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Original Research Article

Evaluation of a hepatic biomarker of nutritional imbalance in juvenile red drum (*Sciaenops ocellatus*) fed 60% soybean meal-based diets using NMR-based metabolomics

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ABSTRACT

A 12-week feeding trial with juvenile red drum (*Sciaenops ocellatus*) fed high-soybean meal (SBM) diets was conducted to investigate a putative biomarker of nutritional imbalance, N-formimino-L-glutamate (FIGLU). Three fishmeal-free, 60% SBM pelleted diets (named B12, Fol, and Met, respectively) were tested to evaluate the effects on growth performance and tissue metabolite profiles of supplementation of vitamin B_{12} (0.012 mg/kg), folate (10 mg/kg), methionine (1 g/kg) respectively, above basal supplementation levels. A fourth SBM-based diet (named B12/Fol/Met) was formulated with a combination of B_{12}, folate, and methionine to attain the above-mentioned target concentrations. A fifth 60% SBM diet (named FWS) with methionine supplementation (1 g/kg above basal supplementation levels), enriched with taurine, lysine and threonine as well as minerals, was also tested. This diet contained formulation targets and additives which have allowed for replacing fishmeal with plant proteins in rainbow trout feeds. Control diets included a fishmeal-based diet (named FM), an unsupplemented basal 60% SBM diet (named SBM60), and a "natural" diet (named N) made up of equal parts of fish (cigar minnows), squid and shrimp as a positive reference for growth performance. Formulated feeds contained approximately 37% total crude protein, approximately 14% total crude lipid and were energetically balanced. Standard growth performance metrics were measured, and tissues (liver, muscle) were collected at week 12 to evaluate diet-induced metabolic changes using nuclear magnetic resonance (NMR)-based metabolomics. Our results show that the FWS diet outperformed all other SBM diets and the FM diet under all performance metrics ($P < 0.05$). FIGLU was not detected in fish fed the N diet but was detected in those fed the SBM diets and the FM diet. Fish fed the FWS diet and the Met diet showed lower hepatic levels of FIGLU compared with the other SBM-based diets ($P < 0.05$), suggesting that among the different supplementation regimes, methionine supplementation was associated with lower FIGLU levels. The FWS

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diet produced tissue metabolite profiles that were more similar to those of fish fed the N diet. Based on our results, the FWS diet constitutes a promising SBM-based alternative diet to fishmeal for red drum.

1. Introduction

N-Formimino-L-glutamate (FIGLU) is a known intermediate in the histidine catabolic pathway that ultimately converts this essential amino acid into L-glutamate (Borek and Waelsch, 1953; Tabor and Mehler, 1954). In turn, L-glutamate can be transformed into glucose for energy production via gluconeogenesis, among other important biological functions. FIGLU carries a one-carbon unit that can be transferred to tetrahydrofolate (THF), the bioactive form of folic acid (folate), thereby generating L-glutamate and 5-formimino-THF (Krebs and Hems, 1976). This metabolic pathway is also known to occur in fish (Itoh, 1970). In mammals, urine levels of FIGLU have been used as a marker of cobalamin (vitamin B_{12}) or folate (vitamin B_9) deficiency (Bennett and Chanarin, 1962; Brown et al., 1960; Knowles et al., 1960; Luhby et al., 1959; Rabinowitz and Tabor, 1958; Tabor and Wyngarden, 1958). In the case of a folate deficiency, THF is not available, thereby inhibiting FIGLU catabolism to L-glutamate and ultimately resulting in FIGLU accumulation (Wade and Tucker, 1998). Vitamin B_{12} is a known cofactor in the recycling of folate (5-methyltetrahydrofolate, 5-MTHF), in a reaction that also requires L-homocysteine as a one-carbon unit acceptor (Froese et al., 2019). L-homocysteine is in turn generated from dietary methionine, another essential amino acid, through the "methionine cycle", and therefore either a vitamin B_{12}-deficiency or a methionine deficiency can also induce increases in FIGLU levels (Batra et al., 1974).

