The mechanism for improving the flesh quality of grass carp (Ctenopharyngodon idella) following the micro-flowing water treatment using a UPLC-QTOF/MS based metabolomics method

The mechanism for improving the flesh quality of grass carp (*Ctenopharyngodon idella*) following the micro-flowing water treatment using a UPLC-QTOF/MS based metabolomics method

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Abstract: The micro-flowing water system can improve the flesh quality of freshwater fish using the traditional pond farming method. However, the mechanism of this phenomenon has not yet been explored. This study intends to examine the changes of metabolites in freshwater fish after treatment with the micro-flowing purification system (MFPS). The UPLC-QTOF/MS based metabolomics method was utilized to screen the metabolites and predict the major possible metabolic pathways after MFPS treatment. There were 377 types of metabolites identified in the fish muscle, of which 54-71 represented significant different metabolites identified during different stages of MFPS treatments. The main mechanism of MFPS treatment in improving the quality of grass carp fish muscle was investigated, and the MFPS treatment was shown to improve the flesh quality and the flavor of grass carp fish muscle. This study could provide the theoretical basis for improving the quality of aquatic products.

***Keywords**:* Micro-flowing purification system; Quality; Grass carp; Metabolomics; UPLC−QTOF/MS

1. Introduction

The annual yield of grass carp (Ctenopharyngodon idella) is about 25 million tons in the world, with 17.9% produced in China (Qiu, et al., 2020). Grass carp plays a major role in aquaculture, as it is the largest freshwater fish species. It is one of the most popular food items in China, and is often used as fresh fish or processed fish fillet products (Liu, et al., 2013). Because the cultivation of freshwater fish often uses a high-density cultivation mode, the cultivation of microorganisms in aquatic environments could easily cause the accumulation of undesirable flavors in the fish, which in turn would affect, to some extent, the degree of acceptance by consumers of the grass carp culture (Fuentes, et al., 2010; Rincón, et al., 2016).

Depuration treatment or short-term aquaculture can effectively improve the quality and acceptability of market-size fish before selling. For example, depuration improved the muscle quality of atlantic salmon (salmo salar), and decreased the content of earth-musty off-flavors (Burr, et al., 2012). By changing nitrate-nitrogen levels in recirculating aquaculture systems, the concentration of off-flavor compounds geosmin and 2- methylisoborneol in rainbow trout was reduced (Schrader, et al., 2013). Common carp had higher levels of polyunsaturated acids after being kept in clear water without feeding for several days (Zajic, et al., 2013). Furthermore, there was a significant improvement in the consumers' acceptability of the flavor of halibut after two weeks of depuration treatment (Drake, et al., 2010). In our previous study, the edible quality of grass carp muscle improved after using treatment of the micro-flowing water depuration system. The texture, taste and flavor of grass carp muscle appeared to significantly change during the purification process (Lv, et al., 2018). But the mechanism of the fish quality improvement through the depuration system treatment has not yet been reported, thus the mechanism of fish quality improvement should be further explored.

Metabolomics is an effective analytical method for the identification and quantification of small molecular endogenous metabolites in organisms (Patti, et al., 2013). It has been widely used in the food industry to investigate the metabolic changes of aquatic products under various physiological or storage conditions, specifically, after discovering the inherent mechanism and potential biomarker of monitoring food quality. Metabolomics has been applied to some aspects of aquatic products processing. For example, the NMR based metabolomics method was used to study changes of metabolites of sea cucumbers and crabs under different stress conditions (Ye, et al., 2016). GC-MS technology was used to characterize the metabolic profiles of crucian carps infected with bacteria, and to screen the important biosynthesis pathway and metabolic biomarkers (Guo, et al., 2014). Owing to its powerful separation ability, high throughput capacity, high resolution and detection sensitivity, HPLC-MS technology has been widely used in the study of aquatic products and metabolomics. For instance, the metabolic changes in zebrafish induced by environmental disturbance were investigated using HPLC-MS technology (De Sotto, et al., 2016). The performance of the HPLC-MS approach is excellent and has been broadly used for quantitative determination of meat quality and related metabolites or nutrients (Gil-Solsona, et al., 2019).

