**¹H-NMR metabolite profiles and discriminate analysis of two different kinds of freshwater
fish soups before and after in-vitro gastrointestinal digestion**

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Abstract: Soups show diverse health functions, which should be related with their nutrient profiles.

NMR spectroscopy is a robust, sensitive and rapid method to unveil or identify the chemical composition of food or food-derived metabolites. In the current study, ^1H -NMR spectroscopy approach was applied to identify the metabolic profiling of two kinds of home-cooked freshwater fish soups (crucian carp and snakehead fish) before and after *in vitro* gastrointestinal digestion. The nutritional profiles of these two kinds of soups were identified at first. With the help of OPLS-DA method (Orthogonal Partial Least Squares Discriminant Analysis), these two different kinds freshwater fish under different states were completely discriminated. The metabolites changes in digested fish soups could reveal the information of chemical compounds which play important roles in the body. Furthermore, the metabolic patterns of different kinds of fish soups could reflect the various nutrition profiling characteristics for dietary therapy.

Key words: Freshwater fish; Soup; Metabolites; ^1H -NMR; *In vitro* digestion;

1. Introduction

Soup is a very popular diet all over the world, it is suitable for people of all ages [1], and fish is always consumed as one common material of soup. Fish is an important protein rich food source for most countries [2]. The freshwater fish are always cooked into fish soup as a common eating habit, due to its high levels of polyunsaturated fatty acids and easy digestibility property.

Different kinds of fish soups show different dietotherapy functions, which are closely related to their special nutritional components [3]. In China, two representative freshwater fish species (crucian carp and snakehead fish) are frequently cooked into nourishing soup, and the dietotherapy function of them are totally different. The snakehead fish soup is usually used in the adjuvant therapy of people with weak body and poor nutrition, especially for the wound healing and burns healing [4, 5]. It can help muscle growth, blood regeneration, post-operative pain reduction, improving microcirculation, *etc.* With the attractive milky white color and rich nutrients, crucian carp soup (CCS) has function of regulating menstruation and lactogenesis, and it is also suitable for consumption specially for lactating women [6]. The diverse health functions should be related with their different metabolic profiles, which were never investigated before.

Food metabolites profiling generated by metabolomics is the key approach to understand the nutritional and functional characteristics of food materials or commercial products [7, 8]. Metabolomics was considered as one of the most powerful approach for exploring the alterations in metabolite profiles among different samples under different factors. It has provided vital information for assessing food nutrition, food quality, food adulteration [9, 10]. Metabolomic analyses have been generally classified as targeted or untargeted approaches. The targeted analyses were focused on a specific or small group of intended metabolites that in most cases require an

accurate quantification [11, 12], and the untargeted or comprehensive metabolomics was focused on the detection of as many metabolites as possible in order to obtain the patterns or fingerprints without focusing on the specific compounds [13, 14]. The NMR method was proved to be one of the most robust methodologies among various technologies for metabolite profiling, especially for a comprehensive analysis of primary food metabolites.

The advantage of ^1H -NMR metabolomics is that it can unambiguously identify a broad range of standard and unknown compounds without any physical or chemical treatment prior to the analysis [15, 16]. Furthermore, ^1H -NMR spectroscopy combined with pattern recognition and related multivariate statistical methods could offer an efficient way for assessing the metabolic functions [17, 18]. It can identify the significant inherent patterns in a set of indirect measurements with classifying objects combined with pattern recognition methods, such as partial least-squares discriminant analysis (PLS-DA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA) [19, 20]. Approaches of PLS-DA or OPLS-DA could reduce the dimensionality of the complex data sets, facilitate the visualization of inherent patterns among the data set and accelerate the interpretation for various functions.

In current study, metabolomics of two genotypes of freshwater fish soups were explored by an untargeted ^1H -NMR approach. To comprehensively understand the different functions of these two kinds of freshwater fish, the metabolite profiles of different digested state of the fish soups (before and after *in vitro* simulated gastrointestinal digestion) were investigated. Multivariate statistical method - OPLS-DA was applied to identify the inherent patterns within ^1H -NMR spectral data, the screened metabolic patterns potentially ascribe to genotypic diversity and digestion effect, and they could be related with various dietary function. The aim of this work was to establish an effective

metabolomics platform for various fish soups, which may partly explain their diversity dietary therapy.

