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Analysis of Healthy Beneficial Nutritional Components from *Fructus mume* and the Response of Intestinal Flora in Mice

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Abstract

Japanese apricot, a deciduous tree belonging to the Rosaceae family, has a plantation history of more than 3000 years in China. Fructus mume, dried from immature Prunus mume fruit, has been used as a constituent of many medicinal formulas and functional foods since ancient China for its beneficial health effects. This study aimed to determine the types and contents of organic acids and mineral elements that played important roles in Fructus mume and to explore the functions of the gut microbiota, liver, and kidneys of mice. Ultra-performance Liquid Chromatography (UPLC), Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-OES), and Inductively Coupled Plasma Source Mass Spectrometer (ICP-MS) were used to analyze the types and contents of organic acid and mineral elements of Fructus mume. 16S rRNA sequencing was performed to assess the impact of the Fructus mume on the intestinal flora of mice. The coefficients of the liver and kidney were tested to evaluate the effect of Fructus mume on the liver and kidney of mice. The results indicated that the main organic acid in Fructus mume was citric acid, and the main mineral element was potassium. We further found that Fructus mume could modulate the Firmicutes to Bacteroides ratio, optimize the composition of intestinal microbiota, enhance the proliferation of beneficial bacteria, improve intestinal flora function based on 16s RNA sequencing data and provide a certain degree of protection to the liver and kidney in mice.



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Introduction

Japanese apricot is a deciduous small tree of the Rosaceae family that originated from the southwest of China. It has a long history of cultivation in China and has been introduced to Taiwan, Japan, Korea, Vietnam, and other countries¹ because of its high ornamental value. Japanese apricot is rich in nutrients and contains a variety of essential minerals and organic acids^{2,3} that have beneficial effects on health. The consumption of Japanese apricots as a health food is widespread in Asian countries such as China and Japan. However, fresh and unprocessed Japanese apricots are randomly consumed because of their severe sourness and astringency.⁴

The Japanese apricot has been used in traditional medicine for more than 3000 years and was used to treat various symptoms. Plant parts, such as branches, leaves, flowers, seeds, and roots, have been used in traditional medicine in China, but most pharmacological studies have been performed with fruit extracts. *Fructus mume*, produced from the unripe fruit of *Prunus mume*, is one of the most famous and ancient medicinal herbs and health foods used in China.⁵

According to traditional Chinese herbal medicine records, Fructus mume has been used to treat various diseases, such as chronic diarrhea, cough, expectoration, ulceration, gastrointestinal diseases, and nocturnal asthma symptoms.6-13 Moreover, it is also used as one component in many formulas, and modern studies have reported that Fructus mume exerts a therapeutic effect on various diseases owing to its anti-inflammatory, antioxidant, antibacterial, and radical scavenging activities.^{12, 14} The components in Fructus mume are complex and diverse. Like the fresh fruit of Prunus mume, it contains various nutritional and functional substances, such as organic acids and minerals. But there is no report on the consistency of organic acids and mineral elements between Fructus mume and fresh fruits of Prunus mume. To the best of our knowledge, ultra-performance liquid chromatography (UPLC), inductively coupled plasma atomic emission spectrometer (ICP-OES), and inductively coupled plasma source mass spectrometer (ICP-MS) were used to determine the types and contents of organic acids and mineral elements in Fructus mume.

The microbiota plays a major role in health and disease, It participates in various metabolic functions such as intestinal homeostasis, and development. and that exists in the intestinal flora is associated with metabolic reactions and immunomodulatory.15-18 It is reported that dietary changes show significant impacts on the microbiota.¹⁹ Previous studies have reported that Prunus mume fruit juice concentrate could affect the gut microbiota in mice.²⁰ However, the effects of Fructus mume on the gut microbiota have not been investigated. Therefore, this study aimed to investigate the effects of Fructus mume on the intestinal flora by 16s rRNA sequencing. The liver and kidney tissues were used to discuss the effects of Fructus mume on the liver and kidney of mice in detail.

