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Life Science

Multi-omics Analysis of Gut Microbiota, Metabolites and Aroma Components by Next-Generation Sequencer and GC/MS

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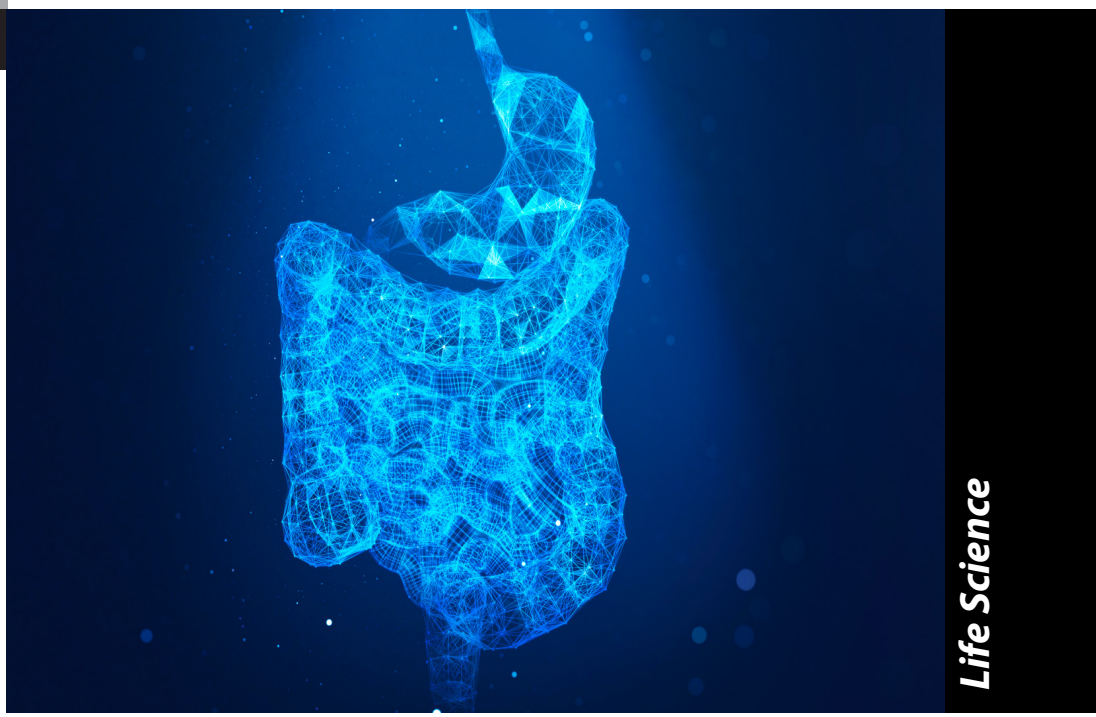


■ Abstract

Human fecal samples were analyzed using a next-generation sequencer and a gas chromatograph mass spectrometer (GCMS-TQ™8040 NX). The gut microbiota (approximately 250 microbial species), metabolites (approximately 500 components), and aroma components (approximately 500 components) were detected. Based on the approximately 800 items detected, an integrated analysis was performed using a Multi-omics Analysis Package, combining principal component analysis and volcano plot, respectively, with metabolic pathway analysis. This article introduces an example of multi-omics analysis sorted by gender and time series.

1. Introduction

The human gut is home to about 1000 different types of bacteria, up to 40 trillion of them[1)]. These bacteria play an essential role in human health. However, changes in modern lifestyle and diet can cause an imbalance in the gut microbiota. For example, excessive stress and a biased diet can negatively affect the gut microbiome. Therefore, a balanced diet and stress management are essential to maintain the health of the intestinal environment.

While the gut microbiota plays a vital role in digestion and the regulation of immune function, metabolites are substances produced by the digestion and metabolism of food and affect the body's physiology. Investigating the correlation between gut microbiota and metabolites can help prevent and treat chronic diseases.

On the other hand, when investigating the correlation between gut microbiota and metabolites, it is necessary to consider the lifestyle and diet of the individual. Because the human gut microbiome varies from person to person and varies with diet and environment, it may not be generalizable.

Therefore, in this study, we collected monthly fecal samples from subject A (male) and observed changes in bacterial flora, metabolites, and aroma components over nine months. The fecal samples from the other seven subjects (two women and five men) were collected only once and used as a comparison for subject A. The measurements were performed using a next-generation sequencer (MiSeq System, Illumina Corporation) and a gas chromatograph mass spectrometer (GCMS-TQ8040 NX), and the analysis was completed using a Multi-omics Analysis Package.

Fig. 1 GCMS-TQ™8040 NX (left) and MiSeq system (right)

1 Shimadzu Corporation
2 Metagen,Inc.
3 Illumina Inc.

Note) In this study, Shimadzu and Illumina did not participate in observing subjects using the data, and Metagen explained subjects, collected samples, performed NGS analysis, and discussed the analysis results.

2. Experiments

1223 compounds and bacterial species were measured using the following two instruments to evaluate the intestinal environment.

