^1H NMR-based metabolomics investigation of dietary soybean meal substitution in hybrid sturgeon (*Acipenser baerii* $\times$ *A. schrenckii*)

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Abstract

Background: Soybean proteins is the most widely used alternative protein sources for fish meal in aquafeed. However, soybean meal (SBM) substitution imposes negative effects such as growth restriction and intestinal inflammation. Limited reports were concentrated on the metabolic alterations in fish. **Results:** A ten-week feeding trial was conducted to examine the effect of dietary SBM substitution on growth performance, serum biochemistry and metabolism in hybrid sturgeon. It was showed that 25% SBM substitution produced the highest weight gain, with hepatosomatic index reduced significantly in the 50% and 100% SBM substitution groups. Besides, SBM diet led to significant increase of the glutamic-pyruvic transaminase concentration in the 100% SBM diet group. Moreover, SBM diets higher than 50% resulted in hepatic injury of lipid droplet accumulation by histological analysis. ¹H NMR method was applied to detect metabolite changes in liver and serum. Totally, 47 and 50 representative metabolites were screened in liver and serum, respectively. The subsequent PLS-DA analysis identified 23 in liver and 10 in serum of metabolites affected by SBM substitution through pairwise comparisons, respectively. The following pathway enrichment revealed 9 in liver and 17 in serum of perturbed pathways, respectively. Among them, pathway of phenylalanine, tyrosine and tryptophan biosynthesis with two differentiated metabolites (phenylalanine and tyrosine) involved deserved the highest impact factor, which indicated severe liver damage by SBM diets.

Background

Fishmeal (FM) is the predominant protein source in aquafeeds because of its well-balanced amino acid profile, digestible energy and minerals [1, 2]. The worldwide demand for FM imposes ever-rising pressure on feed cost and wild fish resources [3]. Therefore, great efforts have been made to replace FM with alternative plant-based ingredients, due to their relative sustainability and low prices [4, 5]. Soybean meal (SBM) as the most widely used plant protein for FM substitution has been studied in many fish species [5-8]. However, the large inclusion of SBM in aquafeeds will cause negative effect on fish growth and health [9, 10] by the presence of anti-nutritional factors (ANFs) such as protease, phytates, lectins and saponins [11]. Current studies on the adverse influences of SBM replacement focused mainly on the growth restriction [12, 13] and intestinal inflammation [14-17], with limited reports regarding the complete metabolomic alterations in fish.

Metabolomics is a bio-analytical approach that allows the investigation of both qualitative and quantitative variation of metabolites in biological matrices in response to biotic or abiotic factors [18, 19]. Low-molecular-weight metabolites, including lipids, sugars and amino acids, can be quantified in tissues and biofluids by adopting metabolomics platforms, such as ¹H-nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS), and liquid chromatography–mass spectrometry (LC–MS) [20-22]. Among them, NMR possesses the advantages of easy sample preparation, universal metabolites detection, fast quantitation and reproducibility [23, 24]. Assessment of urinary metabolomics using NMR indicated that the metabolism dysfunction induced by hypercholesterolemia could partially be recovered by virgin avocado oil treatment in rats [25]. In the field

of fish nutrition, metabolic profiles detected by NMR revealed significant alterations in response to dietary imbalances in *Megalobrama amblycephala* [26]. Furthermore, in the cultured Arctic char (*Salvelinus alpinus* L.), NMR was effective to reveal the mechanism of lower body mass and the tendency of less lipid content observed [27].

Sturgeons are a group of about 25 fish species and mostly appreciated for their unfertilized roe known as the black caviar [28]. In China, the farmed hybrid sturgeons accounted for over 35% of production in the overall sturgeon industry during 2007–2009 [29]. Currently, commercial salmonid diets with FM as the main protein source are utilized for the culture of sturgeon, since no refined and species-specific diets are available for sturgeons [30, 31]. In order to develop economically and nutritionally efficient diets for sturgeons, plant ingredients including SBM were examined for the replacement of FM in several sturgeon species. In beluga (*Huso huso*), SBM substitution caused growth performance impairment, and 700 g/kg SBM in the diet resulted in reductions in the haematocrit, glucose and cholesterol levels in the blood [32]. In Persian sturgeon (*Acipenser persicus*), 40% SBM substitution of FM led to the limited growth with the lack of available phosphorus [33]. Besides, SBM inclusion had no influence on caviar quality or production in White sturgeon (*A. transmontanus*) [34].

