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Integration of ^{1}H-NMR-Based Metabolomics Approach with Traditional Chinese Pulse Diagnosis to Evaluate the Qi-Enhancing Activity of Herbal Tea

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Abstract

Human metabolism is intricately linked to an individual's health status. Regardless of living habits, it will be reflected in the metabolic characteristics of urine. The utilization of the ^{1}H NMR-based metabolomics method has enabled examine the metabolomic changes in urine under various physiology conditions, providing valuable insights into metabolites. In this particular study, volunteers were divided into two groups based on the strength of their spleen pulses, using the pulse diagnosis method employed in traditional Chinese medicine. Subsequently, their urine samples were analyzed, revealing notable variances in urea, creatinine, citric acid, succinic acid, trimethylamine-N-oxide (TMAO), alanine, hippuric acid, and glycine between the two groups. Interestingly, individuals with weak spleen pulses showed significant improvements after consuming herbal tea. Furthermore, we conducted LC-MS analysis on herbal tea and performed adenosine triphosphate (ATP) activity tests on the C2C12 mouse skeletal muscle cell line. The results indicated that within a reasonable concentration range, exposure to herbal tea led to an increase in the mitochondrial ATP production capacity of C2C12 cells. These findings shed light on the relationship between traditional Chinese medicine pulse diagnosis and urine metabolites, highlighting their potential as non-invasive and straightforward health assessment indicators. They can aid in the preliminary determination of necessary dietary and lifestyle changes to enhance overall bodily health.

Keywords

[1]H-NMR, Metabolomics, Traditional Chinese Pulse Diagnosis, Qi-Enhancing, Herbal Tea

1. Introduction

In traditional Chinese medicine (TCM) diagnosis, several main methods of differentiation exist: observation (望, Wang), olfaction/smelling (闻, Wen), inquiry/asking (问, Wen), and palpation/touching (切, Qie). Among them, "切" specifically refers to the observation of pulse in the area approximately 5 centimeters (equivalent to 3 Chinese inches) above the transverse wrist crease on the lateral (radial) side of both wrists. This particular area is divided into three main positions known as "cun," "guan," and "chi," each representing different body systems. The cun, guan, chi positions on the left hand represent the heart, liver, and kidney systems respectively, while on the right hand, they represent the lung, spleen, and vital gate [1]. However, in TCM theory, these positions do not solely represent individual organs but rather an interconnected system. For example, the heart system consists of the heart, small intestine, face, and tongue; the liver system functions primarily in bile secretion and blood storage, including emotional and digestive regulation as well as the circulation of qi, blood, and fluids; the kidney system involves the kidney, bladder, bone marrow, brain, and ears; the lung system is not only responsible for respiration but also closely related to the large intestine, hair, and skin; the spleen system governs digestion and absorption of nutrients from food and transmits them to the heart and lungs for distribution throughout the body. The spleen system is responsible for the transformation and transportation of food essence and fluids, providing energy to maintain the functional state of organs and tissues [2]. Mitochondria, as the central regulators of cellular energy, produce ATP to drive energy-requiring cellular reactions. From these perspectives, TCM theory considers "qi" to be the fundamental energy and driving force of all living organisms. Therefore, whether it is the energy provided by the spleen system or the energy generated by mitochondria, they share similar concepts in terms of energy and regulation of cellular responses [3] [4]. Going further into the discussion of the spleen system in TCM theory, it is responsible for transforming food into qi and blood to provide nourishment to other tissues and organs. The proper functioning of digestion relies on the assistance of intestinal microbiota, they also play a crucial role in regulating key physiological activities, such as processing and absorbing nutrients and the metabolism of many xenobiotic compounds, so an imbalance in gut bacteria can directly affect the breakdown and utilization of nutrients [5].

In our current study, our primary focus lies in utilizing pulse diagnosis as the initial step in distinguish different groups. To carry out this analysis, we have selected individuals base on judgment of TCM practitioners, specifically targeting those with weak and strong spleen pulses (right cun). Urine samples are collected from these individuals for further analysis. Subsequently, individuals with weaker spleen pulses are provided with Qi-nourishing tea to consume for a period of seven days. Following this intervention, we collect urine samples once again for analysis. The presence of a weaker pulse often indicates an abnormal state within the overall system, and our objective is to identify potential clues through the analysis of metabolites present in the urine. For the analysis of urine samples, we employ 1H-NMR spectroscopy, which enables us to compare differences between the groups by observing signals corresponding to various substances in the urine. Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical technique used to determine the content and purity of a sample as well as its molecular structure. It relies on the principles of nuclear magnetic resonance, where nuclei in a strong magnetic field are perturbed by a weak oscillating magnetic field and respond by producing an electromagnetic signal. This signal provides detailed information about the electronic environment surrounding the nuclei, which can be used to infer the structure, dynamics, and chemical environment of molecules in the sample. These substances include urea, creatinine, citrate acid, succinate, TMAO (trimethylamine-N-oxide), DMA (dimethylarsinic acid), glucose, alanine, lactate, hippurate acid, glycine, and taurine. In order to perform data analysis, we utilize 1H-NMR analysis in conjunction with Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA). OPLS-DA is a supervised multivariate statistical analysis method commonly used for analyzing complex biological data, such as gene expression and metabolites. This method effectively handles situations involving small data samples and successfully identifies differences between sample groups.

2. Materials and Methods

2.1. The Significance of Pulse Information by TCM Clinical Doctor

Pulse information is a crucial component of clinical information in TCM. Traditional pulse information is obtained by placing the fingertips on the radial artery, just above the radial bone, where the tissue covering it is relatively thin, making detection easy. It can be classified according to the descriptive adjectives of Float, Sink, Slow, Fast, Thick, Thin, Strong, Weak, Fluent, Astringent and Rhythm. Afterward, we create a form to record the medical condition of each volunteer (Supplementary Data 1). We will select participants with a weaker spleen pulse as the experimental group.

2.2. Urine Preparation Protocol and Metabolic Profiling by NMR

The urine samples were processed using freeze drying. Then, the choice of solvent is critical in NMR spectroscopy as it can affect the chemical shift and peak resolution of the sample signals. Commonly used solvents include deuterated solvents like deuterated water (D2O) or deuterated chloroform (CDCl3), which do not interfere with the NMR spectra of the sample molecules. These solvents are preferred because their hydrogen atoms are replaced with deuterium atoms (which do not exhibit an NMR signal under typical conditions), ensuring that they do not obscure the signals from the sample of interest. So, we redissolve the dried urine with 600 μL 99.9% D_2O solution after drying containing the Sodium 3-(trimethylsilyl) propionate trimethylsilylpropanoic acid (TSP) (0.01% w/v), an internal standard for chemical shift reference as well as quantification of metabolites. Then, the solution was centrifuged at 1500 rpm for 10 min at 4˚C and takes the 500 μl supernatant to analysis. A total of 500 μl samples were transferred into a 5 mm NMR tube for the NMR experiment. The NMR experiments were carried out at 400 MHz NMR spectrometer.

2.3. Herbal Tea Preparation

The herbal tea is made from a combination of Huangqi (*Astragalus membranaceus*), 5 g, and Gouqizi (*Lycium chinense*), 1 g. The mixture of these herbs is boiled for 30 minutes to create the herbal tea. Drink 1000 c.c of this tea per person per day for a week.

2.4. LC-MS Quantification of the Herbal Tea

The herbal tea was freeze-dried for three days, and then the dried material was extracted by dissolving it in water for analysis. A Bruker amazon speed-ion trap mass spectrometer (Bruker, Billerica, MA, USA) coupled with a Thermo Scientific Dionex UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) was used (Bruker Hystar software). The separation was performed on a Waters RP-18, 100 × 2.1 mm, 1.7 micro column. The eluting solvent system was MeOH (Sigma-Aldrich, St. Louis, MO, USA) (A) and 10% (v/v) acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) in water (B). The gradient started with 5% A and 95% B for 10 min, →50% A and 50% B at 15 min, →100% A, 0% B at 25 min. The column temperature was 40˚C. The flow rate was 1 mL. The mass parameters setting was as follows: 4.5 kV of capillary; 1.93 bar of nebulizer pressure; 8.0 $l \cdot min^{-1}$ of dry gas flow at 250˚C.

2.5. ATP Generation Activity Test

Mouse myoblasts (C2C12) cells were obtained from the Bioresource Collection and Research Center and maintained at 37˚C in a humidified atmosphere with 5% CO2. A total of 5 × 105 cells were cultured in 10 cm dishes using high glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and antibiotics. After 2 days, myotube differentiation was induced and maintained for 4 days using differentiation medium containing 2% horse serum. Subsequently, the differentiated C2C12 cells were retreated with a prescription of traditional Chinese medicine for 48 hours. To measure the intracellular ATP content, an ATP luminescence assay kit (Cayman chemical, USA) was emplyed. Cells were washed with phosphate-buffered saline (PBS) and centrifuged at 2500 rpm in 4˚C for 5 minutes. The PBS was then removed and 100 μL of 1X ATP detection sample buffer was added to the cell. The cells were homogenizing the cell by pipetting the 1X ATP detection sample buffer up and down several times. The cell lysate was transferred to an Eppendorf and kept on ice. Since the ATP detection standard configuration is completed, 100 μL of the luminescence reaction mixture and 10 μL of standard or cell lysate to each well of a 96-well. Finally, the plate was incubating at room temperature for 15 to 20 minutes before reading the luminescence signal.