Gas chromatograph mass spectrometer

488 primary metabolites, including organic acids, sugars, nucleic acids, fatty acids, and amino acids, were measured using GCMS-TQ8040 NX and Smart Metabolites Database™ Ver. 2. Area values were corrected with 2-isopropylmalic acid, an internal standard. In addition, 484 aroma components were measured at 40 ˚C by SPME Arrow using Smart Aroma Database™. Approximately 280 primary metabolites and 110 aroma components were detected in each sample.

Next-generation sequencer

The samples were lyophilized using a VD-800R freeze dryer (Titek) for 24 hours. After DNA extraction by the bead method, the V1-V2 variable region of the 16 S rRNA gene was amplified with Tks Gflex DNA polymerase (Takara Bio) using bacterial universal primers 27 F-mod (5' -AGRGT TTGATYMTGGCTCAG-3') and 338R (5' -TGCTGCTCCC GTAGGAGT-3'). Amplicon DNA was sequenced using MiSeq according to the illumina protocol. 251 bacterial species were measured. Approximately 150 bacterial species were identified in each subject, and relative quantification values were calculated.

3. Principle Component Analysis results on metabolic pathways

Metabolites, aroma components, and gut microbiota measurements of eight subjects were analyzed by principal component analysis using a Multi-omics Analysis Package. For subject A, only one sample was used after confirming that a 9-month change over time did not affect principal component analysis (Back Out, Fig. 7).

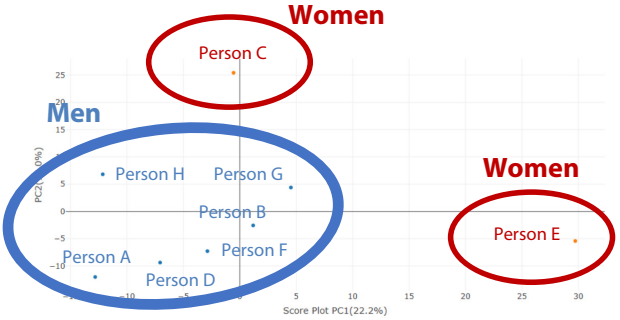
Principal component analysis showed that two females deviated from 6 males despite a cumulative contribution of 40 % (Fig. 2).

Therefore, the lower left of the loading plot (PC1<0 and PC2<0 test item) in men and in the upper right (PC1>0 and PC2>0 test item) was projected onto the metabolic pathways as a group (yellow) that was characteristically detected in females (Fig. 3).

In females (yellow), metabolites of the lysine degradation pathway were accumulated.

This suggests that lysine degradation is accelerated in men, suggesting intestinal bacteria that use lysine as a substrate promote metabolism. Lysine is metabolized by intestinal bacteria in the large intestine and is known to biosynthesize imidazole propionic acid and pipecolic acid[2) 3)].

In men, many compounds of the pyrimidine metabolic pathway were detected (suppression of the metabolic pathway).



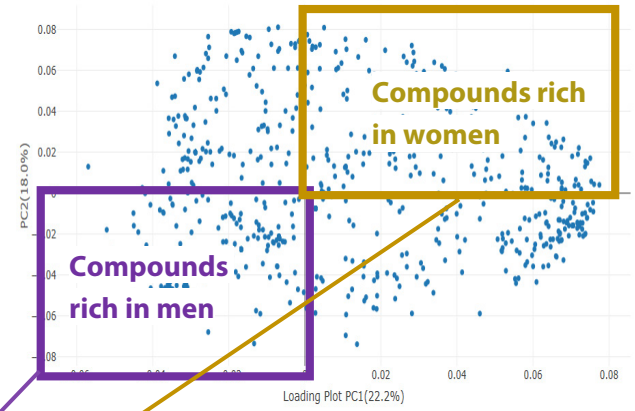


Fig. 2 PCA of 8 individuals
(Metabolites,Aroma,Bacteria Species)

