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# Mice fecal metabolomics by LC-MS/MS: Comparison between young mice and old mice

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Takanari Hattori[1, 2], Shuichi Kawana[1], Yoshihiro Hayakawa[1], Eiichiro Fukusaki[3], Mitsuharu Matsumoto[4]
1 Shimadzu Corporation, Kyoto, Japan.
2 Osaka University Shimadzu Analytical Innovation Research Laboratory, Osaka, Japan.
3 Osaka University, Osaka, Japan.
4 Kyodo Milk Industry Co. Ltd., Tokyo, Japan

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## Introduction

The intestinal microbiome plays an important role in health and/or disease because it influences pathological and normal homeostatic functions. Low-molecular-weight metabolites produced by intestinal microbiome are absorbed constantly from the intestinal lumen and carried to systemic circulation; they play a direct role in health and/or disease. There are limited reports concerning the function of metabolites produced by intestinal microbiome. Furthermore, these studies are targeting specific metabolites such as short chain fatty acids but not global metabolites (metabolome). For clarifying the relationship between health and/or disease and metabolome produced by intestinal bacteria, only free bacterial metabolites in the intestinal luminal content (or feces) should be analyzed. In this study, metabolites in feces of young and old mice were analyzed by LC-MS/MS.

## Methods and Materials

### Sample Preparation

Mice feces were diluted with phosphate-buffered saline and extracted by intense mixing. The upper portion was centrifuged and the supernatant was centrifugally filtered.
Figure 1 shows the detailed procedures.