2.6. Statistical Analyses

Orthogonal projections to latent structures discriminant analysis (OPLS-DA) are a supervised method that enables the identification of differences groups of samples. The objective of OPLS-DA is to partition the systematic variation in the X-block (an input dataset of metabolite concentrations) into two model parts: one part models the covariation between the measured data of X variable (metabolite concentrations) and the response of Y variable (in our case, binary variables of disease status) within the groups. The second part captures systematic variation in X that is unrelated (orthogonal) to Y. This approach is valuable for identifying metabolites that exhibit differential expression between groups of samples and provides insights into metabolic pathways and processes in biological systems. Statistical analysis was performed with GraphPad Prism. Values were expressed as the mean value ± standard deviation (SD) and compared using a one-way ANOVA test. Differences were considered statistically significant at values of $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Diagnosis of Traditional Chinese Pulse Condition

This study primarily focuses on utilizing pulse diagnosis as the initial step to distinguish between groups. We specifically selected individuals exhibiting weak and strong spleen pulses (right guan) and collected their urine for 1H-NMR analysis. Subsequently, we administered Qi-nourishing tea to the individuals with weaker spleen pulse for a duration of 7 day and collected their urine again for analysis. By employing the statistical model of OPLS-DA, we successfully classified the individuals with weak and strong spleen pulses into two distinct clusters (**Figure 1**), indicating significant metabolic differences between the two groups.

3.2. Urinalysis Was Conducted to Differentiate between Strong and Weak Spleen Pulses

^{1}H-NMR analysis was utilized to evaluate the variations in metabolites profile in the urine. The tentative identification of 15 tentative identification off 15 detected metabolites is summarized in **Table 1**. The findings indicated that individuals with weaker spleen pulses exhibited lower relative levels of trimethylamine N-oxide (TMAO), alanine, creatinine, citric acid, phenylacetylglutamine

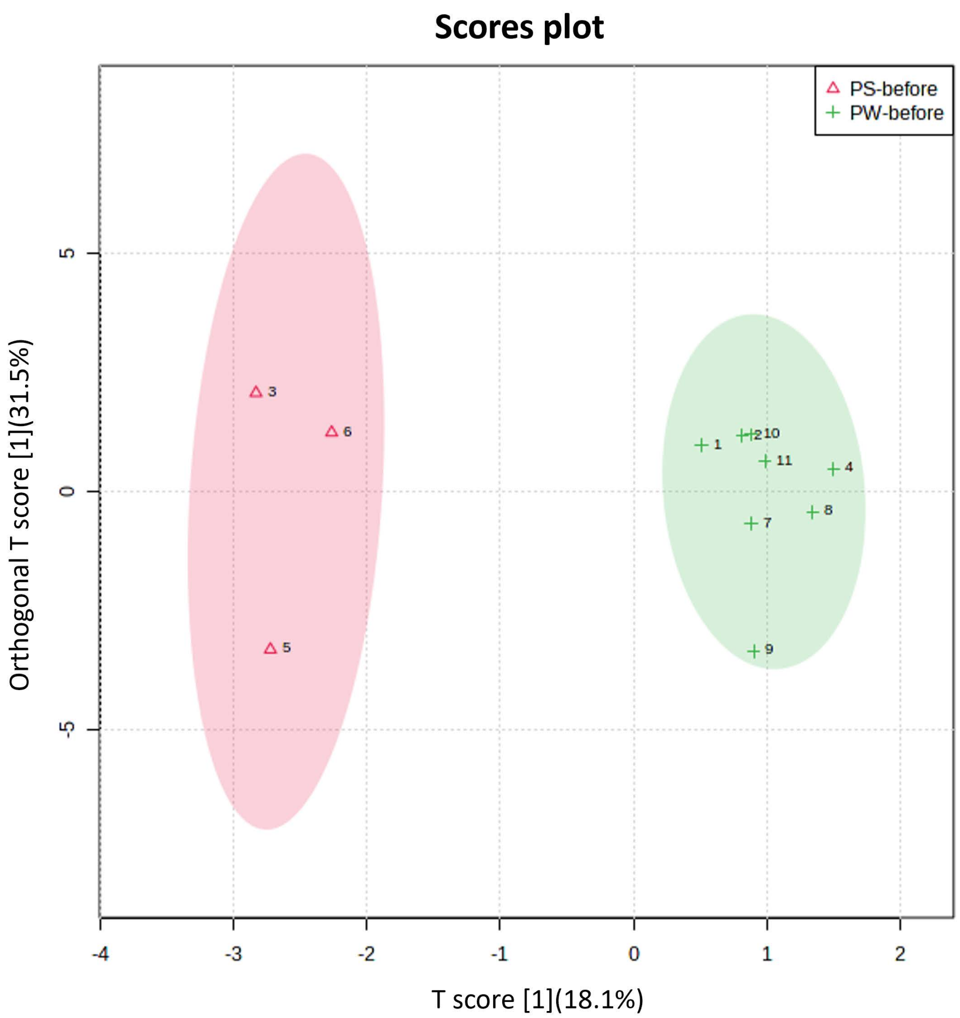


Figure 1. This picture shows the two groups with weaker and stronger spleen veins before drinking tea.

(PAG), glycine, and hippuric acid in their urine. Upon optimizing the data using statistical software, individuals with stronger spleen pulses demonstrated relative levels of TMAO, alanine, creatinine, and PAG that were more than doubled, while hippuric acid and citric acid were approximately three times higher (Figure 2). The Variable Importance in Projection (VIP) score revealed 10 significant metabolites. Among them, urea displayed higher relative concentrations in individuals with weaker spleen pulses, whereas TMAO, glycine, succinate, and glucose exhibited lower relative concentrations in individuals with stronger spleen pulses (Figure 3). Correlation heatmaps provide a visual representation of the strength of the relationships between urine metabolites, depicted through various shades of colors. For instance, creatinine exhibits a positive correlation with TMAO and glycine, while succinate shows a positive correlation with hippuric acid and TMAO. Additionally, citric acid demonstrates a positive correlation with alanine, looking from the perspective of TMAO. Looking at it from the perspective of TMAO, there is positively correlation with glucose and urea

(**Figure 4**). Among these metabolites, only glycine and creatinine are linked in terms of biosynthesis in the human body. Glycine serves as an amino acid, while creatinine is a byproduct creatine metabolism in the muscles. Glycine plays a crucial role in creatine metabolism and contributes to its synthesis. Creatine is stored as energy in muscles and can be converted to creatine phosphate when necessary. When creatine phosphate breaks down, creatinine is produced as a byproduct, released into the bloodstream from the muscles, and eventually excreted by the kidneys. The remaining metabolites are not directly associated with the biosynthetic pathway, but they all reflect potential health risk. The hierarchical clustering heatmap displays OPLS-DA VIP metabolite expression levels. This heatmap visualizes the top 15 metabolites from the PS and PW groups (**Figure 5(A)**). A similar pattern of results was observed using a grouping classification model (**Figure 5(B)**). In clinical medicine, a low concentration of PAG in urine does not indicate immediate danger related to diseases. However, it may suggest an abnormal gut microbiota environment. After tea consumption, there is an observable increase in PAG concentration (**Figure 7**), indicating a positive regulatory effect on the gut microbiota.

Table 1. The tentative assignments of ^{1}H-NMR spectral signals from PS and PW groups.

ID	Metabolites	Chemical shift (ppm)	PS	PW
1	4-Deoxythreonic acid	1.13(d), 3.17(m), 4.19(d)	*	*
2	Alanine	1.48(d), 3.77(q)	+	−
3	Lactate	1.32(d), 4.1(q)	*	*
4	Glucose	3.2(q), 3.4(q), 3.5(q), 3.7(q), 3.8(q), 4.6(s), 5.2(s)	*	*
5	Citric acid	2.66(d), 2.75(d)	+	−
6	Succinate	2.4(s)	*	*
7	Dimethylarsinic acid (DMA)	1.62(s)	*	*
8	Hippurate acid	3.95(d), 7.80(dd), 7.53(m), 7.62(tt)	+	−
9	Trimethylamine (TMAO)	3.25(s)	+	−
10	Creatinine	3.034(s), 4.042(s)	+	−
11	Taurine	3.25(t), 3.42(t)	*	*
12	Glycine	3.54(s)	+	−
13	Urea	5.8(s)	*	*
14	Creatine	3.02(s), 3.92(s)	*	*
15	Phenylacetylglutamine (PAG)	2.58(dt), 4.15(t), 7.35(dd) 7.28(m), 1.91(td)	+	−