Materials and Methods Experimental Materials

The *Fructus mume* juice used in this study was provided by the National Field of Waxberry and *Prunus mume* at the Nanjing Agricultural University, Nanjing, Jiangsu Province.

Chemicals

The reagents used included chromatography-grade standards for oxalic, tartaric, malic, ascorbic, acetic, citric, maleic, and fumaric acid (organic acid); mineral element standards for potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na), selenium (Se), and germanium (Ge); disodium hydrogen phosphate; phosphoric acid; and a 4% polyformaldehyde fixing solution.

UPLC Quantitative Analysis of the Organic Acid

The organic acid content was accurately determined by UPLC, which consisted of a Waters ACQUITY UPLC HSS T3 column (2.1 mm×100 mm, 1.8µm). The mobile phase was 50 mM sodium dihydrogen phosphate (pH 2.4). The flow rate was set at 0.25 mL/min, the column temperature was maintained at 30 °C, the injection volume was 2 µL, the PAD was used as the detector, and the detection wavelength was 214 nm.

ICP-OES And ICP-MS Quantitative Analysis of the Mineral Element

The mineral element content was accurately measured by ICP-OES and ICP-MS.

Preparation and determination of mineral element standard solutions: Standard solutions of Ca, K, and Na with a concentration of 1000 mg/L were diluted to 10 mg/L, 5 mg/L, 2.5 mg/L, 1.25 mg/L, and 0.625 mg/L, respectively. The standard solutions of Mg, P, Se, and Ge with a concentration of 1000 mg/L were diluted to 10 mg/L, 5 mg/L, 2 mg/L, 1 mg/L, and 0.5 mg/L. The blank sample was made, and all the samples were measured on the machine.

Feeding and Gavage of Mice

The SPF male healthy mice (4 weeks) were used in this study with a weight between 18-20 g. All experimental mice were obtained from the Experimental Animal Center of Nanjing Agricultural University and were raised in the barrier environment animal room of the Experimental Animal Center of Nanjing Agricultural University. This study was approved by Animal Ethics Committee of Nanjing Agricultural University Permit (NJAU. No20221031206) and performed in accordance with the guidelines of the Science and Technology Agency of Jiangsu Province and Nanjing Agricultural University.

A total of 12 mice were randomly divided into two groups (n=6): the CK group with treatment of distilled water and the other group with *Fructus mume* juice. They were fed and drank adaptively for one week and then given gavage continuously six times a week for six weeks. The mental state, activity, and foraging amount of the mice were observed after gavage, and the weight of the mice was measured once a week. The fresh manure of mice was collected after 12 h.

High-Throughput Sequencing of Intestinal Flora for Mouse

Stool samples from each group (n=6/group) were collected and stored at -80 °C for further analysis. The samples were completed by Nanjing Zhongke Shikang Biotechnology Co., Ltd. (Nanjing, China). The total DNA of the specimen was extracted from a DNA extraction kit. A database was created for qualified DNA samples. PCR amplification was performed using Pfu high-fidelity DNA polymerase from the Total Gold Company, using microbial ribosomal RNA or specific gene fragments and other target sequences that could reflect the composition and diversity of the flora as the target. The corresponding primers were designed, and the quality of the products was assessed by a Microplate

reader (BioTek, FLx800). The library was sequenced using a two-terminal sequencing strategy on the MiSeq platform, and quality control was performed using QIIME2, USEARCH, and VSearch. The operational taxonomic units (OTUs) were identified by QIIME2. Community bar-pot, alpha and Beta diversity analyses, heat mapping, and species identification were performed using QIIME2 and R software packages (http://www.R-project.org).

Determining the Response of Liver and Kidney in Mice

Mouse eyeball blood was collected, incubated at 4 °C for 30 min, and centrifuged at 3000 r/min for 20 min, and the supernatant was used for testing the ALT, AST, BUN, and CR using test kits.