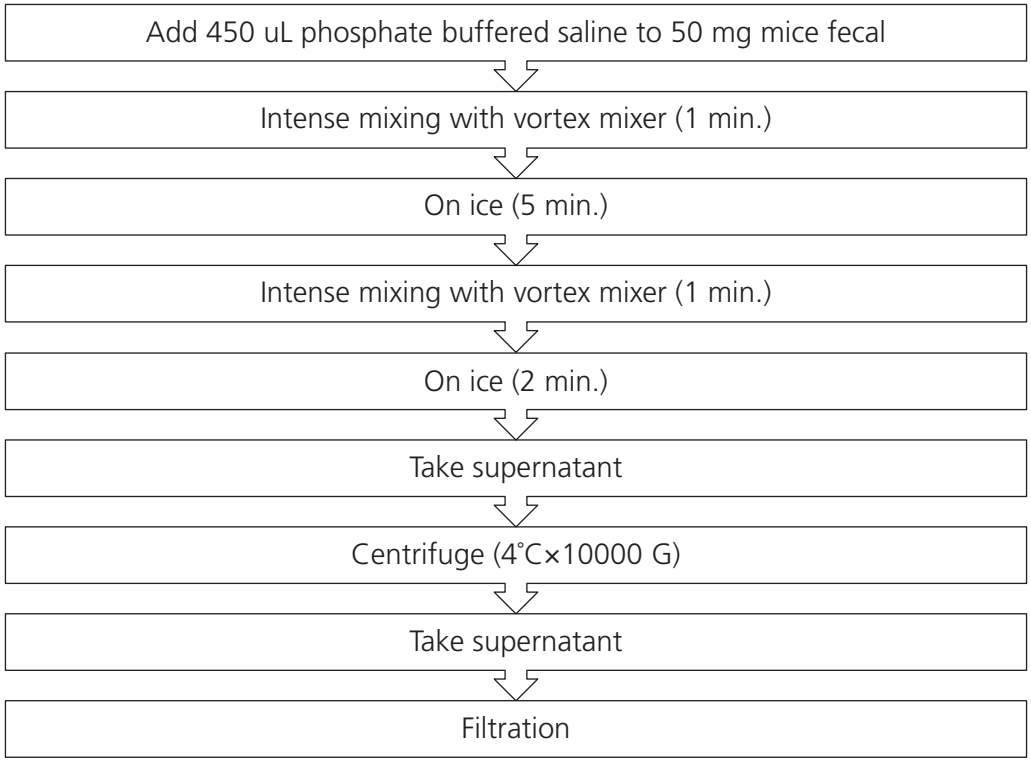


Figure 1 Process flow of sample preparation

# Mice fecal metabolomics by LC-MS/MS: Comparison between young mice and old mice

## Experimental

Two LC-MS/MS methods (ion-pairing and non-ion-pairing method) were used for analysis. Nexera X2 system coupled with a LCMS-8040 or a LCMS-8050 (Shimadzu Corporation, Japan) was used. In ion-pairing method, separation was achieved on Mastro C18 column (Shimadzu GLC Ltd., Japan). The mobile phase consisted of 15 mM acetic acid and 10 mM tributylamine in water (A) and methanol (B) under gradient elution. In non-ion-pairing method, separation was achieved on Discovery HS F5-3 column (Sigma-Aldrich, U.S.A.). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) under gradient elution.

Analytical Condition (Ion-pairing method)

| | | |
|---|---|---|
| **UHPLC (Nexera X2 system)** | | |
| Column | : | Mastro C18 (150 mmL.×2.0 mmI.D., 2.0 µm) |
| Mobile phase A | : | 15 mM Acetate, 10 mM Tributylaimne/water |
| B | : | Methanol |
| Flow rate | : | 0.3 mL/min |
| Injection vol. | : | 3 µL |
| Column temp. | : | 40°C |
| **MS (LCMS-8040)** | | |
| Ionization | : | Negative, MRM mode |
| DL temp. | : | 250°C |
| HB temp | : | 400°C |
| Drying gas | : | 10 L/min |
| Nebulizing gas | : | 2.0 L/min |

Analytical Condition (Non-ion-pairing method)

| | | |
|---|---|---|
| **UHPLC (Nexera X2 system)** | | |
| Column | : | Discovery HS F5 (150 mmL.×2.1 mmI.D., 3.0 µm) |
| Mobile phase A | : | 0.1% Formate/water |
| B | : | 0.1% Formate/acetonitrile |
| Flow rate | : | 0.25 mL/min |
| Injection vol. | : | 3 µL |
| Column temp. | : | 40°C |
| **MS (LCMS-8050)** | | |
| Ionization | : | Positive/Negative, MRM mode |
| DL temp. | : | 250°C |
| HB temp. | : | 400°C |
| Interface temp. | : | 400°C |
| Nebulizing gas | : | 2.0 L/min |
| Drying gas | : | 10 L/min |
| Heating gas | : | 10 L/min |

# Result

## Analysis of Metabolites in Mice Fecal by LC-MS/MS

We evaluated 55 and 97 of metabolites in ion-pairing method and non-ion-pairing method respectively. In ion-pairing method, 17 of metabolites were detected in the extract of mice feces (peak area RSD <20%). Main compounds of 17 metabolites were amino acids.
In non-ion-pairing method, 75 of metabolites were detected in the 10-fold diluted extract of mice feces (peak area RSD <20%). Main compounds of 75 metabolites were amino acids and nucleic acid-related substances. Figure 2 shows the MRM chromatogram of extract of mice feces obtained using LC-MS/MS. The list of detected compounds and their repeatability is shown in Table 1.