2. Materials and methods

2.1 Materials

Snakehead fish (*Channa argus*) (~750g) and crucian carp (*Carassius auratus*) (~250g) were purchased from the local market in Huazhong Agricultural University, Hubei, China. Every specimen was gutted and cleaned. All chemicals used in this work were analytical grade.

2.2 Methods

2.2.1 Preparation of fish soup samples

According to the method of Tang [21], the handled fish was cooked at a suitable raw material/water ratio of 1:4 (w/v) adopting stew soup with induction cooker (RT2134, Midea, China) for 1.5h. At the beginning the power was set at 500W to simmer soup within 20 min, and then the power was kept at 300W and the soup were maintained boiling for 70 min. Then the raw soup samples were prepared for ^1H -NMR analysis and further *in vitro* digestion.

2.2.2 In vitro digestion

A two-step process was used to simulate the gastric and intestinal digestion for fish soup using the *in vitro* enzymatic digestion protocol described by Lin et al. [22] with slight modifications. At first, pH of the samples was adjusted to 2.0 with 1 M HCl. Then pepsin was added (4%, w/w, protein basis), and the mixture was incubated at 37 °C for 2 h in a shaking water bath. Subsequently, the pH value was adjusted to 5.3 with 0.9 M NaHCO_3 and further to 7.5 with 1 M NaOH. Then pancreatin was added (5%, w/w, protein basis) and the mixture was further incubated at 37 °C for 2.5 h. To

terminate the digestion, the test tubes were kept in boiling water for 10 min.

2.2.3 Sample Preparation for ^1H -NMR Analysis

To avoid the presence of proteins in the solution (both raw and digested soup), the prepared sample were mixed with 10% (w/w) trichloroacetic acid (Tca) [23]. To be more specific, the samples of crucian carp and snakehead soup (SS) were mixed Tca in a proportion of 1:1 (v:v), respectively. Then the mixture was centrifuged at 12, 000g for 20 min. The supernatants were filtered with 0.45 μm filter paper under vacuum. At the end, there were five different samples in every group, and the filtered solution in every sample were studied in quadruplicate for further analysis. Both raw and digested fish soup samples were stored at -80 °C for ^1H -NMR detection.

2.2.4 Samples preparation for ^1H -NMR spectra acquisition

The frozen fish soup solution (raw and digested) was thawed at first. Then 300 μL sample was diluted with 240 μL phosphate buffer (pH : 7.2; 90 mM Na_2HPO_4 and 35 mM H_2PO_4) and 60 μL 120mg/L 3-(Trimethylsilyl) propionic - 2, 2, 3, 3, d_4 acid sodium salt (TSP, 269913-1G, Sigma-Aldrich) in D_2O , and TSP was set as the internal standard and transferred to a 5 mm diameter tube for ^1H -NMR spectra detection.

2.2.5 ^1H -NMR Spectra Detection

The measurements of the samples were achieved on at 11.75T BrukerAvance III vertical bore NMR spectrometer (600 MHz for ^1H) equipped with an inverse cryogenic probe (BrukerBiospin, Germany), and the detected temperature was kept in 298 K. The NMR detection was completed with a standard WATERGATE pulse sequence [24], which could be used to suppress the water signal. The parameters were set in the following: 90° pulse length, 10.5 ms; number of scans, 128; whole data points, 64K; spectral width, 20ppm. The nutritional components in the NMR spectrum was

identified with the publications [25-28], chemical shifts and 2D NMR spectrum. The 2D-NMR spectrum included COSY (^1H - ^1H correlation spectroscopy), HSQC (^1H - ^{13}C heteronuclear single quantum correlation), HMBC (^1H - ^{13}C heteronuclear multiple bond correlation) and J-resolved (J-resolved spectroscopy).

2.3 Data analysis

2.3.1 NMR Spectra analysis

All the NMR spectral data was analyzed in the commercial software *Topspin* 3.2 (Bruker Biospin, GmbH, Germany) and a home-made software *NMRSpec* in MATLAB (R2014b, Mathworks Inc. 2014) [29].

