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Metabolomics characterization of energy metabolism reveals glycogen accumulation in gut-microbiota-lacking mice☆

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Abstract

Microbiota in the gut are considered an important environmental factor associated with host metabolism and physiology. Although gut microbiota are known to contribute to hepatic lipogenesis and fat storage, little is known about how the condition influences the deposition of glycogen in the liver. To better understand and characterize the host energy metabolism in guts lacking microbiota, we compared the liver metabolome of specific pathogen-free and germ-free mice by gas chromatography–mass spectrometry combined with partial least-squares discriminant analysis. We identified 30 of 52 highly reproducible peaks in chromatograms of liver tissue extracts from the two groups of mice. The two groups showed significant differences in metabolic profile. Changes in liver metabolism involved metabolites such as amino acids, fatty acids, organic acids and carbohydrates. The metabolic profile of germ-free mice suggests that they synthesize glycogen and accumulate it in the liver through gluconeogenesis and glycogenesis. Our findings shed light on a new perspective of the role of gut microbiota in energy metabolism and will be useful to help study probiotics, obesity and metabolic diseases.

Keywords: Glycogen; Microbiota; Metabolomics

1. Introduction

The human gastrointestinal tract contains an enormous variety and diversity of microorganisms, referred to as gut microbiota. This community is dominated by anaerobic bacteria, which consist of 500~1000 species [1]. It is a complex and dynamic ecosystem that has subsequent profound effects on host nutrition and metabolic function [2,3], development of innate immunity [4], production of essential vitamins [5,6] and the biotransformation of endogenous and exogenous compounds [7–9]. Therefore, the bacterial composition and function of gut microbiota in the digestive tract have been an active research field for many years.

Recent studies revealed that the composition and function of gut microbiota are related to host metabolism and physiology for pivotal roles in the development of metabolic disorders [10]. Transplantation of gut microbiota harvested from normal mice to germ-free (GF) mice led to weight gain, insulin resistance and obesity [11]. Bäckhed et al. [12] indicated that gut microbiota is an important environmental factor in modulating host lipid metabolism and regulating fat storage systemically because microbiota can induce hepatic lipogenesis and, by regulating the expression of the circulating lipoprotein lipase inhibitor, promote storage of triglycerides (TGs) in adipocytes. Germ-free mice are resistant to diet-induced obesity, which reveals that such mice have increased fatty acid oxidation and decreased adiposity and hepatic TG levels as compared with conventional mice [13]. Although three pathways, fasting-induced adipose factor, AMP-activated protein kinase and G-protein-coupled receptor 41, are involved in gut microbiota by regulating host energy expenditure and storage [14], knowledge of gut microbiota affecting host energy metabolism is still limited.

Abbreviations: GC-MS, gas chromatography–mass spectrometry; GF, germ-free; GSD, glycogen storage disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SPF, specific pathogen-free; PAS, periodic acid-Schiff; PLS-DA, partial least-squares discriminant; TC, total cholesterol; TGs, triglycerides; TP, total protein.

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Metabolomics is a powerful global strategy used to understand the change in metabolites and metabolic state of biological systems [15–17]. Plasma metabolome analysis has revealed gut microbiota to greatly affect the host metabolism, especially amino acid metabolism [18]. Recently, metabolomics and lipidomics demonstrated that gut microbiota affect the distribution of lipid species in serum, adipose tissue and liver [19]. Liver is the central organ of metabolism and biosynthesis in mammals; therefore, the composition of endogenous metabolites changes dynamically under different physiologic conditions. Many important functions, including glycogenesis, lipogenesis and decomposition of erythrocytes, take place in the liver for energy translation. Thus, the relation among liver function, energy metabolism and gut microbiota must be characterized.

Gas chromatography–mass spectrometry (GC-MS) is a useful metabolomics tool for toxicology [20], biomarker discovery [21,22] and disease diagnosis and classification [23]. To have more information on energy metabolism between gut microbiota and the host, we analyzed the liver metabolome of specific pathogen-free (SPF) and GF mice by GC-MS–based techniques.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of reagent grade from Sigma-Aldrich-Fluka (St. Louis, MO, USA), unless stated otherwise. Periodic acid-Schiff (PAS) and Oil Red O were purchased from Merck (Whitehouse Station, NJ, USA).