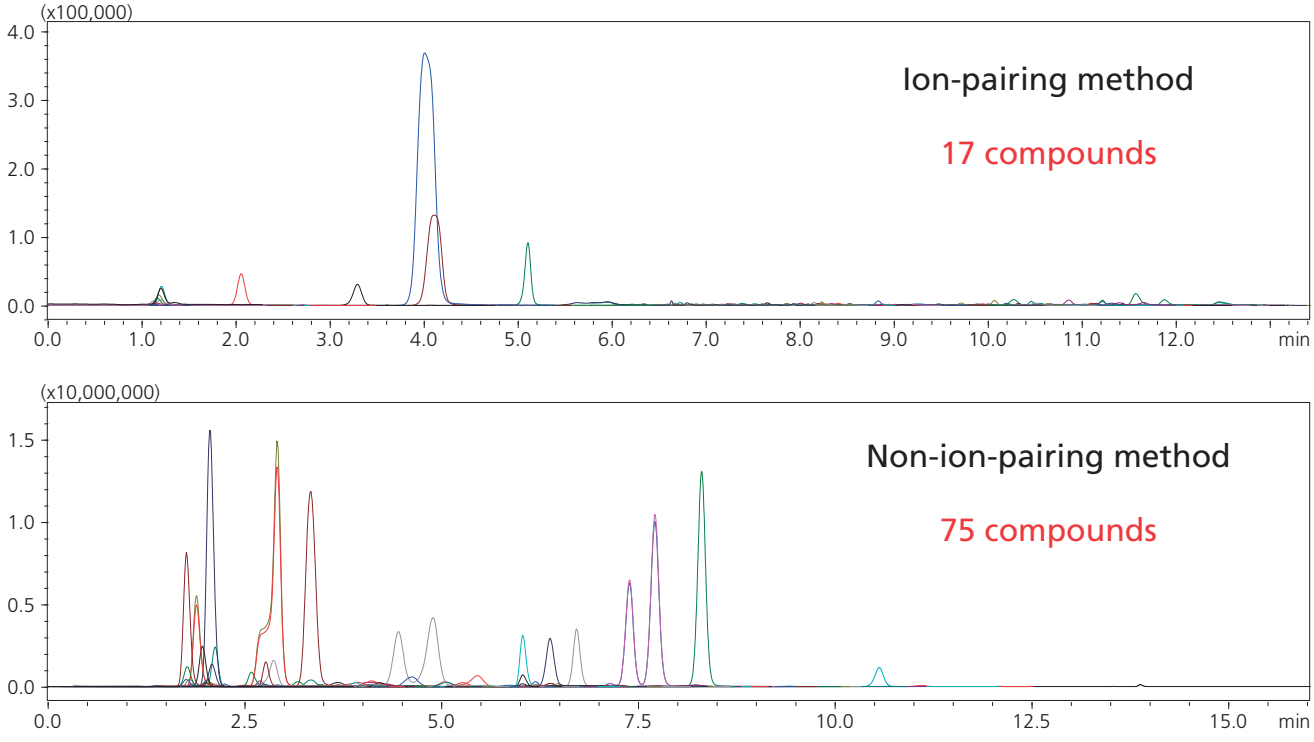


Figure 2 MRM chromatograms of extract of mice feces

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Table 1 Detected compounds and their repeatability