After blood collection, the kidney and liver tissues were collected for pathological observation; the mice were sacrificed by neck removal, and liver and kidney tissues were collected for experiments. The collected tissues were weighed and then fixed in a 4% polyformaldehyde solution and dyed. The formula for calculating the organ coefficient was as follows: The organ coefficient = (organ wet mass/ mouse body mass) *100%.

Statistical Analysis

The statistics were analyzed using Excel and SPSS (version 26.0). All data were expressed as mean \pm standard deviation. Comparisons were based on t-tests of the independent samples. A p-value<0.05 was considered statistically significant.

Results

The Functional Components in *Fructus Mume* The Composition and Content of Organic Acids in *Fructus Mume*

The main nutrition in the fruit of Japanese apricot is organic acid, and there are eight types of organic acid in it. In this study, eight kinds of organic acids were determined in *Fructus mume*, similar to those in the fresh fruit of Japanese apricots. The total organic acid content in *Fructus mume* was measured at 31.73 g/L. The main kind of organic acid in *Fructus mume* was citric acid, and the content was 25.35±0.049 g/L, accounting for 79.89% of the total organic acid. The malic acid was the secondary major with a 2.68±0.01g/L content. The succinic acid content was recorded at 0.019±0.001g/L, the lowest among the eight kinds of organic acid (Figure 1).



Fig. 1: Determination of organic acids in Fructus mume



Fig. 2: Determination of mineral element content in Fructus mume

Mineral Elements in Fructus Mmume

The seven types of mineral element and their content ranges are listed in Figure 2. The result showed that the content of the seven mineral elements in *Fructus mume* followed the order from highest to lowest K>Ca>P>Na>Mg>Se>Ge. Similar to *Prunus mume*

fruit, which is rich in potassium, phosphorus, calcium, magnesium, and selenium.²¹ As is seen, potassium content, recorded at 4566.03±130.76 mg/L, was the highest in *Fructus mume*, accounting for 79.9% of all the determined mineral element content.

Group	0week	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	Weight gain
Control	19.2±0.64	19.6±0.71	19.9±1.26	19.9±1.32	19.6±1.48	20.0±1.54	20.9±0.82	1.7±1.10
Fructus mume	19.3±0.62	19.2±0.82	20.2±0.83	20.4±0.63	20.7±1.08	21.5±1.22	21.6±1.54	2.3±2.00

Table	1: Effects	of	Fructus	mume	on	body	weight	of mice
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Note: * indicates significant differences in the same column.

The Effect of *Fructus Mume* on the Body Weight of Mice

Mice in the control and *Fructus mume* groups showed normal behavior and activity levels. There were no significant differences in mice body weights before the experiment, and the final body weight of mice in the *Fructus mume* group was higher than that of the control group (Table 1). However, no significant differences were observed between the two treatments.



Fig. 3: Distribution diagram of sequence length

Effects of *Fructus Mume* on Intestinal Flora of Mice

Information on Sequencing and Species Composition

To investigate the effects of *Fructus mume* on the intestinal flora of mice, 16S rRNA high-throughput sequencing was used in this study. 3297342 original sequences were obtained. There were 2899159 effective sequences after noise removal and 1681057 high-quality sequences after chimera removal, with an average of 67501 sequences per sample. The length distribution of all high-quality sequences was analyzed, and the results showed that the length was mainly concentrated in the 404-432 bp (Figure 3).

As shown in the OTUs Venn diagram obtained from the cluster of the two groups of samples, the OUTs number of the *Fructus mume* group was 6822, which was higher than that of the control



Fig. 4: Venn diagram illustrating the overlap of OUTs for intestinal flora in mice. Blue represents the *Fructus mume* group (FM) and green represents the control group (CK_m).

group (6306). A total of 354 species were found in both groups (Figure 4). As shown in the map of the specific composition of microbial communities at each taxonomic level, the number of taxa in the two treatments was calculated at each classification level. The results showed that the class, order, family, genus, and species levels of the *Fructus mume* group were higher than those of the control group.