At first, the experimental window function of all the NMR spectra was employed, and the line broadening factor was set to 1 Hz prior to the Fourier transformation, then phase and baseline correction were manually corrected in *Topspin*. Chemical shift is the most important parameter for a chemical, and it was always infected by various factors, such as instrumental factors, pH value, temperature, salt concentrations, and relative concentrations of specific ions. However, the effect of these factors is not uniform for all the peaks.

To proceed the peak alignment, all the phase and baseline corrected spectra were imported into *NMRSpec* [30]. This tool is free for researchers and it has been successfully analyzed the NMR data [31, 32]. The region with strong solvent signal (4.70 - 5.2ppm) was excluded prior to the further spectral analysis, and the peak alignment was automatically completed. Then continuous even spectral bucket (Size: 0.004 ppm) in all spectra was automatically integrated in *NMRSpec*, and all bucketed spectra data were normalized to the total spectral area for comparing the total

concentration differences.

2.3.2 Multivariate Data Analysis

Multivariate data analysis was conducted on the normalized and bucketed NMR data sets in SIMCA (Version 14, Umetrics, Umea, Sweden). The par scaling method was applied for all multivariate analyses. An un-supervised pattern recognition analysis method - Principle component analysis (PCA) was firstly used to reveal the intrinsic variations in the data set and to diagnose any possible outlier if exists. Then, a supervised orthogonal projection to latent structures discriminate analysis (OPLS-DA) was further applied to capture the discriminating components implied in the different sample groups.

Therefore, OPLS-DA models were calculated for finding variables responsible for discrimination among the following groups: crucian fish soup and snakehead fish soup, crucian fish soup and its digested samples, snakehead fish soup and its digested samples. This simple and robust method has a general applicability for data mining in metabolomics and other similar kinds of data. The quality of the model is defined by total variance of the components at a confidence level of 95%. R2X is represented the goodness of fit of the representative model, and the overall predictive ability of the model was assessed by cumulative Q2, which represents the fraction of the variation of the Y component that can be predicted the internal cross-validation of the model. All models were validated applying CV-ANOVA test within SIMCA at $p < 0.05$.

The significantly varying metabolites were extracted from OPLS-DA correlation coefficient color coded loading plots. The extracted variables were then plotted in standard error bar graphs using their normalized relative intensities and explained as unique features for the respective fish soups before and after gastrointestinal digestion.

3. Results and Discussion

3.1 Signal assignment of ^1H -NMR spectra of the fish soup

The identifications of nutritional components in the samples of fish soup were achieved with the help of publications [25-28], chemical shift and 2D-NMR spectra. For the identification of chemical from 2D-NMR spectra, an example of identification of Taurine from COSY, HMBC, HSQC and J-resolved spectra were collected in Fig. 1. Finally, the whole identified chemicals were also verified with several accessible public databases, such as MMCD (URL: <http://mmcd.nmr.fam.wisc.edu/>) and HMDB (URL: <http://www.hmdb.ca/>). The plausible assignments of the signals in ^1H -NMR were given in Fig. 2. They were include: Valine (Val), ethanol, fatty acids (FA), lactic acid, alanine, eicosapentaenoic fatty acid (ω -3; EPA C20:5), acetic acid, unsaturated fatty acids (UFA), glutamate, succinic acid (SA), creatine/phosphocreatine, anserine (Ans), tyrosine (Tyr), choline (Cho), taurine (Tau), glycine (Gly), glycerophosphorylcholine (GPCho), sucrose (Sue). The variation of the concentration of these metabolites could be linked to the difference of genotypes of fish and the condition of *in vitro* simulated digestion.

Fig. 1. A series of 2D-NMR spectra for identification of metabolite Taurine. (A): COSY; (B): HSQC; (C): HMBC; (D): J-resolved.

Fig. 2 Representative ^1H NMR spectra of fish soup samples. J: Crucian carp soup (CCS); H: Snakehead soup (SS); XJ: Digested crucian carp soup (DCCS); XH: Digested snakehead soup (DSS). Note: 1.Valine (Val); 2. Not identified (NI); 3.Ethanol (Eth); 4.Fatty acids (FA); 5. Lactic acid; 6. Alanine; 7. Eicosapentaenoic fatty acid (ω -3; EPA C20:5); 8. Acetic acid; 9. Unsaturated fatty acids (UFA); 10. Unknown; 11. Docosahexaenoic fatty acid (DHA) 12. Succinic acid (SA); 13. Creatine/phospheratine; 14. Anserine (Ans); 15. Tyrosine (Tyr); 16. Choline (Cho); 17. Taurine (Tau); 18. Glycine (Gly); 19. Sucrose (Sue).