Liver is an important organ involved in the metabolism of nitrogen and other metabolites [35], which moved through the whole body via the blood [26]. The primary aim of this study was to assess the metabolic changes induced by different dietary levels of SBM substitution for FM in juvenile hybrid sturgeon *A. baerii* × *A. schrenckii*. The comparative metabolomics analysis was conducted by NMR method in liver and blood samples of fish fed four different diets with partial or complete SBM replacement. After identifying the significantly altered metabolites between groups, metabolic pathways involved were enriched. The results should shadow lights on understanding the metabolomic regulation of SBM replacement for FM, which will further provide theoretical basis for SBM utilization in aquafeeds.

Methods

Diets preparation, experimental fish and sample collection

Four diets were formulated and produced in which 0% (P0, the control), 25% (P25), 50% (P50) and 100% (P100) of FM were replaced with SBM (Additional file 1). Fishmeal (Kilka, Iran), SBM and other macronutrients were homogenized, fully mixed and passed through a 60-μm square aperture mesh. The mixture of each group was pelleted with a 6-mm die and oven-dried at 50°C for 18–24 hr. All diets were stored in nylon bags at –20 °C until use.

For the experiment, 180 juvenile hybrid sturgeons (initial weight 30.00 ± 2.5g) were obtained and reared at Taihu station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. After acclimation for two weeks with the control diet, sturgeons were randomly allotted to 12 cylindrical tanks (diameter of 1.05 m, height of 0.5 m, and water depth of 0.3 m). Each group of diet was assigned to

triplicate tanks. Fish were hand-fed three times daily (08:00, 14:00 and 20:00) to apparent satiation for 10 weeks. During the experimental period, underground water filtered was used with the flow rate of 2 L/min. The temperature was maintained at 18.2~20.0°C, with the pH of 7.8~8.2 and the dissolved oxygen level of approximately 5 mg/L.

After a 12-hour fast, the fish were anesthetized and weighted at the end of the experiment. Six specimens of each group were randomly selected to collect blood and liver samples. Blood samples (n=24) were obtained quickly by venipuncture of the caudal vein using a sterile 5-ml syringe. Supernatants were prepared by centrifugation, divided into two tubes and stored at -80°C. Liver samples of each fish (n=24) were weighed, and separated into two tubes: one to be fixed in 10% formalin for histological examination, and the other one to be immediately dipped into liquid nitrogen and stored at -80°C until use for metabolomics analysis.

Growth performance, blood chemistry measurements and histological analysis

Growth performance was determined and calculated as follows: Weight gain (WG, %) = $100 \times (\text{final body weight} - \text{initial body weight}) / \text{initial body weight}$; Hepatosomatic index (HSI, %) = $100 \times (\text{liver weight} / \text{whole body weight})$. Serum biochemistry parameters including glutamic-pyruvic transaminase (ALT) and glutamic oxalacetic transaminase (AST) by commercial kits produced by Jiancheng Bioengineering Institute (Nanjing, China) using the auto-analyzer (Mindray BS-460).

Formalin-fixed liver samples were embedded in paraffin, sectioned (in 4 µm thickness) and stained with hematoxylin-eosin (HE). Images of sections were observed under the light microscope (BX-51, Olympus) equipped with a digital camera (DP-73, Olympus).

Chemicals and reagents

Double distilled water was used for the preparation of all of the solutions. Deuterium oxide (D₂O, 99.9%) and Anachro Certified DSS Standard Solution (ACDSS) were purchased from Cambridge Isotope Laboratories Inc. (Miami, FL, USA) and AnachroTechnologies Inc. (Calgary, Canada), respectively. Methanol was purchased from Merck (Darmstadt, Germany). The phosphate buffer solution (0.1 M K₂HPO₄/NaH₂PO₄, pH 7.29) used in this study was obtained from Sigma. All of the other chemicals used were of HPLC grade.

Sample preparation for ¹H NMR analysis

After centrifuged for 2 min (13,000 rpm, 4°C), supernatants of each serum sample (five samples per group) was filtered with the 3 kDa ultra-filtration filter (Millipore, USA). Then filtrates of 450 µL were transferred into a 2 ml tube with the addition of 50 µL ACDSS dissolved in D₂O as an internal standard. The twenty liver samples (five replicates per group) were ground in liquid nitrogen with a mortar and a pestle. Then, 50 mg freeze-dried power of each sample was extracted with 1 mL 50% methanol solution (methanol/water, 1/1, v/v) by vortexing for 60 s. Extracts were sonicated on ice for 8 times at 45% amplitude for 4s by intervals of 3s with an ultrasonic processor (Sonics VCX130). After centrifugation for 15 min (13,000 rpm, 4 °C), 700 µL supernatant was collected and concentrated under nitrogen gas stream for approximately 30 min. The mixture was then evaporated to dryness in a vacuum centrifugal evaporator. After freezing at -80°C for 12 h and freeze-drying with a lyophilizer (LABCONCO Freezone25), the dried sample was redissolved with 450 µL ultrapure water and filtered. Similarly, 50 µL ACDSS dissolved in D₂O was added to the 450 µL filtrates. After mixed thoroughly, 480 µL extract from each of the serum and liver sample was transferred to a 5 mm NMR tube for NMR analysis [36].