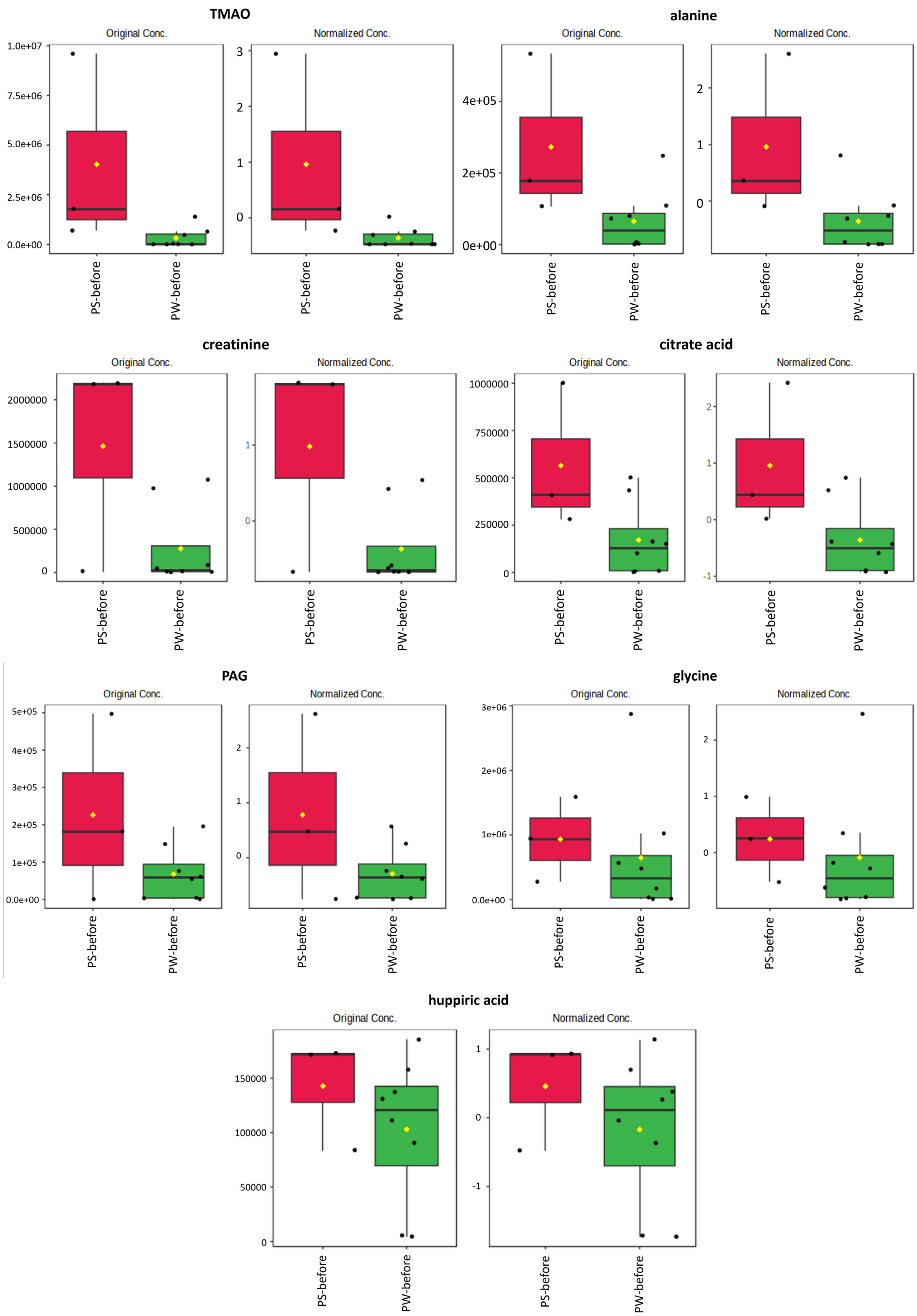


Figure 2. Each metabolite's expression level changes with respect between the PS and PW group. Six metabolites were found with significant fold change, with p-values higher than Green boxes are PW sample, and red boxes are PS sample. Box-and-Whisker plots were plotted by using MetaboAnalyst5.0. https://www.metaboanalyst.ca.

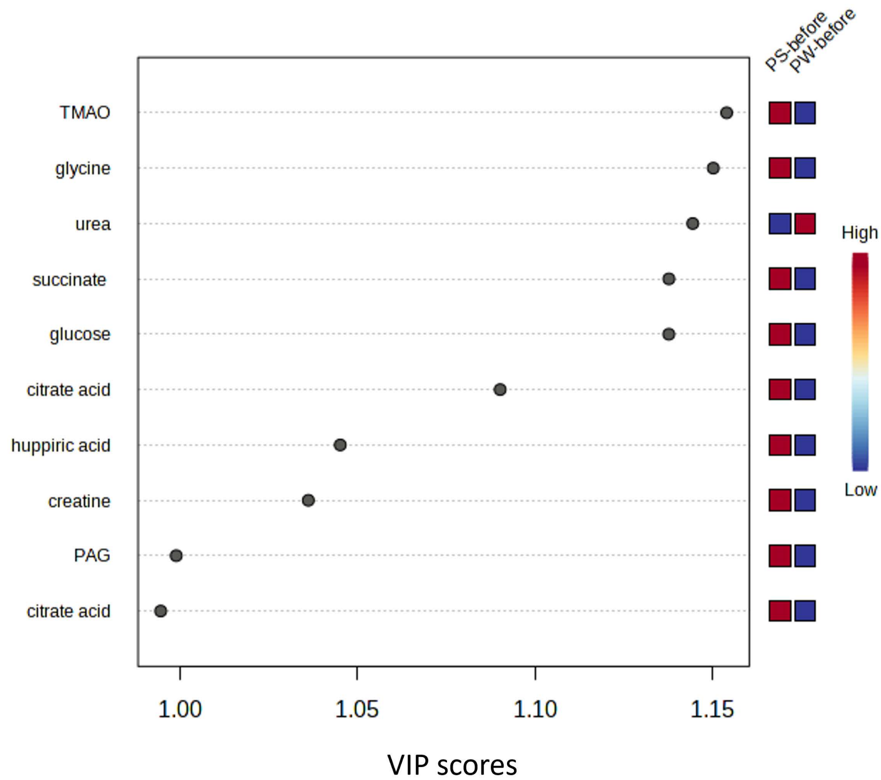


Figure 3. Variable importance in projection (VIP) score plot for the top 10 most important metabolite features.