3.2 PCA analysis of all kinds of fish soups

To explore the comparative interpretations and the relationship of different kinds of fish soups, one common pattern recognition method - principal component analysis (PCA) was initially applied to the NMR spectrum to visualize the metabolic discrimination among different freshwater fish soups before and after *in vitro* gastrointestinal digestion. PCA is a classic classification approach requiring no a prior knowledge of the data set and acts to reduce the dimensionality of complicated original data whilst generating information within it [33].

In the current study, there were four different kinds of fish soups involved. To discriminate these different samples, the nutritional components in ^1H -NMR spectra was divided into even gaps. All the integrated gaps were utilized for PCA analysis. The PCA analysis of all the samples were illustrated in Fig. 3, and the first two major components could totally explain 83.0% of all the information inherent in the ^1H -NMR spectra data set (PC1: 68.9; PC2: 14.1%). The quality of the PCA model was described by two statistical parameters $R^2\text{X}(\text{cum})$ and $Q^2(\text{cum})$. Here, $R^2\text{X}$ represents the goodness of fit and Q^2 means the predictability of the PCA model [34]. The parameters of $R^2\text{X}$ and Q^2 were 98.5% and 96%, respectively.

Fig. 3 PCA score scatter plot derived from ^1H -NMR spectra of all kinds of freshwater fish soup samples before and after *in vitro* gastro-intestinal digestion: Class 1: CCS; Class 2: SS; Class 3: DCCS; Class 4: DSS.

All the samples were divided into four different groups, and they were completely separated in the 2D major components space. The results show that the metabolites released in the crucian carp soup and snakehead soup are different to some extent, which means different genotypes of freshwater fish contains various metabolites in their fish soup. At the same time, the characteristics of the metabolites inherent in fish soup have been changed after *in vitro* digestion processing, so they formed two different groups in 2D PC scatter plot. The nutritional components detected with ^1H -NMR spectra are representative and they could be used to assessment of the differences of the nutritional profiles of different kinds of fish soup, even after *in vitro* digestion simulation. Then all of samples were used in the following analysis to screen the most important information.

3.3 Statistical analysis of the whole nutrition components of crucian carp and snakehead soups

Fish soup is one of the most popular dietary in China, due to its delicious taste and natural nutritional materials source. Various fish species always have different functional roles due to their own nutritional profiles. Thus, it was valuable to compare these two kinds of fish soups at first - crucian carp and snakehead soups. A traditional univariate analysis method -two sample *t*-test was utilized to screen out the major differences among these two kinds of fish soups. The result was drawn in Fig. 4. In this figure, there are three different nutritional patterns collected: 1) crucian carp > snakehead soups; 2) crucian carp < snakehead soups; 3) there is no significant difference between these two groups. It was valuable to discuss the major difference between these two kinds of fish soups.

Fig. 4. Two sample *t*-test comparison map of the ^1H -NMR for both crucian carp soup and snakehead soup. *Note: the labels were same as Fig. 1.*

Among these different nutrition components, several metabolites showed very significantly different in various freshwater fish soups, such as Gly, Tau, Ala and Ethanol (Eth) (Fig. 5). Firstly, Eth was only detected in the crucian carp soup. The reason maybe crucian carp as the most known anoxia-tolerant fish, it is easily to produce ethanol serve as the main anaerobic end - product to avoid lactic acidosis during prolonged periods of anoxia [35]. But this phenomenon is not easily occurred in snakehead fish, so the Eth did not detect in the snakehead soup. Furthermore, crucian carp soup has higher concentrations of Gly and Tau (Fig. 5A and 5C), even twice amounts of the