NMR spectroscopy and data processing

¹H NMR spectra were recorded on a Bruker AV III 600 MHz spectrometer equipped with an inverse cryoprobe (NMR frequency of 600.20 MHz). For each sample, 128 scans over a period of 15min were recorded at 25°C with the spectral width (SW) of 8403.361 Hz. For resonance assignment purpose, two dimensional (2D) NMR spectra was utilized for data acquisition [37]. The *noesygppr1d* sequence was applied to suppress the residual water signal.

All free induction decay (FID) signals were processed automatically by Chenomx NMR Suite v.8.3 (Chenomx Inc., Edmonton, Canada) for Fourier transformation, phase adjustment and baseline correction. Metabolites were identified by assigning spectral signals to Chenomx 600 MHz Library containing 330 metabolites. The peak of DSS-d₆ (set to 0.0 ppm) was referenced as the internal standard for chemical shifts. The quantification of metabolite concentrations was achieved as described [38, 39], and exported as a Excel file for further analysis.

Data analysis

Principal component analysis (PCA) [40] and the partial least square discriminant analysis (PLS-DA) [41] were performed for data visualization and differential metabolites characterization, respectively. Plots were made by the R's ggplot2 [42]. The quality of the PLS-DA model was cross validated by permutation tests, with the R² value described the data fitness and Q² value described the predictive ability as reported [43]. For each metabolite, the Variable Importance in the Projection (VIP) score was calculated [44] to determine the most relevant metabolites by SBM substitution. Besides, metabolite -differences with VIP ≥ 1 was also evaluated by T-test at a significance level of p < 0.05. Differential metabolites were further

assigned to Kyoto Encyclopedia of Genes and Genomes database (KEGG) for metabolic pathways enrichment. Pathways with impact factor > 0 and significance level of $p < 0.05$ were analyzed.

Data of growth performance and blood chemistry measurements described above were presented as means $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests with the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). A probability level of $p < 0.05$ was considered significant.

Results

Growth performance and blood chemistry

After 10 weeks feeding, the greatest weight gain (462.58 ± 18.82 g) was observed in the P25 group, with the least weight gain found in the P100 group (Table 1). Besides, values of HSI showed a continuous decline with the increase of SBM replacement (Table 1). Furthermore, HSI values of sturgeons in the P50 and P100 groups had significant differences with that of P0 and P25 group ($P < 0.05$). As for blood parameters, no difference was detected in terms of AST levels among the four groups ($P > 0.05$). However, ALT levels in the complete SBM P100 group was significantly higher than that in the P0 and P50 groups ($P < 0.05$).

Liver morphology

Hematoxylin and eosin (H&E) staining was performed to visualize the accumulation of lipids in liver (Fig. 1). Liver cells of hybrid sturgeons in the P25 group exhibited mild accumulation of lipid droplets (marked by blue arrowheads), with large and round nuclei in the middle (marked by green triangles) and normal structural integrity of hepatic cells (yellow dot line circled) (Fig. 1B). However, in the P0 and P50 groups, moderated accumulation of lipid droplets and swelling hepatocytes were found by signs of irregular nucleus located at the periphery of the cell (Fig. 1A and C). Moreover, complete replacement of SBM in the P100 group led to large lipid droplets with swollen hepatocytes and nuclear degeneration (Fig. 1D).

Metabolites identification in serum and liver

In liver, 47 metabolites were quantified, and classified into alcohols (3), amino acids and the derivatives (24), nucleic acid components (5), organic acids (7), sugars (3) and others (5) (Additional file 2). Meanwhile, a total of 50 metabolites with the classifications of alcohols (4), amino acids and the derivatives (20), Amines and ammoniums compounds (3), nucleic acid components (6), organic acids (13), sugars (2) and others (2), were identified in serum samples (Additional file 3).

Multivariate analysis of serum and liver metabolites

To evaluate the effects of the test diets on liver and serum metabolic profile, concentrations of all identified metabolites between each group were overviewed by using PCA model. As illustrated in Additional file 5, pairwise-comparisons of P100 vs. P0, P100 vs. P25, and P100 vs. P50 in liver samples showed obvious separation trends (Additional file 5), while no separation tendency were present in P0 vs. P25, P50 vs. P25, and P50 vs. P0 (Additional file 4). In serum samples, only P100 vs. P0 and P100 vs. P25 group pairs demonstrated the separation trend, with the other four group pairs overlapped greatly from each other, respectively (Additional file 6 and 7).