In the present study, market size grass carp were depurated using the micro-flowing water depuration system (MFPS). The UPLC-QTOF/MS based metabolomics method was utilized to explore the metabolites and their contents. The different metabolites were screened out and the key metabolic pathways related to the quality of grass carp muscle were investigated. Thus, the main mechanism of depuration treatment to improve the quality of grass carp fish was explored using UPLC-QTOF/MS based metabolomics and expected to provide the theoretical basis for improving the quality of aquatic products through regulating and controlling the depuration process.

2. Materials and Methods

2.1 Chemical reagents

2-Chloro-L-phenylalanine (Sigma Aldrich, St. Louis, MO, USA) and methanol (A.R.) (Sinopharm Chemical Reagent Co. Ltd, Shanghai, P.R. China) were used in the sample preparation. MS‐222 (3-Aminobenzoic acid ethyl ester methanesulfonate, CAS: 886-86-2, Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, P.R. China) was used as the anesthetic typically administered to induce anesthesia in fresh grass carp. Ultrapure water was obtained from the Milli-Q system (Millipore, Billerica, MA). The mobile phase was composed of the following components: acetonitrile and methanol (HPLC grade, Merck, Darmstadt, Germany), formic acid (Sigma Aldrich, St. Louis, MO, USA), phosphoric acid (Kemiou Chemical Reagent Co. Ltd, Tianjin) and ultra-pure water (Merck, Darmstadt, Germany).

2.2 Fish sample preparation

All animal procedures were approved by the Animal Care and Use Committee of Huazhong Agricultural University and performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Huazhong Agricultural University. The schematic diagram of the whole experiment is illustrated in Fig. S1. Fresh grass carp (1.5~2.0 kg) from the fish farming in Chidong Lake (115.41°E, 30.10°N, Hubei, P.R. China) were transported in troughs (Long×Width×Height: 720cm×200cm×150cm, n=30) in the micro-flowing water purification system (Lv, et al., 2018). There were ~125 kg fish placed in every trough, and the depuration period lasted from October to December (2015). All fish were deprived of food during the whole period. During the period of depuration, five grass carp were collected every 10 days. The sampled fish were anaesthetized using the anesthetic MS‐222 (100 mg/L) and were unconscious before slaughter. Fish were immediately gutted and segmented. At the end, the dorsal

muscle of each fish was sampled and kept at -80°C for further analysis.

2.3 Metabolites extraction

The fish muscle samples (~50 mg) were extracted with 800 μL methanol. Dichlorophenylalanine (10 μL, 2.9mg/mL) was added to the mixture according to the internal standard. All fish samples were grinded for 90s at 65Hz, vortexed for 30s and centrifuged for 15min (12000 rpm, 4°C). At the end, 200 μL supernatant was transferred to sample vials for further detection.

2.4 UPLC-QTOF/MS detection

Chromatographic evaluations were performed using a Waters Acquity UPLC-I Class (Waters Corp., Mil-ford, MA, USA). The UPLC system was equipped with a binary pump, micro degasser, an autosampler and a temperature-controlled column compartment. Chromatographic separations were achieved on an ACQUITY UPLC HSST3 ODS-SP column (1.8 μm, 2.1 mm × 100 mm; Waters, Ireland). The gradient system consisted of 0.1% formic acid in water in mobile phase A and 0.1% formic acid in acetonitrile in mobile phase B (0-2 min, A, 95%; 2-12 min, A, 95–5%; 12-15 min, A, 5%; 15-17 min, A, 5–95%; 17-20 min, A, 95%). The chromatographic separation condition was set as following: oven temperature-40 °C, injection volume-0.6 μL, flow rate-0.35 mL/min.

The separated components were detected with a Vevo G2-S QTOF mass spectrometer (Waters Corp., Mil-ford, MA, USA) equipped with both ESI ion modes (ESI- and ESI+). The detecting parameters were collected as following: Cone gas, 50 L/h; Ion source temperature, 120 °C (+)/110 °C(−); Desolvation gas temperature, 350 °C; Capillary voltage, 1.4 kV (ESI+)/1.3 kV (ESI−); Sample cone, 40V (ESI+) or 23V (ESI−); Gas flow, 600 L/h; Collision energy, 10–40 V; Ion energy: 1V; Scan time: 0.03s; Inter scan time: 0.02s; Scan range 50–1500 m/z. All analyses were performed using lockspray to ensure accuracy and reproducibility, with a concentration of 200 ng/mL of leucine encephalin (m/z

556.2771) in positive ion mode and (m/z 554.2615) in negative ion mode locking quality.

