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Application News

No. C201

Liquid Chromatography Mass Spectrometry

Metabolome Analysis of Japanese Rock Ptarmigan Feces by LC/MS/MS: Application to the Establishment of Breeding Technology for Endangered Species

According to the latest Red List of the International Union for Conservation of Nature issued in 2017, 25,821 species of wildlife are listed in the three high-risk categories (CR, EN and VU). Many endangered species are difficult to breed, and experts in each field are working together to develop breeding techniques.

In recent years, the number of Japanese rock ptarmigans has decreased and they are threatened with extinction. In order to protect them against extinction, efforts have been made to breed them outside zoos, etc., and return them to the wild, but there are a number of issues that need to be resolved. One of them is glomerular nephropathy with diarrhea and oxalate deposition when artificially bred Japanese rock ptarmigans eat the alpine plants consumed by wild Japanese rock ptarmigans. This is considered to be because the artificially bred birds are not given alpine plants containing a lot of oxalic acid while being reared, and consequently they lack the intestinal flora that decomposes oxalic acid. This means that a breeding technique that includes reconstruction of the wild intestinal flora must be developed in order to return the birds to the wild. Some means being used to elucidate the effects of intestinal flora on the host are flora analysis using next-generation sequencers and metabolome analysis using mass spectrometers.

In this article, feces containing metabolites produced by the intestinal flora of wild and artificially bred Japanese rock ptarmigans were analyzed using LC/MS/MS. It was suggested that metabolome analysis could be applied in establishing breeding technology focused on return to the wild, so we are introducing an example of that here.

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Sample and Pretreatment

Japanese rock ptarmigan feces was frozen immediately after excretion and kept frozen at –80 °C before pretreatment and analysis. Fig. 1 shows the pretreatment procedure for extracting metabolites from Japanese rock ptarmigan feces. 500 µL of phosphate buffered saline (PBS) was added to 100 mg of feces then stirred, and the supernatant was centrifuged and ultrafiltered. The filtrate was then diluted 10 × with ultrapure water, which was used as a sample for analysis by LC/MS/MS.

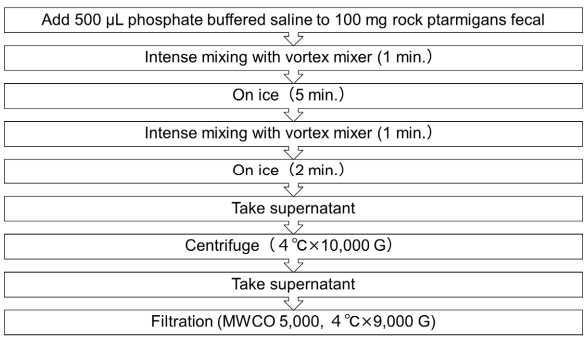

Fig. 1 Pretreatment of Japanese Rock Ptarmigan Feces

Analysis Conditions

LC/MS/MS analysis was performed with an LCMS™-8060 using the ion-pair-free LC/MS/MS method included in the LC/MS/MS Method Package for Primary Metabolites Ver. 2. This enables simultaneous analysis of 97 components of hydrophilic metabolites such as amino acids, organic acids, nucleosides, and nucleotides, which are important for metabolome analysis in the life science field. Please refer to Application Data Sheet No. 49 for more information. Table 1 lists the analysis conditions for HPLC and MS.

Table 1 Measurement Conditions

[HPLC conditions] (Nexera™ X2)	
Column	: Reversed-phase column
Mobile phases	: A) 0.1 % Formic acid in water B) 0.1 % Formic acid in acetonitrile
Mode	: Gradient elution
Flow rate	: 0.25 mL/min
Injection volume	: 3 µL
[MS conditions] (LCMS-8060)	
Ionization	: ESI (Positive and negative mode)
Mode	: MRM
Nebulizing gas flow	: 3.0 L/min
Drying gas flow	: 10.0 L/min
Heating gas flow	: 10.0 L/min
DL temp.	: 250 °C
Block heater temp.	: 400 °C
Interface temp.	: 300 °C

Metabolome Analysis

As a result of analyzing the feces of wild and artificially bred Japanese rock ptarmigans using LC/MS/MS, amino acids, nucleotides, nucleosides and organic acids involved in the TCA cycle were detected. An average of 56 components (n=4) were detected in the wild birds and an average of 60 components (n=6) in the artificially bred ones, and a tendency for the artificially bred birds to have a higher number of components and higher content levels was observed.

Next, a principal component analysis and t-test were performed with the Traverse MS software using the area ratio with respect to the internal standard substance for each component. Fig. 2 shows the results in a score plot and loading plot. Fig. 3 shows a comparison of the area ratios with respect to the internal standard substance of components confirmed to show a significant difference ($p<0.05$) in the t-test results. The score plot results show the formation of clear clusters for the feces of wild and artificially bred Japanese rock ptarmigans. Further, we found from the loading plot and results of the t-test that the characteristic components of the feces of artificially-bred Japanese rock ptarmigans were amino acids such as phenylalanine, leucine, isoleucine and valine, while those of the feces of wild Japanese rock ptarmigans were nucleic acids such as uridine, adenine and cytidine. Based on the fact that there were a lot of free amino acids in artificially bred Japanese rock ptarmigans, it was suggested that they ingested too much protein in their feed and that there was some surplus. It is known that a continuously high protein level makes them susceptible to infectious diseases, leg abnormalities, wing dysplasia, etc.