| Category | Compounds | Repeatability (%RSD, n=6) | |
|---|---|---|---|
| | | R.T. | Peak area |
| Amino acid | 4-Hydroxyproline | 0.11 | 3.8 |
| | Alanine | 0.10 | 3.0 |
| | | 0.06 | 3.0 |
| | Arginine | 0.07 | 2.6 |
| | | 0.05 | 5.0 |
| | Asparagine | 0.10 | 1.4 |
| | | 0.21 | 9.7 |
| | Aspartic acid | 0.07 | 1.7 |
| | | 0.21 | 1.1 |
| | Asymmetric dimethylarginine | 0.18 | 1.5 |
| | Citrulline | 0.09 | 2.1 |
| | Cystine | 0.16 | 7.2 |
| | Dimethylglycine | 0.07 | 5.5 |
| | Glutamic acid | 0.09 | 1.6 |
| | | 0.27 | 0.5 |
| | Glutamine | 0.10 | 1.0 |
| | | 0.06 | 3.1 |
| | Glycine | 0.08 | 4.0 |
| | | 0.19 | 5.9 |
| | Histidine | 0.06 | 4.7 |
| | | 2.12 | 18.0 |
| | Isoleucine | 0.13 | 1.7 |
| | Leucine | 0.10 | 2.4 |
| | Lysine | 0.08 | 0.7 |
| | | 0.06 | 5.0 |
| | Methionine sulfoxide | 0.08 | 2.1 |
| | Ornitine | 0.06 | 2.8 |
| | Phenylalanine | 0.07 | 1.0 |
| | | 0.14 | 0.6 |
| | Proline | 0.04 | 2.3 |
| | Serine | 0.09 | 1.9 |
| | | 0.13 | 9.3 |
| | Symmetric dimethylarginine | 0.22 | 4.3 |
| | Threonine | 0.09 | 2.4 |
| | | 0.09 | 3.5 |
| | Tryptophan | 0.04 | 1.2 |
| | Tyrosine | 0.13 | 0.9 |
| | | 0.10 | 2.4 |
| | Valine | 0.05 | 4.8 |
| Nucleosides and Nucleotides | Adenine | 0.20 | 2.1 |
| | Adenosine | 0.23 | 9.6 |
| | Adenosine 3',5'-cyclic monophosphate | 0.42 | 14.8 |
| | Adenosine monophosphate | 0.14 | 3.6 |
| | Cytidine | 0.23 | 1.2 |
| | Cytidine monophosphate | 0.07 | 1.3 |
| | Cytosine | 0.12 | 9.2 |
| Nucleosides and Nucleotides | Guanine | 0.17 | 5.8 |
| | Guanosine | 0.32 | 2.1 |
| | Guanosine monophosphate | 0.16 | 6.4 |
| | Inosine | 0.33 | 1.5 |
| | Thymidine | 0.26 | 10.1 |
| | Thymidine monophosphate | 0.18 | 13.1 |
| | | 0.03 | 6.6 |
| | Thymine | 0.05 | 6.9 |
| | Uracil | 0.12 | 3.1 |
| | Uridine | 0.07 | 3.7 |
| | Uridine diphosphate | 0.03 | 8.1 |
| | Xanthine | 0.15 | 7.6 |
| Organic acid | 4-Aminobutyric acid | 0.06 | 3.9 |
| | Argininosuccinic acid | 0.14 | 14.1 |
| | Cholic acid | 0.02 | 2.6 |
| | Creatine | 0.28 | 2.5 |
| | Lactic acid | 0.04 | 5.0 |
| | Nicotinic acid | 0.10 | 3.4 |
| | Pantothenic acid | 0.16 | 2.6 |
| | Taurocholic acid | 0.04 | 4.1 |
| | Uric acid | 0.15 | 7.0 |
| TCA cycle | 2-Ketoglutaric acid | 0.07 | 2.9 |
| | Aconitic acid | 0.14 | 2.4 |
| | Citric acid | 0.13 | 6.4 |
| | Isocitric acid | 0.12 | 5.0 |
| | Malic acid | 0.10 | 2.8 |
| | Succinic acid | 0.06 | 2.2 |
| Methylation and Transsulfuration cycle | Cystathionine | 0.14 | 5.1 |
| | Cysteine | 0.20 | 7.1 |
| | Methionine | 0.06 | 2.5 |
| | | 0.04 | 1.6 |
| | S-Adenosylhomocysteine | 0.12 | 8.9 |
| Others | 2-Aminobutyric acid | 0.24 | 4.8 |
| | 6-Phosphogluconic acid | 0.04 | 5.6 |
| | Acetylcarnitine | 0.15 | 3.0 |
| | Allantoin | 0.11 | 4.2 |
| | Carnitine | 0.12 | 6.9 |
| | Carnosine | 0.16 | 2.0 |
| | Choline | 0.05 | 1.6 |
| | Creatinine | 0.06 | 3.0 |
| | Dopamine | 0.26 | 6.4 |
| | FMN | 0.11 | 8.0 |
| | Histamine | 0.22 | 2.6 |
| | Hypoxanthine | 0.04 | 1.4 |
| | Norepinephrine | 0.54 | 1.7 |
| | Serotonin | 0.06 | 2.4 |

* Red: the repeatability of compounds using ion-pairing method
Black: the repeatability of compounds using non-ion-pairing method

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## Comparison between Young Mice and Old Mice

As an application to fecal metabolomics, feces derived from mice that the age was different (10-week-old and 70-week-old) were analyzed by non-ion-pairing method. As results of analyses for the extracts of the mice feces, 66 of metabolites were detected. By Traverse MS software, principal component analyses (PCA) were performed. As shown in Figure 3, two mice feces were successfully classified. The concentrations of 21 in old mice (70-week-old) feces were obviously lower ($p < 0.01$) than those in young mice (10-week-old) feces. The lower concentration of fecal cholic acid in old mice feces indicates that the secretion of bile acid was decreased by the ageing. On the other hand, there is a likelihood that the number of bacteria that deconjugate bile acid (taurocholic acid) to taurine and cholic acid, is more in young mice than old mice. In contrast, the concentrations of 26 in old mice feces were obviously higher ($p < 0.01$) than those in young mice feces. Intestinal luminal arginine is absorbed and converted to polyamines that are essential substances for the proliferation of somatic cells, through ornithine. The lower concentration of arginine in young mice feces indicates that colonocytes of young mice absorb luminal arginine actively to grow compared with old mice. Table 2 shows the list of compounds that showed significant differences between young mice and old mice.