2.5 Data processing

The raw UPLC-QTOF/MS data were initially transformed to CDF files using CDFbridge (Masslynx 4.1, Waters), then imported into XCMS software for peak alignment, peak detection, peak picking, peak filling and isotope elimination. The data were normalized with retention time, MZ and observation and peak intensity. The data matrix was analyzed using Multivariate Analysis (MVA) SIMCA-P 13.0 software (Umetrics AB, Umea, Sweden) for principal component analysis (PCA) and Orthogonal partial least squares discriminant analysis (OPLS-DA). According to the variable importance plot (VIP), the necessary conditions were set (variables scoring>1.5 and $p<0.05$) to identify metabolites with significant differences between the control and micro-flowing water system treated groups.

In order to investigate detailed information of these potential significant metabolic biomarkers, the identification and quantification of these biomarkers were investigated using the detailed information detected by UPLC-QTOF/MS. At first, the accurate *m*/*z* obtained by multivariate statistical analysis was matched with metabolites from the online databases of Metlin (https://metlin.scripps.edu/) and KEGG (http://www.kegg.com/), which initially indicated the possible metabolic mechanism. At the end, the identification of the metabolites was validated with ion fragments, parent ion and the retention time. The quantification of metabolites was determined with the area of the peaks in EIC (Extracted ion chromatograms) extracted from TIC (Total ion chromatograms) (Fig. 1), which was automatically completed with the commercial software MassLynx 4.1 (Waters, Milford, MA, USA).

2.5 Determination of hypoxanthine and inosine

For the pretreatment and determination of the content of hypoxanthine and inosine in grass carp muscle, reference was made to a former method (Kuda, et al., 2008). Separation of hypoxanthine and

111 inosine was conducted with a reverse-phase column (Agilent C18, Agilent Technologies, USA). The
112 mobile phase of phosphate buffer-methanol (85:15, pH 6.0) was used (flow rate: 0.7 ml/min; temperature:
113 35℃). The detection wavelength of eluent was monitored at 254 nm for hypoxanthine and inosine.

114 **2.6 Statistical analysis:**

115 The chemical analyses were repeated at least three times, and the results expressed as means ± SEM.
116 Statistical analysis was performed with SPSS 22.0 (IBM, New York, USA) using one-way ANOVA.
117 LSD adjustment was used to determine the significant difference between different groups. Significant
118 differences were declared at $p < 0.05$. The statistical power analysis was used to validate the sample size
119 for significant differences, which was implemented in the freeware GPower (Erdfelder, et al., 1996).

120

121 **3. Results and discussion**

122 **3.1 UPLC-QTOF/MS analysis**

123 The metabolites extracted from fish samples, which were treated with the MFPS under different
124 purified periods (0d, 10d, 20d, 30d, 40d and 50d), were detected with UPLC-QTOF/MS technology in
125 the positive (ES+) and negative (ES-) ESI modes. As an example, a typical series of peak intensity
126 chromatogram of treated fish samples in the positive ESI mode are illustrated in Fig. 1. From Fig. 1,
127 most endogenous metabolites could be detected in 18 min using UPLC-QTOF/MS technology.

128 According to the peak heights in the spectra, about 4420 peaks of positive ions (ESI+) and 4600
129 peaks of negative ions (ESI-) were detected which were extracted from TIC by the MassLynx (Fig. 1).
130 By comparison with the NIST standard spectrum, there were 377 metabolites identified in the grass carp
131 muscle, including 73 amino acids, 117 lipids, 56 nucleic acids, 123 sugars and carbon sources, and some
132 inorganic acids. From Fig. 1, it is visually evident that there are some differences among these six

different groups in the TIC chromatograms. However, it is rather difficult to screen the potential different metabolites in fish muscle samples except by manually and intuitionally comparing the control and treated chromatograms. Therefore, application of the appropriate pattern recognition approach is very necessary for visualizing characteristic changes.

3.2 Principal component analysis (PCA)

As a non-supervised multivariate data analysis method, PCA is always used to give a comprehensive view of the clustering trend for the multidimensional data (Boyan, et al., 2013). In order to gain an overview of the fish muscle metabolic profiling, PCA was initially used for the analysis of UPLC-QTOF/MS data.