2.2. Animals and sample collection

Male GF and SPF C57BL/6JNarl mice (n=5, respectively), 11 weeks old (National Laboratory Animal Center, Taipei), were maintained in a vinyl isolator in a room kept at a constant temperature (20°C–26°C) and humidity (40%–60%). Mice were fed a commercial diet (5010 LabDiet, Purina Mills, St. Louis, MO, USA) and sterile water *ad libitum*. To confirm GF status, microbiological assays were performed on a monthly basis by culturing feces, bedding and drinking water in thioglycollate medium (DIFCO, Camarillo, CA, USA). The experimental protocol and design were approved by the National Laboratory Animal Centre (NLAC) animal experimentation committee and performed according to the NLAC guidelines for animal experimentation. Before being sacrificed, animals were deprived of food for 6 h and sacrificed after anesthetization with 95% CO_2. The liver and epididymal fat pad were removed and weighed. Blood samples were collected by cardiac puncture for clinical biochemistry analysis. Livers were excised for metabolomics and histology studies.

2.3. Clinical biochemistry analyses of blood

Blood samples were centrifuged at 3000*g* at 4°C for 10 min. The supernatant (serum) was used for the determination of glucose and levels of total protein (TP), TGs, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) with use of an automatic analyzer (HITACHI 717, Hitachi, Tokyo, Japan).

2.4. Histopathological investigation of liver tissue

Fresh liver tissue was embedded in Tissue-Tek 4583 OCT compound (Tissue-Tek OCT Compound, Sakura Finetek, Torrance, CA, USA). Tissue was sectioned at 4 μm by Universal Microtome Cryostat (Leica CM3050S, Leica Microsystems, Nussloch GmbH, Germany) and processed for histological examination by PAS and Oil Red O staining. The PAS staining of liver tissues was as described [24]. Samples were pretreated with 0.5% periodic acid for 5 min, washed in distilled water for 5 min and then incubated in Schiff's reagent at room temperature for 10 min. After the reaction, they were treated with 0.5% sodium metabisulfite and washed with distilled water for 5 min. They were counterstained for 2 min with hematoxylin, washed for 10 min and finally covered with glass coverslips. The Oil Red O staining of liver tissues was as described [25], and lipid deposition was quantified as specified [26]. Images were captured at 200× magnification.

2.5. Liver extract preparation for metabolomics study

Liver tissue extracts were prepared as described [27]. An amount of 1 ml methanol was added to 100 mg liver tissue from each mouse and homogenized using the Bullet Blender (Next Advance, Cambridge, MA, USA) at 4°C for 3 min.

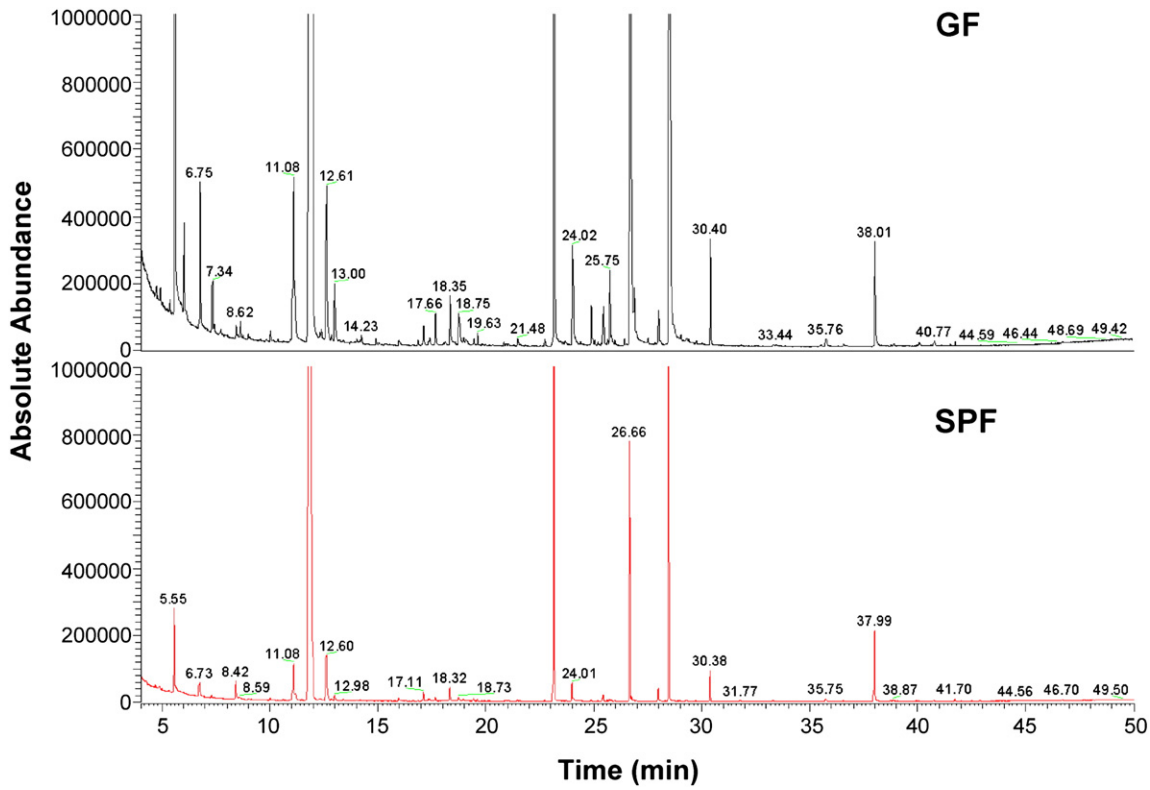

Fig. 1. Metabolite profile of liver tissue extracts from GF and SPF mice in GC-MS TIC chromatographs. Each peak indicates a metabolite, and peaks are identified by comparison of their retention times and MS spectra with data from the NIST and Wiley databases.