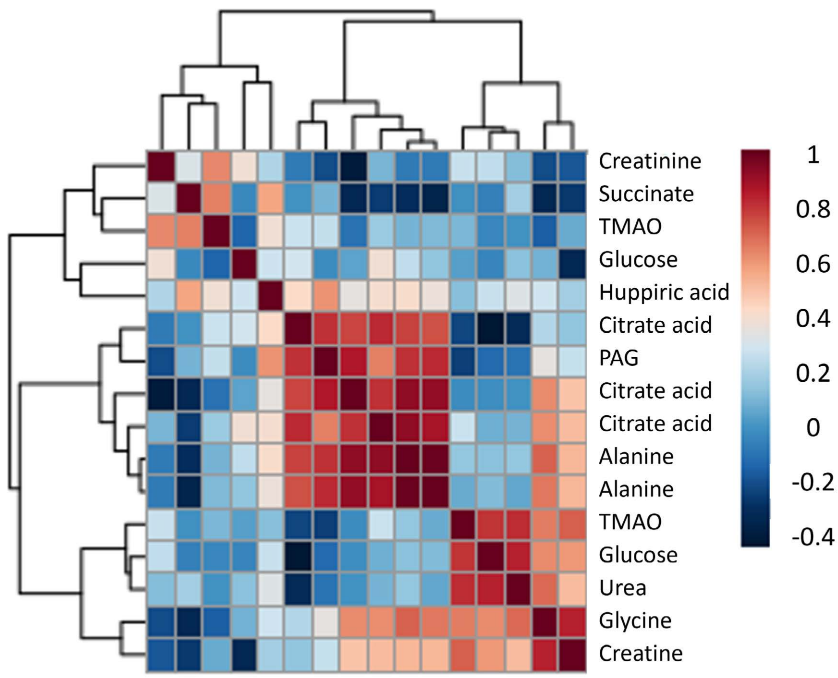
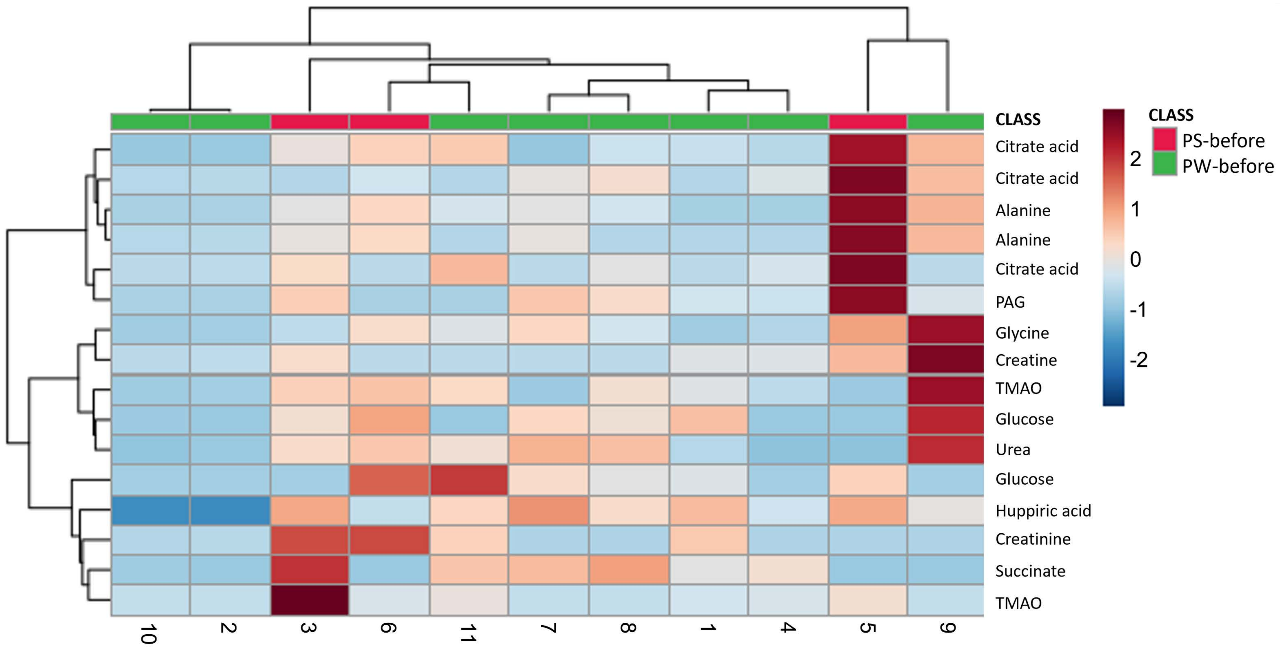
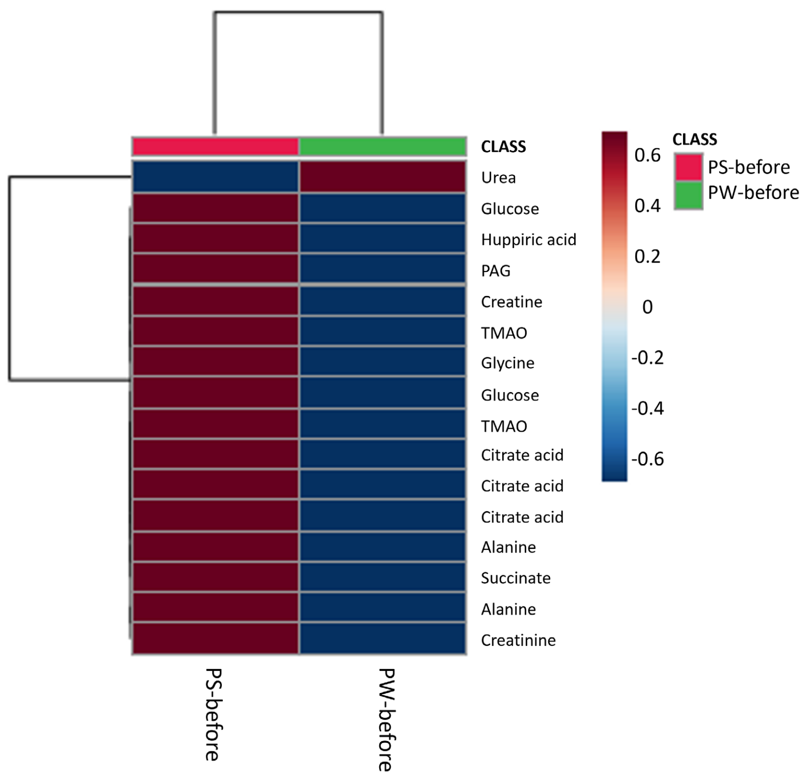


Figure 4. Note the correlation heatmaps will only show correlations for a maximum of 1000 features. For larger datasets, only the top 1000 features will be selected based on their interquantile range (IQR). When the color distribution is fixed, you can potentially compare the correlation patterns among different data sets.



(A)



(B)

Figure 5. A hierarchical clustering heatmap selected OPLS-DA VIP metabolite expression levels. (A) This heatmap visualizes the top 15 metabolites from the PS and PW groups. The degree of variation was color-coded, ranging from dark red (with a positive value of 2) to dark blue (with a negative value of 2), representing the highest computed ratio of metabolites to the lowest. Metabolites are represented in each row, while a column represents each replicate. Ward's method and colored boxes were generated using MetaboAnalyst 5.0. The data are reported as mean ± SD of three independent experiments. (B) This is also the hierarchical clustering heatmap, but it shows only group averages.

3.3. Urine Analysis Following TCM Tea

We conducted a study wherein TCM tea was administered to individuals with weaker spleen pulses, and urine samples were collected for NMR analysis. In the context of traditional Chinese medicine's clinical symptoms, not every prescription is suitable for every person's condition. As a result, only a portion of the volunteers experienced changes in their pulse condition after consuming the tea. However, hose volunteers who did experience changes showed corresponding alterations in their urine metabolites. Notably, individuals who initially had weaker pulses exhibited a significant increase in the strength of their spleen pulse after consuming the tea. These findings were further substantiated by clear statistical differences among the groups (**Figure 6**).

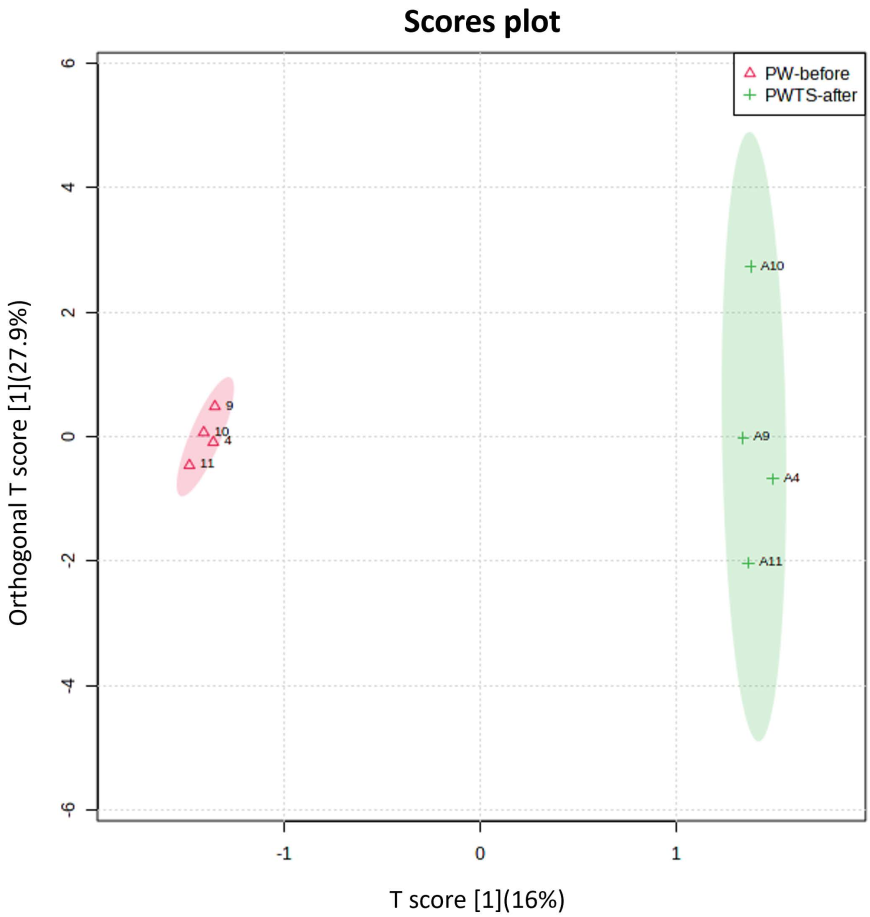


Figure 6. After giving tea to those with weak spleen veins, collect the grouping diagram of their urine.

3.4. Urine Metabolites and Mitochondrial Productivity

In our research findings, we have successfully utilized the VIP score to identify 10 metabolites of significant importance. Remarkably, alanine and urea exhibited a downward trend in relative concentrations following the strengthening of the spleen pulse, while citric acid and creatinine displayed an increase (**Figure**

7). The expression levels of each metabolite showed distinct changes between the PW (Pre-Intervention) and PWTS (Post-Intervention) groups. Notably, three metabolites, namely alanine, citrate acid and PAG, demonstrated a significant 2 - 4 folds change, supported by p-values lower than the specified threshold. In Figure 8, the PWTS samples are duplicated by green box, while PW sample are presented by red box.