To determinate whether the micro-flowing purification treatment is able to influence metabolic patterns of grass carp muscle and to screen the characteristic metabolites with significant concentration changes (*i.e.* potential biomarkers), the PCA approach was utilized to conduct a model with the ES+ and ES-data, respectively. The score plots of PCA are illustrated in Fig. 2A (ES+) and Fig. S2A (ES-). The validity of the PCA model was evaluated using the correlation coefficient R^2 and the cross-validation correlation coefficient Q^2. R^2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit. Q^2 is defined as the proportion of variance in the data predicted by the model and indicates predictability. R^2 and Q^2 of PCA models for the control group and the other five treated groups were 0.514 and 0.384, 0.226 and 0.0198 for the ES+ and ES- modes, respectively. Thus, the PCA method was more useful for the analysis of data in the ES+ mode. It is clearly noticeable that the samples in the control group were completely separated compared with the samples in the micro-flowing purification treated model groups. Thus, the metabolic pattern in the grass carp muscle was

155 significantly altered after the purification process.

156 Furthermore, the PCA method also illustrated the separation of the control group and the other
157 five treated groups under the ES+ mode, respectively. The statistical parameters (R^2 and Q^2) of all PCA
158 models are illustrated in Table S1. Results from the PCA model showed that this method could generate
159 good results for the discrimination between samples from the control and treated groups, and the
160 performance from the positive ion mode was better than from the negative ion mode. With various
161 periods of micro-flowing purification system treatment, the different metabolite compositions were
162 further explored between the purified groups and control group.

163

164 **3.3 OPLS-DA analysis**

165 In order to maximize the separation among the different grass carp groups and focus on metabolic
166 variations at different purification periods, the data for both ESI+ and ESI- acquisition modes were
167 performed using a supervised multivariate data analysis approach OPLS-DA (Aru, et al., 2016) to build
168 more intensive and accurate models and explore the real trend and grouping the multidimensional data.

169 The score plots of OPLS-DA are shown in Fig. 2B-2F and Fig. S2B-S2F. Compared with the
170 control group, the cluster separations are very good at different micro-flowing purification treated time
171 points. The results mean that the metabolic patterns in grass carp were significantly changed during the
172 purification processing. The performance of OPLS-DA was distinctly better than the PCA models. All
173 models exhibited reasonable separation between groups and acceptable goodness-of-fit (*R2Y*) and
174 goodness-of-prediction (*Q2Y*). The statistical parameters of the OPLS-DA models (*R2X*, *R2Y* and Q^2)
175 are summarized in Table S1. The results not only indicated the strong explanatory power of the data and
176 the good predictive ability of all permuted OPLS-DA models, but also demonstrated a robust metabolic

177 difference between the micro-flowing purification systems treated grass carp and the controls.

178

179 **3.4 Identification of different metabolites for micro-flowing system purification treatment**

180 Based on the judgement criteria (VIP > 1.5, $p < 0.05$) for determining the different metabolites of
181 the OPLS-DA model, there were 145 metabolites identified in fish muscle samples compared with the
182 control group and purified groups under different purification periods. The retention time, molecular
183 weight and relative amount of potential different metabolites are demonstrated in Table 1 and Table S2.
184 The majority of identified metabolites were lipids and secondary metabolites of lipids under different
185 purification times (Fig. 3), which indicates that the micro-flowing purification treatment could alter the
186 lipid metabolism in grass carp. The phenomenon that most metabolites were lipid decomposition
187 products shows that the fat consumption increased during the purification process, which is consistent
188 with our previous study (Lv, et al., 2018). There are many phospholipid components involved in the lipid
189 metabolites, which indicates that a large number of signal molecules (such as phosphatidylserine,
190 phosphatidylethanolamine, *etc*.) contributed to the metabolism caused by the purification treatment.

