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Effects of Lactic Acid and Betaine As Feed Additives on Metabolomic Profiles of Juvenile Bester Sturgeon (*Acipenser ruthenus* × *Huso huso*)

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Abstract Lactic acid and betaine have shown promising effects on growth in various animals, yet their impact on the growth performance and metabolomics of sturgeon remains underexplored. Using blood samples, this study investigates how these additives influence growth and metabolomics in juvenile bester sturgeon. Initially, lactic acid alone significantly enhanced growth during the first 100 days, but by Day 169, growth rates in the experimental group lagged behind those in the control group. After 258 days of feeding with 5% lactic acid and betaine, growth rates in both groups converged. However, by Day 377, sturgeons fed with 5% betaine exhibited notably poorer growth compared to the control, while those given lactic acid showed improved performance over time. Importantly, both additives induced distinct metabolic shifts, particularly in amino acids and fatty acyls, with lactic acid primarily up-regulating 71 metabolites, accounting for 87.65% of the total number of differential metabolites, and betaine leading to down-regulation of 83 metabolites, accounting for 93.26% of the total number of differential metabolites. Changes in amino acid and fatty acyl metabolites will have an important impact on the flavor and quality of sturgeon. This study highlights the nuanced role of lactic acid and betaine in sturgeon growth and metabolism, offering new insights into their potential as feed additives.

Keywords: *metabolome, targeted metabolomics, metabolism, growth, hybrid sturgeon*

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1. Introduction

Sturgeon, belonging to the family *Acipenseridae*, are an ancient fish originally distributed in the Northern Hemisphere and known as a "living fossil." There are currently 27 known species of sturgeon [1]. In recent years, sturgeon have become well known because their eggs, as caviar, have appeared on tables as a prestigious dish. However, overfishing of wild sturgeon resources for caviar production has led to a sudden decline in wild sturgeon populations. In addition, an increasing number of wild sturgeon species have become extinct or are at risk of extinction due to the greenhouse effect and habitat destruction [2]. As a result, sturgeon have been protected by The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [3]. As the market demand for caviar grows year by year, the expansion of sturgeon aquaculture has become an indispensable way to meet the growing market demand. Therefore, sturgeon aquaculture has flourished in recent years, providing a reliable and high-quality protein source in the form of sturgeon meat, which is rich in a variety of essential amino acids [4]. In addition to caviar, sturgeon meat is a significant contribution to food security. Sturgeon fat is rich in unsaturated fatty acids, such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), arachidonic acid (ARA, 20:4n-6), alpha-linolenic acid (ALA) and, linoleic acid (LA) [5], further enhancing its nutritional value. The excellent qualities of hybrids, such as their high growth rate and early sexual maturity, have also accelerated the development of sturgeon aquaculture, offering a promising future for the industry. The application of biotechnology has shortened the time required for sturgeons to reach sexual maturity while increasing their growth rate [6,7].

Bester sturgeon is a hybrid species generated by crossing female beluga (*Huso huso*) and male sterlet (*Acipenser ruthenus*) [8]. Bester has inherited the excellent qualities of rapid growth from beluga and rapid sexual maturity from sterlet, making it one of the most well-known and most commercialized hybrids [9]. It has also become the primary variety for caviar production [10]. In addition, studies have shown that the collagen in sturgeon's skin, swim bladder, and notochord, which is present in high amounts, has strong stability, providing it with potential for industrial use [11]. Kim [12] evaluated the changes in the metabolic rate of bester during early

development—based on resting routine metabolic rate—to guide the culture of bester. Bester has become one of the indispensable species in sturgeon aquaculture.

As an emerging scientific tool, metabolomics can establish a direct link between the changes in the types and contents of metabolites and the phenotypic changes in the research object. Through qualitative and quantitative analysis of the metabolome, the metabolic pathways of organisms can be studied, and the relationship between the metabolome and its changes in the organism and life activities, such as biological diseases, growth and development, nutrition, and metabolism, can be further revealed. At the same time, in the field of nutrition research, the analytic method of metabolomics can also reflect the effects of exogenous substances on the growth, development, metabolism, reproduction, and other life activities of organisms.

Feed formulations in sturgeon aquaculture are not specific and targeted; primarily fishmeal-based salmonid feeds are used [13], and nutritious and economical sturgeon feed formulations have been explored. Reducing feed costs and improving feed efficiency are crucial for the development of aquaculture [14]. Feed additives are widely used in aquaculture due to their positive effects on fish growth and metabolism [15]. The addition of soybean meal to reduce fishmeal content in their diet resulted in growth restriction in Persian sturgeon, *A. persicus* [16]. However, in white sturgeon (*Acipenser transmontanus*), the addition of soybean meal did not negatively affect caviar production [17]. Acidifiers, such as formic acid, propionic acid, lactic acid, etc., are often added to fish feed as feed additives [18]. These acidifiers play a crucial role in promoting the growth and development of intestinal villi [19], improving the basal health of fish, and increasing the digestibility of some nutrients [20,21]. They also promote the growth of beneficial microorganisms in the gut and reduce intestinal pH, enhancing the digestive and assimilative functions of fish [22,23]. Some studies have demonstrated that acidifiers are also able to inhibit the growth of pathogenic microorganisms, stimulate the immune system of fish, increase antioxidant capacity, and enhance disease resistance in fish [24].

Betaine, the trimethyl derivative of glycine (*N*,*N*,*N*-trimethylglycine), is a compound of great interest. It acts as a methyl donor in the body for the synthesis of other substances. Originally found as a natural by-product in beetroot, it has found applications as a feed additive in poultry farming and aquaculture. One of betaine's capabilities is to act as a feeding attractant to increase the feed intake of aquatic organisms [25]; in addition, it can act as an osmoregulator to maintain the water balance of aquatic organisms [26]. Studies have shown that betaine as a feed additive can alleviate the occurrence of hepatic steatosis and inflammation in black seabream [27]. In a study of zebrafish, betaine was found to be able to regulate fatty acid synthesis [28]. Betaine, as an aquafeed additive, has been shown to exert beneficial effects on aquatic organisms by promoting growth and survival and indirectly participating in protein and fatty acid metabolism [29].

In this study, we hypothesized that lactic acid as a feed additive would have a positive effect on the lipid, amino acid, and carbohydrate metabolism of bester sturgeon, while increasing its growth rate, and that high concentrations of betaine as a feed additive would have a negative effect. This study aims to investigate the effects of lactic acid and betaine as feed additives on the growth performance and physiological metabolism of bester sturgeon. The results can provide a theoretical basis for the improvement of sturgeon growth performance and the development of the sturgeon aquaculture industry.

2. Materials and Methods

2.1. Fish and Diet

Metabolomics analyses were performed on 12 juveniles bester sturgeon (a hybrid of *Huso huso* × *Acipenser ruthenus*), of which six were 1.2 years old and six were 2.3 years old at the beginning of the study—these were treated as the "younger" group and the "elder" groups, respectively. Juvenile sturgeon represent an early developmental stage in which metabolic pathways are highly active. Studying the metabolome of juvenile sturgeon provides insights into their developmental biology, including changes in energy metabolism, growth, and organ development. Juvenile sturgeon are more sensitive to external changes compared to adult sturgeon. Six individuals of each group were equally divided into two subgroups of three individuals each. One subgroup acted as the control subgroup and the other as the experimental subgroup fed with the additives of *L*-lactic acid and betaine. Hence, four subgroups were used in this study.

Fish of a subgroup were reared in a dedicated recirculated pool (2.09 m × 1.08 m × 0.60 m deep, 1.35 m^3), and four pools were used for the four subgroups. The pools were located side by side in a "patio" of the Hiroshima University campus. The water exchange rate was >1/3 of the pool volume per day; the pH value was 7-8, and the dissolved oxygen level was >6 mg l^{-1} during the study period.

The basal feed was fast-sinking cylindrical pellets, "2P" (ca. 1 mm wide and 2 mm long) for the younger group or "4.5P" (ca. 2 mm wide and 4.5 mm long) for the elder group. The feeds differed only in size; they had the same ingredients of crude protein, >45.0%; crude fat, >6.0%; crude fiber, <3.0%; crude ash, <15.0%; calcium,>1.60%; and phosphorus, >1.20% and were manufactured at Scientific Feed Laboratory Co. Ltd. (Tokyo, Japan).

The feed additives used in this study were *L*-lactic acid and betaine. A miscible 50% solution of *L*-lactic acid was provided by Musashino Chemical Laboratory, Ltd. (Tokyo, Japan). Highly purified (>97%) betaine, i.e., *N*,*N*,*N*-trimethylglycine, was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The feed pellets for the experimental subgroups were sprayed with x1/10 weight of 50% (w/v) solution of *L*-lactic acid or betaine, or both, to prepare 5% added pellets. Pellets that contained less or more than the 5% added pellets were also prepared specifically for the first 23 days of the experimental period, during which experimental fish were acclimatized to the additives. The added pellets were stirred by roll-shaking for homogenization until they were dry.