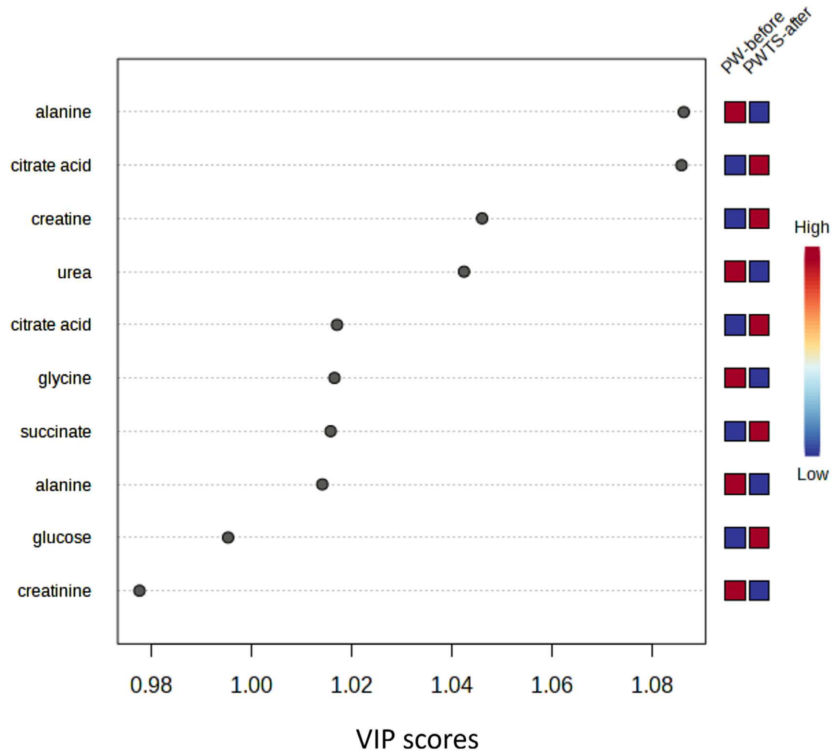
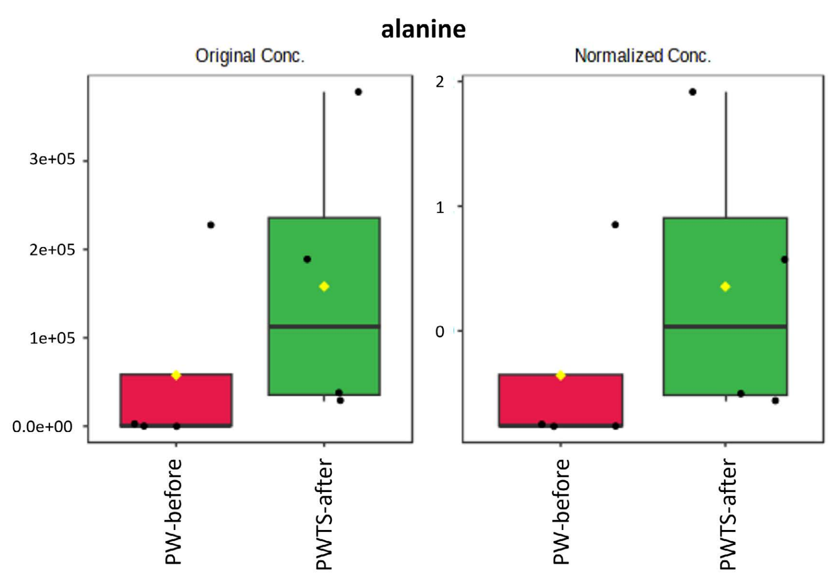


Figure 7. VIP score plot for the top 10 most important metabolite features identified by OPLS-DA.



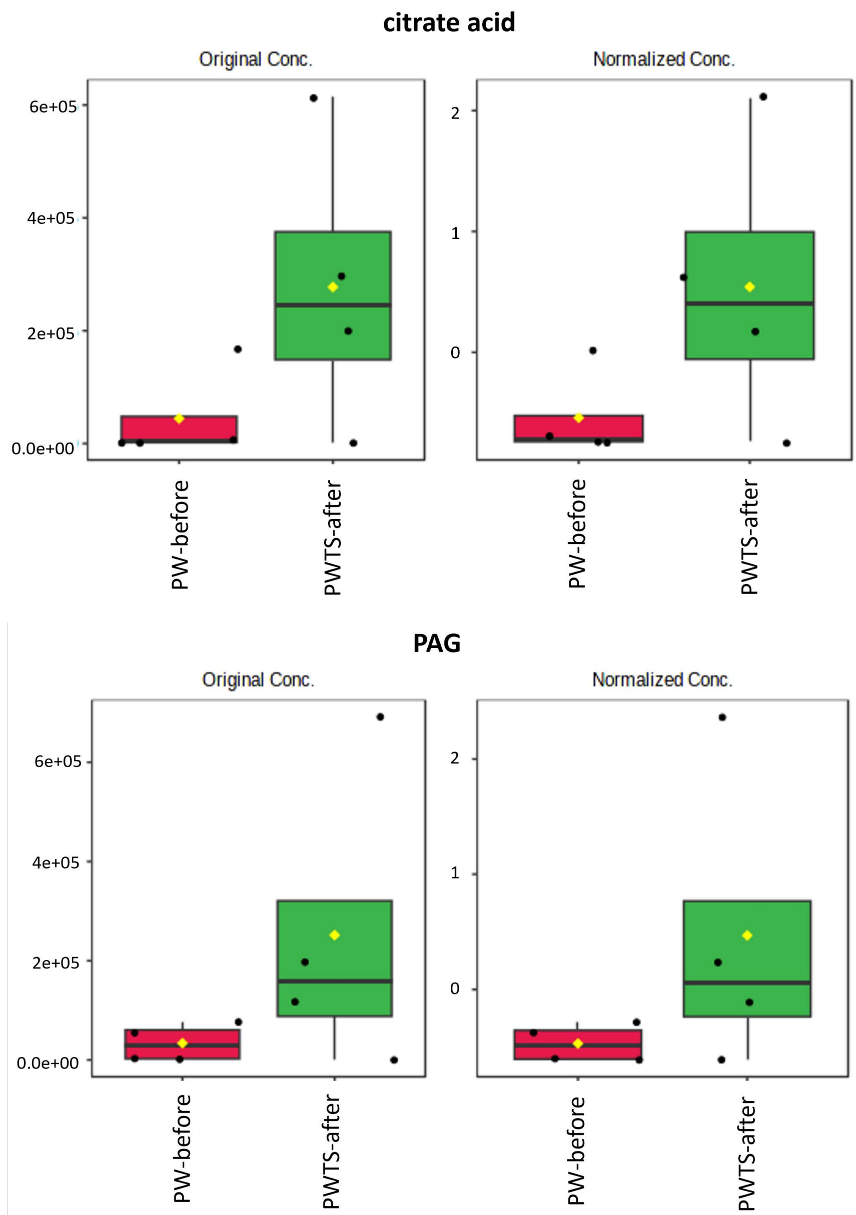
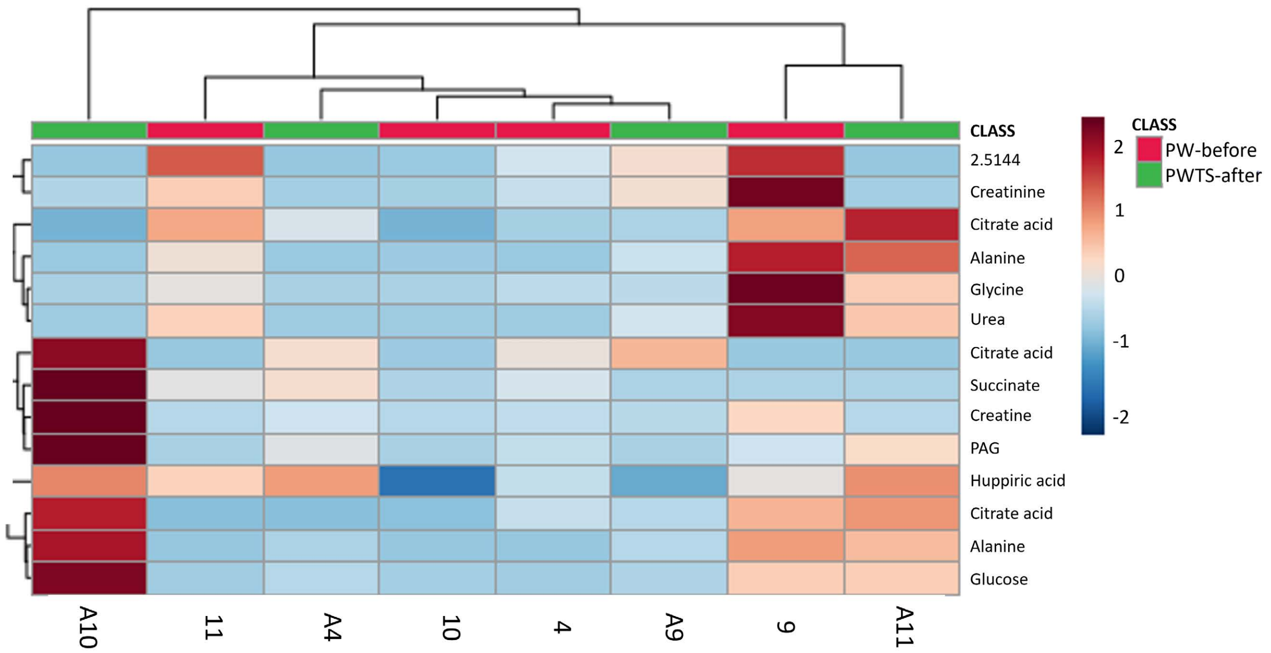