The further PLS-DA model showed a better discrimination between dietary groups. The group pairs of P100 vs. P50 in liver ($R^2=0.97$, $Q^2=0.73$) (Figure 2G), and P50 vs. P0 ($R^2=0.92$, $Q^2=0.18$) and P100 vs. P50 ($R^2=0.56$, $Q^2=0.21$) in serum presented complete segregation (Figure 3G and 3E). Besides, obvious separation tendency were observed in comparison pairs of P50 vs. P0 ($R^2=0.84$, $Q^2=0.31$), P100 vs. P0 ($R^2=0.89$, $Q^2=0.51$) and P100 vs. P25 ($R^2=0.99$, $Q^2=0.66$) in liver, and of P100 vs. P0 ($R^2=0.99$, $Q^2=0.66$) and P100 vs. P25 ($R^2=0.99$, $Q^2=0.58$) in serum (Figure 2 and 3). Based on the above PCA and PLS-DA analysis, four group comparisons of P50 vs. P0, P100 vs. P0, P100 vs. P25 and P100 vs. P50 were chosen for the following assessment in liver and serum samples. The first fifteen differential metabolites with VIP value ≥ 1 were exhibited as well, with the highest VIP scores received by maltose in liver (4.86) and glucose in serum (4.54) both in the P100 vs. P25 comparison group, respectively (Figure 2 and 3).

Totally, 23 metabolites in liver (Table 2) and 16 metabolites in serum (Table 3) were significantly affected by SBM replacement, as assessed by PLS-DA method with VIP score ≥ 1 (marked by black bold). Among them, 16 metabolites in liver and 12 metabolites in serum (marked by asterisk) were significantly altered further evaluated by T-test (marked by asterisks in Table 2 and 3). In the complete SBM diet group P100, metabolic levels of four metabolites in liver (taurine, cholate, inosine and maltose) (Table 2) and two in serum (proline and aspartate) (Table 3) were significantly declined in comparison to the other groups, with all other metabolites increased (VIP ≥ 1). Moreover, compared to the P0 control group, five metabolites (methanol, glycine, serine, lactate and inosine) in liver (Table 2) and one in serum (proline) (Table 3) represented lower levels in the P50 group. Furthermore, the highest distinguished level in pairwise comparisons of liver were the myo-inositol of 11.90, as observed in the P50 vs. P0 group (VIP > 1 , Table 2). In serum, lysine with the increasing metabolic level of 2.84 was the highest in P100 vs. P0 group pairs (VIP ≥ 1 , Table 3).

Metabolite pathway analysis

To further understand the metabolic differences between different SBM-supplemented groups, the differential metabolites data were further analyzed by pathway enrichment. In liver, fifteen pathways were screened, with the five classifications of Amino acid metabolism (6), Carbohydrate metabolism (3), Lipid metabolism (1), Nucleotide metabolism (2) and Other (3) ($P<0.05$) (Table 4). In serum, eighteen pathways allocated to five classifications were obtained as well ($P<0.05$) (Table 5). Most pathways were clustered into the amino acid metabolism (8) and the carbohydrate metabolism (6), with the rest of one pathway

belonged to Lipid metabolism, one to Nucleotide metabolism, and two to Other (Table 5). Among them, the pathway of Phenylalanine, tyrosine and tryptophan biosynthesis with two differentiated metabolites involved (phenylalanine and tyrosine) deserved the highest impact factor of 0.98 both in liver and serum. In addition, the pathway of Alanine, aspartate and glutamate metabolism comprised the maximum number of variable metabolites (alanine, aspartate, fumarate, glutamate, glutamine, succinate) (Table 4 and 5). Based on the above pathway enrichment, schematic metabolite changes involved in metabolic pathways were demonstrated for liver and serum in Fig. 4 and 5, respectively.

Discussion

Successful FM substitution with plant alternatives in fish feed represents the most desired development in the aquaculture industry [5]. In the present study, SBM replacement of FM in diets was conducted in hybrid sturgeons, of which growth performance and serum biochemical index were evaluated. Besides, metabolites variations and metabolic pathways involved in liver and serum by SBM substitution were illustrated for the first time. The results provide valuable metabolomics information for SBM supplementation in fish feed, which is also helpful for further diet development of sturgeons.