For the younger, experimental subgroup, the *L*-lactic acid content of the feed was gradually increased from 0%

to 6% during the first 23 days and set to 5% from Day 24 to Day 169. For the elder, experiment subgroup, the betaine content of the feed was also gradually increased from 0% to 4.3% during the first 23 days and set to 5% from Day 24 to Day 169.

From Day 170 to the end of the study (Day 546), both subgroups were fed with double-added pellets containing 5% *L*-lactic acid and 5% betaine. The differences between the two experimental subgroups were the ages (and age-related body weights) and the history of food additives, i.e., betaine first or *L*-lactic acid first. The feeding timeline is summarized in Table 1.

Table 1. Timeline of feed additives used for the "experimental" subgroups.

	Day 1 to Day 23	Day 24 to Day 169	Day 170 to Day 546
Younger	0%-6% *L*-lactic acid	5% *L*-lactic acid	5% *L*-lactic acid
Elder	0%-4.3% betaine	5% betaine	5% betaine

2.2. Blood Sample Collection and Metabolomic Analysis

Blood samples were collected from sturgeon that had been reared for 101, 169, 427, and 546 days. Sample codes and information on the time of sample collection, age, and weight of the sturgeon at the time of sample collection are summarized in Table S1. Juveniles sturgeon were weighed prior to each blood sample collection, and growth parameters were measured (Table S2 and Figure S1). One bester individual in the betaine experimental group died after sampling on Day 427; as a result, its body weight and daily specific growth rate from sampling on Day 546 could not be calculated. All sturgeon were humanely culled at the last collection (Day 546) of blood samples by a combination of impingement to unconsciousness and nerve stimulation, which prevents unnecessary pain in the fish [30].

Blood was collected from the base of the pectoral fins of the sturgeon. While ensuring that sufficient blood samples are collected, the size of the wound is minimized to lessen physical damage to the sturgeon and ensure its continued survival. Blood samples were aspirated using sterilized beral pipettes and quickly transferred to pre-cooled sterilized 2.0 ml centrifuge tubes on an ice box and sealed with a sealing film. The collected blood samples were immediately stored in a refrigerator at -80°C to ensure the quality of the blood samples and to minimize errors in metabolomics analysis. Finally, the blood samples were transported to the BGI company (formerly Beijing Genomics Institute, Shenzhen, China; https://www.bgi.com/global/home) for metabolomics analysis by dry ice storage. Separation of metabolite molecules was performed by ultra-performance liquid chromatography (UPLC), and metabolite identification was performed by tandem mass spectrometry (MS/MS) (details described in [5]). Blood samples collected on Day 101 and Day 169 were analyzed for targeted metabolomics at the same time using the HM350 panel; the results produced the HM350 dataset. Blood samples collected on Day 427 and Day 546 were analyzed for targeted metabolomics separately using the HM400 panel and the HM400-1 (Day 427) and HM400-2 (Day 546) datasets, respectively.

2.3. Data Acquisition and Preprocessing

Manual inspection was assisted by Skyline (MacCoss Lab Software, https://skyline.ms/project/home/software/Skyline/begin.view) to perform metabolite identification and quantification with default parameters. Then, a data matrix containing information such as metabolite identification results and quantitative results was obtained, and the table was further processed for bioinformatic analyses.

2.4. Statistical and Bioinformatic Analyses

Metabolomic data were standardized or normalized to improve their normality before statistical analysis. As some bioinformatic analyses require triplicate (n = 3) or more data, a synthetic set of data were used for the betaine experimental group of Day 546, in which one individual had died before the blood sampling. The synthetic data were generated by averaging the data of the remaining two individuals.

Principal component analysis (PCA) and orthogonal partial least squares discrimination analysis (OPLS-DA) were performed to visualize the relatedness of the metabolomic profiles of blood samples at Metware Cloud, a public online platform for data analysis (https://cloud.metware.cn). Orthogonal partial least squares discrimination analysis (OPLS-DA) was also used to analyze the differences between different groups. PCA is an unsupervised data dimensionality reduction analysis method with no grouping information when calculating. OPLS-DA is a supervised multivariate statistical analysis method that groups information when calculating. It combines the regression model between metabolite changes and experimental groups while reducing the dimensionality and uses a certain discrimination threshold to perform discriminant analysis on the regression results. Compared with PCA, OPLS-DA analysis can further show the differences between groups.

The differential metabolite screening process was performed using a combination of multivariate statistical analysis based on variable importance in projection (VIP) scores and univariate statistical analysis based on fold-change values, which can help to obtain more accurate analysis results by avoiding false-positive errors or overfitting of the model caused by using only one statistical analysis method. Differential results were visualized in the form of volcano plots [31]. Linear discriminant analysis (LDA) effect size analysis (LEfSe) was also performed online at the Hutternhower Lab, Biostatistics Department, Harvard T. H. Chan School of Public Health (https://huttenhower.sph.harvard.edu/lefse/; [32]) for differential metabolite screening. Differential metabolites were further projected on the metabolic pathway maps generated by the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/pathway.html; [33]). KEGG enrichment analysis was performed on the differential metabolites, and the KEGG metabolic pathways enriched with differential metabolites were visualized in the form of bubble diagrams.

2.5. Animal Ethics

This study on bester sturgeon was carried out following the guidelines established by the Animal Care and Use Committee of Hiroshima University (approval number F24-2) and under the guidelines and regulations relevant to the Sustainable Aquaculture Production Assurance Act, Japan (https://www.japaneselawtranslation.go.jp/en/laws/view/3674).

3. Results

3.1. Fish Growth and Blood Samplings

Sturgeon were in good growth conditions throughout the experimental period (absolute values are shown in Table S1), although one individual in the betaine experimental subgroup died after the sampling on Day 427. Initial body weight (W_0) was measured for each individual sturgeon before the start of the feeding experiment (Day 0), and body weight (WT) was also measured for each individual sturgeon at each blood sample collection. Blood samples were taken four times during the experiment: on Days 101, 169, 427, and 546.

Sturgeon body weight was measured, and growth performance metrics such as daily specific growth rate (SGR) were calculated for each sturgeon individual at each sampling time (Table S2). Because one individual died before the last sampling on Day 546, the growth performance metrics on Day 546 could not be calculated. The mean values of the growth parameters of each group of sturgeon and their corresponding standard deviation (SD) values are summarized in Table 2.

Table 2. The mean values of the growth parameters and their corresponding standard deviation values

		Day 0	Day 101	Day 169	Day 427	Day 546
Betaine	BW (kg)	1.357 ± 0.17	1.797 ± 0.28	1.894 ± 0.35	2.128 ± 0.46	2.925 ± 0.16
	SD of BW	0.292	0.487	0.600	0.795	0.228
	SGR, %/d		0.27 ± 0.11	0.06 ± 0.05	0.04 ± 0.02	0.15 ± 0.11
	SD of SGR		0.190	0.080	0.029	0.161
Lactic acid	BW (kg)	0.3 ± 0.01	0.487 ± 0.02	0.614 ± 0.03	1.013 ± 0.09	1.209 ± 0.05
	SD of BW	0.022	0.036	0.048	0.163	0.081
	SGR, %/d		0.48 ± 0.06	0.34 ± 0.02	0.19 ± 0.03	0.15 ± 0.04
	SD of SGR		0.103	0.035	0.043	0.076
BC	BW (kg)	1.415 ± 0.15	2.007 ± 0.18	2.154 ± 0.15	2.829 ± 0.24	3.13 ± 0.18
	SD of BW	0.252	0.307	0.265	0.412	0.304
	SGR, %/d		0.35 ± 0.03	0.11 ± 0.05	0.1 ± 0.01	0.09 ± 0.03
	SD of SGR		0.046	0.087	0.009	0.057
LC	BW (kg)	0.339 ± 0.02	0.48 ± 0.03	0.701 ± 0.08	1.107 ± 0.12	1.386 ± 0.12
	SD of BW	0.038	0.058	0.132	0.203	0.207
	SGR, %/d		0.34 ± 0.06	0.55 ± 0.06	0.18 ± 0.02	0.19 ± 0.02
	SD of SGR		0.102	0.105	0.037	0.033

As the feeding time increased, no significant differences in growth parameters between the experimental group and the control group were observed following the addition of *L*-lactic acid (*p*-values were higher than 0.05), although, based on the growth results in the first 100 days of feeding, lactic acid promoted the growth of sturgeon (Figure S1). In contrast, based on the growth parameters, the addition of possibly excessive amounts of betaine inhibited the weight gain of sturgeon during the entire feeding period. However, the seemingly inhibitory effect of betaine was nuanced with the co-addition of lactic acid after the 170-th day (Table 1). The *t*-test analysis results (*p*-values were higher than 0.05) of the growth parameters of the samples at each sampling time in the lactic acid and betaine groups showed that the difference in weight gain caused by feed additives was not significant. We can only discern subtle growth differences from the size of the growth parameters of the experimental group and the control group.