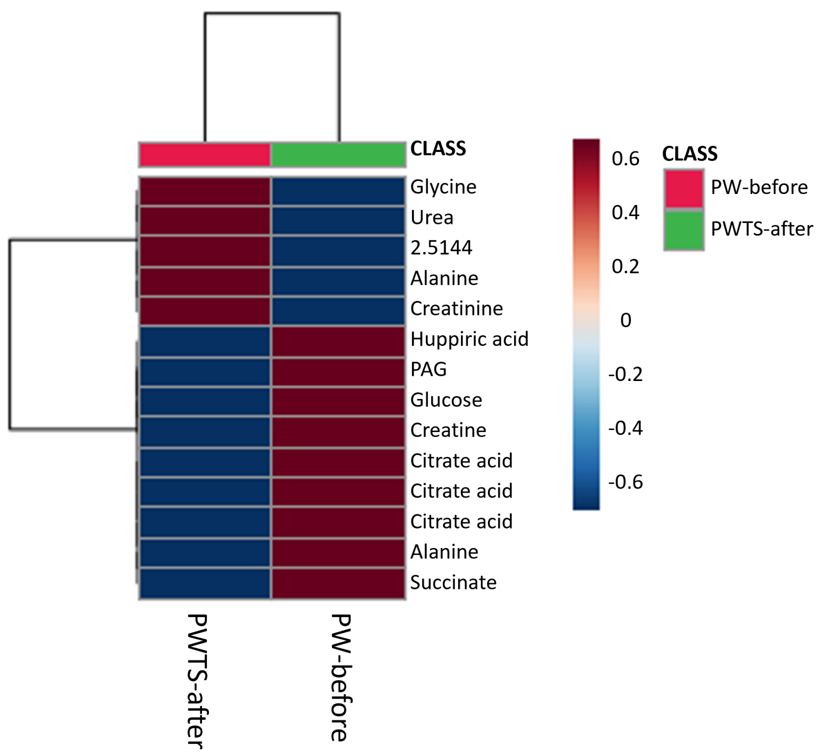
Figure 8. Each metabolite's expression level changes with respect between the PW and PWTS group. Three metabolites were found with significant fold change, with p-values lower than Green boxes are PWTS sample, and red boxes are PW sample. Box-and-Whisker plots were plotted by using MetaboAnalyst5.0. https://www.metaboanalyst.ca.

3.5. Relationship between Urine Metabolites and Physiological Responses

Following the consuming of TCM tea, participants were reclassified based on changes in their pulse, as assessed by a TCM doctor. Remarkably, several volunteers who initially had a weak spleen pulse showed an upward trend in their pulse strength, and this change was accompanied by significant differences in their urine composition (Figure 9). These findings suggest a noteworthy correlation between pulse changes and urinary metabolites.



(A)



(B)

Figure 9. A hierarchical clustering heatmap selected OPLS-DA VIP metabolite expression levels. (A) This heatmap visualizes the top 15 metabolites from the PS and PW groups. The degree of variation was color-coded, ranging from dark red (with a positive value of 2) to dark blue (with a negative value of 2), representing the highest computed ratio of metabolites to the lowest. Metabolites are represented in each row, while a column represents each replicate. Ward's method and colored boxes were generated using MetaboAnalyst 5.0. The data are reported as mean ± SD of three independent experiments. (B) This is also the hierarchical clustering heatmap, but it shows only group averages.

3.6. TCM Tea Increases ATP Production in C2C12 Cells

In order to explore the potential of TCM tea in enhancing ATP production, we conducted cell experiments using freeze-dried TCM tea. The results revealed that the formulation of TCM tea, at appropriate concentrations, effectively increased ATP production capacity in C2C12 mouse skeletal muscle fiber progenitor cells (**Figure 10**). These promising findings suggest that consumption of the tea might contribute to improving ATP production in the human body. Notably, the primary ingredients in TCM tea are Huangqi and Goji berries.

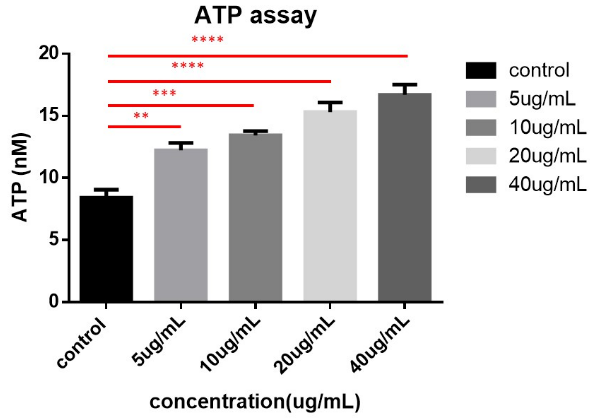
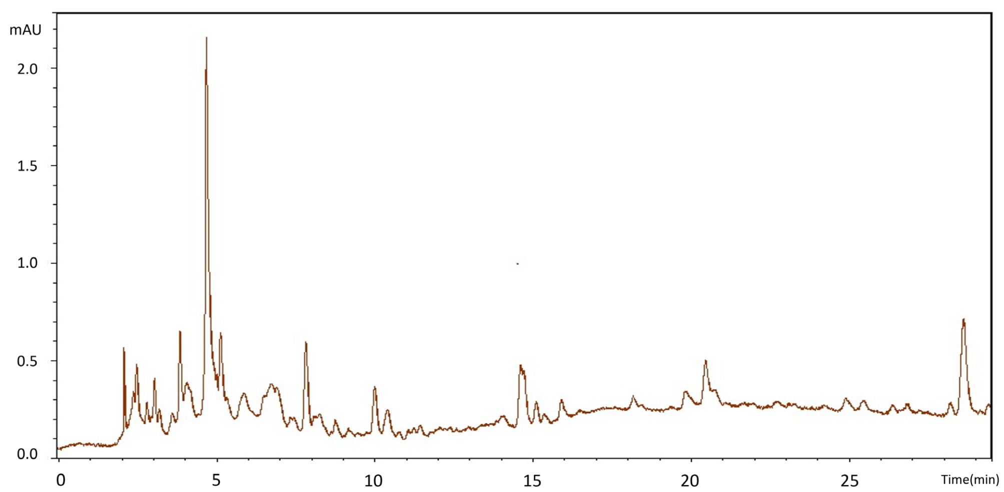


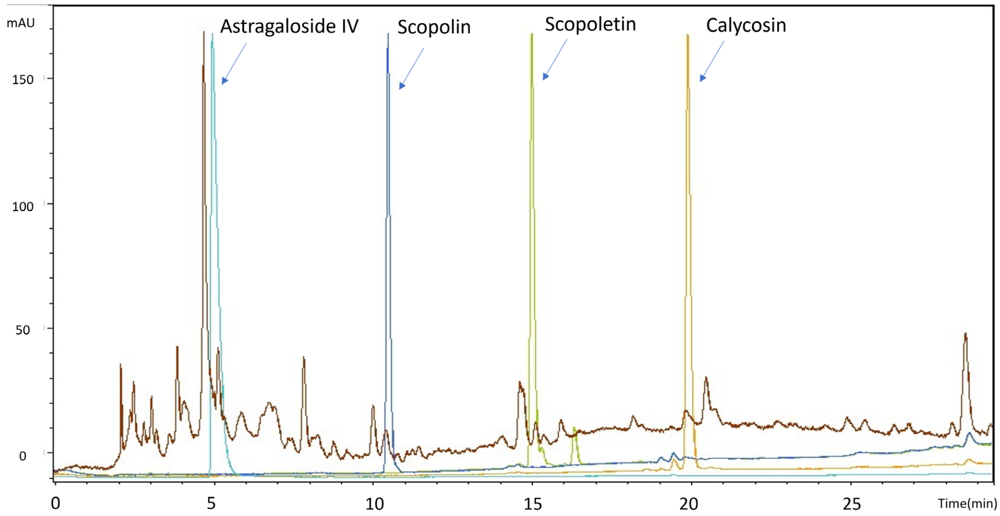
Figure 10. The TCM tea administered to myofibroblast C2C12 cell for ATP assay test.

3.7. Analysis of TCM Tea Composition by LC-MS

In our study, we subjected the TCM tea containing Huangqi and Goji, which was administered to the volunteers, to LC-MS analysis. After a high-temperature decoction, we identified the presence of calycosin, astragaloside IV, scopolin, and scopoletin, which are common markers for Huangqi and Goji (**Figure 11**). These four compounds are considered secondary metabolites of the medicinal herbs.



(A)



(B)

Figure 11. (A) The TCM tea analysis by LC-MS. (B) Overlay comparison of sample analysis chart and four standard products.

