Metabonomics Study on the Effect of Traditional Chinese Medicines Feed Addition on Growth Performance and Serum Metabolic Profile of Juvenile Chinese Softshell Turtle (Pelodiscus Sinensis Wiegmann) using UPLC–Triple/TOF–MS Analysis

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Abstract

Background: Traditional Chinese medicines (TCMs) had been proven to stimulate digestion, promote growth, boost immune response, and reduce inflammatory potential in aquaculture, but its action mechanism was still lack of holistic interpretation.

Results: The UPLC-Triple-TOF-MS/MS based platform was developed to investigate the serum metabolic profile associated with the growth performance and immunity in Pelodiscus sinensis fed with the diet added with TCMs feeding additive. The final weight and specific growth rate of TG2 groups was significantly the highest (p < 0.05), and the feed conversion ratio of TG2 groups was significantly the lowest (p < 0.05) compared to the CG group. In the metabolomics assay, a total of 795 (VIP > 1) out of 3089 variable ions were significantly different between the TG2 group and CG group. 43 potential biomarkers between TG2 and CG groups were finally screened out on the basis of VIP > 1 and p < 0.05. Based on the analysis of MetPA, eleven potential biomarkers and six involved metabolic pathways were significantly regulated with treatment of TCMs. Phenylalanine, tyrosine and tryptophan biosynthesis and Arginine and proline metabolism were the two most heavily affected pathways (Impact >0.2), while Tyrosine metabolism, Cysteine and methionine metabolism and Arginine biosynthesis were relatively lightly affected pathways.

Conclusions: The dietary supplementation nineteen TCMs as feed additives could influence the growth performance of the healthy juvenile P. sinensis, and the optimal dose of TCMs for juvenile turtles was 2% TCMs. The UPLC-Triple-TOF-MS based metabolomics may be a useful tool for elucidating the efficacy and mechanism of complex TCM prescriptions.

Background

The Chinese softshell turtle (Pelodiscus sinensis Wiegrmamn) is an ancient, subaquatic reptile, which has high edible and pharmaceutical values in China and other Asian countries, such as Korea, Japan and Vietnam [1-2]. Due to its high economic value, the turtle has become a commercially important aquaculture species in China [3], and more than 340,000 tons of the turtles per year were produced in Chinese turtle farms [4]. Similar to other aquaculture species, the P. sinensis has suffered from parasitic, bacterial and viral diseases attacks due to the increase in stocking density that threaten the sustainable development of aquaculture of this species [5]. To control disease outbreaks, traditional chemical and antibiotic drugs were used indiscriminately, which are easy to cause residual problems in the surrounding environment affecting higher animals [6]. Traditional Chinese medicines (TCM) are cheaper source for therapeutics, have greater accuracy than chemotherapeutic agents, and offer a viable solution for all problems which aquaculture faces today [7].

TCM is gaining more attention all over the world, due to the reliable therapeutic efficacy [8]. Compared with inorganic chemicals or synthetic antibiotics, TCM had been proven to be less toxic, residue free, natural, antibacterial, immune modulators, antioxidant, etc., and is thought to be ideal growth promoters in animal feeding [9-10]. A lot of TCMs have already been reported to stimulate digestion, promote growth, boost immune response, provide antioxidant and antimicrobial properties, and reduce inflammatory potential in pigs, chickens, fish and other animals [11-14]. As a natural ‘green’ feed additive, TCMs are increasingly used in aquaculture [15-16]. Herbal medicine is considered one of the best ways to boost the fish's growth, immunity and could be suitable alternatives to antibiotics [17-19]. The healthy Trionyx sinensis were randomly divided into five groups, and were fed with basal diet added Astragalus polysaccharides (APS) at the levels of 0.00, 0.25, 0.50, 0.75 and 1.00% for 40 days. The results showed that the specific growth rate, the weight gain rate and the survival rate of Trionyx sinensis fed with the diet added with APS were significantly increased (P<0.05) [20]. Internal environment of metabolism of TCM was a dynamic process, which accorded with the dynamics, integrity and systemic characteristic of metabolonic [21].

Metabolomics is an important part of systemic biology. Metabonomics is concerned with the quantitative understandings of the metabolite component of integrated living systems and its dynamic responses to the changes of both endogenous and exogenous factors [22-23]. As a powerful analytical platform, metabonomics was a useful tool for elucidating the efficacy of TCMs, and exploring its potential mechanisms and identify potential biomarkers [21-22]. Many analytical tools have currently been employed including direct GC–MS, HPLC–MS and 1H NMR spectroscopy [21-24]. To the best of our current knowledge, using UPLC-Triple-TOF-MS/MS to research the mechanism of TCMs feed additives on the growth performance and immunity of P. sinensis can offer more useful information for elucidating the efficacy and mechanism of complex TCM prescriptions. Therefore, in this study, we studied the effect of supplementation with nineteen TCMs as feed additives on the growth performance of the healthy juvenile P. sinensis and involved possible mechanism by the UPLC-Triple-TOF-MS/MS.

Materials And Methods

Investigations and protocols were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with Anhui Science and Technology University Institute of Animal Care and Use Committee. The institutional review board approved this procedure. Our study had been submitted to and approved by the Academic Ethics Committee of Anhui Science and Technology University. All sample collection was undertaken in accordance with relevant Academic Ethics Committee of Anhui Science and Technology University guidelines and regulations.