In sturgeon species, SBM addition induced significantly negative effects on growth performance in beluga [32], Persian sturgeon [33], and Russian sturgeon *Acipenser gueldenstaedtii* [45]. In this study, SBM levels higher than 50% (P50 and P100 groups) also showed severe growth decline compared to the FM group (Table 1). The growth impairment might be ascribed to anti-nutritional factors, the low apparent digestibility of the diet, the imbalanced amino acids and the reduced availability of phosphorus and other minerals in soybean [46, 47]. However, the highest weight gain was present in the P25 group with SBM substitution level of 25%, but not in the complete FM group (P0) in the present study (Table 1). As been reported, the optimum dietary protein levels from 370 g/kg to 405 g/kg were more favorable for the maximal growth of white sturgeon (*Acipenser transmontanus*), Siberian sturgeon (*A. baeri*), Persian sturgeon (*A. persicus*), and juvenile hybrid sturgeon (*A. baeri* × *A. gueldenstaedtii*), respectively [48-51]. Therefore, the protein level of diet in FM group (42.80% in P0 group) might be too high for hybrid sturgeon *Acipenser schrenckii* × *A. baeri* in this study, and diet with lower lipid content was more suitable. It was also indicated that 250 g/kg SBM substitution had no negative influence on the growth parameter of hybrid sturgeon. Similar results were reported previously that 300 g/kg of the mixture of SBM, RM and CSM in the diet did not compromised the growth performance and feed utilization in juvenile hybrid sturgeon [52].

Herein, the HSI reduced significantly along with the soybean protein source levels increased (Table 1), which was in accordance with that in cuneate drum *Nibea miichthioides* [53], tiger puffer *Takifugu rubripes* [54], turbot *Psetta maxima* L. [55] and common carp [56]. The decline in HSI might be due to the impaired liver structure by feeding SBM diets, as confirmed by the following liver histology observation (Fig. 1). In common carp fed 540 g/kg cottonseed meal (CSM), liver damages including shrinkage of hepatocytes and an increased number of smaller hepatic cells were appeared [56]. In addition, hepatocyte nucleus polarization and isolated necrosis were demonstrated in gilthead sea bream

fed SBM [57]. Furthermore, AST and ALT were considered as the nonspecific and specific markers for hepatic injury [58, 59]. Elevation levels of these enzymes and AST/ALT ratio in the serum indicated their leakage from injured hepatocytes and therefore were supposed to be the sensitive indicator of injury in tissues, including the liver [60, 61]. In our study, only the ALT level in P100 group increased greatly compared to other groups, with no significant difference on AST levels among groups (Table 1). It was the other evidence of seriously liver injury by 100% SBM diet.

As been reported, the number of metabolites detected by GC-MS platform in serum is usually less than that in animal tissues [62], although the metabolites in different tissues are secreted, excreted, or discarded into blood under physiological requirements or stress [63]. However, in the present research by NMR method, three more metabolites in serum were detected than that in liver, which suggested more accurate demonstration of the animal metabolism by NMR than that by GC-MS. Meanwhile, most of the metabolites screened in liver and serum belonged to the classification of amino acid with increasing levels by SBM diets. It is known that amino acids play a vital role in the regulation of protein turnover processes as the essential substrates of body proteins [64]. Therefore, the changes measured in the pool of amino acids might be due to the activation of cytoprotective mechanisms in response to the new and less favorable condition such as SBM diet inclusion, by promotion of new protein synthesis [64, 65].

Myo-inositol, the most abundant inositol, serves important functions in organisms by stimulating growth and improving digestive capacity [66, 67]. However, piglets with lower birth weight had higher concentration of myo-inositol [68]. In cultured Arctic Char (*Salvelinus alpinus*), lower final weight was related to higher myo-inositol in plasma in the test diet group compared to the control group [27]. In our study of hybrid sturgeon, concentrations of myo-inositol increased greatly in SBM diets groups (P50 and P100) compared to the FM group (P0), with higher increasing levels in the liver than that in the serum (Table 2 and 3). Besides, the weight gain of P50 group was higher than that in the P0 group (Table 1), which indicated altered endogenous myo-inositol metabolism, and promotion of growth by higher content of myo-inositol in the soybean proteins [27].

Moreover, significant decrease of creatine, taurine, cholate, and maltose were demonstrated in liver of the complete SBM group (P100). Creatine is normally produced by the liver, kidneys and pancreas, and dietary supplementation of creatine resulted in improved muscle strength and increased lean body mass in athletes [69]. Taurine is an essential nutrient for growth and development in juvenile turbot (*Scophthalmus maximus*) [70, 71]. Similar promotion effect of taurine on growth were reported in fish species including yellowtail [72], Japanese flounder [73] and red sea bream [74]. Insufficient taurine may led to anemia [75], and inferior growth [76] in accordance with the result of our study. For cholate, its concentration reduction in the atherogenic diet affected the lipid accumulation in liver [77]. Dietary inosine supplementation effectively provoked growth and health performance of red sea bream (*Pagrus major*) by increasing growth, immune response, oxidative stress resistance and intestinal health condition [78]. Maltose was mainly involved in metabolism of glucose conversion as illustrated in Figure 4. However, the glucose levels in liver and serum increased slightly instead (Table 2 and 3), which suggested multiple sources of glucose production. Therefore, the growth suppression in the complete SBM group

(P100) might be attributed to the severe negative effect including metabolite metabolism alterations by soybean as described above.