4. Discussion

Spleen deficiency is a common syndrome characterized by symptoms such as indigestion, loss of appetite, and fatigue. Although the specific diseases present in individuals with weaker spleen pulses cannot be definitively identified, it is speculated that they may suffer from spleen deficiency. According to TCM theory, the spleen is responsible for digestion and transformation, converting food in the gastrointestinal tract into qi, blood, and nutrients. Spleen deficiency suggest urea, an amino acid metabolite synthesized by the liver and excreted by the kidneys, may indicate potential kidney dysfunction when its excreted concentration is low.

Among the various metabolites found in urine, previous studies have reported that TMAO and PAG are associated with metabolites derived from digestion and gut microbiota. TMAO is a metabolite formed through the microbial metabolism of nutrients like potassium, nitrogen, phosphorus, present in foods such as meat, fish, and shrimp. Gut microbiota, including Vibrio, Aeromonas, Acinetobacter, Shewanella, and Flavobacterium, contribute to the production of TMAO. TMAO is considered a metabolite regulated by both animal and gut microbial metabolism. Research has linked TMAO to an increased risk of cardiovascular and kidney diseases, as it promotes cholesterol accumulation leading to atherosclerosis [6]. Moreover, TMAO can impact inflammation response, intestinal barrier function, and gut microbiota composition, thereby influencing metabolic health. Although some studies suggest potential benefits of TMAO in certain situations, such as protecting the heart from hypoxic damage and positive effects on inflammation response and intestinal barrier function, there is substantial research associating TMAO with an elevated risk of cardiovascular and kidney diseases [7]. Therefore, dietary and lifestyle adjustments are recommended to reduce TMAO production and mitigate the risk of these diseases.

PAG is a naturally occurring metabolite in human urine. It is synthesized in the liver from the amino acids, namely tyrosine and glutamine. Research indicates that PAG may play a role in various aspects of human nitrogen metabolism, inflammatory responses, and gut microbiota balance. The production of PAG is associated with several gut bacteria, including Enterococcus, Veillonella, Akkermansia, and *Staphylococcus aureus*, which are closely linked to gut health. For example, Akkermansia has been found to be associated with conditions like obesity and diabetes [8]. Similarly, the presence of Enterococcus and Veillonella in the gut is linked to gastrointestinal infections and diseases [9]. However, further research is needed to fully understand the exact role of PAG in the human body. Enterococcus is a type of bacteria present in the gut microbiota. Certain strains of Enterococcus can cause infections such as urinary tract infections and cystitis, and they can also weaken the immune system [10]. Some strains of Enterococcus can aid in cholesterol breakdown, reduce its absorption in the intestines, and consequently lower cholesterol levels in the bloodstream. They can also stimulate the immune system, enhancing the body's resistance. Additionally, they can inhibit the growth of other harmful bacteria, thus reducing the risk of intestinal infections [11]. Paracoccus is another genus of bacteria that is less commonly found in the human gut compared to other gut bacteria. Initial studies have suggested that certain strains of Paracoccus can help regulate the gut microbiota by suppressing the growth of harmful bacteria and promoting the proliferation of beneficial bacteria, thereby contributing to gut health. They also possess antioxidant properties, helping to counteract the attack of free radicals and reduce oxidative damage in the body [12]. However, further research is necessary to gain a deeper understanding of the potential benefits of Paracoccus for gut health. Certain strains of Enterobacter provide benefits by producing enzymes that aid in the digestion and absorption of nutrients from food. However, some strains of Enterobacter can cause hospital-acquired infections, particularly in individuals with weakened immune systems who are at a higher risk of infection. Moreover, certain Enterobacter strains exhibit high levels of antibiotic resistance, which can complicate the treatment of diseases [13]. Overall, impaired digestive and absorptive capabilities can result in inadequate nutrition and related symptoms [14].

In the spleen system, besides its vital role in digestion and nutrient absorption, it plays a crucial role in converting nutrients into qi and blood for transportation throughout the body [15]. This function can be likened to that of mitochondria, which serve as energy factories in the body by converting nutrients into ATP to provide cellular energy. Therefore, it can be inferred that individuals with weaker spleen pulses may experience a decline in digestive and absorptive functions, ultimately affecting ATP synthesis in mitochondria and leading to abnormal cellular and tissue metabolism as well as physiological functions. Additionally, research has found a correlation between TCM-defined spleen deficiency and increased oxidative stress and inflammation, which can also impact mitochondrial function [16]. Thus, regulating spleen system function and protecting mitochondrial function are important methods for maintaining overall health. Furthermore, citric acid is an intermediate metabolite that enters the tricarboxylic acid (TCA) cycle, also known as the respiratory cycle, in the human body. The TCA cycle involves the oxidative metabolism of glucose to generate cellular energy (ATP) [17].

Citric acid also aids in digestion and calcium absorption. After entering the intestines, citric acid combines with calcium ions to form soluble substances, facilitating calcium absorption and utilization. Citric acid possesses antioxidative properties, which slow down cellular damage and aging [18]. It also helps regulate the body's acid-base balance to maintain normal physiological functions. The production of citric acid relies on enzymes and coenzymes in the mitochondria, particularly the iron-containing enzyme acontitase, which catalyzes the conversion and production of citric acid [19]. Then, citric acid has the ability to enter the cytoplasm through the mitochondrial transport system and participate in various important metabolic pathways, including lipid metabolism and gluconeogenesis. Its close relationship with mitochondrial energy metabolism underscores the importance of protecting and maintaining mitochondrial function for overall energy and nutrient metabolism in the body [20]. Moreover, citric acid in urine, it can bind with calcium to form insoluble calcium salts, thereby inhibiting the formation of urinary stones. Imbalances in the body's acid-base equilibrium can lead to a decrease in urine acidity, which in turn reduces citric acid levels and increases the risk of stone formation [21].

Creatinine, a vital metabolite used to assess kidney function and monitor muscle metabolism, is primarily produced through the conversion of metabolites in muscles and other tissues into creatine. The liver further metabolites creatine into creatinine [22]. Hence, individuals with higher muscle mass tend to have increased creatinine production. In healthy adults, the typical concentration of creatinine in urine ranges from 14 to 26 mg/kg per day for men and 11 to 20 mg/kg per day for women [23]. Glycine, a nonessential amino acid, plays a crucial role in body growth and development. It is involved in the synthesis of muscle tissue, bones, skin, ligaments, and the liver. Research suggests that glycine can influence neural transmission in the central nervous system, as it can participate in the formation of NMDA receptors alongside aspartate [24]. Glycine is also involved in the synthesis and metabolism of bile acids in the liver. It converts bile acids into glycine-conjugated glycine bile acids, which aid in fat digestion and absorption when secreted into the intestines. Additionally, glycine assists in the liver's detoxification process by binding with methyl compounds and other harmful substances, facilitation their metabolism and excretion. In carbohydrate metabolism pathways, glycine, through the action of glycine amidotransferase, converts into pyruvate or lactate, thereby participating in glucose metabolism [25].

Alanine, another nonessential amino acid, contributes to protein synthesis and maintaining muscle structure. It interacts with other metabolites in the liver and muscles for energy metabolism. In muscles, when energy demand increases, alanine is released by breaking down creatine phosphate to supply the body's energy needs. Alanine also participates in gluconeogenesis, thus ensuring stable blood glucose levels. Moreover, alanine is involved in nitrogen metabolism. During protein metabolism, amino acids, including alanine, are released and converted into urea in the liver for excretion, maintaining nitrogen balance. Hippuric acid, formed by the combination of benzoic acid and glycine, is one of the most abundant metabolites found in human urine. Its levels are influenced by various factors such as physical, emotional and dietary [26].

Hippuric acid primarily functions as an effective detoxification substance, aiding the elimination of harmful substances from the body. When benzoic acid is metabolized by the body, the liver converts it into benzoic alcohol, which then combines with glycine to form hippuric acid. This compound is eventually excreted from the body. Additionally, hippuric acid can serve as an indicator to evaluate kidney function. The kidneys play a crucial role in clearing metabolic substances from the body, and impaired kidney function leads to decreased excretion of hippuric acid. Therefore, the concentration of hippuric acid in urine can be measured to assess kidney function. Previous studies have demonstrated that high levels of hippuric acid and other aromatic compounds observed in animal urine originate from gut microbial metabolism of dietary polyphenols [27]. Furthermore, some studies have indicated that lower levels of hippuric acid are associated with an increased risk of metabolic syndrome and cardiovascular diseases [28]. Patients with kidney disease tend to have lower concentrations of hippuric acid in their urine [29].

In ancient texts, Huangqi is described as having the ability to tonify the middle and augment Qi, specifically in the spleen and lung systems [30]. Qi is considered a vital substance necessary for transporting nutrients and blood throughout the body, according to traditional Chinese medicine concepts. Sufficient and unobstructed Qi flow is believed to be the source of good health in the body [31].