3.2. Metabolomic Profiles

The four collected blood samples were analyzed via metabolomics three times, and finally, three sets of raw metabolomics data were obtained: HM350, HM400-1, and HM400-2. The HM350 panel was used to generate the HM350 metabolomic dataset from the blood samples collected on Day 101 and Day 169. The HM400 panel was used to generate the HM400-1 and HM400-2 datasets separately from the blood samples collected on Day 427 and Day 546, respectively. The identified metabolites were categorized into super-class, class, and sub-class based on the HMDB database (Human Metabolome Database: https://hmdb.ca/). The original datasets are available at https://www.sturgeon-metabolome.hiroshima-u.ac.jp/h0888_Bet-Lac_HM350_Day101_Day169.ods, https://www.sturgeon-metabolome.hiroshima-u.ac.jp/h0888_Bet-Lac_HM400-1_Day427.ods, and https://www.sturgeon-metabolome.hiroshima-u.ac.jp/h0888_Bet-Lac_HM400-2_Day546.ods.

The results of metabolomics analysis (HM350 dataset) of blood samples from Day 101 and Day 169 showed that a total of 244 metabolites were identified, of which 231 metabolites were completed with categorical annotations in the HMDB database. The remaining 13 metabolites were not categorically annotated with the HMDB database (Table S3, Figure 1A). The 231 metabolites were categorized into eight super-classes, 19 classes, and 38 sub-classes. Among the super-class classification, 96 metabolites were annotated as organic acids and derivatives (39.3%), and 73 metabolites were annotated as lipids and lipid-like molecules (29.9%). In the class category, 79 metabolites were annotated as carboxylic acids and derivatives (32.4%), and 58 metabolites were annotated as fatty acyls (23.8%). In the sub-class category, 66 and 36 metabolites were annotated as amino acids, peptides, and analogs (27.1%) and as fatty acids and conjugates (14.8%), respectively.

The results of the metabolomics analysis (HM400-1 dataset) of blood samples from Day 427 showed that a total of 234 metabolites were identified, of which 212 metabolites were completed with categorical annotations in the HMDB database. The remaining 22 metabolites were not categorically annotated with the HMDB database (Table S4, Figure 1B). The 212 metabolites were

categorized into eight super-classes, 18 classes, and 35 sub-classes. Among the super-class classification, 83 metabolites were annotated as organic acids and derivatives (35.5%), and 75 metabolites were annotated as lipids and lipid-like molecules (32.1%). In the class category, 67 metabolites were annotated as carboxylic acids and derivatives (28.6%), and 56 metabolites were annotated as fatty acyls (23.9%). In the sub-class category, 56 and 44 metabolites were annotated as amino acids, peptides, and analogs (23.9%) and as fatty acids and conjugates (18.8%), respectively.

The dataset HM400-2 (Day 546) was similarly comprehensive, consisting of 266 metabolites, of which 241 were annotated and 25 were not annotated (Table S5, Figure 1C). These 241 metabolites were also categorized into eight super-classes, 18 classes, and 35 sub-classes. In the super-class category, 96 were annotated as lipids and lipid-like molecules (36.1%), and 82 were annotated as organic acids and derivatives (30.8%). In the class category, 70 were annotated as fatty acyls (26.3%), and 68 metabolites were annotated as carboxylic acids and derivatives (25.6%). In the sub-class category, 58 and 57 were annotated as fatty acids and conjugates (21.8%) and as amino acids, peptides, and analogs (21.4%), respectively.

3.3. Multivariate Statistical Analyses

According to the type of feed additives and the number of feeding days, the metabolomic profiles of the blood samples collected on four occasions were subjected to principal component analysis (PCA). The results showed that there were differences in blood metabolites between the experimental group and the control group (Figure 2). The experimental group sturgeon in Figure 2A were initially fed with lactic acid as the feed additive. Only lactic acid was used as a feed additive from Day 0 to Day 169, and 5% lactic acid and 5% betaine were used as feed additives from Day 170 to Day 546. The experimental group sturgeon in Figure 2B were initially fed with betaine as the feed additive. Only betaine was used as a feed additive from Day 0 to Day 169, and 5% lactic acid and 5% betaine were used as feed additives from Day 170 to Day 546. The PCA analysis results in Figure 2A show that, when 5% lactic acid was used alone as a feed additive, the metabolite differences between the experimental group and the control group were relatively obvious on Day 101. Still, as the feeding time was extended to Day 169, the differences in blood metabolites between the experimental group and the control group gradually became obvious. When the feed additive was changed to 5% lactic acid and 5% betaine, the difference in metabolites gradually decreased. Still, as the number of feeding days increased, the difference in metabolites gradually became obvious. The PCA analysis results in Figure 2B show that, when 5% betaine was used as a feed additive alone for 101 days of feeding, there were differences in metabolites between the experimental and control groups, but these differences were not particularly obvious. Similarly, as the feeding time was extended to 169 days, the difference in blood metabolites between the experimental group and the control group gradually became clearer. When the feed additive was changed to 5% lactic acid and 5% betaine on Day 170, the difference in metabolites in the blood samples on Day 427 was not obvious. Still, as the number of feeding days increased to Day 546, the difference in metabolites gradually became apparent. Comparing Figure 2A and Figure 2B at the same sampling time, the difference caused by betaine is more obvious than the difference caused by lactic acid. The PCA results reflect the differences within and between groups, which are manifested in changes in metabolites. The clustering of samples within the group indicated that there is no difference in metabolites between biological replicates within the group. The separation of samples between groups indicated that there are differences between the experimental group and the control group, which are mainly reflected in the differences in metabolites. The differences in metabolites between the experimental and control group are mainly caused by lactic acid and betaine as feed additives.

The results of OPLS-DA analysis based on samples from different groups and different sampling times showed that the values of an orthogonal component Q^2 were close to or greater than 0.5, indicating that the OPLS-DA model had better goodness of fit and prediction ability [34]. The results of the OPLS-DA analysis showed that there were differences between the metabolites of the experimental group and the control group (Figure 3). Similarly, the differences became more obvious with the increase in feeding time. However, in the lactic acid group (Figure 3A), the Q^2 of Day 169 was lower than that of Day 101. Comparison between the lactic acid group and the betaine group (Figure 3B) showed that the separation degree between the experimental group and the control group in the betaine group was higher than that in the lactic acid group, indicating that the metabolic differences caused by betaine were more obvious.

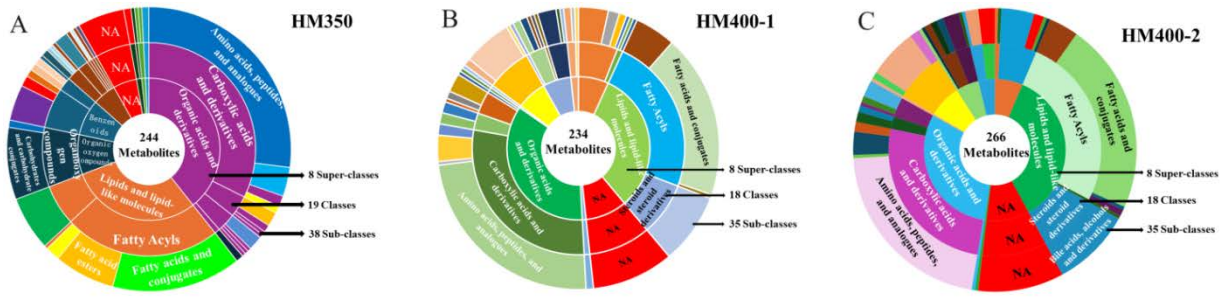

Figure 1. Statistics and hierarchical assignment (annotation) of the detected metabolites from the datasets HM350 (Days 101 and 169), HM400-1 (Day 427), and HM400-2 (Day 546). The innermost, intermediate, and outermost sections of the sunbursts represent the super-class, class, and sub-class categories, respectively. "NA" represents "not annotated."

