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NOTE

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Fecal metabolite analysis of Japanese macaques in Yakushima by LC-MS/MS and LC-QTOF-MS

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ABSTRACT. We performed a comprehensive fecal metabolite analysis using LC-MS/MS and LC-QTOF-MS approaches as a preliminary study. Feces of Japanese macaques on Yakushima Island were collected from five monkeys at two separate locations. Using the former methodology, 59 substances such as free amino acids, nucleotides, nucleosides and nucleic acid bases, and organic acids in the citrate cycle were quantitatively detected and successfully differentiated in two different monkey groups by the concentrations of nucleic acid metabolites and free amino acids. In the latter, around 12,000 substances were detected both by positive and negative mode in each sample. Differences in signal intensities were observed between two monkey groups in the concentrations of plant secondary metabolites such as cyanogenic glycosides, flavonoids, and phenolics.

KEY WORDS: fecal metabolome, liquid chromatography-quadrupole time-of-flight mass spectrometry, liquid chromatography-tandem mass spectrometry, wild monkey

A 16S rRNA genes amplicon sequence analysis, so-called Meta 16S microbiome, became popular in the wildlife sciences due to the recent development of sequencing technology [2, 4, 12]. It is a powerful tool for distinguishing one group of animals, such as captive individuals, from other groups, such as their wild counterparts. However, microbiome analyses are based only on bacterial phylogenetic information that does not reveal any nutritional and biochemical information about the gut environment. The shotgun metagenomic approach was applied to estimate the gut environment with various sequence depth levels [2, 7–9], but it is still based on the presence/absence of particular gene repertoires, which does not suggest the actual biochemical reactions.

The recent development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) approaches enables the comprehensive detection and quantification of metabolites, which suggests the actual gut chemical environment [13, 14]. Food is a major resource to modify the gut chemical environment. If two animal groups consume different food items, their gut chemical environment may differ. In this study, we performed a comprehensive fecal metabolite analysis using LC-MS/MS and LC-QTOF-MS to know whether this approach can successfully distinguish a group of wild monkeys from an adjacent group.

Fresh feces of Japanese macaques (*Macaca fuscata*) on Yakushima Island were collected from four monkeys at two separate locations (the Kawahara area and the Hizukushi area), as indicated in Fig. 1. Feces were collected upon observation of defecation. The sex and age of the monkey were visually estimated. A one night old feces, designated S1, was also collected at the point in the Kawahara-north area. Two fresh feces were collected at each site; S2 and S3 were from monkeys in the Kawahara-south area, and S4 and S5 were from monkeys in the Hizukushi area. Samples S2, S3, S4, and S5 were respectively collected from a juvenile male, another juvenile male, an adult female, and another adult female. Feces were immediately frozen on dry ice and transported to the laboratory. After being thawed, feces were mixed with buffer and extracted according to the protocol of Matsumoto [7]. The extracts were subjected to MS analyses both by LC-MS/MS (LCMS-8060, Shimadzu Co., Kyoto, Japan) and LC-QTOF-MS (LCMS-9030, Shimadzu Co.). Details of the LC-MS/MS analysis were given in our previous report [5]. In brief, hydrophilic metabolites in feces were extracted by Dulbecco's phosphate-buffered saline containing 2-morpholinoethanesulfonic acid (0.1 mol/l) as an internal standard.

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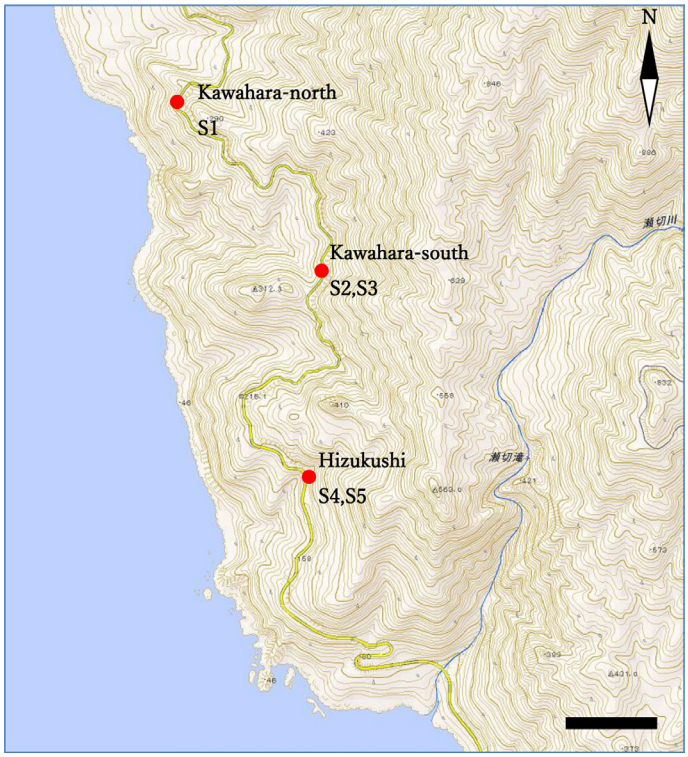

Fig. 1. Sampling locations. Map image was obtained from the Geospatial Information Authority of Japan (https://maps.gsi.go.jp/development/ichiran.html). S1 was an overnight feces, S2 and S3 were fresh feces from juvenile males, and S4 and S5 were fresh feces from adult females. Direction arrow shows the orientation of the figure and bar on the bottom represents 500 m.