snakehead soup. As well know, glycine always regard as an ideal seasoning ingredients especially for the seafood [36], that is why crucian carp soup taste delicious than snakehead soup. Taurine is a semi-essential amino acid that is not incorporated into protein, but it has many diverse physiological effects including osmoregulation, bile salt conjugation, membrane stabilization, calcium modulation, anti-oxidation, and immune stimulation [37]. Furthermore, Taurine level in the liver of female rats turn to very high during pregnancy and lactation [38], that maybe partial interpretation of crucian carp soup is a good diet resource for lactating postpartum women to promoting milk secretion. At the end, snakehead soup has higher concentration of Ala and Lac (Fig. 5B). The concentration of Lac is related with anaerobic oxidation of glucose after the animal dead, thus it could not provide any useful information for the nutrition effect. Alanine is an intrinsic α -helix stabilizing amino acid which can produce glucose in the liver and play a crucial role in glucose-alanine cycle [39], it is benefit to improve body energy.

Fig. 5. The ¹H-NMR spectra of selected significant different metabolites between crucian carp soup and snakehead soup. (A): Gly; (B): Ala; (C): Tau. Note: Blue background, crucian carp soup (CCS); Red background: snakehead soup (SS).

3.4 OPLS-DA analysis for all kinds of fish soups

In order to improve the separation among different freshwater fish soup samples based on maximizing covariance between the measured data (X) and the response variables (Y), OPLS-DA models were also built by SIMCA. The identity of each group of samples is specified, therefore the maximum variance of the groups can be obtained in the multidimensional space. OPLS-DA model applied to visualize the metabolic difference were illustrated in Fig. S1, good separation in the scores plot of PC1 and PC2 of OPLS-DA analysis was obtain between crucian carp soup and snakehead soup before and after *in vitro* simulated gastrointestinal digestion. Moreover, OPLS-DA model with significantly high parameters R^2X , R^2Y , and Q^2 values of 0.932, 0.982 and 0.98, respectively, which indicate that it owns a satisfactory predictive ability.

3.5 Metabolic profiling comparison of different type of freshwater fish soups

To improve the separation among different freshwater fish soup samples based on maximizing covariance between the measured data (X) and the response variable (Y), the loading plot of OPLS-DA model was also utilized for the discriminate of these two kinds of fish soups. The identity of each group of samples is specified, therefore the maximum variance of the groups can be obtained in the multidimensional space. OPLS-DA model applied to visualize the metabolic difference as shown in Fig. 6A and Fig. 6a, good separation in the scores plot of PC1 and PC2 of OPLS-DA analysis was obtain between crucian carp soup and snakehead soup before and after *in vitro* simulated gastrointestinal digestion (Fig. 6A). Moreover, OPLS-DA model with significantly high parameters R^2X , R^2Y , and Q^2 values of 0.840, 0.965 and 0.963, respectively, which indicate that it owns a satisfactory predictive ability. The differences of metabolic profiling among various fish

soup samples are important for key metabolites identification. The color scale corresponded to the NMR model variable weights (Fig. 6a). The relative changes of metabolites with significant correlation coefficients were a major discrimination. Positive and negative peaks indicate a relatively decreased and increased metabolite level in the control groups. Five metabolites, Valine, Tyrosine, Choline, Taurine and Glycine were much higher in the CCS group, whereas higher levels of fatty acids and unsaturated in the SS group which is similar with the univariate analysis.

Fig. 6 OPLS-DA analysis of the NMR spectrum obtain from different kinds of fish soups. Note: A and a: crucian carp soup (CCS) and snakehead soup (SS); B and b: CCS groups under before vs after *in vitro* simulated gastro-intestinal digestion; C and c: SS groups under before vs after *in vitro* simulated gastro-intestinal digestion. Note: Capital(A, B, C): Scores plot from OPLS-DA analysis; Lowercase (a, b, c): Corresponding PC1 loading plot, and the color bar corresponds to the weight of the corresponding variable in the discrimination of statistically significant (red) or no statistically significant (blue). Positive and negative peaks indicate a relatively decreased and increased metabolite level of the first group.