Analysis of Flora composition and Species diversity at Phyla and Genus Levels

According to the results of species annotation, the top 10 species in the relative abundance of each group at the phylum and genus levels were selected (Figure 5) in this study. At the phylum level, the predominant phyla in the mouse gut were Bacteroidetes and Firmicutes, accounting for an average proportion of > 87%. Other dominant phyla were Actinobacteria, TM7, Tenericutes, Proteobacteria, Verrucomicrobia, Cyanobacteria, Deferribacteres, and Acidobacteria. The abundance ratio of Firmicutes and Bacteroidetes of the Fructus mumue group was higher than that of the control group, which is the same as the changing trend of the body mass of the mice. The results showed that *Fructus mume* modulated the Firmicutes to Bacteroides ratio, optimized intestinal microbiota composition in mice, enhanced the proliferation of beneficial bacteria, improved intestinal flora function, and promoted body weight gain in mice.

The dominant bacteria genera at the genus level are Lactobacillus, Bacteroides, Oscillospira, Ruminococcus, Adlercreutiza, Odoribacter. Allobaculum, Parabacteroides, Alistipes, and Enterococcus. Compared with the control group, the relative abundance of Lactobacillus, which played a role in preserving the microecological equilibrium with gastrointestinal traces, was significantly increased. The relative abundances of Oscillospira, Adlercreutzia, Odoribacter, Allobaculum, Alistipes, and Enterococcus were enhanced in the Fructus mume group. At the same time, those of Ruminococcus and Parabacteroides were lower in the Fructus mume group.



Fig. 5: Relative abundance of intestinal flora at phylum(A), genus, and species (B) level in mice

Analysis of Alpha Diversity of Intestinal Flora in Mice

Shannon and Simpson indices and richness of Chao1 and Observed_species of the gut microbiota characterized alpha diversity.

Goods_coverage indices of the two groups were higher than 0.98, suggesting that the sequencing

depth was sufficient to cover all sample species. To evaluate the diversity and richness of intestinal flora, we analyzed alpha diversity through Chao1, Observed Species, Shannon, and Simpson Pielou indices (Table 2), and the boxplot of the alpha diversity index is shown in Figure 6. The richness of the mice in the *Fructus mume* group was lower than that in the control group. There were significant differences in the Pielou indices and no significant differences in the Chao 1, Observed species,

Shannon, and Simpson indices between the two groups.

Group	Chao1	Simpson	Shannon	Pielou	Observed_species	Goods_coverage
Control	2277.51±434.33	0.97±0.00	7.34±0.40	0.67±0.01	1970.4±482.05	0.99±0.00*
Fructus	2692.58±307.68	0.98±0.01	7.76±0.25	0.7±0.02*	2130.93±258.90	0.98±0.00
mume						

Table 2. Richness and microbial diversity index of flora from mice

	Note: 3	* indicates	significant	differences	in the	same co	blumr
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Fig. 6: The boxplot of Alpha diversity index

Analysis of Beta Diversity of Intestinal Flora in Mice

Beta diversity, also known as habitat diversity, refers to the variation in species composition between different communities along environmental gradients or the rate of species turnover along these gradients. Multi-dimensional microbial data can be reduced through unconstrained sorting techniques, such as principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS), to visualize the main trends of data changes by displaying the distribution of samples along continuous sorting axes. Clustering analysis can also be used to identify discrete subsets of objects in an environment and classify the data.



Fig. 7: NMDS two-dimensional sorting diagram

NMDS was performed to determine the beta diversity of the different groups. The greater the distance between various groups, the greater the difference in diversity.

The *Fructus mume* group was separated from the control group, suggesting significant differences

between the two groups in the species structure composition of the intestinal flora. In conclusion, the beta diversity of the *Fructus mume* group was higher than that of the control group, and *Fructus mume* enhanced the beta diversity of the intestinal flora of mice (Figure 7).

	kidney coefficient of mice					
Group	Liver coefficient (%)	Kidney coefficient (%)				
Control Fructus mume	4.98±0.47 1.44±0 e 5.50±0.16 1.57±0).12).07				

Table 3: P Effect of Fructus mume juice on liver and kidney coefficient of mice

Note: * indicates significant differences in the same column.