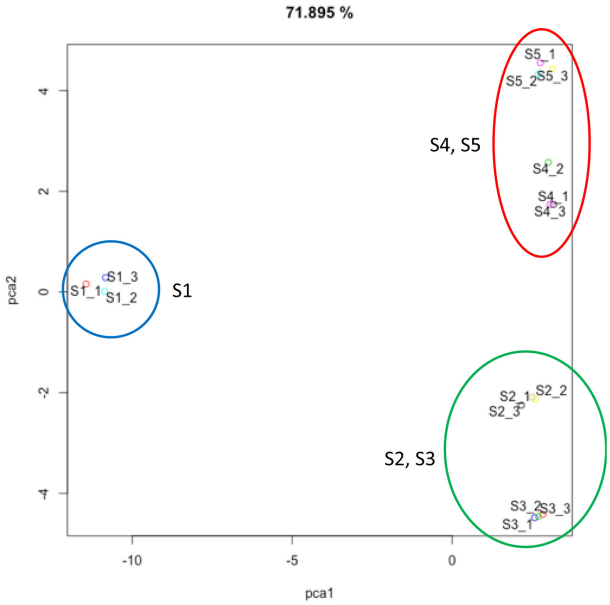

Fig. 2. Principal component analysis (PCA) plot of hydrophilic metabolites detected by Liquid chromatography-tandem mass spectrometry (LC-MS/MS). S1-S5: corresponding to sample IDs shown in Fig. 1. Value in % on the top of the figure shows contribution rate of PC1.

The supernatant was obtained by centrifugation and further subjected to filtration through a 3 kDa cutoff filter. The filtrate was diluted tenfold and analyzed using a Nexera X2 system (Shimadzu Co.) coupled with an LCMS-8060 triple quadrupole mass spectrometer (Shimadzu Co.). Ninety-seven of the target metabolites were analyzed using MRM mode. A Discovery HS F5 column (150 mm × 2.1 mm, 3 µm, Sigma-Aldrich, St. Louis, MO, USA) was used for the separation of metabolites with gradient elution. Peak selection and integration were performed using LabSolutions LCMS (Shimadzu Co.); subsequently, principal component analyses (PCA) were conducted using R version 3.5.2 [10]. The peak area value of each metabolite was normalized to that of the internal standard (2-morpholinoethanesulfonic acid). Triplicate analyses were conducted for each sample, and these were used for the PCA analyses. The levels of these metabolites were compared between two locations (S2 and S3 vs. S4 and S5) by Welch *t*-test. $P<0.1$ is considered as marginally significant.

The same filtrates of sample S2, S3, S4 and S5 were subjected to non-target analysis by LC-QTOF-MS. The Nexera X2 system (Shimadzu Co.), coupled with an LCMS-9030 QTOF mass spectrometer (Shimadzu Co.), was used. A Discovery HS F5 column (150 mm × 2.1 mm, 3 µm, Sigma-Aldrich) was used to separate the metabolites with gradient elution. Scan analysis was performed in ranges of *m/z* 50–1,000 and 50–800, respectively, in positive ion mode and negative ion mode. In mass spectra, protonated analyte molecules are observed in the positive ion mode operation and deprotonated analyte molecules are observed in the negative ion mode operation. Before analysis, external mass calibration using clusters of NaI was performed. Peak annotation and peak alignment were performed using MS-DIAL ver.4.00 (http://prime.psc.riken.jp/compms/msdial/main.html). Single analysis was performed for each sample and the results were subjected to PCA analyses. The levels of these metabolites were compared between two locations (S2 and S3 vs. S4 and S5) by Welch *t*-test.