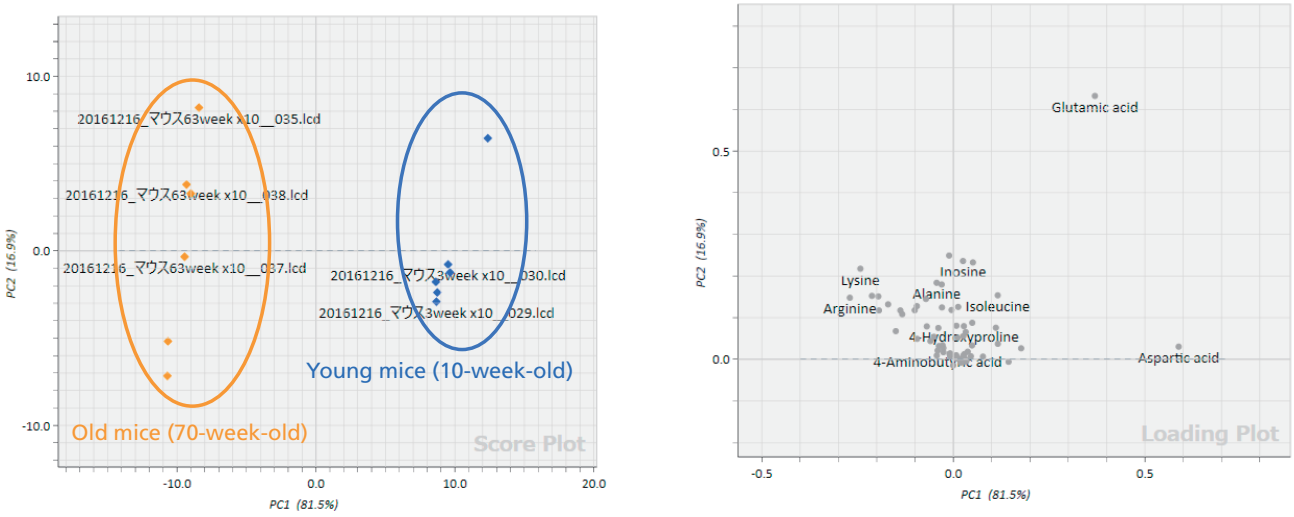


Figure 3 Score plot and loading plot

# Mice fecal metabolomics by LC-MS/MS: Comparison between young mice and old mice

Table 2 Compounds that showed significant differences between young mice and old mice

| Compounds | | | |
|---|---|---|---|
| Young mice > Old mice | | Old mice > Young mice | |
| Adenine | Proline | 4-Aminobutyric acid | Guanosine monophosphate |
| Adenosine | Succinic acid | Adenosine monophosphate | Histamine |
| Asparatic acid | Thymidine | Arginine | Histidine |
| Carnitine | Thymidine monophosphate | Asymmetric dimethylarginine | Hypoxanthine |
| Cholic acid | Thymine | Citrulline | Lactic acid |
| Choline | Uracil | Cystine | Lysine |
| Cystathionine | Uric acid | Cytidine | Methionine |
| Cytosine | Xanthine | Cytidine monophosphate | Methionine sulfoxide |
| Fumaric acid | | Dimethylglycine | Nicotinic acid |
| Glutamic acid | | FMN | S-Adenosylmethionine |
| Glycine | | Glutamine | Serotonin |
| Homosysteine | | Guanine | Symmetric dimethylarginine |
| Isoleucine | | Guanosine | Threonine |

# Conclusions

- We analyzed metabolites in mice fecal by LC-MS/MS (ion-pairing method and non-ion-pairing method) and 17 and 75 of metabolites were detected, respectively.
- As results of analyses by non-ion-paring method and principal component analysis (PCA), two types of mice fecal (young and old) was successfully categorized.
- These analytical methods could be a very powerful tool in fecal metabolomics.

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