Noticeably, the aspartate concentrations declined to zero in serum in the SBM diet group of P50 and P100 (Table 3). Nevertheless, significant promotion of aspartate levels were detected in liver of all the three SBM diet groups (Table 2). Aspartate is mainly involved in the alanine, aspartate and glutamate metabolism (Table 4 and 5) serving as carbon sources for perivenous glutamine synthesis [79], and also plays a substantial role in enhancing immunity and detoxifying ammonia [80]. Addition of aspartate alleviates growth suppression of weaned pigs after the lipopolysaccharide challenge [81]. In liver of hybrid sturgeon, the increase of aspartate might be ascribed to the enhancement of alanine (Figure 4). It is thus indicated that soybean meal destroyed the aspartate transportation from liver to blood, which resulted in its degradation in serum. Proline is another metabolite, which presented decreased level in serum compared with the up-grading tendency in liver by SBM diets (Table 2 and 3). Proline mainly participated in the arginine and proline metabolism as exhibited in Table 4 and 5. It could be synthesized from arginine and glutamine/glutamate (Fig. 4 and 5), and played important roles in anti-oxidative reactions and immune responses [82, 83]. The ascending levels of both glutamate and arginine might alleviate the decrease of proline in liver (Fig. 4). Comparably, proline depression was probably the response of glutamate downregulation in serum.

Metabolite alterations will lead to the fluctuation of metabolomics pathways in organisms. In this study, four main categories of pathways were enriched, including the amino acid metabolism, carbohydrate metabolism, lipid metabolism and nucleotide metabolism (Table 4 and 5). As indicated above, the up-regulation of most of the metabolites by SBM resulted in the overall variations in the amino acid metabolism. Among them, pathway of phenylalanine, tyrosine and tryptophan biosynthesis exerted the highest impact factor of 0.98 (Table 4 and 5). As been previously demonstrated, large quantities of phenylalanine and tyrosine were detected in rats with liver injury [84]. It was also proved that L-phenylalanine metabolism relies on the integrity of liver cell function, since it is catabolized in liver [85]. Pathways including phenylalanine, tyrosine and tryptophan biosynthesis were perturbed by liver injury in dimethylnitrosamine treated rats [86]. Thus, it was suggested that liver cell injury by SBM addition (Fig. 1) in this study caused the elevation of phenylalanine concentration (Table 2 and 3) as well as the disturbance of phenylalanine, tyrosine and tryptophan biosynthesis. Amino acids contributed to the carbohydrate metabolism by expanding the pool of TCA cycle (Fig. 4 and 5). Intermediate metabolites of the TCA pathway can be converted to glucose, fatty acids and amino acids for use into biosynthetic pathways [87]. In general, the integrated networks of different pathways with combined metabolites regulated the overall metabolism in animals.

Conclusions

The above results including parameters of growth performance, blood chemistry, liver histology and metabolism detection strongly support the conclusion that 250 g/kg of SBM substitution from fish meal is feasible in diets of hybrid sturgeon. Severe liver damage and metabolism disorder will occur by SBM

addition more than 500 g/kg. The data is meaningful for comprehending the metabolic regulation by SBM diets, which will further be helpful for SBM utilization in the development of diets for sturgeons.

Abbreviations

ACDSS: Anachro Certified DSS Standard Solution; ANFs: anti-nutritional factors; ANOVA: one-way analysis of variance; ALT: glutamic-pyruvic transaminase; AST: glutamic oxalacetic transaminase; CSM: cottonseed meal; D₂O: Deuterium oxide; FID: free induction decay; FM: Fishmeal; GC–MS: gas chromatography–mass spectrometry; HE: hematoxylin-eosin; HSI: Hepatosomatic index; KEGG: Genes and Genomes database; LC–MS: liquid chromatography–mass spectrometry; NMR: ¹H-nuclear magnetic resonance; PCA: Principal component analysis; PLS-DA: partial least square discriminant analysis; SBM: soybean meal; SD: means ± standard deviation; VIP: Variable Importance in the Projection; WG: Weight gain; 2D: two dimensional.

Declarations

Ethics approval and consent to participate

All fish were handled with the approval of the Animal Care and Use Committee of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. Efforts were made to minimize the suffering of fish in the experiment as much as possible.