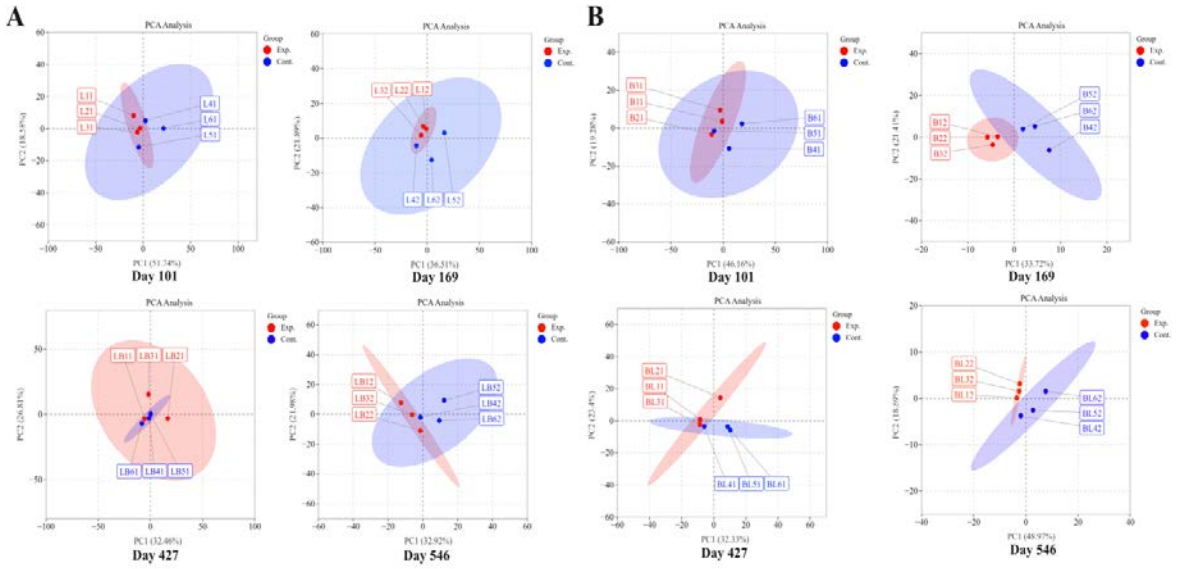

Figure 2. Principal component analysis (PCA) analysis based on samples from different groups and different sampling times shows that the metabolite differences caused by a single feed additive gradually emerge with the extension of feeding time. When compound feed additives are used for feeding, the differences are not obvious under short-term feeding, but as the feeding time reaches 546 days, the metabolite differences gradually emerge. **A** and **B** represent the PCA results of the lactic acid group and the betaine group, respectively

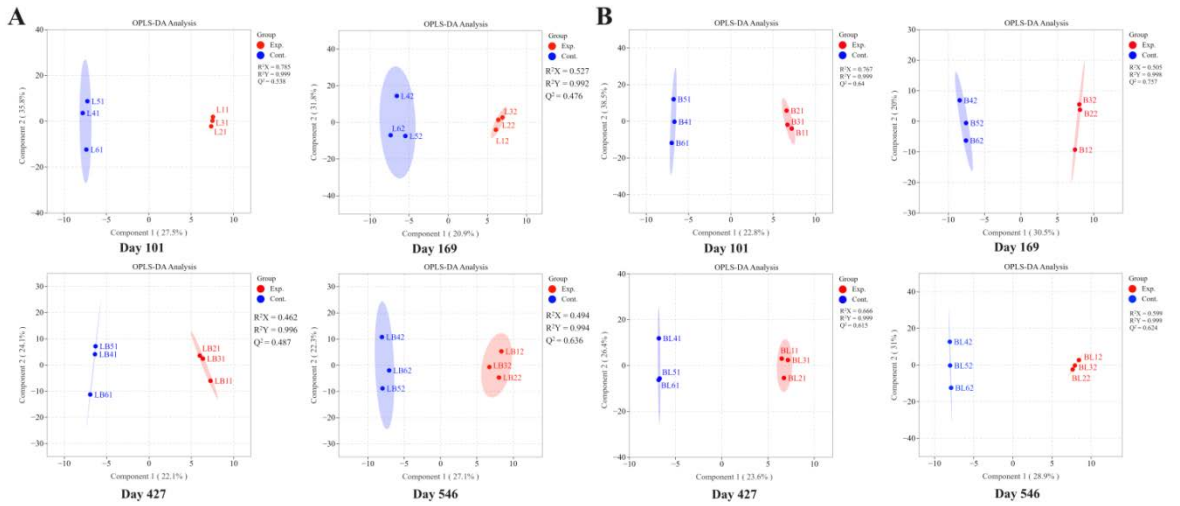

Figure 3. Orthogonal partial least squares discrimination analysis (OPLS-DA) based on feed supplement composition and feeding days to show separation between the experimental groups and the control groups. **A** and **B** represent the OPLS-DA results for the lactic acid group and the betaine group, respectively

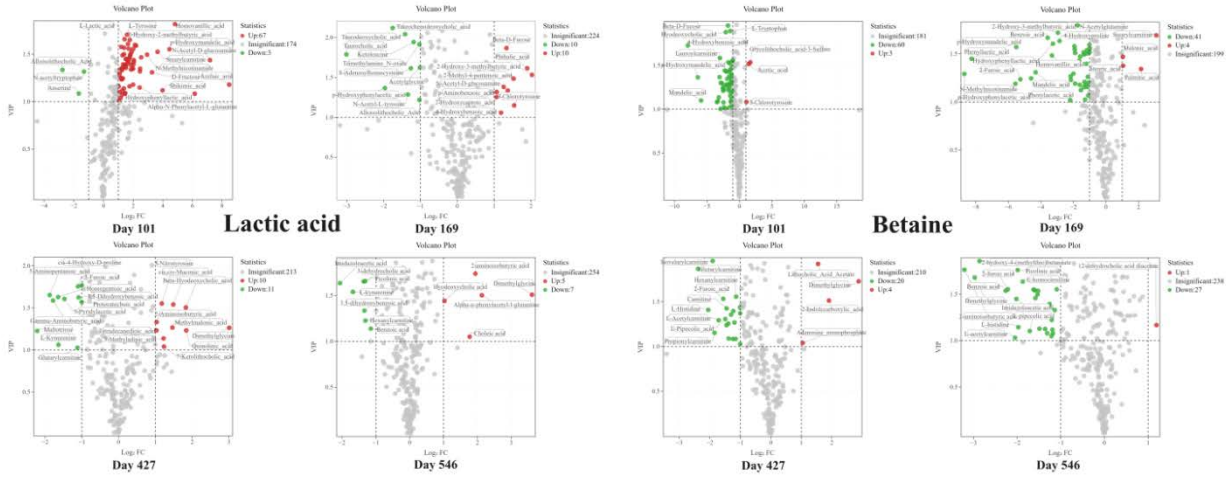

Figure 4. Differential metabolites visualized by volcano plots. Red (up) and green (down) represent the upregulated and downregulated metabolites in the set experimental groups, respectively

3.4. Differential Metabolite Analysis

Differential metabolites were screened with multivariate and univariate statistical significance criteria, i.e., VIP score ≥1 and fold change >2 or <0.5, respectively, and the results were visualized using a volcano plot (Figure 4) and heat maps (Figures S2 and S3).

Table 3. The top ten differential metabolites based on fold change at each sampling time in the lactic acid group.