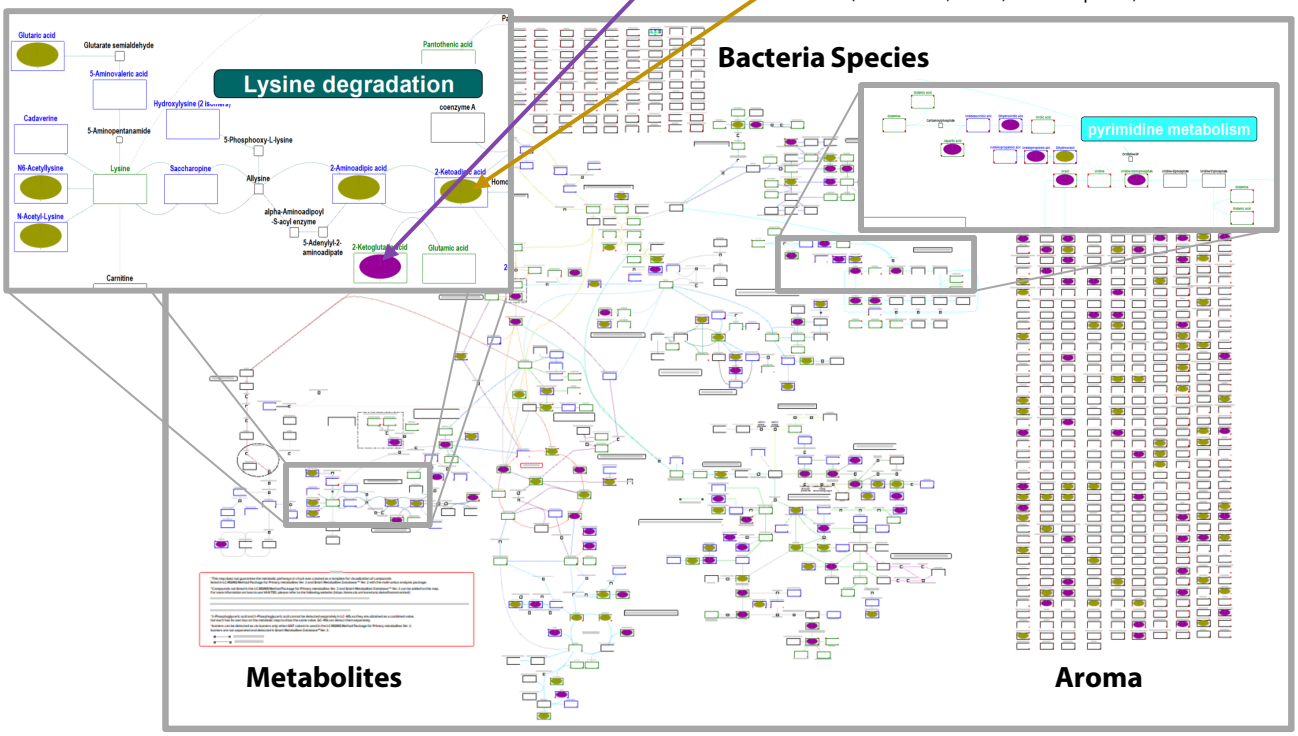


Fig. 3 Metabolic pathway analysis based on the loading plot of principal component analysis
(In purple, compounds specifically detected in males; in yellow, compounds specifically detected in females)

Compound or species	P-value
Dihydroxyacetone	0.003
2-Hydroxyglutaric acid	0.003
Lachnospiraceae UCG-004	0.01
Octanoic acid	0.01
Desulfovibrio	0.01
4-Coumaric acid	0.02
2,5-Dimethylpyrazine	0.03
Lachnospira	0.03
2-Hydroxybutyric acid	0.03
Guanine	0.03
Lachnospiraceae NK4A136 group	0.03
Succinic acid	0.03
Ruminococcaceae UCG-004	0.04