3.6 Influence of the digestion on the nutrition components of fish soups

To explore the influence of simulated gastrointestinal digestion on the nutrition components of fish soups, OPLS-DA approach was also applied to distinguish the metabolites difference between CCS and SS before and after digestion processing. The OPLS-DA model of different states of CCS samples was illustrated in Fig. 6B and 6b, it was established using one predictive and one orthogonal component in Fig. 6B, and these two groups was completely separated. The parameters of OPLS-DA model were in the following : $R^2X = 0.967$, $R^2Y = 0.989$, $Q^2 = 0.988$, which mean they show good stability and predictability. There are much more difference among these two kinds of samples. It is clearly to see that the positive signals indicated the higher levels metabolites in the digested crucian carp soup in comparison with crucian carp soup, including Val, Leucine, EPAC20:5, Acetate, Tau, Gly, GPCho and Creatine. (Fig. 6b).

At the same time, OPLS-DA loading plot was also applied to discriminate the different state of snakehead soup. Similar with the CCS group, these two groups were also completely separated with one predictive and one orthogonal component (Fig. 6C and 6c). The OPLS-DA classification model showed good stability and predictability with parameters of $R^2X = 0.915$, $R^2Y = 0.986$, $Q^2 = 0.985$ (Fig. 6c). Similar with the digested CCS group, most of nutrition components were also increased, such as Val, Leucine, EPA C20:5, Acetate, Tau, GPCho and Creatine. However, there also have some metabolites decreased after digestion, such as Tyrosine and lactate.

4. Conclusion

In present study, the metabolomics approach based on $^1\text{H-NMR}$ spectra was applied to analyze the nutritional characteristics of two kinds of freshwater fish soups before and after *in vitro*

simulated digestion. With the help of OPLS-DA methods, different groups of samples were completely discriminated. To our knowledge, this is the first study using ^1H -NMR based metabolomics to explore the characteristics of nutrition profiling of different kinds of freshwater fish soups and the state of *in vitro* digestion simulation. The metabolites changes in digested fish soups could reveal the information of chemical compounds which play important roles in the body. Furthermore, the metabolic patterns of different kinds of fish soups could reflect the various nutrition profiling characteristics for dietary therapy.

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Figure legends:

Fig. 1. A series of 2D-NMR spectra for identification of metabolite Taurine. (A): COSY; (B): HSQC; (C): HMBC; (D): J-resolved.

Fig. 2 Representative ^1H NMR spectra of fish soup samples. J: Crucian carp soup (CCS); H: Snakehead soup (SS); XJ: Digested crucian carp soup (DCCS); XH: Digested snakehead soup (DSS). *Note: 1. Valine (Val); 2. Not identified (NI); 3. Ethanol (Eth); 4. Fatty acids (FA); 5. Lactic acid; 6. Alanine; 7. Eicosapentaenoic fatty acid (ω -3; EPA C20:5); 8. Acetic acid; 9. Unsaturated fatty acids (UFA); 10. Unknown; 11. Docosahexaenoic fatty acid (DHA) 12. Succinic acid (SA); 13. Creatine/phosphatine; 14. Anserine (Ans); 15. Tyrosine (Tyr); 16. Choline (Cho); 17. Taurine (Tau); 18. Glycine (Gly); 19. Sucrose (Sue).*

Fig. 3 PCA score scatter plot derived from ^1H -NMR spectra of all kinds of freshwater fish soup samples before and after *in vitro* gastro-intestinal digestion: Class 1: CCS; Class 2: SS; Class 3: DCCS; Class 4: DSS.

Fig. 4. Two sample *t*-test comparison map of the ^1H -NMR for both crucian carp soup and snakehead soup. *Note: the labels were same as Fig. 1.*

Fig. 5. The ^1H -NMR spectra of selected significant different metabolites between crucian carp soup and snakehead soup. (A): Gly; (B): Ala; (C): Tau. *Note: Blue background, crucian carp soup (CCS); Red background: snakehead soup (SS);*

Fig. 6 OPLS-DA analysis of the NMR spectrum obtain from different kinds of fish soups. *Note: A and a: crucian carp soup (CCS) and snakehead soup (SS); B and b: CCS groups under before vs after in vitro simulated gastro-intestinal digestion; C and c: SS groups under before vs after in vitro simulated gastro-intestinal digestion. Note: Capital (A, B, C): Scores plot from OPLS-DA analysis; Lowercase (a, b, c): Corresponding PC1 loading plot, and the color bar corresponds to the weight of the corresponding variable in the discrimination of statistically significant (red) or no statistically significant (blue). Positive and negative peaks indicate a relatively decreased and increased metabolite level of the first group.*