Lactic acid group							
	Differential Metabolites	Class	Control (umol/L)	Experimental (umol/L)	VIP	Fold change	Type
Day 101	Azelaic acid	Fatty Acyls	4.67E-04	0.17	1.18	357.79	up
	N-Acetyl-D-glucosamine	Organooxygen compounds	0.02	3.13	1.44	145.50	up
	Alpha-N-Phenylacetyl-L-glutamine	Carboxylic acids and derivatives	3.77E-03	0.27	1.08	71.42	up
	Homovanillic acid	Phenols	2.43E-03	0.07	1.82	28.34	up
	p-Hydroxymandelic acid	Phenols	4.67E-04	0.01	1.55	21.86	up
	Shikimic acid	Organooxygen compounds	4.97E-03	0.08	1.12	15.80	up
	Stearylcarnitine	Fatty Acyls	0.02	0.19	1.52	12.54	up
	D-Fructose	Organooxygen compounds	0.23	2.17	1.31	9.65	up
	N-Methylnicotinamide	Pyridines and derivatives	1.17	8.96	1.50	7.68	up
	Alloisolithocholic Acid	NA	0.05	0.01	1.33	1/7	down
Day 169	2-Hydroxy-3-methylbutyric acid	Fatty Acyls	0.73	2.99	1.53	4.09	up
	Phthalic acid	Benzene and substituted derivatives	0.02	0.06	1.61	3.74	up
	3-Chlorotyrosine	Carboxylic acids and derivatives	2.64	7.72	1.15	2.92	up
	2-Methyl-4-pentenoic acid	Fatty Acyls	1.07	3.09	1.48	2.89	up
	3-4-Dihydroxyhydrocinnamic acid	Phenylpropanoic acids	0.59	1.52	1.34	2.59	up
	N-Acetyl-L-tyrosine	Carboxylic acids and derivatives	0.17	0.07	1.29	1/2.4	down
	Taurodeoxycholic acid	Steroids and steroid derivatives	0.08	0.03	2.04	1/2.7	down
	Taurochenodeoxycholic acid	Steroids and steroid derivatives	0.09	0.03	2.12	1/3	down
	p-Hydroxyphenylacetic acid	Phenols	0.22	0.06	1.37	1/3.7	down
	Taurocholic acid	Steroids and steroid derivatives	2.67	0.33	1.79	1/8.1	down
Day 427	Dimethylglycine	Carboxylic acids and derivatives	1.42	11.41	1.26	8.05	up
	Quinolinic acid	Pyridines and derivatives	28.47	102.02	1.23	3.58	up
	Beta-Hyodeoxycholic acid	Steroids and steroid derivatives	0.01	0.03	1.50	3.56	up
	cis,cis-Muconic acid	Fatty Acyls	0.25	0.71	1.54	2.80	up
	3-Pyridylacetic acid	Pyridines and derivatives	0.04	0.01	1.61	1/4	down
	L-Kynurenine	Organooxygen compounds	1.78	0.57	1.06	1/3.1	down
	Homogentisic acid	Benzene and substituted derivatives	0.32	0.10	1.63	1/3.2	down
	Gamma-Aminobutyric acid	Carboxylic acids and derivatives	41.61	12.08	1.59	1/3.4	down
	5-Aminopentanoic acid	Carboxylic acids and derivatives	34.90	9.51	1.65	1/3.7	down
	Maltotriose	Organooxygen compounds	454.19	97.83	1.23	1/4.6	down
Day 546	Dimethylglycine	Carboxylic acids and derivatives	0.49	6.03	1.51	12.23	up
	Alpha-n-phenylacetyl-l-glutamine	Carboxylic acids and derivatives	0.09	0.38	1.50	4.38	up
	2-aminoisobutyric acid	Carboxylic acids and derivatives	127.88	490.36	1.74	3.83	up
	Choleic acid	Steroids and steroid derivatives	0.01	0.04	1.05	3.41	up
	Hexanylcarnitine	Fatty Acyls	0.01	2.35E-03	1.23	1/2.5	down
	Picolinic acid	Pyridines and derivatives	1.35	0.54	1.66	1/2.5	down
	3,5-dihydroxybenzoic acid	Benzene and substituted derivatives	0.85	0.34	1.33	1/2.5	down
	3-dehydrocholic acid	Steroids and steroid derivatives	0.30	0.12	1.65	1/2.5	down
	L-kynurenine	Organooxygen compounds	0.43	0.13	1.54	1/3.3	down
	Imidazoleacetic acid	Azoles	12.63	3.03	1.63	1/4.2	down

Table 4. The top ten differential metabolites based on fold change at each sampling time in the betaine group

Betaine group							
	Differential Metabolites	Class	Control (umol/L)	Experimental (umol/L)	VIP	Fold Change	Type
Day 101	N-Acetyl-D-glucosamine	Organooxygen compounds	1.73	0.24	1.30	1/7	down
	p-Hydroxyphenylacetic acid	Phenols	1.51	0.20	1.00	1/7	down
	Lauroylcarnitine	Fatty Acyls	2.13E-03	2.67E-04	1.58	1/8	down
	Chenodeoxycholic acid	Steroids and steroid derivatives	0.02	2.27E-03	1.37	1/8	down
	L-Methionine	Carboxylic acids and derivatives	10.56	1.17	1.13	1/9	down
	Phenylacetic acid	Benzene and substituted derivatives	159.14	15.68	1.09	1/10	down
	N-Methylnicotinamide	Pyridines and derivatives	128.75	11.70	1.08	1/11	down
	Mandelic acid	Benzene and substituted derivatives	0.01	2.00E-04	1.10	1/60	down
	p-Hydroxymandelic acid	Phenols	0.02	2.00E-04	1.36	1/84	down
	4-Hydroxybenzoic acid	Benzene and substituted derivatives	0.05	2.00E-04	1.73	1/244	down
Day 169	Hydroxyphenyllactic acid	Phenylpropanoic acids	0.45	0.05	1.61	1/9	down
	Homovanillic acid	Phenols	0.17	0.02	1.48	1/9	down
	Benzoic acid	Benzene and substituted derivatives	18.27	1.53	1.67	1/12	down
	Mandelic acid	Benzene and substituted derivatives	0.01	3.67E-04	1.31	1/20	down
	Phenylacetic acid	Benzene and substituted derivatives	4.40	0.18	1.25	1/24	down
	2-Furoic acid	Furans	0.20	0.01	1.24	1/38	down
	p-Hydroxymandelic acid	Phenols	0.02	3.67E-04	1.57	1/46	down
	N-Methylnicotinamide	Pyridines and derivatives	3.83	0.08	1.19	148	down
	Phenyllactic acid	Benzene and substituted derivatives	0.11	3.67E-04	1.45	1/303	down
	p-Hydroxyphenylacetic acid	Phenols	0.15	3.67E-04	1.29	1/417	down
Day 427	Dimethylglycine	Carboxylic acids and derivatives	1.01	7.47	1.73	7.36	up
	2-Indolecarboxylic acid	Indoles and derivatives	0.07	0.27	1.51	3.75	up
	Lithocholic Acid Acetate	NA	2.67E-03	0.01	1.92	2.93	up
	L-Pipecolic acid	Carboxylic acids and derivatives	7.91	2.81	1.31	1/3	down
	2-Furoic acid	Furans	0.23	0.08	1.53	1/3	down
	L-Acetylcarnitine	Fatty Acyls	236.58	72.22	1.29	1/3	down
	Propionylcarnitine	Fatty Acyls	4.79	1.33	1.15	1/3.6	down
	Isovelarylcarnitine	Fatty Acyls	0.06	0.02	1.96	1/4	down
	L-Histidine	Carboxylic acids and derivatives	1060.87	255.60	1.41	1/4	down
	Glutarylcarnitine	Fatty Acyls	0.22	0.04	1.87	1/5	down
Day 546	L-homocitrulline	Carboxylic acids and derivatives	5.65	1.54	1.63	1/3.7	down
	L-histidine	Carboxylic acids and derivatives	145.77	37.17	1.14	1/3.9	down
	Picolinic acid	Pyridines and derivatives	0.27	0.07	1.76	1/4	down
	Imidazoleacetic acid	Azoles	3.38	0.84	1.45	1/4	down
	L-acetylcarnitine	Fatty Acyls	69.02	16.69	1.03	1/4	down
	2-furoic acid	Furans	1.06	0.23	1.55	1/4.6	down
	Benzoic acid	Benzene and substituted derivatives	14.25	2.97	1.54	1/4.8	down
	2-hydroxy-4-(methylthio)butanoate	Fatty Acyls	3.89	0.53	1.86	1/7.3	down
	Dimethylglycine	Carboxylic acids and derivatives	3.68	0.47	1.68	1/7.8	down
	2-aminoisobutyric acid	Carboxylic acids and derivatives	662.70	71.78	1.76	1/9.2	down

In the lactic acid group (Table S6), the blood samples of sturgeon fed for 101 days were screened for differential metabolites, and a total of 70 differential metabolites were screened, of which 67 differential metabolites were up-regulated and 3 differential metabolites were down-regulated. Twenty-seven differential metabolites belonged to fatty acyls, showing differences in lipid metabolism. Eleven differential metabolites belonged to amino acids, peptides, and analogs, showing differences in amino acid metabolism. Nine differential metabolites belonged to carbohydrates and carbohydrate conjugates, showing differences in carbohydrate metabolism. The blood

samples of sturgeon fed for 169 days were screened for differential metabolites, and a total of 20 differential metabolites were screened, of which 10 differential metabolites were up-regulated and 10 differential metabolites were down-regulated. The up-regulated differential metabolites were mainly attributed to fatty acids and conjugates, carbohydrates and carbohydrate conjugates, and benzoic acids and derivatives, and the down-regulated differential metabolites were mainly attributed to amino acids, peptides, and analogs and to bile acids, alcohols, and derivatives. Differential metabolites of sturgeon fed for 427 days were screened, and a total of 21 differential metabolites were screened, of which 10 were up-regulated, and 11 were down-regulated. Four differential metabolites belonged to fatty acyls, showing differences in lipid metabolism. Six differential metabolites belonged to amino acids, peptides, and analogs, showing differences in amino acid metabolism. Differential metabolites of sturgeon fed for 546 days were screened and a total of twelve differential metabolites were screened, of which five were up-regulated, and seven were down-regulated. The differential metabolites were mainly classified into amino acids, peptides, and analogs; bile acids, alcohols and derivatives; and benzoic acids and derivatives. The top ten differential metabolites based on fold change at each sampling time in the lactic acid group are presented in Table 3. Most of the differential metabolites screened in the lactic acid group were up-regulated in the experimental group, and most of the upregulated differential metabolites belonged to amino acid and fatty acyl groups. Up-regulated amino acid and fatty acyl metabolites may not only improve the flavor and quality of sturgeon, but also promote sturgeon growth. Most of the differential metabolites screened in the betaine group were down-regulated in the experimental group, indicating that, as a feed additive, 5% betaine played a negative role in improving the quality of sturgeon.