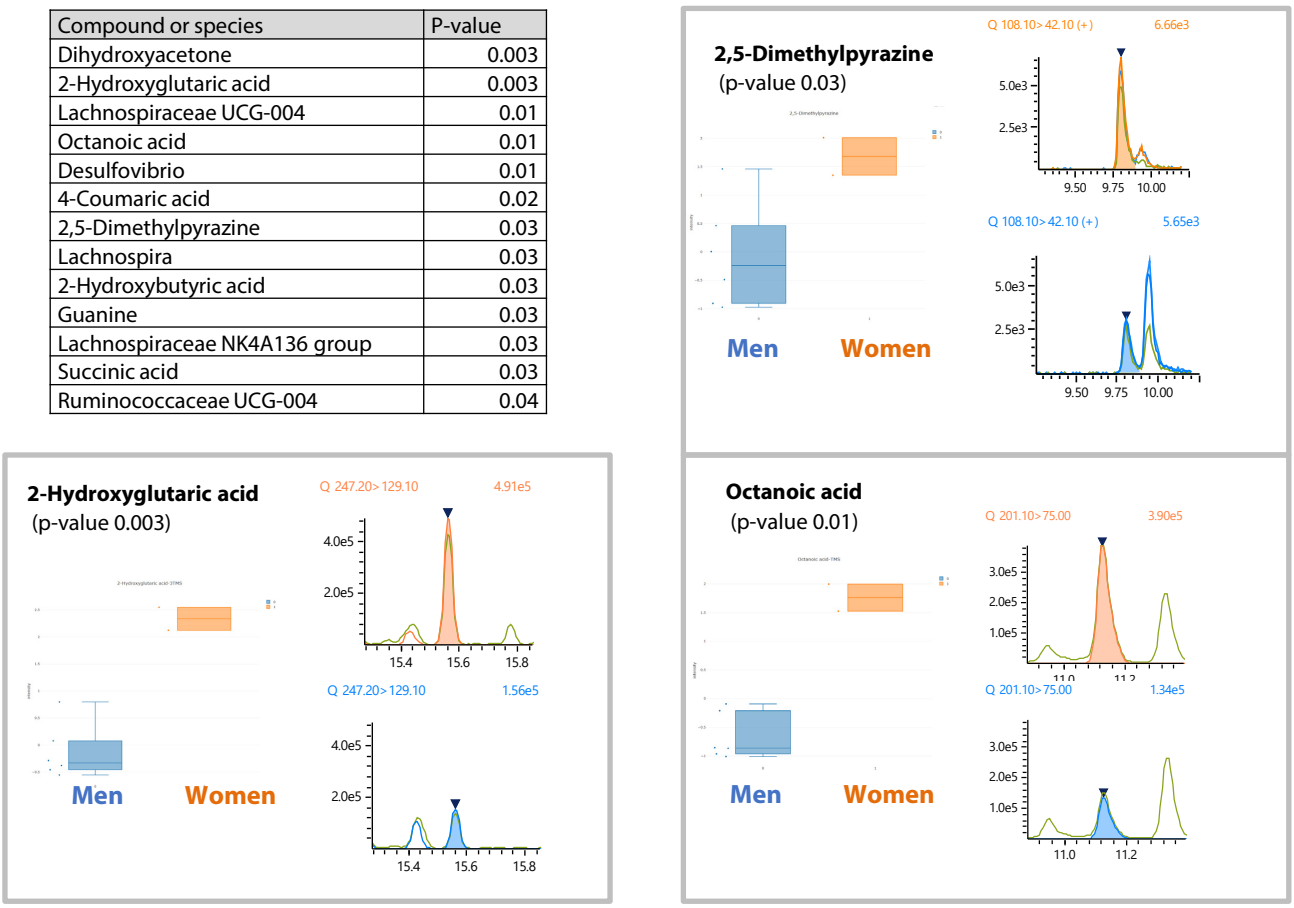
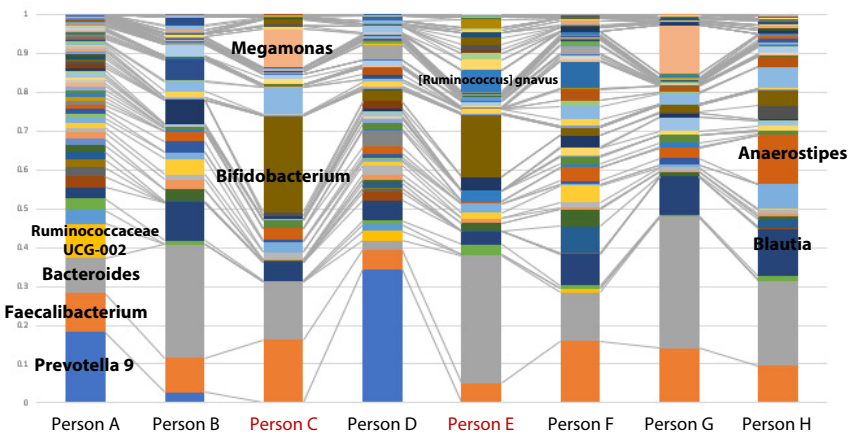


Fig. 4 Compounds with significant gender differences



Type B **Abundant in Bacteroides**

High intake of fermented foods and fiber

- Person B (32-year-old man)
- Person G (28-year-old man)
- Person H (35-year-old man)

Type D **Abundant in Bifid**

Those with short sleep time

- Person C (34-year-old woman)
- Person E (34-year-old woman)

Type E **Abundant in Prevotella**

Common with healthy individuals

- Person A (45-year-old man)
- Person D (34-year-old man)

Fig. 5 Next-Generation Sequencer Results and Enterotype[4] (Black Text: Male, Red Text: Female)

After being catabolized, pyrimidines are essential in maintaining health because they lead to sugar and lipid metabolism pathways. These results suggest that dihydropyrimidine dehydrogenase, a rate-limiting enzyme in pyrimidine metabolism, may differ between sexes.

Other compounds that show gender differences include 2-hydroxyglutaric acid, octanoic acid, and 2,5-dimethylpyrazine. 2-Hydroxyglutarate is a compound that affects immune function and is also known as an oncometabolite (cancer-specific metabolite) at high concentrations. Octanoic acid (caprylic acid) is a medium-chain fatty acid known to improve the intestinal environment and is sold as a supplement. 2,5-Dimethylpyrazine (a precursor of aminoacetone produced by threonine metabolism) is produced by Bacillus subtilis.

4. Correlation Analysis of Intestinal Bacterial Species and Metabolites

The gut microbiome varies from person to person and is affected by factors such as genes, living conditions, and diet. Individual differences in the gut microbiome significantly impact human health, so it is vital to maintain that balance.

The results of the next-generation sequencer were visualized in a bar graph (Fig. 5). Subjects A (45-year-old male) and D (34-year-old male) had more Ruminococcus UCG-002 than the other subjects, while the relative abundance of Bacteroides was lower.

Ruminococcus can break down dietary fiber components, such as indigestible dextrin, and lives in the stomachs of herbivores. It is also known that some species of Ruminococcus contribute to promoting health and are involved in developing diseases.

