metabolites and the active excretion of exogenous hydrophilic metabolites by the kidney.

### 3.3 Enhancement of host digestive activities

Profiling of the 20 constitute amino acids in PI, DI, PC, DC, and FC are shown in Fig. 3. While only minor increasing trends were observed in PI (Fig. 3a), the 20 constitute amino acids commonly and drastically increased in the DI

(Fig. 3b). Since a major part of dietary protein is digested in the small intestine, the observed common increase may result from the enhancement of proteolysis by colonized bacteria in ExGF mice. However, many of the constitute amino acids were dramatically decreased in the colon and feces (Fig. 3c–e). As described in the following section, these diminishments in constitute amino acids seem to be due to efficient utilization by colonic bacteria. While only relatively small changes were observed in plasma, liver and urine, many of the significant decreases were detected in kidney (Supplemental Fig. 2a–d).

It is well known that the most dietary lipids possess FFAs with C16 or C18-based acyl chains such as C16:0 (palmitoyl), C18:0 (stearoyl), and C18:1 (oleoyl). Therefore, we assessed the bacterial effect on lipolysis focused on C16- and C18-based FFAs. Supplemental Fig. 3 shows the fold changes and significance of C16- or C18-based FFAs in ExGF per GF mice. In PI and DI, the major region for lipolysis, marked increases of all molecules



**Fig. 3** Bacterial effects on constitute amino acids in the luminal contents and feces. **a** Proximal intestine; jejunum, **b** distal intestine; ileum, **c** proximal colon; cecum, **d** distal colon; colon, **e** feces.

White and black bars represent the amino acid levels in GF and ExGF mice, respectively. Asterisks represent the significance of differences between GF and ExGF (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ )

except stearate were evident in ExGF mice. The same increasing trends in ExGF mice were observed in colonic and fecal regions, although C18-based FFAs showed more enhanced elevation. In plasma, liver, and kidney, all free fatty acids showed increasing trends by fecal inoculation. No detection of these FFAs in urine reflects the accumulative nature of these molecules in tissues.

### 3.4 Distinct bacteria-dependent metabolism

Bacteria own their specific metabolism and a number of bacteria-specific metabolites (bacterial markers) have been reported to date; however, many of them remain to be identified (Walker et al. 2014). In this study, both known and novel bacterial markers were clearly

**Table 1** Apparent bacterial effects observed in this study

Pathway	Bacterial biomarkers
Lysine degradation	Pipecolate, cadaverine
Polyamine biosynthesis	N1, N12-Diacetylspermine
Histidine degradation	Imidazole derivatives, <i>N</i> -acetylhistamine
Tryptophan	Indole derivatives
Phenylalanine, tyrosine	Benzoate derivatives, cinnamate derivatives, phenol derivatives, phenylpyruvate derivatives
Creatine degradation	Sarcosine
Valine degradation	3-Methyl-2-oxobutyrate, alpha-hydroxyisocaproate
Leucine degradation	3-Methyl-2-oxovalerate, 2-hydroxy-3-methylvalerate, ethylmalonate
Isoleucine degradation	4-Methyl-2-oxopentanoate, alpha-hydroxyisovalerate, methylsuccinate
Terpenoid backbone biosynthesis	Mevalonate, mevalonolactone
Bile acids	Free forms of primary bile acids, secondary bile acids
Inflammatory and oxidative reaction	9- and 13-HODE, 12,13-and 9,10-DiHODE, kynurenine derivatives
NADI salvage pathway	Nicotinate, nicotinamide, nicotinamide ribonucleotides, NMN
Neurotransmitters	Serotonine, acetyl-CoA
Isoflavonoids	Equol