In the betaine group (Table S7), differential metabolites were screened for sturgeons fed for 101 days, and a total of 63 differential metabolites were screened, of which three differential metabolites were up-regulated and 60 differential metabolites were down-regulated. The differential metabolites were mainly classified into fatty acyls (15 metabolites); carbohydrates and carbohydrate conjugates (10 metabolites); amino acids, peptides, and analogs (10 metabolites); benzene and substituted derivatives (7 metabolites); and steroids and steroid derivatives (5 metabolites). Differential metabolites were screened for sturgeons fed for 169 days, and a total of 45 differential metabolites were screened, of which 4 differential metabolites were up-regulated and 41 differential metabolites were down-regulated. The differential metabolites were mainly classified into amino acids, peptides, and analogs (11 metabolites); fatty acyls (9 metabolites); benzene and substituted derivatives (7 metabolites); and dicarboxylic acids and derivatives (5 metabolites). Differential metabolites of sturgeons fed for 427 days were screened, and a total of 24 differential metabolites were screened, of which 4 differential metabolites were up-regulated and 20 differential metabolites were down-regulated. The differential metabolites were mainly classified into fatty acyls (9 metabolites) and amino acids, peptides, and analogs (5 metabolites). Differential metabolites of sturgeons fed for 546 days were screened, and a total of 28 differential metabolites were screened, of which 1 differential metabolite was up-regulated and 27 differential metabolites were down-regulated. The differential metabolites were mainly classified into amino acids, peptides, and analogs (14 metabolites) and fatty acyls (6 metabolites). The top ten differential metabolites based on fold change at each sampling time in the betaine group are presented in Table 4.

3.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis of Differential Metabolites

KEGG metabolic pathway enrichment analysis was performed on the screened differential metabolites (Figure 5). The results of the lactic acid group showed that 70 differential metabolites in the samples of Day 101 were enriched in 91 metabolic pathways, of which 20 metabolic pathways with high significance are displayed in the form of bubble charts (Figure 5A). According to the *p*-value, the most abundant metabolic pathway are thiamine metabolism, pentose and glucuronate interconversions, phenylalanine metabolism, pyruvate metabolism, pentose phosphate pathway, phenylalanine tyrosine, and tryptophan biosynthesis, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, tyrosine metabolism, biosynthesis of nucleotide sugars, glycerolipid metabolism, terpenoid backbone biosynthesis, glycolysis/gluconeogenesis, fatty acid degradation, primary bile acid biosynthesis, phosphonate and phosphinate metabolism, and glutathione metabolism. A total of 20 differential metabolites from samples from Day 169 were enriched in 20 metabolic pathways; these are presented in bubble plots (Figure 5A). The most enriched metabolic pathways based on *p*-values, were folate biosynthesis, primary bile acid biosynthesis, biosynthesis of nucleotide sugars, amino sugar and nucleotide sugar metabolism, ubiquinone and another terpenoid–quinone biosynthesis, tyrosine metabolism, biosynthesis of cofactors, taurine and hypotaurine metabolism, lipoic acid metabolism, valine leucine, and isoleucine biosynthesis, valine leucine and isoleucine degradation, phenylalanine metabolism, cysteine and methionine metabolism, ABC transporters, biosynthesis of amino acids, and 2-oxocarboxylic acid metabolism. A total of 21 differential metabolites from Day 427 samples were enriched in 30 metabolic pathways; these are presented in bubble plots (Figure 5A). The most enriched metabolic pathways, based on *p*-values were biosynthesis of cofactors, propanoate metabolism, butanoate metabolism, pyrimidine metabolism, alanine aspartate and glutamate metabolism, glycine serine, and threonine metabolism, valine leucine and isoleucine degradation, lysine degradation, arginine and proline metabolism, tyrosine metabolism, tryptophan metabolism, phenylalanine tyrosine and tryptophan biosynthesis, beta-alanine metabolism, D-amino acid metabolism, nicotinate and nicotinamide metabolism, ubiquinone and other terpenoid-quinone biosynthesis, ABC transporters, cAMP signaling pathway, and carbohydrate digestion and absorption. Twelve differential metabolites in the samples from Day 546 were enriched in

eight metabolic pathways and displayed in the form of bubble diagrams (Figure 5A); these metabolites were biosynthesis of cofactors, glycine serine and threonine metabolism, histidine metabolism, phenylalanine metabolism, tryptophan metabolism, and bile secretion.

The results of the betaine group showed that 63 differential metabolites in the samples from Day 101 were enriched in 82 metabolic pathways; the result in 20 metabolic pathways with high significance are displayed in the form of bubble charts (Figure 5B). According to the *p*-value, the most abundant metabolic pathways were amino sugar and nucleotide sugar metabolism, folate biosynthesis, pentose and glucuronate interconversions, galactose metabolism, phenylalanine tyrosine and tryptophan biosynthesis, carbohydrate digestion and absorption, ubiquinone and other terpenoid-quinone biosynthesis, phenylalanine metabolism, glycolysis/gluconeogenesis, starch and sucrose metabolism, biosynthesis of nucleotide sugars, 2-oxocarboxylic acid metabolism, arginine biosynthesis, monobactam biosynthesis, and biosynthesis of cofactors. Forty-five differential metabolites in the samples from Day 169 were enriched in 59 metabolic pathways; the 20 most significant metabolic pathways are displayed in the form of bubble charts (Figure 5B). According to the *p*-value, the most abundant metabolic pathways were fatty acid biosynthesis, fatty acid metabolism, propanoate metabolism, sulfur metabolism, fatty acid degradation, cAMP signaling pathway, tyrosine metabolism, fatty acid elongation, arginine and proline metabolism, galactose metabolism, phenylalanine metabolism, pyrimidine metabolism, carbohydrate digestion and absorption, oxidative phosphorylation, and folate biosynthesis. Twenty-four differential metabolites in the samples from Day 427 were enriched in 55 metabolic pathways; the 20 most significant metabolic pathways are displayed in the form of bubble charts (Figure 5B). According to the *p*-value, the most abundant metabolic pathways were lysine degradation, beta-alanine metabolism, fatty acid metabolism, oxidative phosphorylation, nicotinate and nicotinamide metabolism, nucleotide metabolism, fatty acid degradation, histidine metabolism, citrate cycle (TCA cycle), glycine serine and threonine metabolism, sulfur metabolism, propanoate metabolism, purine metabolism, tryptophan metabolism, glyoxylate and dicarboxylate metabolism, pyruvate metabolism, biosynthesis of cofactors, and D-amino acid metabolism. Twenty-eight differential metabolites in the samples from Day 546 were enriched in 34 metabolic pathways; the 20 most significant metabolic pathways are displayed in the form of bubble charts (Figure 5B). According to the *p*-value, the most abundant metabolic pathways were arginine and proline metabolism, D-amino acid metabolism, histidine metabolism, beta-alanine metabolism, glycine serine and threonine metabolism, lysine degradation, tryptophan metabolism, cysteine and methionine metabolisms, nicotinate and nicotinamide metabolism, butanoate metabolism, biosynthesis of unsaturated fatty acids, phenylalanine metabolism, biosynthesis of amino acids, alanine aspartate and glutamate metabolisms, 2-oxocarboxylic acid metabolism, and biosynthesis of cofactors.

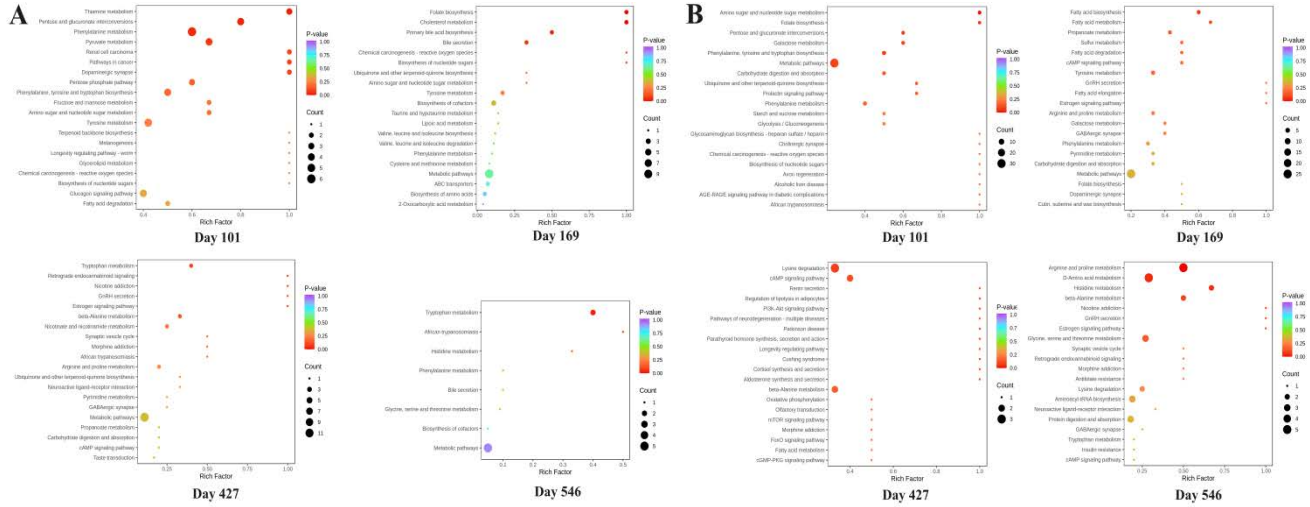

Figure 5. KEGG enrichment analysis showed the most abundant KEGG pathways of differential metabolites. Rich factor refers to the ratio of the differential metabolites in corresponding pathways to the total number of metabolites detected in this pathway. The size of the bubbles in the figure represents the number of differential metabolites enriched in the metabolic concentration of the pathway, and the color of the bubbles represents the magnitude of different enrichment significance *p*-values