In Subject C (female in her thirties) and Subject G (male in his twenties), Megamonas spp., a Gram-negative anaerobe indigenous to the human intestine, was frequently detected. In subjects C and E, Gram-positive anaerobic bacilli of the genus Bifidobacterium (Bifidobacterium), which produce lactate and acetate from glucose, were frequently detected. Regarding the physiological effects of bifidobacteria on humans, there are reports of immunomodulatory effects, antiallergic effects, and infection-protective effects[4) 5)].

In subject H (male in his 30s), Anaerostipes, which metabolizes dietary fiber and oligosaccharides that reach the large intestine and produce butyrate, was detected in large numbers. The presence of bacteria that produce butyrate in the intestine, such as Anaerostipes, is essential for maintaining health because the stomach absorbs butyrate in the small intestine and rarely reaches the large intestine. In contrast, butyrate helps prevent excessive immune responses that can lead to food allergies and inflammation [6)].

It is known that the composition of the gut microbiota varies not only from person to person but also from person to person depending on the living environment, diet, and health conditions. Therefore, we visualized the time-series changes in the bacterial flora composition of subject A from December 2022 to September 2023 as a bar graph (Fig. 6). Principal component analysis showed that time series changes (Diet and health) were more minor than individual differences (Genetics and Environment) (Fig. 7). Principal component analysis was also used to identify the bacterial species that were characteristically detected in subject A and correlations with metabolites and aroma components were analyzed (Fig. 8).

The abundance of Christensenella is high in lean people. Animal studies have shown that it reduces weight gain[7)]. Correlation analysis revealed that Christensenella is associated with taurine and lysine degradation pathways. Taurine is a non-protein amino acid that has a wide range of physiological functions, including detoxification of conjugated bile, antioxidant, and neurotransmitter-like effects and is an important metabolite for maintaining health.

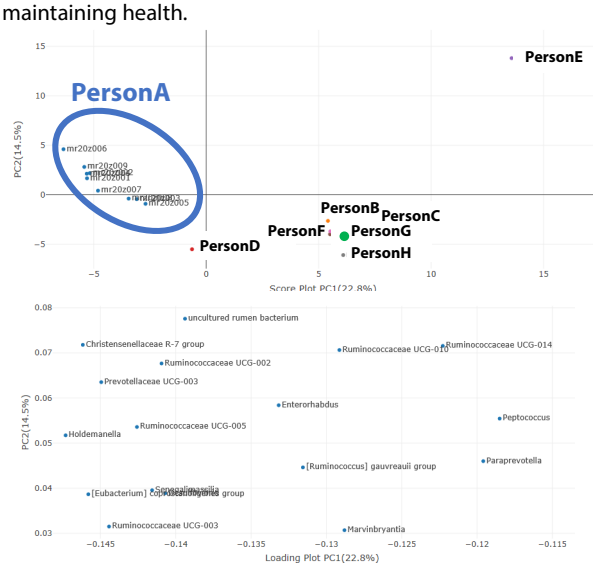


Fig. 7 Next-Generation Sequencer Results
(Comparison of Time Series Changes in PersonA and Other Persons by Principal Component Analysis)

* The loading plot is the part of PersonA that is characteristically detected (Zooming in on PC1>0 and PC2<0).

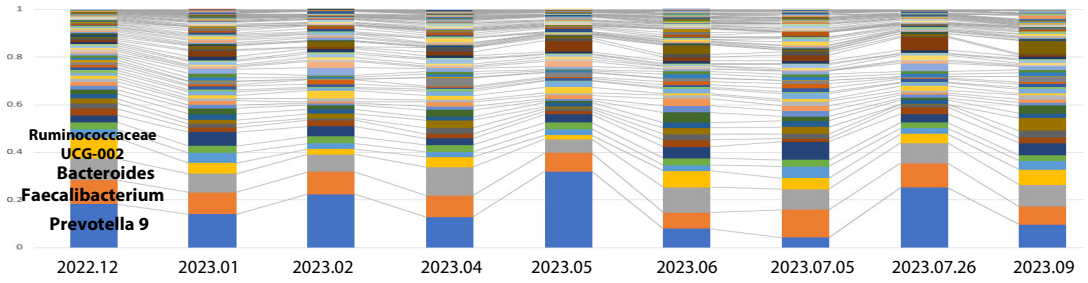


Fig. 6 Next-Generation Sequencer Results (Person A Time Series Changes)