Then, 0.5 ml of Milli-Q water was added and mixed thoroughly; 0.3 ml of the homogenate was transferred to another tube and partitioned with 0.2 ml chloroform. The samples were centrifuged at 15,000*g* for 15 min at 4°C, and the 300-μl upper aqueous layer was centrifugally filtered through a 10-kDa cutoff filter (Pall Life Sciences, NY, USA) to remove proteins. An amount of 20 μl ribitol stock solution (0.2 mg/ml) was added to the filtrate (100 μl) as an internal standard and then lyophilized to dryness. Metabolites in liver tissue extracts were derivatized before GC-MS analysis.

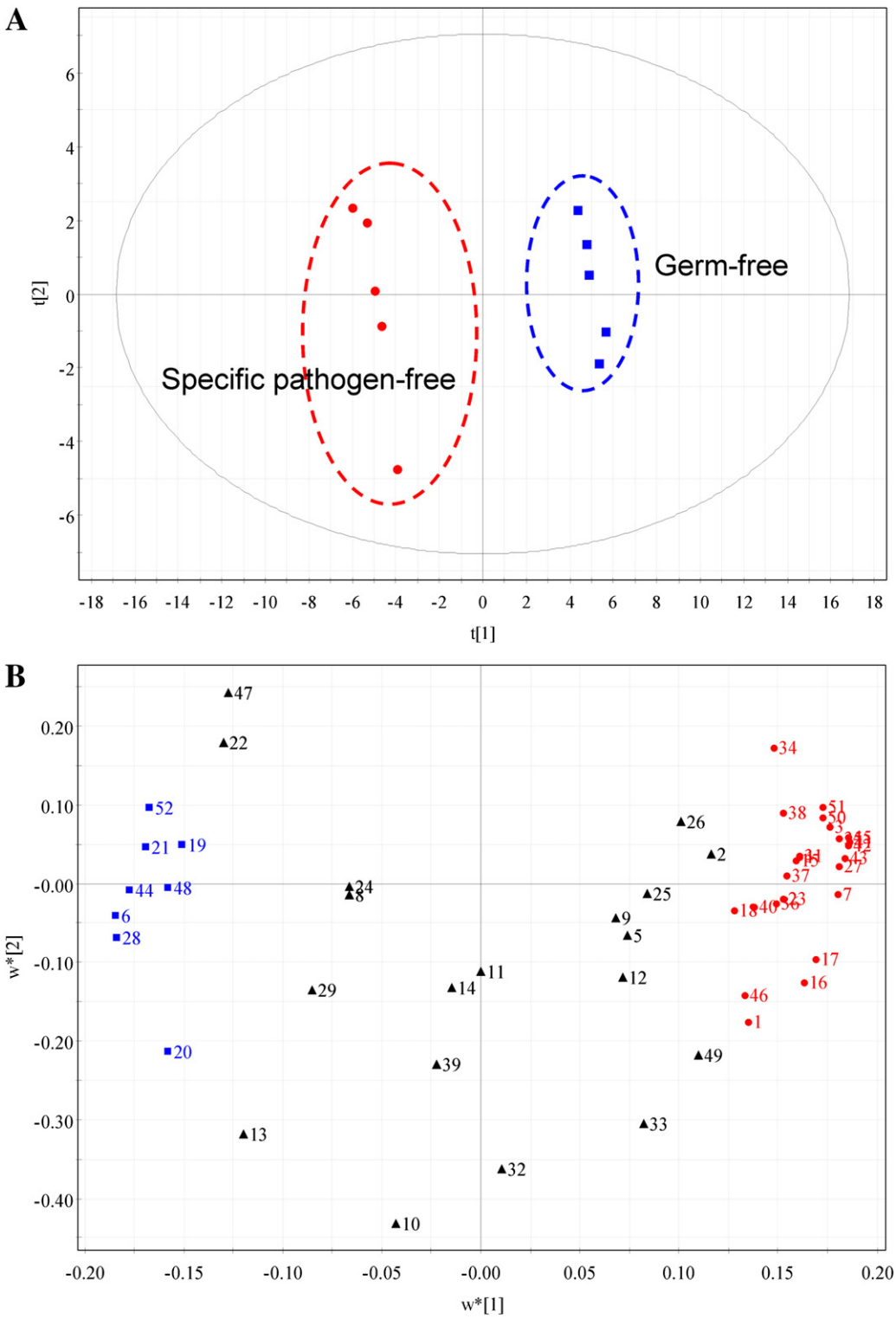

Fig. 2. Partial least-squares discriminant analysis model for nontargeted approach comparing metabolites in GF and SPF mice. (A) Score plot shows distinct clustering of mice by amount in different-colored circles. The *x*-axis (t1) and *y*-axis (t2) indicate the first and second principle components, respectively. One point represents one sample. (B) The loading plot displays parameters related to variables positively correlated with score plots. The *x*-axis (w1) and *y*-axis (w2) indicate the two highest *x* scores of principal components 1 and 2. Each point represents one variable (metabolite). One black triangle indicates one metabolite. The red dot indicates the metabolite that significantly increased in level, and the blue square indicates those that significantly decreased in level in GF mice.

2.6. Liver metabolome analysis by GC-MS

All liver samples from SPF and GF mice were analyzed in a random order. The derivatization procedure was described previously [27]. Briefly, the dried sample was derivatized using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and heated at 70°C for 60 min to form trimethylsilyl derivatives. The GC-MS analysis involved use of Thermo Finnigan Trace GC 2000 installed with a Polaris Q mass detector and Xcalibur software system at the Division of Research and Analysis, Taiwan Food and Drug Administration, Taiwan. One microliter of derivatized sample was injected into a 30-m×0.25-mm (internal diameter)×0.25-μm (film thickness) DB-5-fused silica capillary column, which was chemically bonded with a 5% diphenyl-95% dimethylpolysiloxane cross-linked stationary phase (J&W Scientific). The injector and ion source temperatures were 230°C and 200°C, respectively. The oven temperature program was initiated at 80°C for 5 min, then increased at 5°C/min to 300°C and held for 1 min. Helium was used as the carrier gas at 1 ml/min. The mass spectrometer operated in electron impact mode (70 eV). Acquisition of total ion currents (TICs) was performed in the full scan mode from 50 to 650 *m/z* with a scan time of 0.58 s. All GC-MS-detected peaks were identified by comparing both the MS spectra and the retention index with those available in libraries (NIST and Wiley) and commercially available reference compounds.

2.7. Data processing and pattern recognition