191 In order to further determine the biomarkers in different metabolites of grass carp during the
192 micro-flowing purification treatment, the repeated metabolites and their corresponding relative contents
193 in different metabolites of each comparable group were searched and analyzed. The selected metabolites
194 were further identified by the molecular weight (m/z) in the Metabolites and Tandem MS database
195 (METLIN) and KEGG database. Based on the variety and content changes of different metabolites of
196 grass carp muscle and the reported metabolic pathway of each metabolite in KEGG database, the
197 metabolic pathway changes of grass carp muscle during purification were obtained (Fig. 4). A total of
198 16 significant differential endogenous metabolites were found which showed an increasing and

199 decreasing trend from the beginning to the end of the purification process (Table 1). Among these
200 different metabolites, xanthine, hypoxanthine, trimethylamine oxide, alanine and IMP (Inosine
201 monophosphate) are the most common precursor substances. The changes in their amount affected the
202 quality of fish muscle. The causes of these metabolic differences are further discussed. The species and
203 the changes in the amount of the identified metabolites, combined with the metabolic pathway reported
204 from the KEGG database, the potential metabolic pathways of micro-flowing purification system treated
205 grass carp are all illustrated in Fig. 4.

206 The trend of phospholipid metabolites, such as phosphatidylserine and phosphatidylcholine,
207 indicate that the synthesis and metabolism of signal metabolites are enhanced and kept up-regulating in
208 the early stage of purification. From the decrease in the amount of arachidonic acid, docosanoic acid,
209 leukotriene B4 and the increase in short chain alkyl coenzyme A, it can be seen that the decomposition
210 and metabolism of fatty acids increased in grass carp muscle after purification, and the consumption of
211 fatty acids was the main source of energy. The trend of histidine, homocysteine, alanine and other amino
212 acid metabolites showed that the decomposition rate of amino acid was greater than its synthesis rate
213 during the purification process, which led to the accumulation of alanine in fish muscles. After 20 days
214 of purification, the level of IMP synthesis was enhanced, and its conversion rate of hypoxanthine and
215 xanthine was inhibited. The content of trimethylamine oxide and its related metabolites decreased during
216 the purification process, its metabolic substrate carnitine did not change significantly, while the content
217 of choline increased, indicating that the generation pathway of trimethylamine was inhibited.

218 In order to validate the results of metabolic analysis, the content of hypoxanthine and inosine in
219 grass carp muscle during purification was measured using the HPLC method. The corresponding results
220 are shown in Fig. 5. After the micro-flowing system purification treatment, the contents of these two

kinds of endogenous metabolites in fish muscle significantly decreased, which is consistent with the results of metabolomics. It indicates that the purification treatment could induce the inhibition of catabolism of IMP to hypoxanthine in the muscle of grass carp. Combined with the trend in different metabolites mentioned above which are shown in Table 1 and Table S1, it can be seen that the main metabolites related to fish flavor changed significantly in 20 days during the purification process. The content of the major metabolites can remain stable for a relatively long time, which indicates that the micro-flowing purification treatment can improve the quality and flavor of fish meat, as long as the period of purification is not less than 20 days.

3.5 Metabolic changes in the grass carp muscle during MFPS treatment

Due to grass carp not feeding during the MFPS treatment period, the fish protein decomposed and stored lipids in tissues in order to maintain the energy supply and daily life activities, hence the contents of protein and lipids in the fish muscle showed a consistent downward trend. Basically, the consumption of protein and lipids in grass carp fish muscle is according to the regulation of energy metabolism under prolonged starvation in fish. In other words, the fish metabolism can be divided into three stages during the MFPS treatment: (I) In the first 20 days of the MFPS treatment, the metabolic rates of both protein and lipids are very fast; (II) During 20d-40d of the MFPS process, the catabolism of grass carp muscle is dominated by lipids until reaching the limit in the body of the fish; (III) In the last 10 days of MFPS treatment, protein catabolism plays an important role in the fish body metabolism in supplying the energy (Bar, 2014).