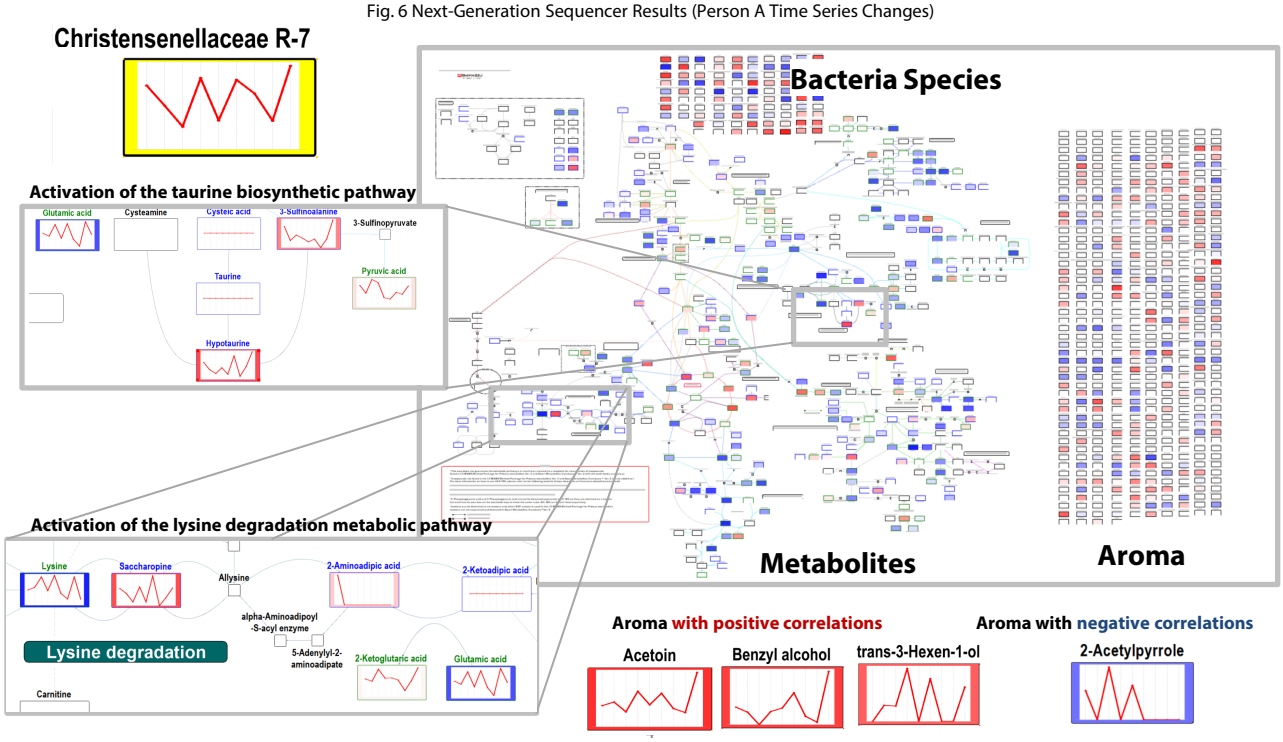


Fig. 8 Search for metabolites and aroma components positively and negatively correlated with increases and decreases in Christensenella bacteria
(white map function of Multi-omics Analysis Package)

Lysine is essential because it stimulates the uptake of fatty acids into mitochondria, which play a crucial role in energy production, and it not only facilitates the energy conversion of fats but also serves as a precursor for forming pipecolic acid, etc. [3].

Regarding aroma components, Christensenella was positively correlated with acetoin, benzyl alcohol, and trans-3 hexene -1 -ol, while negatively correlated with 2-acetylpyrrole. Acetoin is an aromatic compound with a buttery fermentative odor and is known to be produced by bacteria through decarboxylative condensation of pyruvic acid[8].

Benzyl alcohol is a floral scent and is an intermediate metabolite of toluene. It is metabolized to benzaldehyde by alcohol dehydrogenase, then benzoic acid by aldehyde dehydrogenase, and is conjugated with glycine to form hippuric acid[9].

5. Volcano Plot results on Metabolic Pathways

Ruminococcus sp. UCG-003 was one of the bacteria characteristically detected in subject A, shown in the enlarged loading plot of Fig. 7 in the previous section. Ruminococcus is a bacterium that degrades dietary fiber components such as indigestible dextrin. When dietary fiber components are insufficient, the intestinal barrier function is thought to decrease, and the bacteria become more susceptible to infection by consuming mucin, the main component of intestinal mucus[10) 11)]. However, research is ongoing to determine how Ruminococcus affects human metabolic pathways and aroma components.

Therefore, we conducted a Volcano plot analysis of the fecal samples of Subject A with high (2022.12, 2023.01, 2023.02) and low (2023.07.05, 2023.07.26, 2023.09) levels of Ruminococcus UCG-003. In addition, metabolites, aroma components, and species characterized by the Volcano plot were projected onto a white map, and correlation analysis was performed.