Fish housing and feeding

Nineteen TCMs were purchased from Bozhou Qingyi Chinese Medicinal Materials Technology Co., Ltd. (Bozhou, China). The TCMs combined with Glycyrrhiza uralensis, Angelica sinensis, Wolfiporia cocos, Cynanchum otophyllum, Atractylodes macrocephala, Bupleurum chinense, Pericarpium Citri Reticulatae, Saposhnikovia divaricata, Astragalus membranaceus, Lonicerà japonica, Schisandra chinensis, Crataegus pinnatifida, Dendranthema morifolium, Codonopsis pilosula, Ophiopogon japonicus, Massa Medicata, Hordeum vulgare, Eucommia ulmoides, Cinnamomum cassia. Each herb was crushed by DFY-400 high speed disintegrator (Shanghai Bilang Instrument Co., Ltd., Shanghai, China), and then screened by a standard sieve (100 mesh), the powder was called fine powder. Equal amounts of all Nineteen CHMs fine powders were mixed together as CHMs feeding additive and stored at ~20°C for later use.
The healthy juvenile *P. sinensis* were produced by the Anhui Huanghuai Turtle Breeding Co., Ltd. (Bengbu, China). Three hundred the turtles (with 3.92±0.08 g initial mean body weight) were acclimated to laboratory conditions for 7 days before distributed randomly into five groups. Fifteen turtles per plastic tank (75 cm × 50 cm × 55 cm, water volume: 75 L) were randomly assigned to one of five experimental groups, in quadruplicate. The 5 treatment groups were as follows: the control group (CG), in which the turtles were received the basal diet. The TCMs treatment groups (TG1, TG2, TG3 and TG4), the basal diet was supplemented with 1.0%, 2.0%, 3.0%, 4.0% of TCMs feeding additive, respectively. During the experiments, water temperature was controlled at 30 ± 1°C using a thermostat, water depth at 25 - 30 cm, dissolved oxygen at 7.0 - 9.0 mg/L (water were aerated continuously using air compressor), pH at 7.5 - 8.5, ammoniacal nitrogen at 0.2 mg/L. Approximately 30% of the water volume in each tank was renewed with fresh water (30 ± 1°C) every 4 days. The surface water and bottom of the tank were cleaned.

The turtles were fed with a formulated diets manufactured by Haining Hexin Feed Co., Ltd. (Haining, China). The basic experimental diets contained approximately 48% crude protein, 3% crude lipid, 1.2% crude fiber, 9 % moisture, 16 % ash, 2 % salinity, 5 % Calcium, 3 % gross phosphorus. The basic experimental diets supplemented with 0.0%, 1.0%, 2.0%, 3.0%, 4.0% of TCMs feeding additive, respectively. The basic experimental diets and TCMs feeding additive were mixed homogeneously in a helical mixer, and were stored in a -20 °C freezer. A portion of trial diets were periodically transferred to room temperature for 4 h before feeding, and then mixed with ultrapure water and make it suitable for cold-pressure extrusion. The 3 mm pellets were molded, and then were used to feed the turtles [3]. The turtles were fed at 3-6% of their body weight per day in two equal meals (09:00 and 17:30). The experimental diets were maintained on for 7 weeks, and final body weight of the experimental turtles was measured to the nearest 0.01 g using a digital scale. The half of the juvenile *P. sinensis* of each experimental group was euthanized by decapitation and the blood was sampled as described by Zhou et al [3]. Serum was separated described by Zhou et al., and then stored at -80 °C for later analysis[3].

### UPLC-MS/MS analysis

Twenty serum samples (4 samples per group) were slowly thawed at room temperature for calibration curves and quality control (QC) samples. As a part of the system conditioning and quality control process, a pooled quality control sample (QC) was prepared by mixing equal volumes of all samples. Metabolites were profiled using a UPLC-Triple-TOF-MS-based platform. Chromatographic separation of the metabolites was performed on a ExionLC™AD system (AB Sciex, USA) equipped with an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm; Waters, Milford, USA). The sample injection volume was 20 µL and the flow rate was set to 0.4 mL/min. The column temperature was maintained at 40°C. During the period of analysis, all these samples were stored at 4°C. The UPLC system was coupled to a quadrupole-time-of-flight mass spectrometer (Triple TOF™5600+, AB Sciex, USA) equipped with an electrospray ionization (ESI) source operating in positive mode and negative mode. Data acquisition was performed with the Data Dependent Acquisition (DDA) mode. The detection was carried out over a mass range of 50-1000 m/z.

### Data preprocessing and annotation

After UPLC-TOF/MS analyses, the raw data were imported into the Progenesis QI 2.3 (Nonlinear Dynamics, Waters, USA) for peak detection and alignment. The preprocessing results generated a data matrix that consisted of the retention time (RT), mass-to-charge ratio (m/z) values, and peak intensity. Mass spectra of these metabolic features were identified by using the accurate mass, MS/MS fragments spectra and isostructure ratio difference with searching in reliable biochemical databases as Human metabolome database and Metlin database. Furthermore, metabolic features detected at least 80 % in any set of samples were retained. After filtering, following normalization procedures and imputation, statistical analysis was performed on log transformed data to identify significant differences in metabolite levels between comparable groups.

### Multivariate statistical, differential metabolites analysis and Network Construction

Data were expressed as mean ± S.D, and then analyzed using single-factor (ANOVA) to assess the effect of TCMs supplementation on the growth responses and serum metabolomics of *P. sinensis*. The significance of differences between the experimental groups has been compared by Duncan's multiple range tests using the software SPSS 22.0, and *P* < 0.05 was considered significant. A multivariate statistical analysis was performed using ropls (Version1.6.2) R package from Bioconductor on Majorbio Cloud Platform (https://cloud.majorbio.com). Principle component analysis (PCA) using an unsupervised method was applied to obtain an overview of the metabolic data, general clustering, trends, or outliers were visualized. All of the metabolite variables were scaled to unit-variances prior to conducting the PCA. Orthogonal partial least squares discriminate analysis (OPLS-DA) was used for statistical analysis to determine global metabolic changes between comparable groups. All of the metabolite variables were scaled to Pareto Scaling prior to conducting the OPLS-DA. Variable importance in the projection (VIP) was calculated in OPLS-DA model.