From Table 1 and Table S2, it can be observed that the content of NADP in the tissues significantly increased at 10 days after purification, which indicates that the energy supply of grass carp

243 was insufficient during the starvation period. As well as at the end of purification, NADP accumulated
244 due to the decrease in energy supply caused by the reduction in lipid metabolism. This phenomenon is
245 consistent with the expression trend of NADPH related enzymes synthesis found in liver cells of rainbow
246 trout during the starvation phase, as the activities of glucose-6-phosphate dehydrogenase and malic acid
247 enzyme in fish significantly decreased, and the synthesis of NADPH decreased (Nagana Gowda, et al.,
248 2016). After 20-40 days of purification, the degree of metabolic protein and lipid was enhanced, and the
249 synthesis of NADPH returned to normal level. The contents of intermediate products of fatty acid
250 metabolism in fish muscle, such as 3-oxhexyl coenzyme A and 3-oxlauroyl coenzyme A increased
251 significantly after 10 and 20 days' purification, respectively. At the same time, the contents of fatty acids
252 including docosanoic and arachidonic acid decreased, and so did the contents of secondary products of
253 lipid synthesis such as leukotriene, indicating that the consumption of fat was increased and the
254 anabolism weakened. The fat content in the grass carp back muscle was measured but did not
255 significantly change during the first two stages, it however slightly increased in the first 10 days of
256 purification. These results are similar to the purification study of the cod, which reported that during the
257 purification process, the fish consumed the lipid stored in the fish body in the following order, the liver
258 and other internal organs, the abdomen and the digestive tract and the lipid reserved in the muscle
259 (Palmeri, et al., 2008). This style of lipid metabolism also occurred in the carp (Cyprinus carpio) and the
260 roach under the prolonged starvation condition, but for the rainbow and brown trout the opposite was
261 true (Regost, et al., 2001).

262 In this study, the grass carp duration of stage I is similar to that of the striped bass and rainbow
263 trout, while the stage II is shorter (Jönsson, et al., 2007). During the purification process, the content of
264 amino acids such as homocysteine, histidine and homotyrosine decreased (Table 1 and S2), indicating

that the catabolism of protein and amino acids is also very active, and may be related to the synthesis of phosphatidylserine except when involved in supplying energy to the body.

The synthesis of phosphatidylserine is up-regulated to a greater extent during the purification process and accompanied by significant changes in the contents of other phospholipids such as phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol and ceramide metabolites. Phosphatidylserine is an important component of the membrane structure in cells, and it is widely involved in the regulation of the cell signaling pathway, cell proliferation and apoptosis (Fadok & Henson, 2003). In this experiment, the relative content of phosphatidylserine was significantly increased, which is consistent with the phenomenon of grass carp myoblast reported by Sousa et al (Sousa, et al., 2016). In other words, under the condition of diminished nutrient supply, phosphatidylserine is redistributed in the membrane and entered during apoptosis, while proteins, amino acids, nucleic acids and other substances in cells are reused to maintain life activities (Zhou, et al., 2016). The up-regulation of phosphatidylserine indicates that stress starvation during the purification process makes the regulation of cell apoptosis of grass carp more active. Thus, the accumulation of phosphatidylserine can play an important role in the protection of nerve cells (Akbar, et al., 2006). The data in this experiment show that the grass carp body can protect the nervous system by up-regulating the synthesis of phosphatidylserine under stress starvation.

3.6 Differential metabolites related to the flesh quality of grass carp muscle

Flesh quality is defined by the nutritive value and organoleptic properties as determined by consumers (Grigorakis, 2007). The quality and flavor of the fish should be based on the flesh protein (Grigorakis, 2007), peptides (Jürss & Bastrop, 1995), fatty acids (Grigorakis, 2007), and other

metabolites, which have been studied in our study, and protein and amino acids not only belong to the nutritional components, but also influence the flavors of fish products (Jiang, et al., 2016). A period of purging and food deprivation before harvesting is always the common procedure used for improving the quality and flavor of the fish, and the purged fish (2-4 weeks purging) were found to have a consistent and desirable flavor (Palmeri, et al., 2008). Thus, changes in metabolites, especially amino acids, which play important roles in determining the quality and flavor of the fish were investigated in the current study.

3.6.1 Inosine monophosphate (IMP)

The content of IMP was significantly increased after 20 days of purification, while the contents of jaundice and hypoxanthine in fish muscle were considerably reduced. IMP may be degraded into xanthine and hypoxanthine through safrole and inosinein converted in the fish body, respectively (Fig. 4). The trend of the relative contents of IMP, jaundice and hypoxanthine which changed in the muscle cells of the grass carp indicates that the decomposition of IMP was inhibited after the treatment of MPFS. Therefore, the amount of IMP was accumulated in the fish muscle.