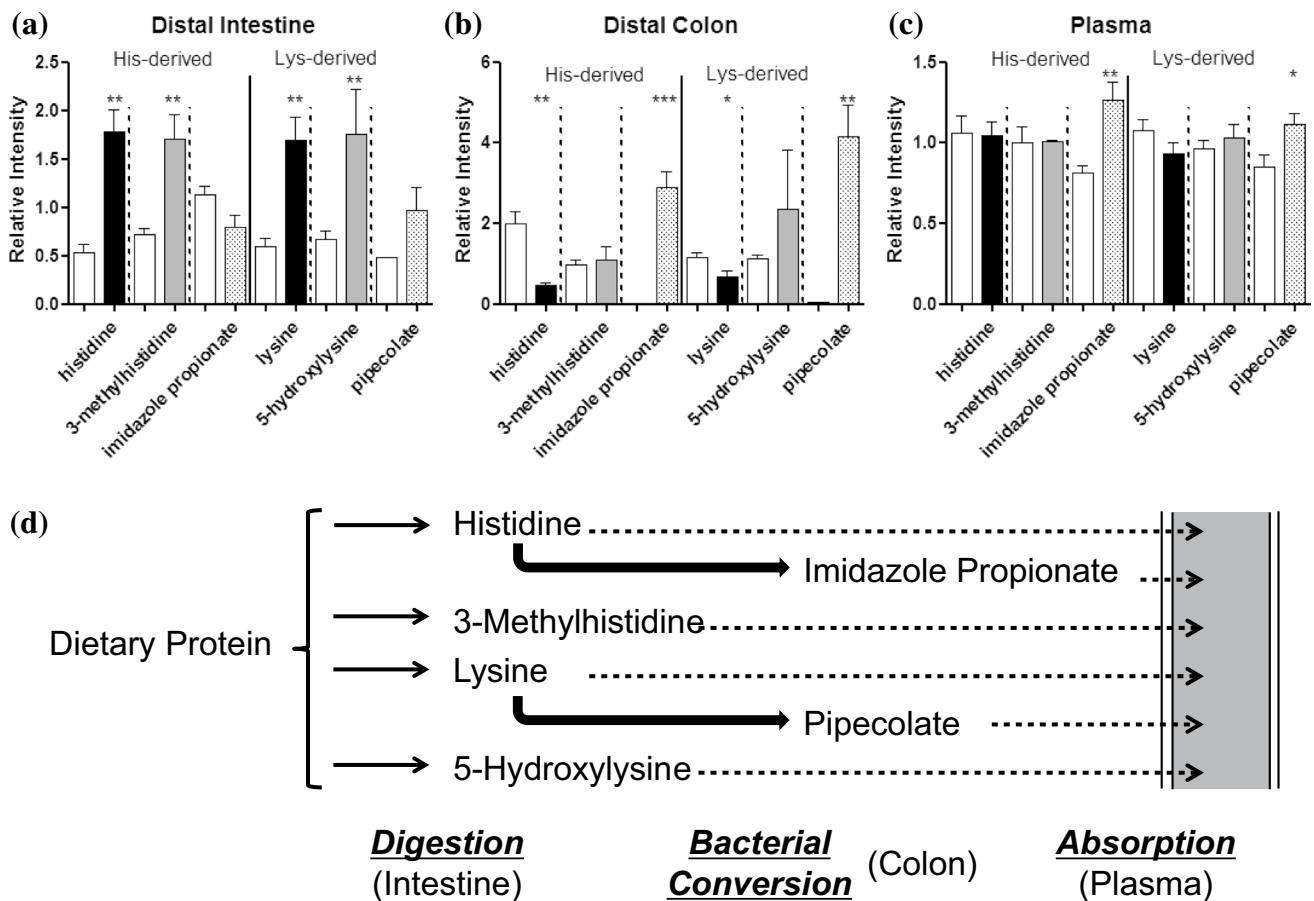
revealed. Bacterial-specific metabolites extracted in this study are summarized in Table 1. This list includes food-derived molecules, such as equol, which is a derivative of soybean isoflavone (Kawada et al. 2016).