In the LC-MS/MS analyses, 59 substances such as free amino acids (19 substances), amino acid derivatives or metabolites (16 substances), nucleic acid metabolites (16 substances), organic acids in the TCA cycle (5 substances), and vitamins (3 substances) were quantitatively detected (Supplementary Table 1). The metabolite profiles successfully differentiated two different monkey groups by PCA plot (Fig. 2). However, due to the small sample size, significant differences were not detected except for several substances, mostly nucleic acid metabolites such as adenine, guanine, thymine, uracil, adenosine, flavin mononucleotide (FMN), nicotinic acid, and uric acid. One overnight feces (sample S1) showed a very different metabolite profile compared to those of fresh feces. Cystathionine and ophthalmic acid were only detected in old feces; on the contrary, creatine, creatinine, dopamine, serotonin, guanine, and FMN were not detected. In addition, carnitine and acetylcarnitine were high in the overnight feces and uric acid was low in the overnight feces. The reasons for such a difference between this overnight feces and other fresh feces are inconclusive due to the sample size. It is possible that food

consumed by the host monkey for the sample S1 may have originated from an animal source rather than plant, because the higher content of carnitine and acetylcarnitine in the feces. Both metabolites are normally high in animal products and an omnivorous diet contains 20 to 100 times larger amount of carnitine than a strict vegetarian diet [11]. Time after defecation may also have an effect on the composition due to the metabolism of aerobic microorganisms. For example, uric acid was very low in sample S1. In fact, fecal uric acid can be degraded by many aerobic uricase-positive bacteria and fungi [1]. In the present study, S1 data was considered as an out group in cluster analyses, because one-night old feces must be very different from those collected upon defecation.

In the latter, around 5,000 and 7,000 substances were detected, respectively, in positive mode and in negative mode in each sample. However, there were 992 and 585 successfully identified substances for the positive mode and negative mode, respectively. Differences in signal intensities were observed between two monkey groups in the concentrations of plant secondary metabolites such as cyanogenic glycosides, flavonoids, and phenolics (Supplementary Tables 2 and 3). These differences separate one group from another on a PCA plot (Fig. 3A–C).

Since this study is of a preliminary nature, we did not follow the feeding behavior of these two different groups. Therefore, we could not conclude the reason for such differences in metabolite profiles. However, food eaten in the previous 24 hr might be different between two groups of monkeys, because we detected the major differences in concentrations of plant secondary metabolites in fecal metabolite profiles. In fact, Japanese macaques in Yakushima feed mainly on plants [6] and secondary metabolite concentration varies with plant species [3].

The present study clearly shows the usefulness of metabolomic analyses of fresh feces using LC-MS/MS and LC-QTOF-MS in ecological studies, particularly to distinguish the feeding behavior of hosts. Fresh fecal samples are generally considered as the best samples for the fecal metabolite analysis due to the decay of volatile metabolites such as short chain fatty acids, ammonia and hydrogen sulfides. Although the present analyses targeted the hydrophilic metabolite seemed to be less influenced by the time of exposure to air, we followed the general consensus about superiority of immediately frozen freshly defecated samples. Further elucidation is required to validate the present analytical approach by increasing the number of fecal samples from different monkey groups.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

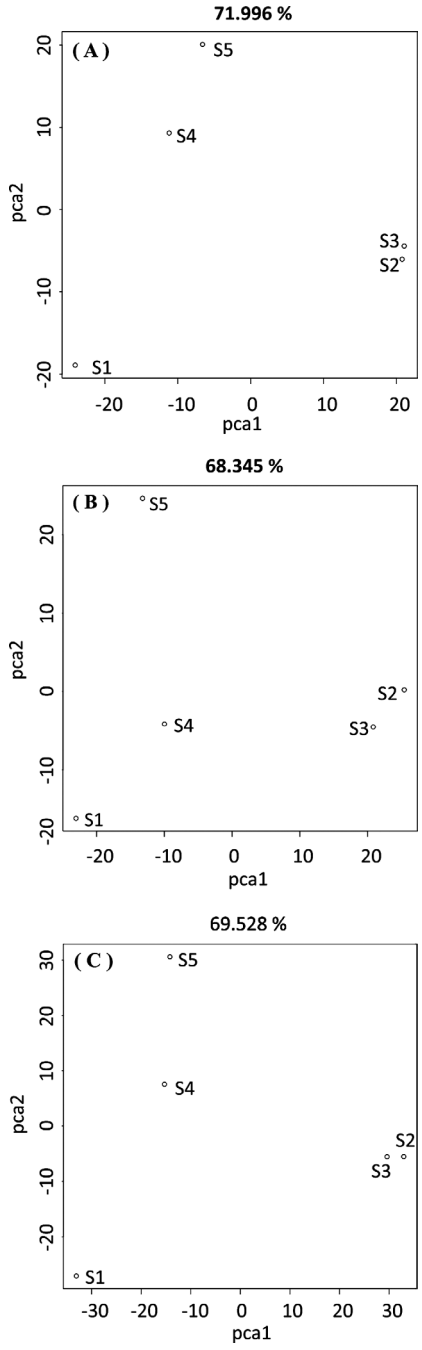

Fig. 3. Principal component analysis (PCA) plot for identified metabolites by Liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). (A) Metabolites identified by a positive mode, (B) metabolites identified by a negative mode. (C) PCA plot for compiled data with positive and negative mode analyses. Sample IDs are shown in Fig. 1. Values in % on the top of the figure show contribution rate of PC1.

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