At the same time, IMP can be decomposed by the metabolite AICAR of 5-phosphate-α-ribose-1-diphosphate (PRPP). The other aspect of PRPP metabolism is histidine, and the content of histidine in muscle was decreased (Table S2). It can be seen that the PRPP metabolism during the purification procedure was mainly carried out with the synthesis of AICAR to IMP. When Kuhla et al studied the expression of dairy cow protein, they found that the expression of AICAR methyltransferase/IMP cyclase was up-regulated under starvation, and the synthesis of IMP was enhanced, thereby supplementing and maintaining the total amount of adenine nucleotides with IMP (Kuhla, et al., 2007).

Furthermore, Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) (HGPRT) is one of the

309 main enzymes that catalyze the synthesis of IMP and XMP from hypoxanthine and xanthine. It has been
310 reported that the increased expression of HGPRT can enhance the reuse of purines in walrus under
311 starvation during the purification process, and maintain a stable amount of purine nucleotides (Sonanez-
312 Organis, et al., 2012). The senegalese sole also showed the same tendency of expressing HGPRT under
313 repeated stress (Cordeiro, et al., 2012). In this study, the increase of IMP content in grass carp muscle
314 might be the result of the joint action between the increased expressions of IMP cyclase and HGPRT,
315 and also the reduced substrate content of hypoxanthine and xanthine.

316 IMP is the major source of umami in meat products, therefore, the accumulation of IMP in muscle
317 can effectively improve the flavor of food (Hong, et al., 2017). At the same time, xanthine and
318 hypoxanthine are regarded as the sources of bitterness in foods (Bedini, et al., 2013). Therefore, the
319 MFPS treatment is beneficial in improving umami substances and reducing the contents of bitter
320 substances in the fish muscle that may partly explain the improvement of grass carp quality after the
321 purification process.

322 **3.6.2 Trimethylamine N-Oxide (TMAO)**

323 After the MFPS treatment, the content of TMAO in the grass carp muscle was lower than the
324 detection limit, and the contents of secondary metabolites, dimethylamine and methyl coenzyme M, fell
325 by more than 40% and 60%, respectively. The potential substrates of TMAO include carnitine,
326 trimethyllysine and choline (Narasimhan, et al., 2018). It has been reported that TMAO is mainly derived
327 from the metabolism of carnitine and choline in foods through the action of intestinal microbes, further
328 metabolized and accumulated in various tissues of the body (Cho, et al., 2017). Intestinal bacteria could
329 influence the production of TMAO from carnitine, and carnitine the composition of gut bacteria. The
330 gut bacteria environment was seriously influenced by the depuration procedure (Oliveira, et al., 2011),

such as MPFS in the current study. Therefore, while the grass carp was under starvation during the purification process, the major source of TMAO was interrupted. The amount of TMAO and the corresponding downstream metabolites were significantly reduced in the metabolic process. The same phenomenon has also been observed in the crab aquaculture, as starvation contributed to the decrease of TAMO content in muscles (Ye, et al., 2016).

TAMO can generate trimethylamine and dimethylamine catalyzed by TAMO reductase, which are the two main off-flavor components in fish or aquatic products (Fu, et al., 2006). In this study, the content of TAMO and its corresponding metabolites were decreased during the process of MFPS treatment, indicating that the purification process helped to reduce the off flavor in muscle and thereby improved the quality of grass carp.

3.6.3 Alanine

Considered as the primary metabolites of amino acid metabolism, such as glutamate, aspartic acid and cysteine, alanine can be further metabolized into pyruvic acid and enters the TCA cycle and energy metabolism. During the purification process, the degree of amino acid decomposition was high, and the content of alanine obtained enriched (Table S2). The major form of energy supply is fatty acid metabolism and the limit of fatty acids in the organism was reached after 50 days of purification. After that, the style of energy supply changed into protein and amino acid, while the content of alanine recovered in comparison to the control group. Furthermore, the enriched alanine could also be caused by the reduction in the activation of AlaAT (Alanine Aminotransferase) due the starvation and purification procedure by the MPFS treatment, which could catalyze alanine into α-ketoglutarate in the cycle to transform alanine to pyruvate and glutamate (Jürss & Bastrop, 1995).