Statistically significant among groups were selected with VIP value more than 1 and p value less than 0.05. Differential metabolites among two groups were summarized, and mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on database search (KEGG, http://www. genome.jp/kegg/). These metabolites can be classified according to the pathways they involved or the functions they performed. Enrichment analysis was usually to analyze a group of metabolites in a function node whether appears or not. The principle was that the annotation analysis of a single metabolite develops into an annotation analysis of a group of metabolites. Scipy.stats (Python packages) was exploited to identify statistically significantly enriched pathway using Fisher's exact test. The identified biomarkers were subsequently confirmed by the p-values with a critical value of 0.05 from a Student’s t-test. Receiver operating characteristics (ROC) curves were plotted and areas under the curve (AUC) were calculated to evaluate the accuracy of the metabolic biomarkers in distinguishing different groups. The sensitivity and specificity of the trade-offs were calculated for the selected metabolites by using the area under the ROC curve (AUC) [25]. The construction, interaction and pathway analysis of these identified potential biomarkers were performed with MetPA [26]. In addition, the network of potential biomarkers-metabolic pathways-targets was constructed, and the corresponding network analysis was conducted using Cytoscape 3.5.1 software (http://www.cytoscape.org/).
Results

Growth performance

The results of the TCMs feed additives on the growth performance indices of *Pelodiscus sinensis* is presented in Table 1. The survival rates of the TG1, TG2, TG3, TG4 and control groups were 93.333%, 94.998%, 91.665%, 88.333% and 90.000%, respectively. The survival rate of TG2 group was the highest but no significant difference from other treatments. Compared with the CG group, the TG2 group supplemented with 2% TCMs feed addition showed the highest final weight and specific growth rate (SGR) (p<0.05), the TG1 group supplemented with 1% TCMs feed addition gave higher final weight and SGR (P<0.05), the TG3 group supplemented with 3% TCMs feed addition increased final weight and SGR, whereas TG4 group supplemented with 4% TCMs feed addition showed lower final weight and SGR (P<0.05). The TG1, TG2 and TG3 groups showed significantly (p<0.05) lower feed conversion ratio (FCR) compared to the CG group (Table 1).

Method validation of LC–MS analysis and potential biomarkers responsible

In total, 3089 variables (1845 peaks in ESI+ mode, and 1244 peaks in ESI- mode) were identified in serum samples from CG, TG1, TG2, TG3, and TG4 groups for subsequent analyses (Supplement Table S1). Due to individual differences, it was difficult to directly observe the changes of serum metabolites in the five groups. Thus, we performed multivariate data analysis to determine the metabolic markers in *P. sinensis*. The PCA performed on the whole samples revealed that the QC samples were tightly clustered in PCA score plots (shown in Figure S1), which indicated that the system stability was accommodate for this metabonomic study. Figure 1 (A and B) showed PCA scores plot of serum, but the discriminations of five groups were not very distinct. In positive ion mode of UPLC-Triple-TOF-MS/MS analysis (Figure 1A), the metabolic profile of the TG2 group deviated away from the control group along both PC1 and PC2, which suggested that apparent biochemical changes were induced by TG2 treatment. The TG4 group was far away from the TG2 group, and much closer to the control group (Figure 1), indicating that the metabolic disturbances induced by TG2 were significantly improved with TG4 treatment. Similar results were observed when TG1 and TG3 were subtracted (TG1 and TG3 groups), except that the metabolic profile of *P. sinensis* in the TG4 group was much closer to the control group than that in the TG1 and TG3 groups. Thus, the OPLS-DA model was established to maximize the distinction between groups (Figure 2 A, B). Meanwhile, variable importance in the projection (VIP) was used to identify the potential biomarkers obtained from five groups.

The VIP values were larger than 1 and the p values of an independent test < 0.05 as the potential biomarkers. According to the results of OPLS-DA, a total of 795 (VIP > 1) out of 3089 variable ions were significantly different between the TG2 group and CG group. 43 potential biomarkers between TG2 and CG groups were finally screened out on the basis of VIP > 1 and p < 0.05 (Table 2). As shown in Table 2, compared with the control group, twenty seven metabolites were up-regulated, and sixteen metabolites were down-regulated by TCMs stimulus. Further, hierarchical cluster analysis of resulting 30 identified metabolites revealed the existence of distinct differences between the control and TG2 group (Figure 3). In this study, we tentatively used the term "potential biomarkers" to acknowledge their potential value and simultaneously to indicate its uncertainty.

Metabolic Pathways, biological pathway and network analysis

The 43 identified potential biomarkers were further confirmed by the univariate ROC curve analyses (AUC > 0.8) and Student's t-tests (p < 0.05). Nineteen potential biomarkers were ultimately considered to exhibit the greatest sufficient utility for discrimination of the TG2 and CG groups (Figure S2). To further investigate the recovery condition of the 19 potential biomarkers, the relative peak areas between the four TCM-dosed groups and the control group were tested based on univariate ROC curve analyses and Student's t-tests (Table S2). For TG1 group, only 4 biomarkers were increased, 3 biomarkers were decreased comparing with the TG2 group. Based on the 19 identified potential biomarkers between TG3 and TG2 groups, the level of Salicylaldehyde and 2-(5-Methyl-2-furanyl)-3-piperidinol were improved, while the level of Ampeloside Bf2, Benzofuran and 2-Hydroxycinnamic acid were decreased. However, for the TG4 group, only one biomarker was decreased comparing with TG2 group. Similarly, fifty of the total metabolites were identified when compared with the metabolomes among the CG, TG1, TG2, TG3 and TG4 groups, as shown in the Heatmap visualization (Figure 4).