As described in the previous section, most of the constitute amino acids were decreased by bacterial degradation in colon and feces, although their levels were dramatically increased in intestinal contents due to the bacterial enhancement of proteolysis (Fig. 3). Derivatives of amino acids such as 3-methylhistidine (3MH) and 5-hydroxylysine (5HL), digestive products derived from dietary proteins (Dragsted 2010; Walrand et al. 2008), were increased as histidine and lysine in DI (Fig. 4a). Imidazole propionate and pipecolate, bacteria-specific products derived from histidine and lysine, respectively (Antener et al. 1983; Fujita et al. 1999), were dramatically increased in DC (Fig. 4b); however, increases were not evident in DI (Fig. 4a). In plasma, these bacteria-specific metabolites were also elevated, likely due to the efficient absorption into blood (Fig. 4d). As described for histidine and lysine, aromatic amino acids (Phe, Tyr, Trp) and branched-chain amino acids (Leu, Val, Ile) were converted into secondary metabolites by colonic bacteria and absorbed into blood (Table 1). In addition to the above mentioned bacterial effects, most of the well-known bacterial markers, such as secondary bile acids and vitamins were clearly detected. Furthermore, bacteria-dominant conversion such as de-taurination and de-glycation of primary bile acids were clearly demonstrated.

### 3.5 Alteration of metabolites in host tissues

One of the most surprising effects of bacterial transfer on host regions was a decrease in sphingolipids. As shown in Fig. 5, abundant sphingomyelin molecules and their biosynthetic intermediates were generally decreased, particularly in liver. Supplemental Fig. 5 shows all of the fold changes in ExGF/GF mice for sphingolipids. While plasma sphingomyelins were similarly decreased by bacterial colonization, the effects on biosynthetic intermediates were limited, and those on kidney were relatively small. On the contrary, colonic sphingolipids were largely suppressed, although the effects in intestine were not evident (Supplemental Fig. 5). It is possible that this drastic decrease in colon affected intra-tissue levels. However, the overall suppression of sphingolipids, including biosynthetic intermediates in liver, suggests the downregulation of both de novo and/or salvage synthesis of sphingolipids in liver (Chaurasia and Summers 2015, see “Discussion”).

An increase in short chain acylcarnitines was the other specific observation in host regions. As shown in Supplemental Fig. 6, the concomitant decrease in long chain acylcarnitines and the increase in the ketone body, 3-hydroxybutyrate, indicate the enhancement of beta-oxidation in the host. Indeed, the same and opposite metabolic changes in plasma are generally observed after exercise and food intake, respectively (Zhang et al. 2017; Shrestha et al. 2015). The decrease in relatively long chain dicarboxylic acids and increase in short and medium chain ones in host regions supports the elevation of omega- and beta-oxidation



**Fig. 4** Bacterial effects on lysine, histidine, and their derivatives. **a–c** Non-colored (*white*) and colored (*black*, *gray*, and *hatched*) bars represent the amino acid levels in GF and ExGF mice, respectively. Color difference represents the amino acid categories as followings: *black bars* (constitute amino acids), *gray bars* (those modified

in dietary proteins), and *hatched bars* (those modified by gut microbiota) for distal intestine (**a**), distal colon (**b**), and plasma (**c**); *asterisks* represent the significance of differences between GF and ExGF ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ). **d** Schematic view of the metabolism of biochemicals described in this figure

by the gut microbiota (Supplemental Fig. 7) (Westin et al. 2005).