Multivariate statistical analysis involved use of SIMCA-P12+ software (Umetrics, Sweden). Partial least-squares discriminant analysis (PLS-DA), a supervised statistical method to maximize the variance matrix, was used to process the acquired GC-MS TIC chromatography data. Ribitol was used as an internal standard to calibrate the variations in sample preparation and analysis. The relative intensity of each peak was normalized in terms of peak area to peak area of ribitol on the same chromatograph and expressed as 100 times the ratio. The data matrix was arranged with the samples as observations and peaks as the response variables. Three parameters, *R2X*, *R2Y* and *Q2Y*, were used for evaluation of the models to indicate the goodness of fit and the predictive ability. *R2X* explains the cumulative variation in the GC-MS response variables, and *R2Y* is the current latent variables of the sums of squares of all *X*s and *Y*s. *Q2Y* reflects the cumulative cross-validated percentage of the total variation that can be predicted by the current latent variables. High coefficient values of *R2Y* and *Q2Y* represent good discrimination.

2.8. Statistical analysis

Data are expressed as mean±S.D. The results were analyzed by one-way analysis of variance (ANOVA). Results were considered significant at $P<.05$.

3. Results

3.1. GC-MS analysis of liver tissue

The typical GC-MS TIC chromatogram of liver tissue metabolites from SPF and GF mice is zoomed in and displayed in Fig. 1. The number of peaks is particularly high for GF mice. Magnifying the low-intensity regions revealed a large number of individual peaks, which indicated significant microbiota-dependent differences in metabolites in mice liver. These metabolites, including amino acids, organic acids, carbohydrates, fatty acids and lipids, are involved in multiple biochemical processes in the liver. Their variation in presence can clarify the physiological and pathological effects related to the gut microbiota in the biosystem of the liver.

3.2. Multivariate statistical analysis of metabolites by PLS-DA

We detected 52 peaks in chromatographic profiles and normalized them by the internal standard (ribitol) to remove systematic differences for further investigating the possible metabolic differences between the samples. To understand which variables carry the class-separating information, PLS-DA was used to model and cluster the maximum covariance between groups of observations. Two principal-component models could explain 99.8% (*R2Y*) and predicted 98.5% (*Q2Y*) of the data according to the cross-validation, which indicated significant differences in distribution of liver metabolites in the two groups of mice. This model also explained 60.7% of the variation (*R2X*). As shown in Fig. 2A, the *x*-axis and *y*-axis indicate the first principal component and second principal component, respectively. One dot represents one observation sample, and the distances between dots represent the similarity of the sample's metabolite composition. In general, the two groups of samples cluster separately. The SPF mice tended to cluster to the left part of the figure, whereas the GF mice are on the right.