Consent for publication

Not applicable

Availability of data and material

Data was available from the corresponding authors by reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The authors' responsibilities were as follows: XC and CL conceptualized the study; HY and JW designed and conducted the research; RR and HY helped with the fish collection; HY and JW collected and analyzed the data; HY wrote the manuscript; XC and CL revised the manuscript. All authors read the final version of the manuscript, and provided comments for improvements.

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Tables

Table 1 Phenotypes and serum biochemical parameters for sturgeons fed the four groups of diets

Parameters	Treatments			
	P0	P25	P50	P100
Initial weight (g)	30.30±1.08	29.91±0.60	30.84±1.37	30.79±0.52
Weight gain (g)	380.98±43.49b	462.58±18.82a	456.79±45.91a	245.84±15.27c
HSI (%)	3.62±0.47b	3.08±0.42b	3.07±0.50a	2.47±0.49a
AST (mmol/L)	279.73±43.77a	245.90±20.30a	305.03±56.10a	241.17±23.68a
ALT (mmol/L)	5.10±0.87a	5.90±0.95ab	4.80±0.20a	7.17±1.53b

Values within the same raw with different superscripts are significantly different (P<0.05)

Table 2 Significantly different metabolites between group pairs in liver extracts with fold changes

Classifications	Metabolites	P50 versus P0	P100 versus P0	P100 versus P25	P100 versus P50
Alcohols	Methanol	0.84	1.11	1.24*	1.31*
	Myo-inositol	11.90*	11.12	1.97	0.95
Amino acid derivatives	Creatine	1.14	0.53	0.49	0.47
Amino acids	Alanine	1.42	1.56	1.24	1.10
	Aspartate	3.31	4.76	2.06	1.44
	Betaine	0.87	1.54	2.15	1.78
	Glutamate	2.04*	2.15	1.44	1.05
	Glutamine	1.75*	1.86	1.20	1.06
	Isoleucine	2.83*	4.18	2.03	1.47
	Leucine	2.70*	3.86*	1.94	1.43
	Lysine	2.47*	2.90	1.43	1.17
	Phenylalanine	2.44	3.69*	1.87	1.51
	Proline	2.06	2.58	1.85	1.25
	Taurine	1.13	0.77	0.71*	0.68*
	Threonine	2.11*	2.63	1.63	1.25
	Tyrosine	2.60	3.73*	1.76	1.43
	Valine	2.62*	3.49*	1.78	1.33
Bile acids	Cholate	1.80	0.15	0.13	0.08*
Nucleic acid components	Inosine	0.69	0.47*	0.51	0.68
Organic acids	Malate	1.06	0.43	0.38	0.41
	Succinate	1.25	0.23	0.22	0.18
Sugars	Glucose	1.35	1.26	1.03	0.93
	Maltose	1.01	0.33	0.39*	0.33*

Black bold represents metabolite differences with VIP ≥ 1 by PLS-DA analysis

*shows metabolites changes between two groups with VIP ≥ 1 by PLS-DA analysis and significant differences (P<0.05) by T-test

Table 3 Significantly different metabolites between group pairs in serum extracts with fold changes

Classifications	Metabolites	P50 versus P0	P100 versus P0	P100 versus P25	P100 versus P50
alcohols	Ethanol	1.59	1.34	0.83	0.84
	Methanol	1.27	1.08	0.92	0.85
	myo-Inositol	1.48	1.73	1.31	1.17
amino acids	Arginine	1.98*	1.63	1.16	0.82
	Aspartate	0.04*	0.03	0.04	/
	Betaine	0.85	1.67	2.49	1.97
	Glutamine	1.77	1.81	2.46*	1.03
	Isoleucine	1.54*	2.03*	1.66*	1.32
	Leucine	1.45	1.79*	1.57*	1.23
	Lysine	1.69*	2.84*	1.75*	1.68*
	Methionine	1.79*	3.55	2.31*	1.99
	Proline	0.42*	0.38*	0.92	0.90
	Valine	1.33	1.66*	1.52*	1.25
organic acids	Citrate	1.38*	1.32	1.31	0.96
	Lactate	1.19	1.44*	1.12	1.21
sugars	Glucose	0.95	1.58	1.32	1.67*

Table 4 Pathway enrichment in liver extracts

Black bold represents metabolite differences with $VIP \geq 1$ by PLS-DA analysis

*shows metabolites changes between two groups with $VIP \geq 1$ by PLS-DA analysis and significant differences ($P < 0.05$) by T-test