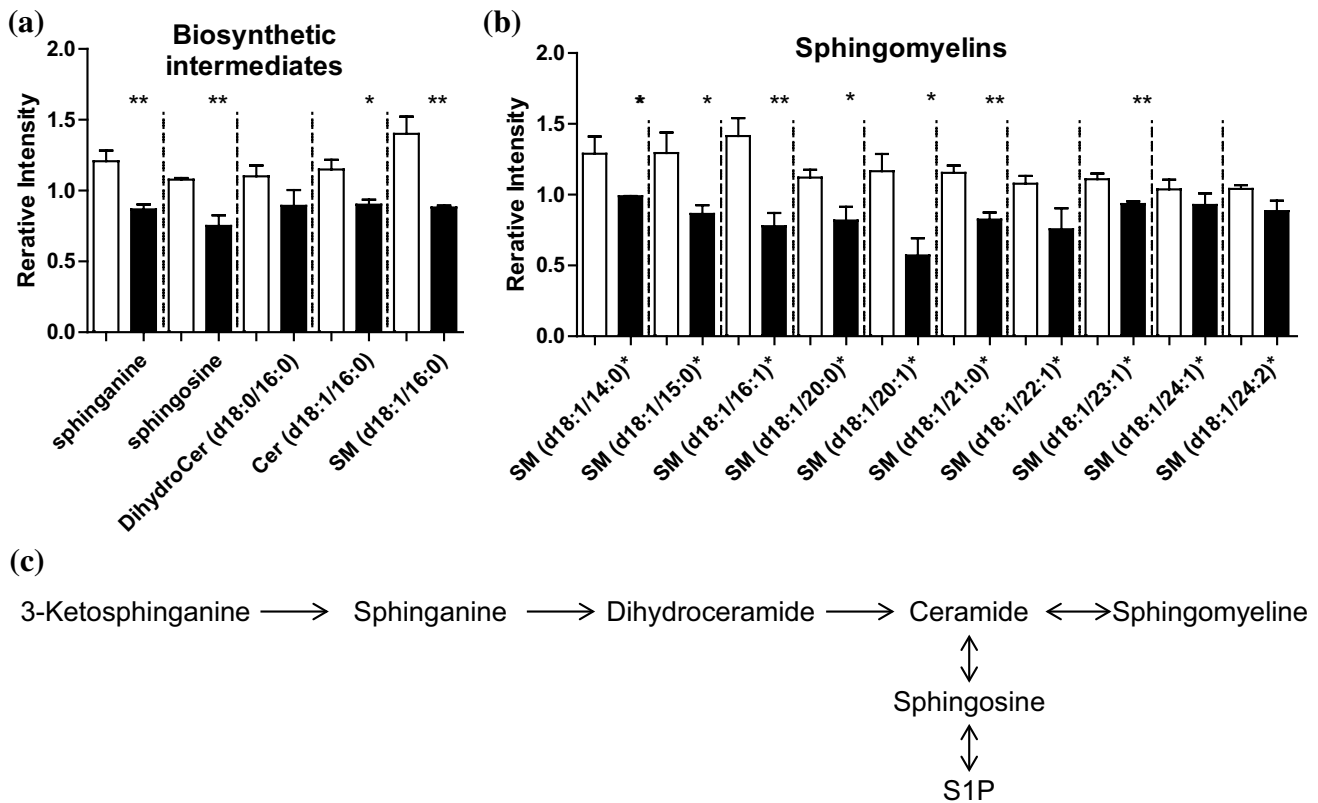
#### 4 Discussion

Expanding gut microbiome research has begun to clarify the effects of microbiota on host physiology through the secretion of endogenous small metabolites, peptides, and proteins (Johnson et al. 2016; Mamo 2016; Petriz and Franco 2017). In particular, small molecules play important roles not only in the gastrointestinal tract, but also in distal regions (e.g., the central nervous systems) through their absorption into the circulation. To evaluate the effects of gut microbiota on each metabolite, comparative metabolomics between GF and ExGF mice have been conducted; however, only limited regions (e.g., feces and plasma) were studied (Matsumoto et al. 2012, 2013). As demonstrated in these studies, Ex-GF mice were usually compared with

GF mice, since GF mice are usually established from SPF mice through different processes, including nursing by foster parents of different lineage. Precise estimation requires the reproduction of gut microflora in the jejunum, ileum, cecum and colon of donor SPF in the corresponding gut sections of recipient ExGF individuals. The conformity of the microflora profiled by 16S rRNA genome sequencing indicates the successful transfer of the whole microbiome and is evident by the simple microflora in small intestine and the diverse community in colon and feces. In addition, this observation enabled us to evaluate bacterial effects on the metabolome of each matrix.

The total 1716 metabolite profiling demonstrated the effects of bacteria being dependent on the metabolites and regions. One of these features is the high number of increased metabolites in the intestine (Fig. 2b). As observed from the analysis of amino acids and lipids, the increased metabolites include many of the degradative products derived from foods. The metabolic feature





**Fig. 5** Bacterial effects on sphingolipids in liver. **a** Effects on de novo biosynthetic intermediates, **b** effects on sphingomyelin molecules, **c** de novo and salvage sphingolipid biosynthesis pathway;