3.3. Significant metabolic perturbation in livers of GF mice

We created a PLS-DA loading plot to display the specific metabolites that differed between the two groups of mice. The variables in the right side of Fig. 2B indicate higher metabolite level in GF mice as compared with SPF mice, and vice versa. In comparison with SPF mice, the GF mice showed the most significantly higher level in monosaccharides, such as glucose (var. 42, 45) and fructose (var. 35), and a significantly higher level of organic acids, such as lactate, succinate, fumarate, malate and citrate. This finding suggested that the efficacy of the tricarboxylic acid (TCA) cycle activities was highly elevated in the liver of GF mice. The GF mice showed high contents, although not significant, of alanine, serine and threonine. According to these findings, we presumed that GF mice might exhibit gluconeogenesis. In contrast, the variables in the left part of the plot in Fig. 2B indicated the metabolites with differential expression in SPF than GF mice. Metabolites of β-hydroxybutyric acid (var. 6), β-aminoisobutyric acid (var. 20), 2,3,4-trihydroxybutyric acid (var. 28),

Table 1
Metabolites observed on GC-MS to have higher, lower or no difference in content in GF mice than in SPF mice

Identified metabolites	GF mice	SPF mice	Peak ID
Higher content in GF mice			
Alanine	3.78±0.58 [b, *, †]	1.79±0.24 [a]	3
Serine	0.26±0.08 [b]	0.03±0.04 [a]	17
Threonine	0.16±0.05 [b]	0.04±0.09 [a]	18
Lactate	31.9±3.2 [b]	15.5±12.3 [a]	1
Succinate	1.49±0.29 [b]	0.87±0.14 [a]	15
Fumarate	0.17±0.06 [b]	0.02±0.04 [a]	16
Malate	1.08±0.38 [b]	0.39±0.12 [a]	23
Citrate	0.06±0.03	ND	37
α-Fructose	1.44±0.32 [b]	0.13±0.08 [a]	35
β-Fructose	2.97±1.98 [b]	0.28±0.23 [a]	40
α-Glucose	346.0±55.1 [b]	22.4±7.86 [a]	42
β-Glucose	441.4±65.8 [b]	37.3±12.5 [a]	45
Glucitol	0.19±0.04	ND	43
Caprylic acid	1.66±0.35 [b]	1.02±0.13 [a]	38
Glycerol phosphate	3.38±0.45 [b]	2.39±0.44 [a]	34
Creatinine	0.19±0.05	ND	27
Lower content in GF mice			
2-Hydroxybutyric acid	0.68±0.26 [a]	3.71±0.45 [b]	6
2,3,4-Trihydroxybutyric acid	0.17±0.03 [a]	0.40±0.03 [b]	28
β-Aminoisobutyric acid	0.79±0.21 [a]	1.25±0.11 [b]	20
Gluconic acid	0.83±0.19 [a]	1.96±0.27 [b]	44
Niacinamide	0.11±0.02 [a]	0.25±0.05 [b]	21
Stearic acid glycerol	0.09±0.01 [a]	0.18±0.04 [b]	52
No significant difference			
Acetate	1.10±0.73 [a]	0.35±0.18 [a]	2
Valine	0.28±0.02 [a]	0.37±0.20 [a]	8
Glutamate	0.97±0.14 [a]	0.80±0.23 [a]	25
Aspartate	0.29±0.02 [a]	0.26±0.03 [a]	26
Phosphoric acid	980.8±295.4 [a]	1111.8±333.9 [a]	10
Dodecanoic acid	0.06±0.01 [a]	0.06±0.06 [a]	32
Urea	7.40±1.62[a]	6.34±1.44 [a]	9
Inositol	2.62±0.13 [a]	3.24±0.52 [a]	47

ND: not detected.

* The relative intensity of each metabolite was expressed as 100 times the ratio of its peak area to that of ribitol (internal standard) on the same chromatograph.

† Data are means±S.D. One-way ANOVA was used for analysis. The same letter indicates no significant difference, and different letters indicate significant difference ($P<.05$).

gluconic acid (var. 44), niacinamide (var. 21) and stearic acid glycerol (var. 52) were detected with higher levels in SPF mice but were significantly lower in GF mice. Variables closer to the center in the loading plot show less differentiation in level. We identified 30 metabolites from the similarity between our mass spectrometry data and the NIST database (Table 1).