Effect of *Fructus Mume* on the Liver and Kidney of Mice

Coefficient of Liver and Kidney

The coefficients of the liver and kidney were tested in this study to evaluate the effect of the *Fructus mume*. As is seen in Table 3, the liver coefficient of the *Fructus mume* group was 5.50±0.16%, higher than that of the control group; the kidney coefficient of the *Fructus mume* group was 1.57±0.07 %, and it was higher, too. However, the two treatments had no significant differences in liver and kidney coefficients.

Pathological Observation of Liver and Kidney

The liver and kidney tissues of mice treated with distilled water (Control group) and *Fructus mume* juice (*Fructus mume* group)were examined in

this study (Figures 8 and 9). The liver and kidney structures of mice in the control and *Fructus mume*

groups were normal. In this study, we found no obvious damage.



Fig. 8: HE staining of mouse liver tissue sections (×100). Blood stasis, black arrow; bleeding, brown arrow; lymphocyte infiltration, blue arrow; tissue shedding, red arrow. C and D represent *Fructus mume* and distilled water control groups, respectively



Fig. 8: HE staining of mouse kidney tissue sections (×100). Blood stasis (black arrow), bleeding (brown arrow), lymphocyte infiltration (blue arrow), and tissue shedding (red arrow). C and D represent Fructus mume and distilled water control groups, respectively. From left to right, there are cortex, renal column between cortex and medulla, and medulla

The renal tissues of mice in the two treatments showed no apparent damage, and their structures were typical. Some differences were observed in the mouse cortex. However, a small amount of tissue bleeding in some areas of the cortex and lymphocyte infiltration in some areas were observed in the *Fructus mume* group. There was a small area of lymphocyte infiltration in the medullary substance of mice in the *Fructus mume* group. Determination of Serum Biochemical Factors

Glutamic-pyruvic transaminase(AST) and glutamic oxaloacetic transaminase (ALT) are commonly used to assess liver damage. Both the ALT and AST increased in the case of liver cell membrane permeability or liver cell necrosis, and blood urea nitrogen (BUN) and creatinine (CR) were used to evaluate the extent of kidney damage. In this study, we determined ALT and AST levels to evaluate liver damage and BUN and CR levels in the kidneys.

Table 4: Effect of Fructus mume juice on serum biochemical factors of mice

(Group)	ALT (U/L)	AST (U/L)	BUN (mg/dl)	CR (µmol/L)	
Control	49.49±7.13	118.78±14.44	13.61±2.23	22.72±1.27	
Fructus mume	47.64±19.87	98.18±12.56*	11.74±1.28*	18.42±1.34	

Note: * indicates significant difference in the same column.

Based on the liver function indices, the ALT and AST levels of the mice in the *Fructus mume* group were lower than those in the control group. However, there were no significant differences between the ALT levels of the two groups, but there was a significant difference in the AST levels between the two groups. Compared with the control group, the levels of BUN and CR in the *Fructus mume* group were lower, and the CR content of that group was significantly lower. After the experiment, the mice were in good physical and mental condition.

Discussion

Advances in food and nutrition have shifted consumer preferences towards nutraceutical and functional foods.²² Fructus mume, processed from the unripe fruit of Japanese apricot by drying at low temperatures,^{8,23} is a natural food with nutritive and pharmaceutical value and has been used as a medicinal and functional food in Asian countries for more than 3000 years to treat a series of diseases.⁶⁻¹³ The functional components in the fruit of Prunus mume include organic acids, mineral elements, fiber, polysaccharides, flavonoids, and polyphenol compounds.^{3,24} In our study, the concentrations of eight kinds of organic acids in the fruit of Fructus mume were evaluated quantitatively by UPLC to determine the differences between the processed and fresh fruit. The results showed that the content of organic acids in Fructus mume was higher than that in the fruit of Prunus mume. Similar to Prunus mume fruit, citric acid, the Q-marker of Fructus mume in the Chinese Pharmacopoeia,9,23 was the primary type of organic acid in Fructus mume, accounting for 79.89% of the total content determined (Figure 1).