White and black bars in **a** and **b** represent the sphingolipid levels in GF and ExGF mice, respectively. Asterisks represent the significance of differences between GF and ExGF (\* $p < 0.05$ , \*\* $p < 0.01$ )

of the distal intestine (DI) indicates not only the production of bacteria-specific metabolites but also the enhancement of host digestive activities. The much higher number of decreased metabolites in colon and feces indicates the conversion of degradative products into other compounds by bacterial metabolic activities (Fig. 2b). The numbers of changed metabolites were lower in plasma, liver, and kidney, although they remained high in urine. Since many bacteria-specific metabolites including amino acid derivatives were detected in the urine of ExGF mice (Supplemental Fig. 1), hydrophilic bacterial metabolites may actively be excreted by the kidney. Therefore, the hydrophilic bacterial metabolites absorbed into the circulation may be more than those estimated by the accumulation in tissues. The longer stabilizing period after fecal gavage (>4 weeks) may result in a greater bacterial effect in liver and kidney.

The enhancement of host digestive activities by gut microbiota has been reported, so far (Nyangale et al. 2012); however, the unexpectedly equal and dramatic fold increase of constitute amino acids in DI by bacterial colonization was demonstrated in this study (Fig. 3). The well-recognized bacterial digestive activity was mainly reported for the conversion of fibers into short

chain fatty acids (Hamaker and Tuncil 2014); however, the clear elevation in degradation products derived from lipids, as well as proteins in DI, showed a large contribution of gut microbiota to host digestive activities. These observations should be noted when considering the gut microbiome association with various diseases related to the gastrointestinal tract such as celiac disease caused by gluten-digestion (Girbovan et al. 2017). The enhancement of proteolytic activities in ExGF mice is likely due to the contribution of bacterial enzymes or the stimulation of secretion of host pancreatic ones. A clinical study revealed that *Bacteroides* levels are strongly correlated with tryptic activities in the feces of Crohn's disease patients (Midtvedt et al. 2013). Similarly, lipolysis alteration by bacteria is also an important observation for diseases related to lipid metabolism, because elevated molecules such as free fatty acids and other degradation products are considered as diagnostic biomarkers (Yoshida et al. 2015). The increase of dicarboxylic acids in the colonic and fecal samples of ExGF mice indicates the elevation of oxidation and inflammation (Lee et al. 2010). The detailed examination of lipids different in chain lengths, desaturation, and oxidation levels should

give more mechanistic evidence for the importance of gut microbiota in disease status.

A number of known and unknown bacterial effects were evident (Table 1), and their production, absorption, accumulation, and excretion were clarified in this study. Cadaverine is a polyamine produced mainly through the decarboxylation of lysine and is mediated by bacteria (Chen et al. 1979). In addition, it is known to contribute to the fishy amine odor in women with bacterial vaginosis. The levels of cadaverine and its downstream metabolites such as *N*-acetyl-cadaverine, 5-aminovalerate, and glutarate showed the greatest elevations in the PC of Ex-GF mice (Table 1). The vast majority of these biochemicals were excreted in the feces, whereas small amounts of *N*-acetyl-cadaverine and 5-aminovalerate were excreted in the urine (data not shown). Pipecolate, another metabolite of lysine, may originate from the catabolism of dietary lysine by intestinal bacteria (Fujita et al. 1999). Its levels were elevated by over 100-fold in the PC of Ex-GF mice and remained high in almost all tissues measured, potentially serving as a good bacterial marker.

Some of the most typical bacterial markers were derivatives derived from aromatic amino acids. Generally, the specificity of these metabolites in ExGF mice was maintained in host tissues and even in urine. Of interest is the fact that these metabolites are well-reported to be toxic factors in both acute and chronic kidney disease (Ramezani et al. 2016). In addition, both beneficial and harmful effects have been reported in metabolic and neural dysfunctions (Wang et al. 2014).

Branched-chain amino acids (BCAAs) are among the other nutrients well metabolized by gut microbiota (Dai et al. 2011), although the ExGF specificity is less in host tissue than in luminal or fecal samples. This observation may reflect the contribution of the original production by the host itself. Bacteria catabolize the three amino acids to generate branched-chain fatty acids (valerate, isobutyrate, and isovalerate), which are essential for bacterial growth. The relatively high plasma levels of BCAAs in obese patients, and their suppression by bariatric surgery, suggest the importance of these amino acids in metabolic disease (Magkos et al. 2013). Since the degradation of BCAAs lead to the production of SCFAs, well-known bacterial markers with beneficial effects on metabolic disease, the elevation of BCAAs in the metabolism of ExGF mice may provide some key aspects of gut microbiota-related mechanisms in obesity (Saad et al. 2016).