3.4. Relationship between biochemical parameters and endogenous metabolites

To verify our results, we further examined the blood biochemical parameters in these mice. Before the experiment, we confirmed that the GF and SPF groups had equal daily dietary intake and water consumption (food: 4.01±0.25 vs. 3.87±0.58 g/day; water: 5.58±0.44 vs. 6.27±1.54 g/day) and that body weights were similar (25.1±0.88 vs. 25.8±0.45 g). However, epididymal fat pad weights in the SPF group were significantly greater than those in the GF group (SPF group, 0.44±0.08 g; GF group, 0.21±0.03 g). The serum glucose and TG content was significantly higher in GF mice than in SPF mice (Fig. 3), with no difference between the mice in TP, TC, LDL-C and HDL-C concentrations.

3.5. Hepatic morphology and histological evaluation in SPF and GF mice

The SPF and GF mice did not differ in liver weight (0.94±0.08 vs. 0.92±0.04 g). Fig. 4A shows the Oil Red O staining of liver sections; the red particle in the cytoplasm indicates the hepatocytes containing lipid droplets in the pericentral areas in the SPF group, which was similar to previous studies [11]. The TG levels were lower in both serum (Fig. 3) and liver of SPF mice (Fig. 4A) combined with higher epididymal fat pad weights in the SPF group, which is consistent with increased lipid clearance. The PAS staining is a main method used to detect glycogen in tissues; pink color indicates the presence of hepatic glycogen. Liver sections of GF mice showed a significantly greater number of PAS-positive hepatocytes (arrow), which suggests glycogen accumulation in hepatocytes of GF more than SPF mice (Fig. 4B). Our findings indicate that gut microbiota significantly affect host metabolism.

4. Discussion

Recently, the relation between the mammalian host and gut microbiota has received much attention. Research has indicated