Based on the 43 identified potential biomarkers between TG2 and CG groups, the relevant metabolic pathways were assigned using MetaboAnalyst 4.0. Based on the analysis of MetPA, 43 potential metabolites were involved into thirteen different metabolic pathways, including Aminoacyl-tRNA biosynthesis (L-Methionine, L-Proline, L-Tyrosine), Phenylalanine, tyrosine and tryptophan biosynthesis (L-Tyrosine), Arginine and proline metabolism (Ornithine, L-Proline), Phenylalanine metabolism (2-Hydroxycinnamic acid, L-Tyrosine), Ubiquinone and other terpenoid-quinone biosynthesis (L-Tyrosine), Arginine biosynthesis (Ornithine), Sphingolipid metabolism (SM(d18:0/16:1(9Z)(OH))), Glutathione metabolism (Ornithine), Cysteine and methionine metabolism (L-Methionine), Glycero-phospholipid metabolism (LysoPC(22:1(13Z))), Pyrimidine metabolism (5-Methylcytosine, Deoxycytidine), Tyrosine metabolism (L-Tyrosine), Purine metabolism (Adenosine 3'-monophosphate, Xanthosine) (Table 3). Five different metabolic pathways with Impact >0.05, including Phenylalanine, tyrosine and tryptophan biosynthesis, Arginine and proline metabolism, Tyrosine metabolism, Cysteine and methionine metabolism, and Arginine biosynthesis (Table 3). Phenylalanine, tyrosine and tryptophan biosynthesis, Arginine and proline metabolism, Tyrosine metabolism, Cysteine and methionine metabolism, and Arginine biosynthesis were considered as the most relevant compounds involved in TCMs, which could be serve as potential biomarkers of growth promotion. Taking the different metabolites and metabolic pathways into consideration, we found that TG2 group supplemented with 2% TCMs
increased mainly the amino acid contents, whereas TG4 group added to 4% TCMs reduced mainly the contents of amino acid and fatty acids in P. sinensis. Overall the results suggest that TCMs could improve protein synthesis and regulate immune function, and the most effective concentration was 2%.

**Discussion**

**Effects of dietary TCMs on growth of P. sinensis**

Feed additives were products used in animal nutrition for purposes of getting better the quality of feed and the quality of food from animal origin, or to improve the animals' performance and health [10]. In aquaculture survival and growth rate of the selected species play vital role in maintaining a commercially viable farm [27]. In recent years, TCMs were generally considered safe and natural "green" feed additive, which were increasingly used in aquaculture [15-16]. Applications of compound formulations in aquaculture were believed to be more acceptable and beneficial as different herbs with diverse mechanisms showed complementary effects between herbs in the formulation [10,12]. TCMs are added in fish feed to improve feed conversion efficiency that result in fish growth [28]. Many studies showed that inclusion of herbs in fish diet has positive effect on growth and disease free fishes [29-30]. The aim of this study was to elucidate the effect of supplementation with nineteen TCMs as feed additives on the growth performance of the healthy juvenile P. sinensis. The final weight and SGR of TG1 and TG2 groups were significantly higher (p<0.05), and the FRC of TG1 and TG2 groups were significantly lower (p<0.05) compared to the CG group. However, the final weight and SGR of TG4 group supplemented with 4% TCMs feed addition were the lowest (P<0.05), and the FRC of TG4 group was the highest compared to the CG group (P<0.05) (Table 1). The present results suggested that supplementation of 1% - 3% TCMs feed additives could improve feed utilization rate and promote the growth of juvenile P. sinensis, and the optimal dose of TCMs for juvenile P. sinensis was 2% TCMs feed additives, which might be related to the wide variety of active components in TCMs. The naturally occurring components of TCM and/or their degradation products in the gastrointestinal tract might be metabolized by metabolic enzymes in the intestinal epithelium, and/or influence their activities. After the TCM components were absorbed into the bloodstream through the intestinal epithelium, they were first delivered to the liver via the portal vein [31]. Most of the herbs and spices could stimulate the function of pancreatic enzymes, some also increase the activity of digestive enzymes of gastric mucosa, and enhance gastrointestinal digestion and absorption capacity [9]. Thus, our results were consistent with the previous literature reports [20,32-34].