The glutamic acid, glycine, alanine and histidine in fish muscle contributed directly to the flavor

353 of fish, thus the higher the content of these four types of amino acids, the better the quality of fish meat
354 (Luo, et al., 2017). Therefore, it is reasonable to conclude that the purification treatment can improve
355 the flavor of fish meat through the enrichment of alanine.

356 **3.6.4 Flesh quality and metabolites**

357 Through analysis of the major differential metabolites and the reported metabolic pathway
358 database, the mechanism of the MFPS treatment to improve flesh quality of grass carp deduced the
359 following: not feeding during purification produced starvation stress which led to the ATP level of grass
360 carp muscle down-regulating and induced significant changes in the purine metabolism pathway in
361 muscle cells, which transferred from hypoxanthine to IMP synthesis and caused the lower concentration
362 of hypoxanthine. At the same time, the decrease in ATP level induced a large number of apoptosis signal
363 molecules to synthesize, thereby accelerating the apoptosis process of some cells, which utilized the
364 stored protein and lipid to generate energy, and finally fed back together with IMP to maintain the normal
365 ATP level. UPLC-QTOF/MS based metabolomics explored whether starvation is the main driving force
366 of metabolic pathway changes which promotes the accumulation of IMP and the results indicate that
367 some of the flavors of amino acids in fish muscle improved significantly. However, the consumption of
368 lipids affected the texture and taste of fish.

369 Due to the lack of supply of carnitine which is one of TMAO substrates during starvation, the
370 accumulation of TMAO and the corresponding downstream metabolites were reduced, which caused the
371 off flavors of fish muscle to decrease. Among the differential metabolites, hypoxanthine, IMP and
372 alanine showed the greatest influence on the flavor of fish muscle, and their content changed
373 significantly during the micro-flowing purification process, which can be used as the potential
374 biomarkers for grass carp purification treatment.

4. Conclusions

There were 377 metabolites detected in grass carp muscle using UPLC-QTOF/MS, and 54~71 different metabolites were screened out during the MFPS treatment process. During the purification process, the catabolism of protein and fat released energy to the body and provided substrates for various metabolic activities of the body. The starvation stress during purification significantly changed the relative contents of phosphatidylserine and other phospholipid metabolites in grass carp muscle, while enhancing the synthesis of phosphatidylserine. Under the joint action of amino acid catabolism and phosphatidylserine synthesis, the contents of histidine, cysteine and other amino acids in fish muscle decreased, and in the end, alanine was enriched. Alanine contributed directly to the flavor of fish or fish products. Due to the accumulation of IMP in fish muscle after the purification treatment, the content of bitter substances like xanthine and the off-flavor substances such as TMAO decreased, which could have improved the flavor and quality of grass carp. Hypoxanthine, IMP and alanine are potential biomarkers of grass carp in the process of micro-flowing water system purification treatment. These results confirm the potential use of metabolomics as a reliable method to explore the mechanism of fish quality improvement during the purification treatment process.

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518 **Figure legends:**

519 Fig. 1. A series examples of chromatograms of representative fish muscle samples detected using UPLC-
520 QTOF/MS under positive ion mode at different depuration processing times (It was arranged as 0d,
521 10d, 20d, 30d, 40d, 50d in depuration from top to bottom). *Note: TIC: Total ion chromatograms;*
522 *EIC: Extracted ion chromatograms.*

523 Fig. 2. Results of PCA analysis (A) and score plots (B-F) of PLS-DA analysis of metabolites detected
524 using UPLC-QTOF/MS equipped with both ESI+ mod which was obtained from grass carp muscle
525 during different depuration periods. Note: B: 0 day *vs* 10 days; C: 0 day *vs* 20 days; D: 0 day *vs* 30
526 days; E: 0 day *vs* 40 days; F: 0 day *vs* 50 days.

527 Fig. 3. Effect of depuration time on the composition of metabolic types in grass carp muscle. Note:
528 *A/B/C/D/E/F represent samples under depuration for 0/10/20/30/40/50 days*.

529 Fig.4. Effect of depuration on metabolic pathways of grass carp muscle. Note: *Different colors of*
530 *metabolites represent change of comparative content, red: increased; blue: not significantly*
531 *changed; green: decreased. Reference: KEGG database, 2018*.

532 Fig. 5. Effect of depuration time on content of hypoxanthine (A) and inosine (B) in grass carp muscle