While TCM diagnoses are sometimes perceived as subjective judgments lacking scientific objectivity, our goal is to interbrate a variety analytical approaches with the millennia accumulated experience in traditional medicine to derive meaningful evidence. Calycosin, a flavonoid compound mainly found in herbs such as *Isatis indigotica* and *Carthamus tinctorius*, is known for its antioxidant [32], anti-tumor [33], and anti-inflammatory [34] activities. Astragaloside IV, renowned for its antioxidant activity, has been studied for its potential in enhancing the immune system and protecting the cardiovascular system [35]. Additionally, scopolin and scopoletin, found in Goji berries, belong to the coumarin class of compounds and have extensively documented their anti-inflammatory and antioxidant effects. Notably, scopoletin, demonstrates significant antimicrobial activity, inhibiting the growth of certain bacteria and fungi [36]. Various studies have highlighted that polysaccharides known as Astragalus polysaccharides,, are the main water-soluble bioactive components in Huangqi (Astragalus root), responsible for its physiological effects [37]. These polysaccharides play crucial roles in regulating physiological functions, adjust constitution, enhancing immune function, and maintaining mental vigor. They also contribute to increase gastric acid secretion and digestive enzyme activity, promoting food digestion and absorption [38]. Furthermore, Astragalus polysaccharides have been found to protect the gastric mucosa, thereby reducing the occurrence of gastric ulcers and gastritis [39]. Studies have indicated that polysaccharides derived from Goji berries possess prebiotic properties, meaning they selectively promote the growth and activity of beneficial bacteria in the gut [40]. These polysaccharides act as a source of nutrients for specific strains of bacteria, thereby influencing the composition and diversity of the gut microbiota. By supporting the growth of beneficial bacteria, Goji berry polysaccharides contribute to a healthy gut environment and overall digestive function. In traditional Chinese medicine, Goji berries are often associated with supporting spleen and stomach health, both of which play vital toles in the digestion and absorption of nutrients [41]. They can enhance digestive enzyme activity, improve nutrient absorption, and regulate gastric emptying and intestinal motility [42]. These effects contribute to better digestion and overall gastrointestinal function. Furthermore, consuming this TCM tea can influence urine metabolism and pulse condition by positively impacting the intestinal flora, highlighting the interconnectedness of various bodily processes affected by the tea's properties.

5. Conclusion

In summary, based on the pulse diagnosis of traditional Chinese medicine, we comprehensively evaluated the status of the spleen system, urine metabolites and intestinal flora. After that, the prebiotic properties of total polysaccharides support beneficial gut bacteria, which, in turn, improve digestive health. Additionally, their positive effects on the spleen and stomach further contribute to enhanced digestion and nutrient absorption. Moreover, they promote the smooth operation of the entire qi system, increasing the generation of ATP by mitochondria, and contributing to overall circulation and vitality.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Authors' Contributions

Conceptualization, S.Y. Wang. Methodology, C. H. Lin. and M. R. Xu Investigation, C. H. Lin., P. J. Huang and C. H. Wang Validation, C. H. Lin. Formal analysis, C. H. Lin. Writing original draft, S.Y. Wang Writing review & editing,

Funding, S.Y. Wang.

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Conflicts of Interest

The authors declare that they have no competing interests.

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Supplementary

Table A1. Physical constitution questionnaire.

Physical Constitution Questionnaire：

1. Do you often experience abdominal pain or bloating? If yes, please indicate the frequency (e.g., three times a week, once a day...).
2. Stool consistency (e.g., normal and smooth, loose, slightly hard but smooth, constipated, diarrhea...). Please indicate the frequency (e.g., twice a day, once every three days...) and describe the color based on the Bristol Stool Scale or international color chart.
3. Do you have any edema (swelling) conditions? If yes, please indicate the level according to the following table.

Grade	Depth	Rebound time
1	2 millimeter (mm) depression, or barely visible	immediate
2	3-4 mm depression, or a slight indentation	15 seconds or less
3	5-6 mm depression	10-30 seconds
4	8 mm depression, or a very deep indentation	more than 20 seconds

4. How is your sleep quality? How many hours do you sleep per day? (e.g., vivid dreams, frequent awakenings, able to sleep through the night, difficulty falling asleep). Do you wake up in the morning feeling tired and lacking energy?
5. When you are on an empty stomach, do you frequently experience dizziness, headaches, ringing in the ears, or nasal congestion?
6. Do you often feel muscle weakness or overall physical weakness?
7. Do you frequently experience dry mouth, headaches, or mouth sores?
8. This question is for female respondents only. Do you often have menstrual periods that last more than seven days?

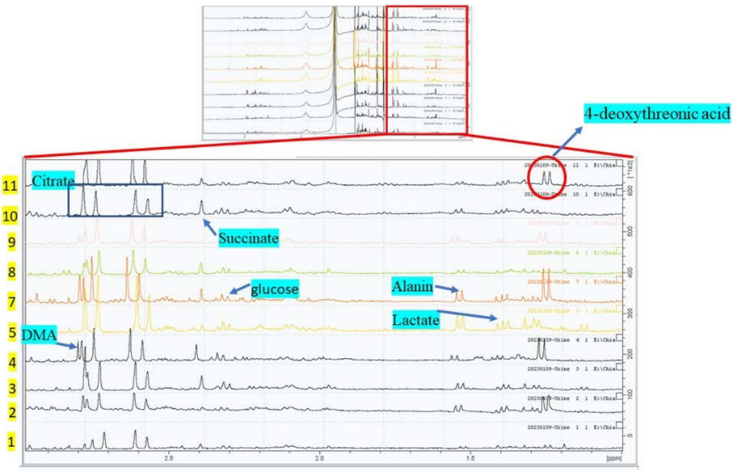
Results of Pulse Diagnosis：

Cun寸 (right hand side)	Guan關 (right hand side)	Chi尺 (right hand side)	Cun寸 (left hand side)	Guan關 (left hand side)	Chi尺 (left hand side)

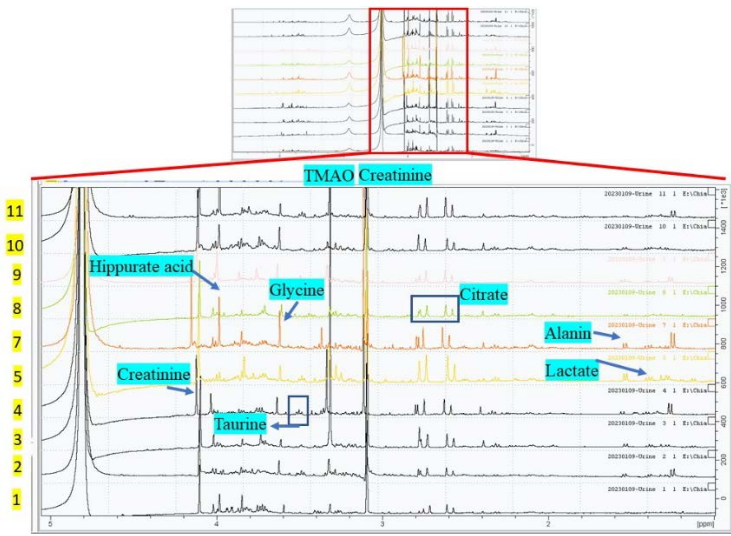
Table A2. Volunteer pulse classification form.

Volunteer number	Cun (right)	Guan (right)	Chi (right)	Cun (left)	Guan (left)	Chi (left)
1	+	−	+	+	+	−
2	−	−	+	+	−	+
3	+	+	−	−	+	+
4	+	−	+	−	+	−
5	+	+	−	+	−	+
6	−	+	−	+	−	+
7	+	−	+	+	−	+
8	+	−	+	+	−	+
9	−	+	+	−	+	+
10	+	−	+	−	+	+
11	+	−	+	+	−	+

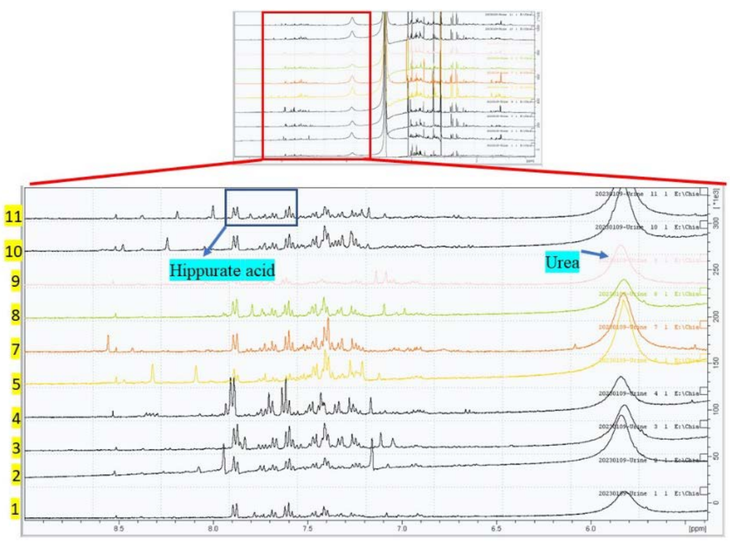
* + indicates a stronger pulse, − indicates a weaker pulse.



(a)



(b)



(c)

Figure A1. The spectrum of urine analyzed by NMR.