Some plant-based ingredients for aquafeeds such as soybean meal (SBM) constitute very promising, cost-effective and sustainable alternative protein sources to fishmeal; however, they are known to be deficient in sulfur-containing amino acids, namely methionine and cysteine as well as vitamins such as vitamin B_{12} (Craig and Gatlin, 1992; Gatlin et al., 2007; Moon and Gatlin, 1991). Vitamin B_{12} and folate are usually supplemented in formulated feeds as part of a multivitamin pre-mix. Methionine is also supplemented in aquafeeds when utilizing soy products (Gatlin et al., 2007). Nutrient deficiencies might still occur when feeding diets with high levels of soybean products, such as SBM, due to unknown interactive effects of ingredients/components either on the bioavailability of the nutrient or changes in demands based on physiological needs. The potential decreases in bioavailability or increased nutrient demand by other physiological processes can contribute to poor growth. Reduced growth outcomes have been demonstrated in several fish species when fed high plant protein diets, particularly when utilizing SBM when nutritional needs are not met. In fact, vitamin B_{12} is not synthesized in higher animals or plants. Vitamin B_{12} supplementation occurs at the trace level (ppm range), since this vitamin appears to not be synthesized in adequate quantities by symbiotic bacteria hosted in the intestine in optimal growth conditions. Vitamin B_{12} deficiency and its biological and physiological effects have been studied in fish species such as salmon, trout, channel catfish and Japanese eel with symptoms varying from anemia to decreased appetite and growth (Hansen et al., 2015; Waagbø, 2010).

Dietary models of methionine and choline deficiency (MCD) in mammals have shown to be characterized by high levels of FIGLU in liver and urine samples due to the impaired recycling of THF, which in turn is required for the conversion of FIGLU to glutamate (Kyriakides et al., 2014). Methionine deficiency in fish has been observed to negatively affect growth and feed intake, in addition to being responsible for other detrimental physiological effects (Ahmed et al., 2003; Elmada et al., 2016). Based on these observations, identification of biochemical markers for vitamin B_{12}, folate and/or methionine deficiency is extremely valuable in aquaculture, especially at early stages in the fish grow-out process.

Previous nuclear magnetic resonance (NMR)-based metabolomic studies conducted in our laboratory on juvenile red drum, *Sciaenops ocellatus*, using graded levels of SBM (0% to 60%) allowed us to identify FIGLU as an important metabolic feature in liver tissue. FIGLU levels allowed differentiation between fish fed a SBM-free diet and those fed diets containing SBM. Additionally, FIGLU levels showed a strong positive correlation with SBM inclusion levels in the feeds with the highest levels detected in fish fed the 60% SBM diet (Casu et al., 2019). Fish grown on these diets displayed significantly lower growth rates and weight gain when compared to a control cohort of red drum fed a standard fishmeal-based commercial diet, and they showed signs of metabolic distress characterized by an energy-deficient state and altered lipid metabolism. Importantly, this metabolite was not detected in feed extracts within our limits of detection, and red drum fed a natural diet (cigar minnows, squid and shrimp) in a subsequent study exhibited healthy growth with FIGLU not detected in liver extracts (Casu et al., 2017).

In all these studies experimental feeds met or exceeded established nutritional requirements for red drum (National Research Council, 2011). We speculate that the histidine catabolic pathway is somehow adversely affected in fish fed diets with high inclusion levels of SBM, thus leading to an accumulation of FIGLU. Our hypothesis is that either a direct vitamin B_{12} or folate deficiency or decreased folate levels induced by a primary methionine deficiency, which leads to decreased levels of L-homocysteine and therefore impaired recycling of 5-MTHF, or possibly a combination of these might be responsible for the observed metabolic signature in red drum fed high levels of SBM. Additionally, FIGLU can be used as a biomarker of this nutritional stress in red drum. To test this hypothesis, we conducted a 12-week feeding study with juvenile red drum (*S. ocellatus*) to evaluate FIGLU as a possible biomarker of vitamin B_{12}/folate/methionine deficiency and address direct dietary interventions using higher nutrient levels.

2. Materials and methods

2.1. Experimental diets

Notations for the feeds used in this study are summarized in Table 1. Formulations and proximate composition of the seven experimental diets used in this study are shown in Table 2. Soybean meal or fishmeal (FM) was used as the protein source. Menhaden oil was used as the lipid source. All experimental diets were formulated to contain 37% digestible protein and 14.4% digestible fat. Digestible energy was allowed to range between 3,600 and 4,500 cal/g with the variation largely due to wheat flour contribution to the FM control diet. Vitamin B_{12}, folate and methionine supplementation levels were formulated to be 5×, 5×, and 2× the general nutrient requirements for red drum (National Research Council, 2011), respectively.