**Identification of potential biomarkers associated with TCMs feeding additive**

Metabolomics is one of the high-throughput "omics" techniques, which beside genomics, proteomics, and transcriptomics play an important role in systems biology [35]. Metabolomics is defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification [36], which has been proposed as a powerful tool for exploring the global metabolic state of the entire organisms, and is coincident with the integrity and systemic features of TCM [22]. Therefore, in this study, the integrated metabolic approach was applied to elucidating the effect of supplementation with TCMs as feed additives on the metabolomics of the healthy juvenile P. sinensis. 43 potential biomarkers between TG2 and CG groups were finally screened out on the basis of VIP > 1 and p < 0.05 (Table 2), twenty seven metabolites were up-regulated, and sixteen metabolites were down-regulated by TCMs stimulus, which suggested TCMs could effectively regulate the metabolomics of the healthy juvenile P. sinensis supplemented with 2% TCMs feed addition, and improved the growth performance of P. sinensis. Thirteen pathways related to the metabolites of TG2 group compared with the control group were shown by the analysis of MetPA (Figure 6 and Table 3), which were mainly involved in amino acid metabolism (Aminoacyl-tRNA biosynthesis, Phenylalanine, tyrosine and tryptophan biosynthesis, Ubiquinone and other terpenoid-quinone biosynthesis, Arginine biosynthesis, Glutathione metabolism, Cysteine and methionine metabolism, Tyrosine metabolism, Phenylalanine metabolism), nucleotide metabolism (Purine metabolism, Pyrimidine metabolism) and lipid metabolism (Sphingolipid metabolism, Glycerophospholipid metabolism). Among them, eleven potential biomarkers, including L-Methionine, L-Proline, L-Tyrosine, Omithine, 2-Hydroxycinnamic acid, SM(d18:0/16:1(9Z)(OH)), LysoPC(22:1(13Z)), 5-Methyltetrahydrofolate, Deoxycytidine, Adenosine 3’-monophosphate and Xanthosine were screened to characterize the potential biomarkers of TCMs. Among thirteen metabolic pathways, Phenylalanine, tyrosine and tryptophan biosynthesis with the impact-value 0.5, Arginine and proline metabolism with the impact-value 0.2039, Tyrosine metabolism with the impact-value 0.1397, Cysteine and methionine metabolism with the impact-value 0.1045 were filtered out as the most heavily affected metabolic pathways, because the pathway with the impact-value threshold above 0.10 was regarded as potential target pathway involved in the growth promoting agents of TCMs [37].

**Phenylalanine, tyrosine and tryptophan biosynthesis**

Amino acids play important roles in many metabolic pathways as main substrates and as regulators [38]. In the current study, Figure 6 shows the network of the potential biomarkers changing for TG2 group according to the KEGG Pathway database, and peptides were found to be the main contents associated with TCMs, which mainly involved in five important metabolism pathways (Figure 5 and Figure 6). In TG2 group, improved levels of amino acids (L-Methionine, L-Proline, L-Tyrosine and Omithine) suggested a high demand and rapid utilization of metabolites to protein producing pathways (Figure 6), which may be relate to TCMs formulation contained different active ingredients that may have a synergistic effect on growth, combating viral infection, appetite stimulation, and stress relief [12]. Phenylalanine and tryptophan are two essential amino acids which cannot be synthesized in vivo and can be obtained only from the daily foods, they are the precursors of tyrosine and serotonin, respectively [38]. Phenylalanine which is one of the essential aromatic amino acids, under normal circumstances, can be used as amino acids to produce various proteins in cells of the body tissue. The steady state of phenylalanine metabolism can maintain the body's normal growth, development and physiology [39]. Tyrosine is an aromatic amino acid that serves as a precursor for a variety of biologically important substances; e.g., melanin pigments, catecholamines, thyroid hormones, and protein [40]. Tyrosine production via phenylalanine hydroxylation by phenylalanine hydroxylase is a major metabolic pathway for phenylalanine. Phenylalanine and its metabolite tyrosine, as catecholamine precursors, participate in dopamine synthesis and are associated with excessive stimulation of the sympathetic nervous system [41]. A significant fate of tyrosine is conversion into catecholamine, e.g., dopamine, norepinephrine and epinephrine [42]. Based on the present study, L-Tyrosine, Omithine and L-Proline were all up-regulated prominently in P. sinensis supplemented with 2% TCMs feed addition. In the role of L-tyrosine, L-phenylalanine, glutathione and coenzyme could produce fumaric acid, which was involved in the TCA, and finally promoted the metabolism of hemoglobin and heme [22]. The up-regulated
trend of L-tyrosine in TG2 group could be indicative for the improvement in phenylalanine metabolism which is associated with the phenylalanine 4-hydroxylase, a responsible enzyme for phenylalanine conversion into tyrosine [43]. The content of L-tyrosine was increased in the TG2 group compared with CG group, which suggested that the synthesis of glutathione was improved because of the inhibition of glucose-6-phosphate dehydrogenase activity after P. sinensis supplemented with 2% TCMs feed addition [22], therefore, TCMs could contribute to increasing the metabolism of hemoglobin.

Arginine and proline metabolism

Arginine and proline are two important fatty acid amides (FAAs) that play important roles in osmotic stress [44]. It has been reported the arginine and proline metabolism was closely related to the progression of oxidative stress [45]. Arginine could exert its potential protection from the gastric mucosal damage through inhibition of oxidative stress derived via xanthine-XO [46]. Arginine is synthesized from citrulline through the sequential action of the cytosolic enzymes argininosuccinate synthetase (EC 6.3.4.5, ASS) and argininosuccinate lyase (EC.4.3.2.1, ASL) [44]. Ornithine is an amino acid that may be generated from glutamic acid or produced in the urea cycle by the hydrolysis of urea from arginine [47]. Glutamic acid is one of the 20 proteinogenic amino acids and a key molecule in cellular metabolism. Meanwhile, proline and citrulline, two metabolites of arginine, were also involved into the progression. In animals, intracellular proline levels are mainly controlled via biosynthetic and catabolic pathways [44,48]. Proline, the only proteinogenic secondary amino acid, is metabolized by its own family of enzymes responding to metabolic stress and participating in metabolic signaling. Metabolism of proline generates electrons to produce ROS and initiates a variety of downstream effects, including blockade of the cell cycle, autophagy, and apoptosis [49]. Under osmotic stress condition, proline is synthesized and degraded mainly through the glutamate metabolism pathway [48]. In this work, the increase of proline and arginine, were observed in TG2 group (Table 2), which may be explained due to the accumulation of proline, as a compatible solute, resulting in an increase in cellular osmolarity that can drive the influx of water or reduce its efflux [44]. Arginine and proline metabolism and cysteine and methionine metabolism has previously been found to be closely associated with metabolic syndrome including dyslipidemia, obesity, hypertension and elevated plasma glucose level [49]. The analysis of MetPA proved the importance of arginine and proline metabolism involved into the formation and development of TCMs.