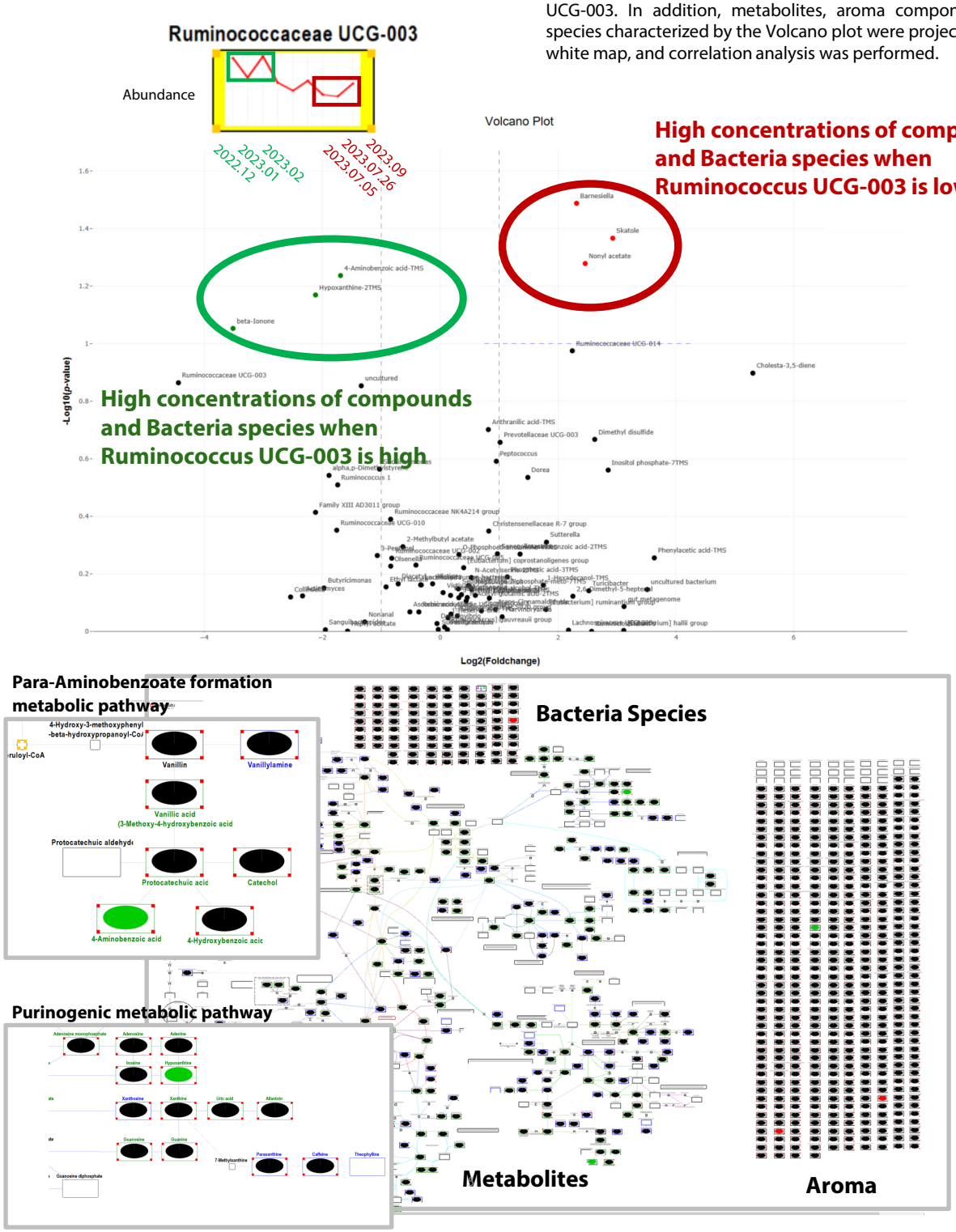


Fig. 9 Search for metabolites and aroma components positively and negatively correlated with increases and decreases in Ruminococcus UCG-003 (map function of Multi-omics Analysis Package)

An increase in Ruminococcus UCG-003 was found to increase the amounts of products in the para-aminobenzoate metabolic pathway and to accumulate intermediate metabolites in the purine metabolic pathway. We also discovered that a decrease in Ruminococcus UCG-003 led to a higher concentration of skatole, a fecal odor. Skatole is produced by the decarboxylation reaction of triprofane to indole -3 acetic acid. It was suggested that it is essential to maintain the abundance of Ruminococcus UCG-003 because it is a compound that has been suggested to damage the intestinal epithelium[12].

Volcano plots also revealed a negative correlation between Ruminococcus UCG-003 and Barnesiella. Barnesiella is an intestinal bacterium suggested to affect human cognitive and physical functions[13) 14)].

In addition to projecting the Volcano plot of only Ruminococcus UCG-003 onto the metabolic pathway, Marvin Briancia, Holdemania, and Rumen bacteria, which were characteristic of subject A, were also analyzed by the volcano plot, and the results were all projected onto a single metabolic pathway map (Fig. 10).

Metabolites of the glycerolipid metabolism pathway (dihydroxyacetone phosphate and glycerol diphosphate) were found to accumulate when rumen bacteria and Marvin briancia spp. Conversely, an increase in these bacteria was associated with an increase in the products of the para-aminobenzoate metabolic pathway[14)].

Dihydroxyacetone phosphate is one of two products of the breakdown of fructose 1,6-diphosphate with glyceraldehyde 3-phosphate, which is rapidly and reversibly isomerized to glyceraldehyde 3-phosphate. It is a metabolite used not only as part of the energy supply but is also involved in the function of essential tissues such as the brain and muscles and is vital in maintaining normal brain function.