The negative control diet (SBM60) consisted of 60% SBM as the primary protein source and was formulated to meet all previously established nutrient requirements for red drum. This formulation was used in previous red drum feeding trials in our laboratory (Casu

Table 1
Experimental diet notations and descriptions for this study.

Feed ID	Notation	Description	Notes
1	N	Natural diet composed of cut fish, squid and shrimp	Reference diet for growth performance, interstudy reference
2	SBM60	Formulated diet with 60% (mass) hexane extracted soybean meal	Reference diet, interstudy reference
3	FM	Pelleted fishmeal-based diet, 0% soybean meal	Reference diet, commercial formulation
4	B12	SBM60 supplemented with vitamin B_{12} (5× nutrient requirement)	Experimental diet
5	Fol	SBM60 supplemented with folate (5× nutrient requirement)	Experimental diet
6	Met	SBM60 supplemented with methionine (2× nutrient requirement)	Experimental diet
7	B12/Fol/Met	SBM60 supplemented with vitamin B_{12}, folate and methionine	Experimental diet
8	FWS	SBM60 diet supplemented with methionine, lysine, threonine and taurine	Experimental rainbow trout (freshwater) diet

Table 2
Formulation, proximate composition and formulated nutrient targets of experimental diets for juvenile red drum (dry matter basis, g/kg).

Item	FM	SBM60	Fol	Met	B12	B12/Fol/Met	FWS
Ingredients							
Soybean meal[1]	0.0	590.0	590.0	590.0	590.0	590.0	590.0
Fish meal[2]	334.0	0.0	0.0	0.0	0.0	0.0	0.0
Squid meal[3]	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Blood meal (poultry)[4]	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Fish oil (menhaden)[5]	104.6	132.5	132.5	132.5	132.5	132.5	132.5
Wheat flour[6]	419.3	120.2	120.2	119.2	120.2	119.2	64.3
Dry lecithin[7]	0.0	0.0	0.0	0.0	0.0	0.0	10.0
Vitamin C[8]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin premix[9]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Sodium chloride	0.0	0.0	0.0	0.0	0.0	0.0	2.8
Magnesium oxide	0.0	0.0	0.0	0.0	0.0	0.0	0.6
Potassium chloride	0.0	0.0	0.0	0.0	0.0	0.0	5.6
Monocalcium Phosphate	26.0	24.0	24.0	24.0	24.0	24.0	42.0
Choline chloride (50%)	6.0	6.0	6.0	6.0	6.0	6.0	6.0
DL-Methionine	2.1	7.5	7.5	8.5	7.5	8.5	8.5
Lysine HCl	1.3	13.0	13.0	13.0	13.0	13.0	23.2
Threonine	3.7	3.8	3.8	3.8	3.8	3.8	6.5
Taurine	0.0	0.0	0.0	0.0	0.0	0.0	5.0
Trace mineral premix[10]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin B_{12}, mg/kg	0.00	0.00	0.00	0.00	0.012	0.012	0.00
Folate, mg/kg	0.00	0.00	10.00	0.00	0.00	10.00	0.00
Total	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate composition							
Crude protein	380.0	426.0	402.0	427.0	420.0	421.0	424.0
Crude lipid	112.0	84.0	115.0	107.0	116.0	111.0	122.0
Fiber (ADF)	11.0	44.0	39.0	48.0	54.0	57.0	52.0
Ash	46.0	69.0	64.0	70.0	67.0	70.0	84.0
Dry matter	919.0	929.0	916.0	885.0	855.0	889.0	926.0
Formulated nutrient targets, %							
Crude protein	42.9	42.6	42.6	42.6	42.7	42.7	43.0
Crude lipid	14.4	14.4	14.4	14.4	14.4	14.4	14.9
Digestible protein	38.7	37.0	37.0	37.0	37.1	37.1	37.5
Energy	4531	3765	3765	3765	3762	3762	3638
Total phosphorus	1.03	1.06	1.06	1.06	1.06	1.06	1.44
Available phosphorus	0.57	0.35	0.35	0.35	0.35	0.35	0.54
Lysine	3.0	3.0	3.0	3.0	3.0	3.0	3.8
Methionine	1.20	1.20	1.20	1.20	1.30	1.30	1.30
Threonine	1.9	1.9	1.9	1.9	1.9	1.9	2.1

FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal.