4. Discussion

Following antibiotics, acidifiers are becoming common as feed additives in aquaculture [14]. Lactic acid, as a typical acidifier, is widely used in the feed of aquatic organisms. Dietary acidifiers are mainly used to improve digestion and absorption of nutrients and reduce intestinal emptying by lowering the pH value in the diet, which not only inhibits the growth of pathogenic microorganisms and increases probiotics in the gastrointestinal tract but also enhances digestive enzyme activity, mineral absorption, and protein metabolism [35,36]. It has been reported that 15 g kg $^{-1}$ Na-lactate as a feed additive can promote the growth of Arctic charr (*Salvelinus alpinus*) [37]. However, Gislason et al. [38] found that 15 g kg $^{-1}$ Na-lactate as a feed additive promoted the growth of Arctic charr (*Salvelinus alpinus*) but did not lead to an improvement in the growth performance of Atlantic

salmon. The use of 15 g kg $^{-1}$ calcium lactate as the feed additive can reduce gastrointestinal pH and improve growth performance and feed utilization of red drum (*Sciaenops ocellatus*). However, when the concentration of calcium lactate is increased to 30 g kg $^{-1}$, the growth performance of fish will decrease. Other studies have shown that 10 g kg $^{-1}$ of lactic acid as the feed additive did not cause significant differences in the growth performance of red sea bream [39] and rainbow trout [40]. For different fish species, the use of the correct type and dietary concentration of organic acids is essential to enhance growth performance [14].

In this study, when only 5% *L*-lactic acid was used as a feed additive, the average growth rate of bester sturgeon juveniles in the experimental group was 0.48, which was higher than the average growth rate of 0.34 in the control group during the first 100 days of feeding, implying that lactic acid had a growth-promoting effect on sturgeon. The use of 5% lactic acid as a feed additive caused a total of 67 differential metabolites to be up-regulated. Among these up-regulated metabolites, *L*-methionine, as an essential amino acid, is essential for protein synthesis and muscle growth. Other up-regulated amino acids, such as *L*-phenylalanine, *L*-tyrosine, and *L*-asparagine, are also essential for energy metabolism and protein synthesis. Ornithine plays a role in the urea cycle, reducing ammonia toxicity and improving protein utilization. Oleic acid can provide energy and support cell growth. Docosapentaenoic acid (DPA) is an omega-3 fatty acid that improves growth, immune function, and stress tolerance to promote growth. S-adenosylhomocysteine, as a precursor of S-adenosylmethionine (SAMe), is important for epigenetic regulation of growth-related genes. These up-regulated metabolites also confirmed that lactic acid as a feed additive has a promoting effect on improving the growth rate of sturgeon. However, as the feeding time increased to 169 days, the growth rates of the experimental group and the control group were basically equal. It has been shown that the effect of using an acidifier would be different for fish at different growth stages, and the need for an acidifier dose would increase accordingly with the increase in body size [41].

Betaine plays an important role as a methyl donor in tissues for the synthesis of methionine, carnitine, phosphatidylcholine, and creatine, which play a role in protein and energy metabolism. Studies have shown that betaine as a feed additive promotes growth and increases the growth rate in fish [42,43]. Betaine acts as an inducer at different concentrations for different aquatic animals. A moderate amount of betaine can act as a growth promoter, and when the concentration is too high, the promotional effect is diminished; growth inhibition can even occur. The general amount of betaine added to feed is 0.5% to 1.5%. An amount of 0.5 g kg $^{-1}$ of betaine promoted the growth of Nile tilapia. The specific growth rate of Nile tilapia in the experimental group was reduced compared to the control group at betaine levels higher than 1 g kg $^{-1}$ [29]. The use of 1.0% and 1.5% betaine as feed additives did not increase the specific growth rate of beluga (*Huso huso*) [44]. The use of 0.4% betaine as a feed additive significantly increased the specific growth rate of gibel carp (*Carassius auratus gibelio*), while 0.08% and 2% betaine did not increase its specific growth rate [45]. In this study, 5% betaine was used as a feed additive, and the results showed that the growth rates of the sturgeon in the experimental group were significantly lower than those of the control group. When both lactic acid and betaine were used as feed additives for 170 days, the growth performance of the sturgeon in the experimental group improved. As the feeding time reached 546 days, the growth rates of the sturgeon in the experimental group were very close to those of the sturgeon in the control group. The result suggested that high concentrations of betaine as a feed additive inhibit fish growth, and the simultaneous use of lactic acid in the later period counteracted the inhibitory effect of high concentrations of betaine.

Fatty acids can maintain fish health and enhance immunity. In this study, metabolomics analysis of sturgeon fed with lactic acid as a feed additive for 101 days showed that lactic acid as a feed additive could cause up-regulation of 27 metabolites belonging to fatty acyls, including monounsaturated fatty acids, polyunsaturated fatty acids, and saturated fatty acids. Fatty acids are not only an important energy source for the body but also an important component of cell membranes and an important source of fish flavor. Some polyunsaturated fatty acids are essential for the human body to maintain health, such as linoleic acid and linolenic acid. With the increase in feeding time, the lipid differential metabolites decreased, which may be related to the increase in the size of sturgeon. Fatty acid metabolism provides part of the energy source for body activities.

Adding betaine to the diet can achieve the purpose of lowering lipids by improving bile acid metabolism [27]. Betaine, as a methyl donor, can enhance the ability to synthesize carnitine. The increased methyl content in the animal body can promote the conversion of carnitine to acid-insoluble carnitine. Long-chain fatty acids can only be transported into mitochondria for β-oxidation after combining with carnitine to form fatty acylcarnitine. The increased synthesis of free carnitine in the liver enhances the transport of fatty acids, thereby promoting fatty acid oxidation and accelerating fat decomposition [46]. A review paper summarized by Cholewa et al. [47] also showed that excess betaine can occasionally stimulate mitochondrial activity and enhance fatty acid oxidation, leading to the consumption of circulating fatty acyl groups. In this study, the use of betaine as a feed additive caused the down-regulation of various carnitine metabolites, such as 2-methylbutyroylcarnitine, butyrylcarnitine, isovelarylcarnitine, linoleylcarnitine, and stearylcarnitine, confirming their involvement in fatty acid oxidation. At the same time, excess betaine can promote the conversion of fatty acids into ketone bodies, which will cause fatty acyl groups in the blood to be transferred to the ketogenic pathway. A review paper summarized by Zhao et al. [48] also showed that betaine can stimulate the expression of fatty acid transporters on cell membranes, such as CD36 [49] and FATP [50], thereby enhancing the absorption of fatty acyl groups by tissues such as muscle and liver. This increased absorption will reduce the level of circulating fatty acyl groups. Betaine can also regulate lipid metabolism by inhibiting key lipogenic enzymes such as acetyl-CoA carboxylase and fatty acid synthase. This inhibition reduces the synthesis of new fatty acids, thereby reducing the level of fatty acyl groups in the blood. In this

study, the analysis results of differential metabolites also showed that 5% betaine caused the down-regulation of a large number of differential metabolites belonging to the fatty acyl group, such as 2-hydroxycaproic acid, 2-methyl-4-pentenoic acid, methylsuccinic acid, ricinoleic acid, and stearic acid.

Studies have shown that, when the concentration of exogenous betaine is too high, the rate of the body's methylation process will increase, resulting in more amino acids being consumed. Similarly, high levels of betaine can up-regulate the trans-sulfurization pathway, and related amino acids are converted into cysteine, taurine, and glutathi-one[48]. At the same time, excessive betaine will accelerate the methionine cycle, increase the demand for precursor amino acids, and ultimately lead to an imbalance in amino acid metabolism. In this study, the results also showed that betaine as a feed additive caused the down-regulation of amino acid metabolites, including *L*-homocitrulline, *L*-homoserine, *L*-proline, *L*-threonine, *L*-methionine, *L*-phenylalanine, *L*-tryptophan, and *L*-tyrosine. High concentrations of betaine will also affect the absorption and transport of amino acids [51]. Betaine can also act as an osmotic regulator to change its internal metabolic environment to maintain the body's balance, and its energy comes from the decomposition of proteins and amino acids [47].