Para-aminobenzoic acid is a precursor of folic acid, and a lack of it is known to cause anemia and stress. Regarding the effects of para-aminobenzoic acid in the intestinal environment, in addition to the correlation between metabolites and aroma components, future research is needed on the direction of causation.

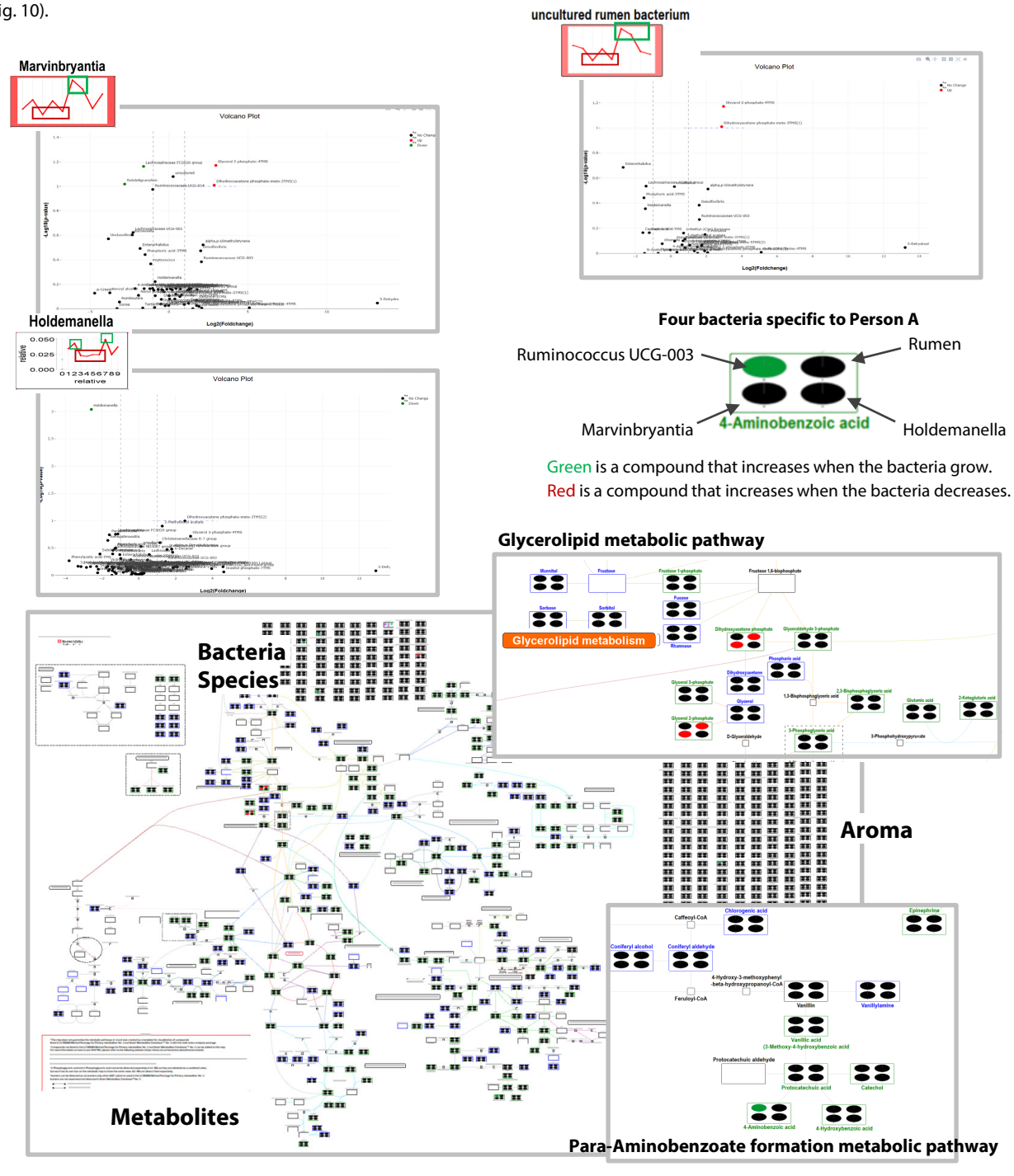


Fig. 10 Search for metabolites and aroma components positively and negatively correlated with increases and decreases in 4 species (Ruminococcus UCG-003, Marvin Briancia, Holdemania, Rumen bacteria) (white map function of Multi-omics Analysis Package)

6. Conclusion

16 human fecal samples were measured using a next-generation sequencer and gas chromatograph mass spectrometer (GCMS-TQ8040 NX) to assess the gut microbiota (approximately 250 microbial species), metabolites (approximately 500 components), and aroma components (approximately 500 components). Based on the approximately 800 items detected, an integrated analysis was performed using a Multi-omics Analysis Package, combining principal component analysis and volcano plot, respectively, with metabolic pathway analysis.
Time series and principal component analyses showed that enterotypes were constant regardless of diet or health status. By correlating the increase and decrease of bacteria with metabolites and aroma components, we identified the affected metabolic pathways and aroma components.

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