[1] Soybean meal (480 g/kg crude protein) supplied by Archer Daniels Midland (ADM).
[2] Fish meal (SeaPro-75) supplied by BioOregon Protein.
[3] Squid meal supplied by Rangen Inc.
[4] Blood meal supplied by Rangen Inc.
[5] Fish oil (Virginia Prime menhaden oil) supplied by Omega Protein.
[6] Wheat flour (120 g/kg crude protein) supplied by Manildra Milling.
[7] Dry lecithin (Yelkinol AC) supplied by Alliance Nutrition, ADM affiliate.
[8] Vitamin C (Stay-C, 35%) supplied by DSM Nutritional Products.
[9] Vitamin premix (Agricultural Research Service [ARS] 702) provides the following (per kilogram of diet): vitamin A, 9650 IU; vitamin D, 6600 IU; vitamin E, 132 IU; vitamin K_3, 1.1 mg; thiamin mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate DL-calcium, 46.5 mg; cyancobalamin, 0.03 mg; nicotinic acid, 21.8 mg; biotin, 0.34 mg; folic acid, 2.5 mg; inositol 600 mg.
[10] Trace mineral premix (Agricultural Research Service [ARS] 1440) provides the following (per kilogram of diet): zinc, 37 mg; manganese, 10 mg; iodine, 5 mg; copper, 3 mg; selenium, 0.4 mg.

et al., 2019; Watson et al., 2019). Three SBM-based test diets (Fol, B12, Met) were formulated to supplement folate, vitamin B_{12} and methionine at 10 mg/kg, 0.012 mg/kg and 1 g/kg, respectively, above basal supplementation levels. The fifth SBM-based diet (B12/Fol/Met) was formulated with a combination of vitamin B_{12}, folate and methionine to attain the prescribed target concentrations. The sixth diet was a fishmeal control diet (FM) to provide the majority of crude protein from fishmeal in place of SBM protein. The seventh diet U.S.

Fish and Wildlife Service (FWS) was a SBM-based diet and was formulated to meet the dietary requirements with an elevated methionine target of 13 g/kg digestible methionine, available phosphorus of 5.4 g/kg, available lysine of 38.2 g/kg and taurine, sodium chloride, magnesium oxide and potassium chloride supplemented at 5.0, 2.8, 0.6, 5.6 g/kg, respectively. These formulation targets and additives have shown promise when utilized in rainbow trout feeds with reduced fishmeal. Additionally, the eighth diet (N) was a natural diet made up of equal parts of fish (cigar minnows), squid and shrimp as a positive reference for growth performance (Casu et al., 2017).

Dry ingredients were weighed, mixed in a Marion paddle mixer (Marion Process Solutions, Marion, IA, USA), and co-ground in an air-swept pulverizer (model 18-H, Jacobson, Minneapolis, MN, USA). Diets were manufactured using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18-s exposure in 6 extruder barrel sections. Slow-sinking pellets (3.5 mm) were dried in a pulse-bed drier (Buhler AG, Uzwil, Switzerland) at 107 °C until a moisture level below 7% was attained. A forced air table was used to cool the pellets prior to fish oil being applied with a Phlauer vacuum infusion topcoater (A.J. Mixing, Oklahoma City, OK, USA). Diets were stored in a cool dry room until use.

2.2. Animal husbandry

Captive, wild red drum broodstock were volitionally spawned at the Marine Resources Research Institute (MRRI) in Charleston, South Carolina by South Carolina Department of Natural Resources (SCDNR) personnel. Larval fish from a single unique genetic family were transported and stocked into earthen ponds at the Waddell Mariculture Center (WMC) in Bluffton, South Carolina. Fish were transported back to the MRRI after approximately 30 d in ponds and cultured in a recirculating aquaculture system consisting of twenty-four 1,600-L tanks equipped with sand filters, fluidized bed filters, and protein fractionation for mechanical and biological filtration and UV sterilizers. Fish density was 25 fish per tank. Fish were acclimated from natural pond conditions to 25 °C over a period of 1 week at 1 °C/d and held at a salinity of 30% to 32%. During a 4-week conditioning period, fish were fed 2 times daily to apparent satiation using the same pelleted FM feed used throughout the trial. Excess feed was removed from the tanks after 10 min of no visible feeding.