Classification	Pathway Name	Metabolites Involved	Impact Factor
Amino acid metabolism	Arginine biosynthesis	Fumarate; Glutamine; Glutamate	0.07
	Arginine and proline metabolism	Arginine; Glutamate; Proline	0.12
	Alanine, aspartate and glutamate metabolism	Alanine; Aspartate; Fumarate; Glutamate; Glutamine; Succinate	0.63
	Histidine metabolism	Glutamate; Histidine	0.23
	Phenylalanine metabolism	Phenylalanine; Tyrosine	0.31
	Phenylalanine, tyrosine and tryptophan biosynthesis	Phenylalanine; Tyrosine	0.98
Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	Formate; Glutamate; Glutamine; Glycine; Malate; Serine	0.18
	Inositol phosphate metabolism	Myo-inositol	0.12
	Starch and sucrose metabolism	Glucose; Maltose	0.30
Lipid metabolism	Glycerophospholipid metabolism	Choline; O-Phosphocholine	0.08
Nucleotide metabolism	Pyrimidine metabolism	Glutamine; Hypoxanthine; Inosine; Xanthine	0.02
	Purine metabolism	Glutamine; Inosine; Xanthine	0.12
Other	D-Glutamine and D-glutamate metabolism	Glutamate; Glutamine	0.48
	Glutathione metabolism	Glutamate; Glycine	0.16
	Taurine and hypotaurine metabolism	Taurine	0.48

Table 5 Pathway enrichment in serum extracts

Classification	Pathway Name	Metabolites Involved	Impact Factor
Amino acid metabolism	Alanine, aspartate and glutamate metabolism	Alanine, Glutamine, Aspartate, Citrate, Glutamate, Oxoglutarate, Pyruvate, Succinate	0.63
	Arginine biosynthesis	Arginine; Aspartate; Glutamine; Glutamate; Oxoglutarate	0.20
	Arginine and proline metabolism	Arginine; Creatine; Glutamate; Proline; Pyruvate	0.12
	Cysteine and methionine metabolism	Aminobutyrate; Methionine; Pyruvate; Serine	0.12
	Glycine, serine and threonine metabolism	Betaine; Creatine; Choline; Glycine; Pyruvate; Serine	0.50
	Phenylalanine metabolism	Phenylalanine; Tyrosine	0.31
	Phenylalanine, tyrosine and tryptophan biosynthesis	Phenylalanine; Tyrosine	0.98
	Valine, leucine and isoleucine degradation	Hydroxyisobutyrate; Isoleucine; Leucine; Valine; Oxoisocaproate	0.05
Carbohydrate metabolism	Glycolysis/ Gluconeogenesis	Acetate; Ethanol; Lactate; Pyruvate	0.08
	Glyoxylate and dicarboxylate metabolism	Citrate; Formate; Glutamine; Glutamate; Glycine; Pyruvate; Serine	0.19
	Pyruvate metabolism	Acetate; Lactate; Pyruvate	0.38
	Butanoate metabolism	Acetoacetate; Glutamate; Oxoglutarate; Succinate	0.06
	Citrate cycle (TCA cycle)	Citrate; Oxoglutarate; Pyruvate; Succinate	0.28
	Starch and sucrose metabolism	Glucose	0.26
Lipid metabolism	Glycerophospholipid metabolism	Choline; Ethanolamine	0.12
Nucleotide metabolism	Pyrimidine metabolism	Alanine; Cytidine; Glutamine; Uracil; Uranine	0.03
Other	D-Glutamine and D-glutamate metabolism	Glutamine; Glutamate; Oxoglutarate	0.48
	beta-Alanine metabolism	Aspartate; Alanine; Uracil	0.40

Figures

Figure 1

Histopathological assessment of livers of fish in four diets groups by HE staining. A-D represent the P0, P25, P50 and P100 group, respectively. Lipid droplet is marked by the blue arrowhead; the whole hepatocyte is marked by the yellow dot line circle; Nucleus is labeled by the green triangle. Scale bar is 20 μm .

Figure 2

¹H NMR data-derived PLS-DA score plots (A, C, E and G) and the corresponding first fifteen metabolites with VIP >1 (B, D, F and H) in liver.

Figure 3

^1H NMR data-derived PLS-DA score plots (A, C, E and G) and the corresponding first fifteen metabolites with VIP >1 (B, D, F and H) in serum.

Figure 4

Metabolic pathways affected by SBM in hybrid sturgeon liver extracts. Metabolites in red and green represent higher or lower levels in liver of pairwise groups. Metabolites in blue represent fluctuated changes, whereas italicized font shows the metabolite not detected.

Figure 5

Metabolic pathways affected by SBM in hybrid sturgeon serum extracts. Metabolites in red and green represent increased or decreased levels in serum of pairwise groups. Metabolites in blue represent fluctuated changes, whereas italicized font shows the metabolite not detected.

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