Bacterial biomarkers were also evident in the histidine metabolic pathway; histamine and imidazole-related compounds were drastically elevated by bacterial colonization. The source of imidazole propionate remains unclear, but it shares a very similar structure with urocanate and is believed to be a histidine catabolite. Histidine can be

degraded via histidase, forming urocanate, or transaminase, forming imidazole pyruvate. It is noted that a recent clinical metabolomics study revealed a sharp increase of imidazole propionate in plasma after Roux-en-Y gastric bypass and vertical sleeve gastrectomy surgeries (Sarosiek et al. 2016).

Bile acids are derived from cholesterol in the liver and are released into the small intestine to facilitate dietary lipid emulsification and absorption. The primary bile acids cholate and chenodeoxycholate are conjugated with either taurine or glycine to decrease its toxicity and increase its solubility (Qi et al. 2015; Martin et al. 2007). The sole detection of tauro-conjugated primary bile acids in GF mice has demonstrated that all of the secreted forms in the biliary duct were tauro-conjugated (Dai et al. 2011). In mouse liver, chenodeoxycholate may also be converted to  $\alpha$ -muricholate and  $\beta$ -muricholate, which can be conjugated with taurine. In the intestine, gut bacteria de-conjugate and de-hydroxylate these primary bile acids into the secondary bile acids deoxycholate, lithocholate, hyodeoxycholate, ursodeoxycholate, hyocholate, and murideoxycholate. These secondary bile acids are reabsorbed and circulated to the liver, whereas some may be excreted in the feces. The importance of bile acids was recently highlighted since farnesoid X-activated receptor agonists showed efficacy in clinical trials as anti-NASH agents (Jahn et al. 2016).

Surprisingly, the robust suppression of sphingolipids is one of the novel findings revealed in this study. It is reported that dietary sphingolipids are degraded to sphingoid bases (e.g., sphingosine) for intestinal absorption (Nilsson 1969). Therefore, a possible explanation of the microbiota effect on their tissue levels is a decrease of sphingosine in the proximal and distal colon. Accordingly, the limited availability of sphingosine may affect salvage synthesis in host cells. Another explanation may be the bacterial contribution of de novo synthesis through an unknown mechanism. To clarify the detailed mechanisms, targeted metabolomics of sphingolipids after administration of stable isotope-labeled sphingolipids or serine, an initial molecule of sphingolipid biosynthesis, is needed. Sphingolipids are stress-causing lipid classes related to cellular lipotoxicity leading to impaired insulin secretion, vascular dysfunction, and neurodegenerative disorders (Zeidan and Hannun 2007). Therefore, the observed suppression by gut microbiota may lead to the discovery of novel molecules/strains for disease intervention.

## 5 Concluding remarks

The effects of gut microbiota on the metabolome of nine different matrices in the host were clarified through the comparison of the corresponding regions in GF and ExGF

mice. The scrutiny of each metabolite level in four different luminal contents, feces, plasma, liver, kidney, and urine demonstrate the dominancy of bacterial metabolism in the levels of each metabolite for each region. These observations also provide evidence of each metabolite's fate including the production site in the gut, absorbability into the plasma, accumulation in tissues, and excretion as urine or feces. Furthermore, some metabolite changes suggest bacterial effects on host digestive and metabolic function. Therefore, results obtained in this study should contribute to the understanding of disease mechanism leading to microbiome-based drug discovery.

### Compliance with ethical standards

**Conflict of interest** 'Takeo Moriya, Yoshinori Satomi, Shumpei Murata, Hiroshi Sawada, and Hiroyuki Kobayashi' declare that they have no conflict of interest.

**Ethical approval** All animal studies were performed in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the Institutional Animal Care and Use Committees (IACUC) of Sankyo-labo Service Co.

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