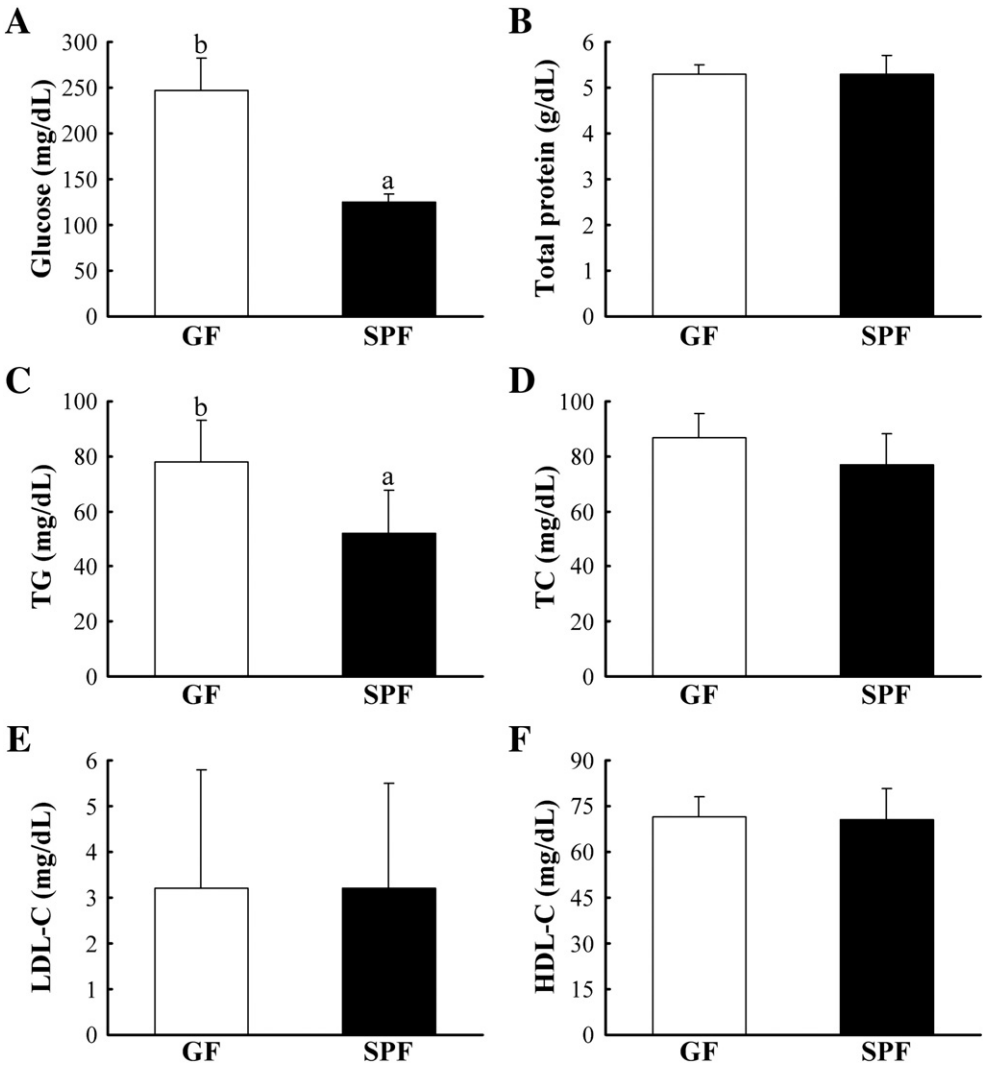

Fig. 3. Serum content of glucose (A), TP (B), TGs (C), TC (D), LDL-C (E) and HDL-C (F) in GF and SPF mice. Data are means ± S.D. for five mice of each group. One-way ANOVA was used for analysis. Different letters indicate significant difference at $P<.05$.

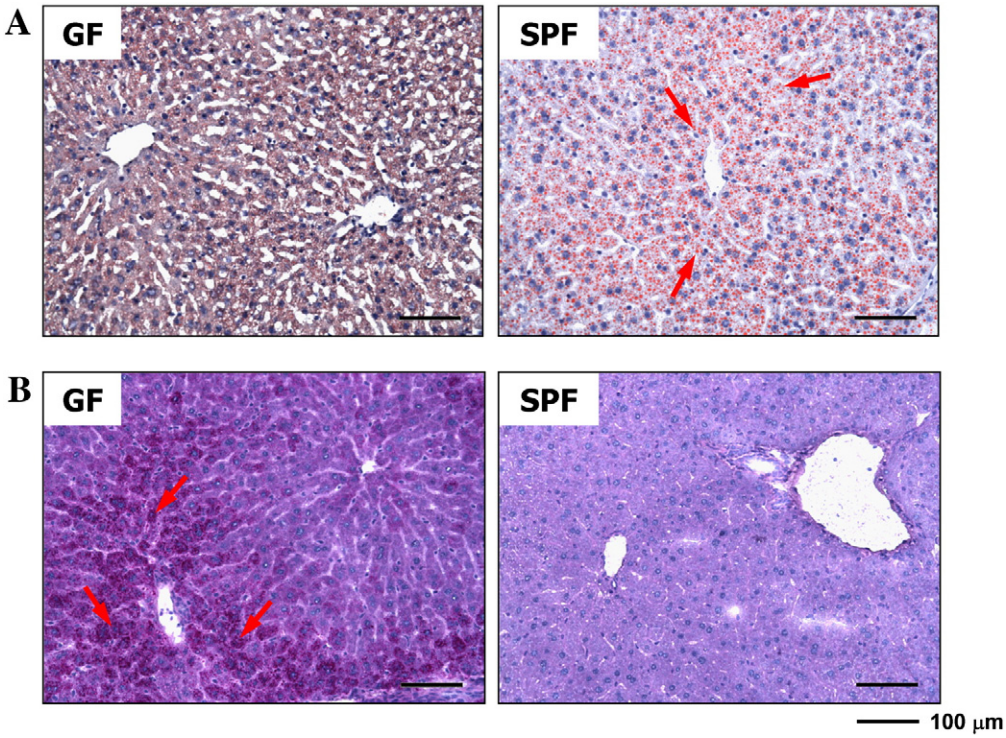

Fig. 4. Histological evaluation of lipids and glycogen in livers of SPF and GF mice. (A) Representative Oil-Red-O-stained liver sections showing greater oil body expression (arrows) in SPF mice. (B) Light micrographs of liver tissue specimens stained with PAS. The PAS-stained hepatocytes (arrows) in specimens from GF mice. Left panel: GF mice; right panel: SPF mice. Magnification ×200. Scale bars, 100 μm.

that gut microbiota modulate host energy and lipid metabolism; therefore, gut microbiota are now considered a "microbial organ" within host organisms. Gut microbiota are known to contribute to hepatic lipogenesis and fat storage. To better understand and characterize the host energy metabolism in the gut lacking microbiota, we compared the liver metabolome of SPF and GF mice by GC-MS combined with PLS-DA to investigate metabolic differences in livers of SPF and GF mice. As compared with previous studies demonstrating that microbiota promote hepatic production of TGs and storage TGs in adipocytes [12], our study demonstrates that glycogen accumulation in liver is the main energy storage form for mice without microbiota.