Cysteine and methionine metabolism

To all organisms, L-cysteine and L-methionine are important as sulfur-containing amino acids. Animals uptake these amino acids as the sulfur sources, and catabolizes them to various cellular components [50-51]. Methionine occupies a central position in the cellular metabolism, as it is involved in the protein synthesis and synthesis of S-adenosylmethionine, the primary source of the methyl groups in the cell. Methionine biosynthesis involves three metabolic pathways: carbon backbone, sulfur utilization, formation and methylation [52]. L-Methionine is converted to S adenosylmethionine under catalysis by methionyladenosine transferase, which is produced by synthesis of S-adenosine homocysteine catalyzed by S-adenosylmethionine transylase [53]. The increase in S-adenosine homocysteine level affected the cystathionine metabolic pathway of methionine, which ultimately affected the production of its metabolite taurine [22]. Taurine (2-aminoethanesulfonic acid), a sulfur-containing amino acid, is one of the most abundant free amino acids in many tissues [54]. Taurine metabolism occurs mainly via two pathways: through synthesis from cysteine and through taurine uptake facilitated by a high-affinity transport system [44]. In bivalves, the system involved in taurine synthesis from cysteine is well-documented [55]. Synthesis of cysteine as a product of the transsulfuration pathway can be viewed as part of methionine or homocysteine degradation, with cysteine being the vehicle for sulfur conversion to end products (sulfate, taurine) that can be excreted in the urine [56]. The lack of methionine intake make the organ index decreased which would result in immunosuppression [57]. Compared to CG group, the content of methionine in P. sinensis of TG2 group was increased, which indicated TCMs supplementation to the basic experimental diets could improve methionine intake of P. sinensis, and increase immune-stimulants and the resistance to tumors. In the present study, TCMs could enhance the formation of taurine by regulating the content of L-methionine in P. sinensis. The main enzyme for taurine synthesis is mainly distributed in the liver, which is considered to be the main organ capable of taurine synthesis, suggesting that taurine may play vital roles in the liver protection [58]. Methionine, which is vital for mammalian metabolism, such as one-carbon metabolism, the transsulfuration pathway, and protein synthesis, is an essential, sulfur-containing amino acid required for human and animal health [59].

Conclusion

In summary, compared with CG group, TG1, TG2 and TG3 gave higher final weight and SGR (P<0.05), whereas TG4 group showed lower final weight and SGR (P<0.05), and the optimal dose of TCMs for juvenile P. sinensis was 2% TCMs feed additives. 43 potential biomarkers between TG2 and CG groups were finally screened out on the basis of VIP > 1 and p < 0.05, twenty seven metabolites were up-regulated, and sixteen metabolites were down-regulated. Eleven metabolites mainly involved in amino acid metabolism, nucleotide metabolism and lipid metabolism. These results illustrated that Phenylalanine, tyrosine and tryptophan biosynthesis, Arginine and proline metabolism, Tyrosine metabolism and Cysteine and methionine metabolism were regarded as potential target pathways involved in the growth promoting agents of TCMs (Impact >0.10). It is concluded that TCMs can be used as feeding additive for better growth of healthy juvenile P. sinensis.

Declarations

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Authors’ Contributions

Mingsong Xiao designed the study. Mingsong Xiao and Fangyin Bao performed the experiments. Mingsong Xiao and Jixiang He collected and analyzed the data. Yuliang Wang and Mingsong Xiao edited Figures. Mingsong Xiao and Jixiang He contributed to the discussion and reviewed/editing the manuscript.

Ethics approval

Investigations and protocols were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with Anhui Science and Technology University Institute of Animal Care and Use Committee. The institutional review board approved this procedure. Our study had been submitted to and approved by the Academic Ethics Committee of Anhui Science and Technology University. All sample collection was undertaken in accordance with relevant Academic Ethics Committee of Anhui Science and Technology University guidelines and regulations.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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### Tables

**Table 1**: Growth performances of *P. sinensis* fed with TCMs feed additive in five groups for 7 weeks.

| Experimental diets     | Initial weight | Final weight | Survival rate (%) | Weight gain | SGR     | FCR     |
|------------------------|----------------|--------------|-------------------|-------------|---------|---------|
| control group (0 %)    | 3.909±0.085    | 22.624±4.636c | 90.000±3.845      | 4.789±1.183c | 3.541±0.427c | 1.963±0.034c |
| TG1 group (1 %)        | 3.921±0.090    | 24.509±3.613b | 93.333±5.442      | 5.252±0.917b | 3.719±0.301b | 1.843±0.024ab |
| TG2 group (2 %)        | 3.929±0.070    | 26.799±4.704a | 94.998±3.335      | 5.824±1.205a | 3.887±0.367a | 1.785±0.015a  |
| TG3 group (3 %)        | 3.917±0.080    | 23.135±4.856bc | 91.665±3.330      | 4.912±1.267bc | 3.581±0.434bc | 1.885±0.031b  |
| TG4 group (4 %)        | 3.923±0.081    | 19.193±2.767d | 88.333±6.381      | 3.897±0.704d | 3.220±0.301d | 2.147±0.070d  |

Data represent means from four replicates per treatment. Values are presented as the mean ± SD. Values in a row with the different superscript letters denote significant difference (p < 0.05).