In the betaine group, when only 5% betaine was used as a feed additive, the common differential metabolites in the blood samples of Day 101 and Day 169 were *p*-hydroxyphenylacetic acid, phenylacetic acid, mandelic acid, *p*-hydroxymandelic acid, and *N*-methylnicotinamide. *p*-hydroxyphenylacetic acid and phenylacetic acid can participate in the metabolism of phenylalanine. *N*-methylnicotinamide is a metabolite of vitamin B3. Compared with the control group, the down-regulation of *N*-methylnicotinamide in the experimental group indicated that the metabolism of vitamin B3 was inhibited. Vitamin B3 can promote the health of the digestive system, reduce cholesterol and triglycerides, and promote blood circulation [52]. At the same time, vitamin B3 has a positive significance for maintaining human health and can be obtained through diet. Nicotinamide is a component of coenzymes I and II and is involved in lipid metabolism, the oxidation process of tissue respiration, and the anaerobic decomposition of carbohydrates in the body [53]. *N*-methylnicotinamide can inhibit the transport of choline [54]. The down-regulation of *N*-methylnicotinamide in the experimental group can promote the normal transport and metabolism of choline. Choline belongs to the B vitamins and is necessary for liver function and lecithin formation. Choline can protect cardiovascular health by lowering blood pressure, changing blood lipids, and reducing plasma homocysteine levels [55]. The results showed that the down-regulation of *N*-methylnicotinamide in the experimental group promoted the health of sturgeon.

When 5% lactic acid and 5% betaine were used as feed additives at the same time, the common differential metabolites in the blood samples from Day 427 and Day 546 were *N*,*N*-dimethylglycine (DMG), 2-furoic acid, *L*-acetylcarnitine, and *L*-histidine. DMG is a derivative of the amino acid glycine and participates in the metabolism of glycine, *L*-serine, and *L*-threonine. DMG is also a byproduct of homocysteine metabolism. Homocysteine and betaine are converted into methionine and DMG by betaine-homocysteine methyltransferase [56]. DMG has physiological and biochemical effects such as improving the body's immunity and antioxidant capacity. The up-regulation of dimethylglycine in the experimental group plays an important role in maintaining the health of the sturgeon's body. *L*-acetylcarnitine is the acetate ester of carnitine, which promotes the entry of acetyl-CoA into the matrix of mitochondria during fatty acid oxidation. The main role of *L*-acetylcarnitine is to help transport fatty acids into the mitochondrial matrix, where fatty acid metabolism occurs [57]. Studies have found that high levels of *L*-acetylcarnitine in the blood (>12 μmol/L) may be associated with inflammation or infection. Increased levels of *L*-acetylcarnitine, a representative member of short-chain acylcarnitines, appear to be due to the release of these compounds from the liver during infection or stress/trauma [58]. *L*-aceftylcarnitine can be a metabolic marker of inflammation or infection.

In this study, it was speculated that the significant decrease in differential metabolites, such as amino acids and fatty acyl in the blood samples of sturgeons fed with betaine in the experimental group, might be related to the excessively high betaine content in the feed.

5. Conclusions

This study demonstrates that lactic acid, when used as a feed additive positively influences metabolic pathways, regulating amino acid and fatty acid metabolism, which enhances the nutritional profile and flavor of the fish. Therefore, during commercial feed production, adjusting the feed ratio and adding an appropriate amount of lactic acid to the feed should be considered. In contrast, excessive betaine was found to hinder growth and disrupt metabolic regulation. Notably, the inclusion of lactic acid mitigates these negative effects, suggesting its potential to counterbalance the drawbacks of high betaine levels. Betaine is often added to feed as an attractant. According to the results of this study, during the production of commercial feed, when betaine is used as an attractant, its concentration should be fully considered to avoid harm to the farmed animals. It is also possible to consider adding an appropriate amount of lactic acid to inhibit the negative effects caused by high concentrations of betaine. These findings highlight lactic acid's dual role in promoting growth and optimizing metabolic function in bester sturgeon.

Supplementary Materials: The following supporting information can be downloaded at:

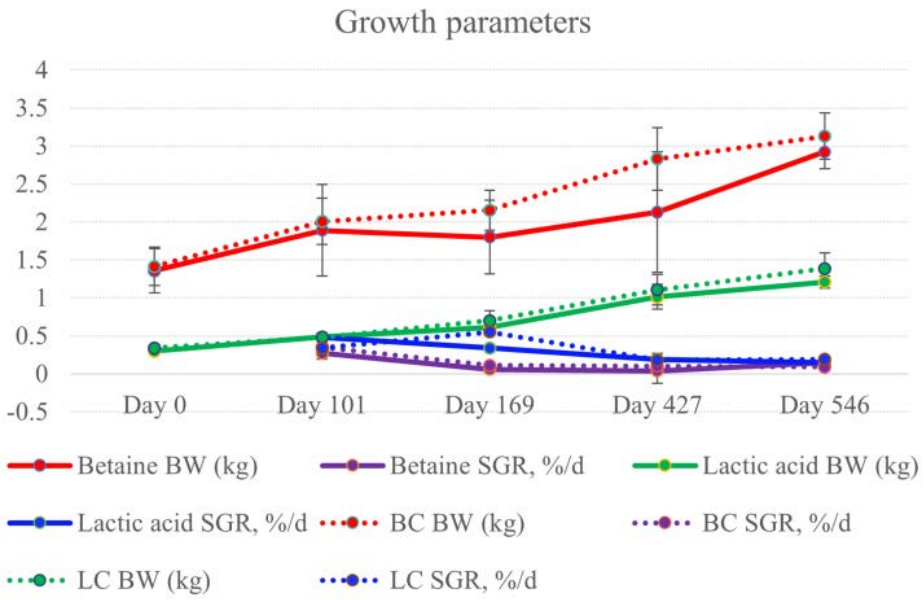

Figure S1. Line chart of body weight (BW, Table 1) and specific growth rate (SGR, Table 2) of bester sturgeon juveniles in each subgroup during the study period. Subgroups were divided into the experimental betaine (Betaine), the betaine control (BC, no betaine), the experimental lactic acid (Lactic acid), and the lactic acid control (LC, no lactic acid)

Figure S2. Cluster heat map of differential metabolites in the lactic acid group

Figure S3. Cluster heat map of differential metabolites in the betaine group

Table S1: Body weights of Bester sturgeon juveniles recorded during the study period (Day 0 to Day 546). * Metabolomic profiles on Day 0 were not determined. ** Metabolites of Days 427 and 546 identified by the HM400 panel were not completely overlapped, and thus, the resulting metabolomes are designated as HM400-1 and HM400-2, respectively. *** WT, water temperature approximately averaged from the day of sampling and several days before and after sampling. **** One individual of the betaine experimental subgroup died after sampling on Day 427, and thus, its body weight on Day 546 was not determined.

Table S2: Daily specific growth rates (SGR, %) of bester sturgeon juveniles during the study period (Day 0 to Day 546). SGR was calculated as: $(\mathrm{Ln}(BD_{N+1})-\mathrm{Ln}(BD_N))/(\mathrm{Day}_{N+1}-\mathrm{Day}_N)\times 100$, where Ln represents the natural logarithm, BD represents body weight shown Table S1, and *N* and *N+1* represent sampling occasions, i.e., Days 101, 169, 427, and 546. * One bester individual of the betaine experimental subgroup died after sampling on day 427, and thus its SGR from Day 427 to Day 546 was not calculated.

Table S3: Summary of the 244 metabolites of the HM350 dataset generated from the Days 101 and 169 bester sturgeon juveniles. "NA" represents "not annotated" and were excluded from the category counts.

Table S4: Summary of the 234 metabolites of the HM400-1 dataset generated from the Day 427 bester sturgeon juveniles. "NA" represents "not annotated" and were excluded from the category counts.

Table S5: Summary of the 266 metabolites of the HM400-2 dataset generated from the Day 546 bester sturgeon juveniles. "NA" represents "not annotated" and were excluded from the category counts.

Table S6: All differential metabolites screened in the lactic acid group at different sampling times.

Table S7: All differential metabolites screened in the betaine group at different sampling times.

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Data Availability Statement: Original data for analysis are available at:

https://www.sturgeon-metabolome.hiroshima-u.ac.jp/h0888_Bet-Lac_HM350_Day101_Day169.ods

https://www.sturgeon-metabolome.hiroshima-u.ac.jp/h0888_Bet-Lac_HM400-1_Day427.ods

https://www.sturgeon-metabolome.hiroshima-u.ac.jp/h0888_Bet-Lac_HM400-2_Day546.ods

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