Levels of metabolites provide integrated information on cellular function and define the phenotype of cells or organs in response to genetic and environmental changes [28]. Our metabolomics study of livers of GF and SPF mice based on GC-MS and multivariate statistical analysis involved use of PLS-DA, a chemometric procedure for discriminating a two-class data set to define the maximum classification and separation of independent samples. The score plot (Fig. 2A) revealed that the first principal component distinguished clusters of metabolites for SPF and GF mice by visual inspection of metabolic patterns and considering the values of *R2Y* and *Q2Y*. By integrating the results from the loading plot (Fig. 2B) and statistical analysis of identified metabolites (Table 1), we observed that the level of glucose in liver was more than 10-fold increased in GF mice as compared with SPF mice. Additionally, the contents of lactate and alanine were elevated about twofold in GF mice. Lactate and alanine are two important precursors of gluconeogenesis, which takes place mainly in the liver and results in the generation of glucose from noncarbohydrate carbon substrates. Among the identified metabolites, TCA cycle-related components, including succinate, fumarate, malate and citrate, were significantly increased in GF mice. These results indicate the high efficacy of gluconeogenesis in GF mice, which can be supported by the twofold increase in serum glucose in these mice (Fig. 3A).

In general, excess glucose can be converted to fatty acid and then form triacylglycerol via lipogenesis or is stored as glycogen through glycogenesis. Our histology results further demonstrated that glucose is channeled along glycogenesis to glycogen in GF mice; therefore, the livers of GF mice showed glycogen accumulation (Fig. 4B). In contrast, β-hydroxybutyric acid is a ketone body, is produced in liver mitochondria from fatty acids and provides acetoacetyl-CoA and acetyl-CoA for synthesis of cholesterol, fatty acids and complex lipids [28,29]. The GF mice showed low levels of β-hydroxybutyric acid and stearic acid glycerol, which suggested low lipogenesis in the livers of these mice. This finding is further supported by the Oil Red O staining results of low evidence of lipids in GF mice (Fig. 4A). High-resolution ^{1}H-NMR spectroscopy was used to study the metabolic phenotype of liver tissue from SPF and GF mice in a previous study, but revealed no difference in glucose and glycogen signals in profiles [18]. Velagapudi et al. [19] used GC-MS to compare the serum metabolome profiles of SPF and GF mice but found that only energy metabolites increased in level in SPF mice. However, we used the different strategies and found interesting observations. Our study successfully demonstrated that glycogen should be the dominant energy storage form of mice lacking a microbiome.

The obesity has been demonstrated to be associated with an altered gut microbiota in rodent model, and their relative contributions vary considerably according to body composition [34]. We examined the microbial species counts in the fecal of our SPF C57BL/6JNarl mice; the result showed that the composition of gut microbiota profile in our SPF mice contained Streptococcaceae, lactobacilli, Bacteroidetes, and *Clostridium*-other. Compared with the *Lep*$^{ob/ob}$ mice (both of them were housed at the same

environment and fed the same food; unpublished data), our results resemble the study of Turnbaugh et al [34], in which the population of Bacteroidetes in C57BL/6JNarl is more than that in obese mice. Ley et al. have demonstrated that the obese *Lep*$^{ob/ob}$ mice have 50% fewer Bacteroidetes and a corresponding increase in the proportion of Firmicutes than their lean littermates [35]. Furthermore, Turnbaugh and collaborators transplanted cecal microbiota from *Lep*$^{ob/ob}$ and lean mice to GF mice recipients. These mice harboring the microbiota from obese mice had a modest fat gain and extracted more calories from their food compared to the lean mice having received the gut microbiota from lean mouse donors [34]. These studies suggested that Bacteroidetes and Firmicutes should be the significant gut microorganisms, which affect lipogenesis and lipid accumulation in the SPF mice.

A number of studies have revealed that gut microbiota influences fat storage, obesity and metabolic disease [30,31]. The gut microbiota in obesity can increase lipogenesis and reduce insulin sensitivity, and vice versa [14]. Germ-free animals have been reported to show protection against diet-induced obesity by an increase in fatty acid metabolism via two complementary but independent mechanisms [13]. Here we demonstrated that GF mice showed significantly increased glycogen synthesis in liver. Abnormal glycogen stored in the liver can result in the liver's failure to regulate the metabolism of glycogen and glucose, named glycogen storage disease (GSD) [32]. Glycogen storage disease is an inherited disorder caused by defective enzymes involved in glycogen synthesis or breakdown. Therefore, lack of gut microbiota resulting in glycogen accumulation needs further investigation in relation to inducing GSD.

In conclusion, we used a metabolomics approach to identify metabolites and to compare groups of metabolites that change in level in response to presence or absence of gut microbiota in mice. We demonstrated that the host gluconeogenesis and glycogenesis processes synthesize glycogen for accumulation in the liver lacking gut microbiota. Recently, increasing studies have investigated the composition of gut microbiota and its possible relationship with obesity [33–35]. Our findings shed light on a new perspective of the role of gut microbiota in energy metabolism and will be useful to help study probiotics, obesity and metabolic diseases.

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