- Weight gain (WG %) = 100 × (final body weight–initial body weight)/initial body weight.
- Specific growth rate (SGR, %g/day) = 100 × [ln(final weight)–ln (initial weight)]/days of the experiment.
- Feed conversion ratio (FCR) = Gd / Gi, Gd: Dry feed intake, Gi: Final weight, Gd: Initial weight.
- Survival rate (%) = 100 × *P. sinensis* number in each group remaining at the end of the experiment/initial number of *P. sinensis*. 
Table 2: Potential biomarkers identified in the Pelodiscus sinensis serum samples between TG2 and CG groups.

| No. | Compound | RT (min) | m/z | Adduct ion | Formula | VIP | Metabolites | Trend |
|-----|----------|----------|-----|------------|---------|-----|-------------|-------|
| 1   | C06475   | 0.5140   | 433.2418 | M+FA-H | C20H36O7 | 2.2046 | 5,6-Dihydroxyprostaglandin F1a | ↑     |
| 2   | C00148   | 0.5771   | 116.0699 | M+H     | C6H8NO2  | 1.5328 | L-Proline | ↑     |
| 3   | C00515,C01602,C00077 | 0.5976 | 177.06 | M+2Na-H | C3H12N2O2 | 1.5614 | Ornithine | ↑     |
| 4   | C01225   | 0.6160   | 333.0592 | M-H     | C7H10O1P | 1.6285 | 1-(sn-Glycero-3-phospho)-1D-myo-inositol | ↑     |
| 5   | C00881   | 0.8142   | 455.1888 | 2M+H, 2M+Na, M+Na, M+H | C6H13N3O4 | 1.0132 | Deoxycytidine | ↑     |
| 6   | C03592   | 0.9251   | 242.1131 | M+H, M+H-2H2O | C10H13N3O4 | 1.0950 | 5-Methyldeoxycytidine | ↑     |
| 7   | C00073   | 0.9251   | 150.0574 | M-H     | C6H11NO2S | 1.0476 | L-Methionine | ↑     |
| 8   | C02376   | 0.9356   | 126.0655 | M-H     | C3H7N3O  | 1.1828 | 5-Methylcytosine | ↑     |
| 9   | C00082   | 1.0497   | 182.0806 | M+H, 2M+H | C6H11NO3 | 1.1755 | L-Tyrosine | ↑     |
| 10  | C14512   | 1.0497   | 119.0483 | M+H     | C6H6O    | 1.2982 | Benzofuran | ↑     |
| 11  | C06202   | 1.0497   | 123.0441 | M-H     | C7H12O2  | 1.1362 | Salicylaldehyde | ↑     |
| 12  | C01772   | 1.0604   | 165.0538 | M+H+H2O, M+H | C6H9O3 | 1.1546 | 2-Hydroxycinnamic acid | ↑     |
| 13  | C01367   | 1.0604   | 348.0702 | M+H, 2M+H | C16H11N2O7P | 1.0269 | Adenosine 3'-monophosphate | ↑     |
| 14  | NA       | 1.0914   | 253.0925 | M+H, M+ACN+H | C6H10NO4 | 1.1263 | Topaquinone | ↓     |
| 15  | C00760   | 1.5559   | 569.1587 | M+H2H2O, M+HCHO+H+H | C16H22O18 | 1.5476 | 1,4-beta-D-Glucan | ↑     |
| 16  | C01762   | 1.5559   | 285.0827 | M-H     | C13H12N2O6 | 1.0672 | Xanthosine | ↑     |
| 17  | NA       | 1.7594   | 131.0356 | M-H     | C5H8O4   | 1.3048 | Monoethyl malonic acid | ↓     |
| 18  | NA       | 1.9905   | 339.1553 | M-H     | C16H22N2O6 | 1.8546 | Nicotine glucuronide | ↑     |
| 19  | C04076,C01475 | 3.2725 | 144.0672 | M-H     | C6H11NO3 | 2.0693 | Allysine | ↓     |
| 20  | NA       | 3.4232   | 138.0566 | M-H2O-H | C7H11NO3 | 1.4977 | N-Acetylproline | ↓     |
| 21  | NA       | 3.4827   | 151.1222 | M+H     | C9H12N2  | 1.1451 | 2,5-Diethyl-3-methylpyrazine | ↑     |
| 22  | NA       | 3.6649   | 477.2473 | M+2H    | C45H17O21 | 1.4747 | Ampeloside Bf2 | ↑     |
| 23  | NA       | 4.2203   | 449.1194 | 2M+FA-H | C7H10N2O5 | 2.1435 | Penmacric acid | ↓     |
| 24  | C02710   | 4.337    | 174.1117 | M+H     | C6H11NO3 | 1.2585 | Acetyl-DL-Leucine | ↑     |
| 25  | NA       | 4.4130   | 182.1169 | M+H+ACN+Na | C10H12NO2 | 1.8490 | 2-(5-Methyl-2-furanyl)-3-piperidinol | ↑     |
| 26  | C16635   | 4.4914   | 280.0496 | M+Cl    | C8H12FN3O4 | 2.0148 | 5'-Deoxy-5-fluorocytidine | ↓     |
| 27  | NA       | 4.6914   | 237.0429 | M-H     | C6H10OS3 | 2.4396 | 1-Propenyl 1-(propylsulfanyl)propyl disulfide | ↓     |
| 28  | NA       | 5.9164   | 855.4596 | M+H2O2  | C47H16O15 | 1.6671 | Pectenotoxin 3 | ↑     |
| 29  | NA       | 5.9223   | 284.139 | M+HCHO+H+H | C15H13N3O | 2.0314 | 7-Aminonitrazepam | ↓     |
| 30  | NA       | 7.7337   | 468.3099 | M+H+M+Na, 2M+H | C22H26NO3P | 1.8861 | PC(14:0/0:0) | ↓     |
| 31  | NA       | 8.0701   | 628.3637 | M-H     | C20H31NO6 | 1.1010 | Janthitrem G | ↑     |
| 32  | C09537   | 8.4625   | 247.1343 | M-H     | C13H20O3 | 2.4700 | Quadrone | ↑     |
| 33  | NA       | 8.4638   | 496.3407 | M+H+M+Na | C24H26NO5P | 1.8431 | PC(16:0/0:0)[U] | ↓     |
| No. | Pathway Name                                           | Total | Hits | Raw p        | Holm adjust | FDR          | Impact        |
|-----|--------------------------------------------------------|-------|------|--------------|-------------|--------------|---------------|
| 1   | Aminoacyl-tRNA biosynthesis                            | 48    | 3    | 0.030486     | 1           | 1            | 0             |
| 2   | Phenylalanine, tyrosine and tryptophan biosynthesis    | 4     | 1    | 0.057245     | 1           | 1            | 0.5           |
| 3   | Arginine and proline metabolism                        | 38    | 2    | 0.10436      | 1           | 1            | 0.20389       |
| 4   | Phynelalanine metabolism                               | 8     | 1    | 0.11136      | 1           | 1            | 0             |
| 5   | Ubiquinone and other terpenoid-quinone biosynthesis    | 9     | 1    | 0.12442      | 1           | 1            | 0             |
| 6   | Arginine biosynthesis                                  | 14    | 1    | 0.18702      | 1           | 1            | 0.06091       |
| 7   | Sphingolipid metabolism                                | 21    | 1    | 0.26754      | 1           | 1            | 0             |
| 8   | Glutathione metabolism                                 | 28    | 1    | 0.34042      | 1           | 1            | 0             |
| 9   | Cysteine and methionine metabolism                     | 33    | 1    | 0.3882       | 1           | 1            | 0.10446       |
| 10  | Glycerophospholipid metabolism                         | 38    | 1    | 0.43267      | 1           | 1            | 0.01505       |
| 11  | Pyrimidine metabolism                                  | 41    | 1    | 0.45785      | 1           | 1            | 0.00558       |
| 12  | Tyrosine metabolism                                   | 42    | 1    | 0.46601      | 1           | 1            | 0.13972       |
| 13  | Purine metabolism                                     | 66    | 1    | 0.54300      | 1           | 1            | 0             |