On the other hand, based on the fact that there were a lot of intermediate products of nucleic acid metabolism in the wild Japanese rock ptarmigans, it could be surmised that there is a metabolic system that uses these intermediate products in amino acid synthesis in these birds. It is known that such a metabolic system exists in the gastrointestinal tract of grazing animals, and that it actively metabolizes the nucleic acids derived from their diet and those derived from dead bacterial cells. It is thought that wild Japanese rock ptarmigans have the same kind of metabolic system as grazing animals and therefore synthesize amino acids well even in the nutritionally poor alpine environment.

As a result of the metabolome analysis, it became clear that the feed currently used in the artificial breeding has excessive protein, so the feed has to be improved.

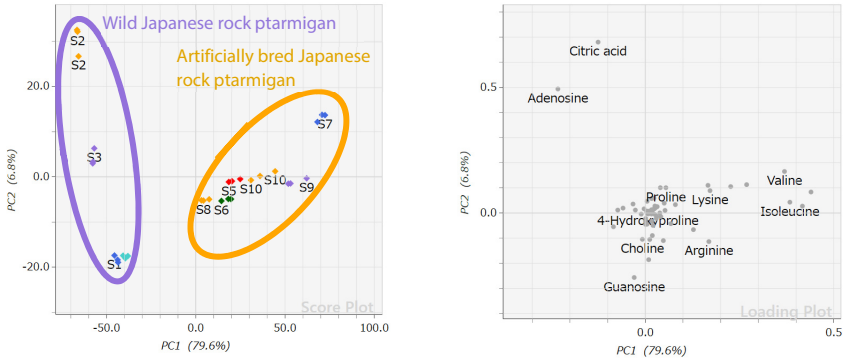

Fig. 2 Principal Component Analysis of Japanese Rock Ptarmigan Fecal Extract

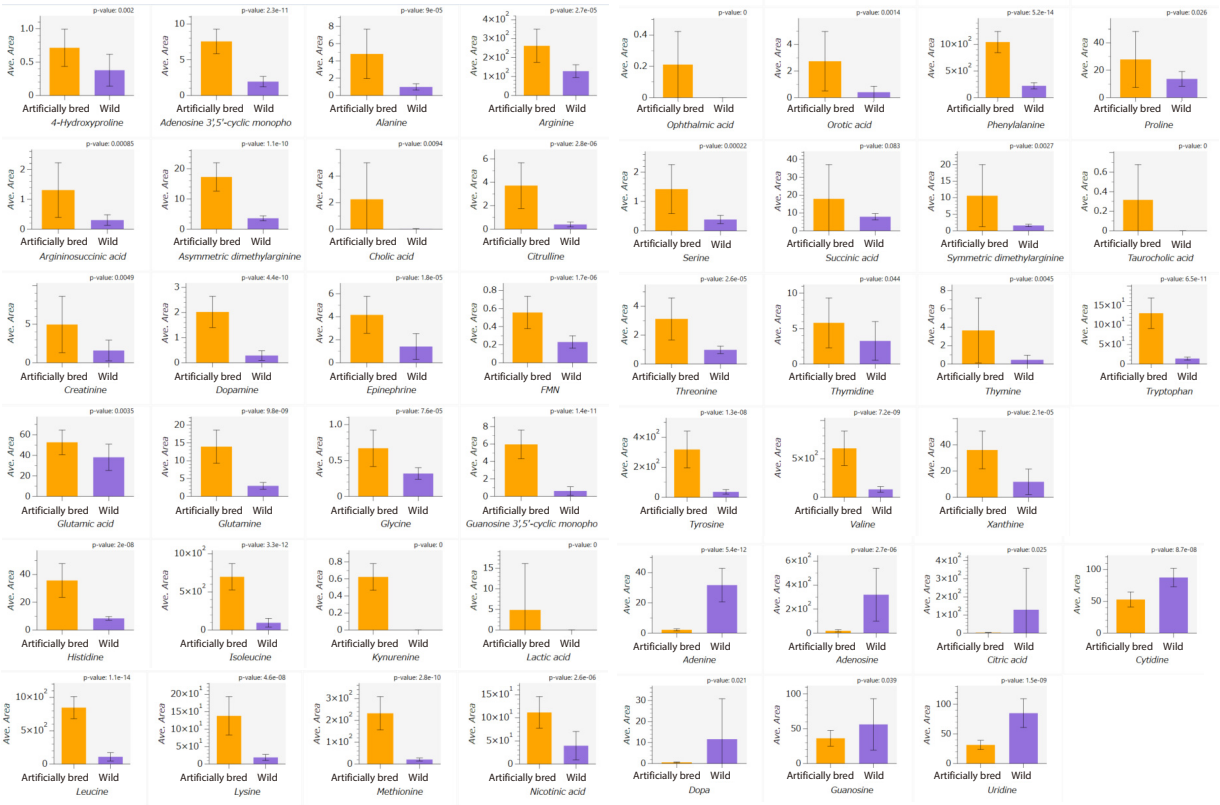

Fig. 3 Area Ratio with Respect to Internal Standard Substance of Components for Which Significant Differences Were Confirmed in the t-test

In creating this article, we benefited from the cooperation of Professor Kazunari Ushida of the Chubu University Academy of Emerging Sciences, who provided the Japanese rock ptarmigan feces and gave guidance.

The product described in this document has not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan.
It cannot be used for the purpose of medical examination and treatment or related procedures.

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