It also has antioxidant, antibacterial, antithrombotic, and anti-inflammatory effects.²³ Malic acid, which accounted for 8.44%, was the secondary main kind of organic acid and higher than that of *Prunus mume* fruit.

Minerals are required as essential nutrients by humans to carry out the functions required for health.²⁵⁻²⁶ The contents of mineral elements in *Fructus mume* were higher than those in *Prunus mume* fruit. The K content was the highest among the seven mineral elements determined in this study. Potassium is involved in regulating water, electrolyte, and acid-base balance in the body.²⁷⁻ ²⁹ This study found that K was the main mineral element, accounting for 70% of the total content.

It has been confirmed that ten phylogenetic bacterial groups exist in the intestinal microbiota.30 The composition and diversity of gut microbiota are related to health and disease.³¹ The gut microbiota performs essential metabolic functions in conjunction with the host's defense and immune systems to protect against pathogen infiltration and colonization.^{19,32} The imbalance of it can lead to the occurrence of many diseases, such as obesity,33-34 hypertension,³⁵ and cardiovascular disease.³² Therefore, 16S rRNA sequencing was used to analyze the composition of the intestinal flora of the mice in this study. The results showed that the intestinal flora composition of Fructus mume was different from that of the control group. Firmicutes and Bacteroidetes are the main bacterial phyla, and the F/B ratio plays a crucial role in maintaining normal intestinal homeostasis.18,36

The genus *Bacteroides* is enriched in the colon of chronic kidney disease patients with CKD.^{20,37} Our study found that the abundance of Bacteroides of *Fructus mume* group was lower than that in the control group, and the BUN and CR contents of the former group were also significantly lower than those of the control group (Table 4). In conclusion, we can deduce that *Fructus mume* is beneficial in protecting the kidneys from damage.

Lactobacillus is known to have probiotic effects in animals,³⁸⁻³⁹ the abundance of which could slow the progression of kidney disease by improving the intestinal environment.^{20,40} We found that the abundance of this species was greater in the *Fructus mume* group, which implies that *Fructus mume* might promote the multiplication of *Lactobacillus* species. *Oscillospira* has a positive effect on human health, low fat, leanness, and inflammatory disease of the liver,⁴¹⁻⁴² and in this study, we find that the relative abundance of Oscillospira is significantly higher compared with control.

Conclusion

In summary, *Fructus mume* intake could increase the body weight of mice and enrich the abundance of intestinal flora at the phylum and genus levels. It was beneficial to protect the liver and kidney from damage by reducing the serum biochemical factors. This study quantitatively analyzed the organic acids and minerals of *Fructus mume*, which are the main functional components in Japanese apricot fruit, using UPLC, ICP-OES, and ICP-MS. The organic acid in *Fructus mume* was higher than in *Prunus mume* fruit. Similar to *Prunus mume*, the primary mineral element in *Fructus mume* is K. These findings provide theoretical support for further exploration of the medicinal value of *Fructus mume*.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The manuscript incorporates all datasets produced throughout this research study.

Ethics Statement

This study was approved by Animal Ethics Committee of Nanjing Agricultural University Permit and performed in accordance with the guidelines of the Science and Technology Agency of Jiangsu Province and Nanjing Agricultural University.

Informed Consent Stetement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author Contributions

- Jing Shao: Writing original draft, Investigation, Formal analysis, Data curation.
- Shasha Wang: Writing original draft, Investigation, Formal analysis, Data curation.
- Jiangfeng Song: Formal analysis and reviewing multiple versions of the manuscript.
- Xueming Zhang: Formal analysis, Writing-Review& Editing.
- **Faisal Hayat**: Investigation, Data curation.
- Hengguang Li: Providing funding support and suggestions.
- Wenjin Li: Providing funding support and suggestions.
- Zhihong Gao: Formal analysis, Conceptualization, Funding acquisition.

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