The "Total" is the number of compounds in the pathway; the "Hits" represents the actual matched number from the user uploaded data; the Raw p is the original p value calculated from the enrichment analysis; the Holm p is the p value adjusted by Holm-Bonferroni method; the FDR p is the p value adjusted using False Discovery Rate; the Impact is the pathway impact value calculated from pathway topology analysis.

NA indicates that there is no information in the database. Arrow (↑) indicates relative increase in signal. Arrow (↓) indicates relative decrease in signal.

a Change trend compared with control group.

Table 3: Results of pathway analysis using MetaboAnalyst database.
Figure 1

PCA score plots of urine samples from all groups detected by UPLC-Q-TOF/MS (A and B). (A) in positive ion mode of UPLC-Q-TOF/MS technique (R^2x = 0.348, Q^2 = 0.117); (B) in negative ion mode of UPLC-Q-TOF/MS technique (R^2x = 0.317, Q^2 = 0.134) (CG: control group, TG1: TG1 group, TG2: TG2 group, TG3: TG3 group, TG4: TG4 group).
Figure 2

OPLS DA model score plot and permutation test plot. (A) Score plot and (B) plot of the permutation test (200 times) of the OPLS DA model obtained from the CG and TG2 groups under positive (R2X = 0.261, R2Y = 0.688, Q2= 0.456) and negative mode (R2X = 0.208, R2Y = 0.597, Q2= 0.255).
Figure 2

OPLS DA model score plot and permutation test plot. (A) Score plot and (B) plot of the permutation test (200 times) of the OPLS DA model obtained from the CG and TG2 groups under positive ($R^2_X = 0.261$, $R^2_Y = 0.688$, $Q^2 = 0.456$) and negative mode ($R^2_X = 0.208$, $R^2_Y = 0.597$, $Q^2 = 0.255$).
Figure 3

The change of 30 biomarkers in CG and TG2 group of *P. sinensis* for serum.
Figure 3

The change of 30 biomarkers in CG and TG2 group of P. sinensis for serum.
Figure 4

The heatmap of metabolites among CG, TG1, TG2, TG3 and TG4 groups.
Figure 4

The heatmap of metabolites among CG, TG1, TG2, TG3 and TG4 groups.
Figure 5

Summary of pathway analysis with MetPA. (a) Phenylalanine, tyrosine and tryptophan biosynthesis, (b) Arginine and proline metabolism, (c) Tyrosine metabolism, (d) Cysteine and methionine metabolism, (e) Arginine biosynthesis.
Figure 5

Summary of pathway analysis with MetPA. (a) Phenylalanine, tyrosine and tryptophan biosynthesis, (b) Arginine and proline metabolism, (c) Tyrosine metabolism, (d) Cysteine and methionine metabolism, (e) Arginine biosynthesis.
The metabolic pathway network of the potential biomarkers for TG2 group according to the KEGG PATHWAY database. The blue boxes are the most heavily affected metabolic pathways found.
Figure 6
The metabolic pathway network of the potential biomarkers for TG2 group according to the KEGG PATHWAY database. The blue boxes are the most heavily affected metabolic pathways found.

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