To assess potential gross changes in composition, 10 fish were euthanized using tricaine methanesulfonate (MS-222, Argent Chemical Laboratories Inc., Redmond, WA, USA) at a concentration of 500 mg/L buffered with sodium bicarbonate for analysis of whole-body ($n = 5$) and fillet composition ($n = 5$) prior to beginning the feeding trial. At the start of the feeding trial fish had a mean individual weight of 99.1 ± 3.1 g. The 8 diets were randomly assigned to 3 tanks per treatment. All the fish in tanks were batch weighed at each sampling point.

Fish were fed to apparent satiation twice per day during the week or once per day on weekends, and total feed weight consumed was recorded. Water temperature, dissolved oxygen (DO), pH and salinity were recorded 3 times per week on a subset of tanks ($n = 12$ tanks/sampling) and ammonia, nitrite and nitrate measured weekly ($n = 12$ tanks/week) using Hach spectrophotometer reagents (Hach Inc., Loveland, CO, USA). Additional fish ($n = 5$ fish/treatment) were euthanized at the conclusion of the 12-week feeding trial for whole-body and fillet proximate analyses (Tables 3 and 4).

2.3. Tissue sample collection for metabolomics

Sampling was performed at week 12, at the end of the feeding trial. Sampled tissues included liver and muscle. Nine fish per treatment were sampled. Fish were euthanized with a lethal dose of MS-222 for 3 min prior to dissection. Fish were dissected anteriorly from anus to gills and viscera were removed. The liver was excised, quickly rinsed with chilled 3% saline solution, placed into pre-labeled 5-mL cryovials, flash frozen in liquid nitrogen, and stored at −80 °C until further processing. For muscle tissue collection, the right fillet was dissected, and upon skin removal, rinsed with chilled 3% saline solution. Specifically, 5 plugs of fillet were collected from above the lateral line, near the dorsal fin and head utilizing 8-mm disposable biopsy punches (VWR, Radnor, PA, USA). Subsequently, the muscle plugs were placed into pre-labeled 5-mL cryovials, flash frozen in liquid nitrogen, and stored at −80 °C until further processing.

Quality control samples were used in each extraction batch, including a pooled liver control material (LCM) and a pooled muscle control material (MCM) prepared from excess tissue, a NIST Standard Reference Material, SRM 1946 ("Lake Superior Fish Tissue"), and a blank.

2.4. Proximate composition, growth performance, feed utilization and morphological indices

Ten fish were euthanized at the beginning of the feeding trial and 5 fish per treatment at the end of the trial for proximate analysis of whole-body and fillet composition (dry matter, crude protein, crude lipid, ash, minerals) (5 whole-fish samples and 5 fillet samples).

Table 3
Whole-body proximate composition of juvenile red drum (per kg DM).

Chemical composition	FM	SBM60	Fol	Met	B12	B12/Fol/Met	FWS	*P*-value
Crude protein, g	655.0 ± 11.4	687.0 ± 7.4	683.0 ± 12.5	671.0 ± 13.2	674.0 ± 3.0	684.0 ± 8.1	642.0 ± 13.3	0.0413
Crude lipid, g	183.0 ± 10.2	155.0 ± 8.1	158.0 ± 12.7	172.0 ± 7.4	175.0 ± 5.7	151.0 ± 7.5	190.0 ± 11.8	0.7861
Ash, g	133.0 ± 3.9	133.0 ± 3.9	142.0 ± 5.2	124.0 ± 4.7	128.0 ± 3.2	142.0 ± 4.5	141.0 ± 2.3	0.0244
Dry matter, g	284.0 ± 2.2	285.0 ± 3.7	286.0 ± 3.2	286.0 ± 4.1	292.0 ± 1.3	283.0 ± 5.5	301.0 ± 7.0	0.1609
Phosphorous, g	23.3 ± 4.9	21.9 ± 9.1	23.4 ± 6.3	22.8 ± 5.0	25.0 ± 4.5	25.2 ± 1.7	24.7 ± 8.3	0.1120
Potassium, g	12.2 ± 2.6	12.6 ± 5.3	12.6 ± 2.2	13.6 ± 2.5	13.3 ± 2.6	12.9 ± 6.7	12.0 ± 4.1	0.1324
Sodium, g	4.7 ± 0.1	4.4 ± 0.2	4.5 ± 0.1	4.8 ± 0.2	4.8 ± 0.1	4.6 ± 0.2	4.4 ± 0.2	0.1611
Calcium, g	41.5 ± 1.2	37.0 ± 1.2	41.4 ± 2.6	37.9 ± 1.1	42.5 ± 0.6	44.4 ± 3.6	41.6 ± 1.6	0.1469
Magnesium, g	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	0.2920
Sulfur, mg	6.1 ± 0.1^{a}	6.2 ± 0.2^{a}	6.3 ± 0.0^{a}	6.9 ± 0.1^{a}	7.2 ± 0.1^{b}	6.6 ± 0.3^{a}	7.8 ± 0.2^{b}	<0.0001
Zinc, mg	34.6 ± 1.50^{a}	37.3 ± 0.81^{a}	38.4 ± 1.21^{ab}	38.9 ± 2.14^{ab}	44.6 ± 1.40^{b}	43.6 ± 2.26^{ab}	38.4 ± 0.66^{ab}	0.0005
Copper, mg	2.0 ± 0.2^{a}	1.9 ± 0.3^{a}	2.0 ± 0.1^{a}	2.4 ± 0.2^{a}	3.5 ± 0.4^{b}	2.2 ± 0.2^{a}	1.6 ± 0.1^{a}	<0.0001
Manganese, mg	12.5 ± 0.7	12.7 ± 1.2	15.0 ± 1.1	13.3 ± 0.8	14.3 ± 1.2	13.5 ± 1.7	14.7 ± 0.7	0.6273
Iron, mg	34.4 ± 1.8^{a}	30.2 ± 0.9^{a}	30.9 ± 1.8^{a}	34.9 ± 1.3^{a}	60.2 ± 6.5^{b}	34.4 ± 1.7^{a}	29.9 ± 1.4^{a}	<0.0001

FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal.
Data represent mean ± SEM. One-way ANOVA is performed to test for significant differences between dietary treatments (Natural diet excluded). Tukey's HSD test is used for multiple comparisons of the means. Mean values in the same line with different superscripts are significantly different ($P < 0.05$).

Table 4
Fillet proximate composition of juvenile red drum (per kg DM).

Chemical composition	FM	SBM60	Fol	Met	B12	B12/Fol/Met	FWS	*P*-value
Crude protein, g	890.0 ± 19.0^{ab}	913.0 ± 10.0^{b}	909.0 ± 6.0^{ab}	909.0 ± 0.0^{ab}	908.0 ± 4.0^{ab}	912.0 ± 8.0^{b}	885.0 ± 19.0^{a}	0.0034
Crude fat, g	36.0 ± 13.0^{ab}	17.0 ± 7.0^{a}	25.0 ± 5.0^{ab}	19.0 ± 5.0^{a}	27.0 ± 8.0^{ab}	19.0 ± 5.0^{a}	42.0 ± 16.0^{b}	0.0009
Ash, g	48.0 ± 2.0^{a}	51.0 ± 1.0^{b}	51.0 ± 1.0^{b}	47.0 ± 1.0^{a}	50.0 ± 1.0^{ab}	50.0 ± 1.0^{ab}	49.0 ± 2.0^{ab}	0.0013
Dry matter, g	231.0 ± 0.0	231.0 ± 0.0	234.0 ± 0.0	232.0 ± 0.0	225.0 ± 0.0	226.0 ± 0.0	232.0 ± 0.0	0.5993
Phosphorous, g	8.9 ± 0.8^{a}	9.9 ± 0.2^{b}	9.6 ± 0.4^{b}	10.0 ± 0.8^{b}	10.3 ± 0.7^{b}	10.1 ± 0.6^{b}	7.7 ± 1.1^{a}	<0.0001
Potassium, g	18.2 ± 1.4^{a}	20.1 ± 0.6^{b}	19.4 ± 0.6^{b}	20.2 ± 1.7^{b}	20.6 ± 1.5^{b}	20.5 ± 1.0^{b}	15.9 ± 2.0^{a}	<0.0001
Sodium, g	1.2 ± 0.1^{a}	1.4 ± 0.0^{b}	1.3 ± 0.0^{b}	1.5 ± 0.2^{b}	1.5 ± 0.2^{b}	1.4 ± 0.6^{b}	0.9 ± 0.1^{a}	<0.0001
Calcium, g	0.8 ± 0.4	0.8 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.6242
Magnesium, g	1.3 ± 0.1^{a}	1.4 ± 0.0^{b}	1.4 ± 0.0^{b}	1.4 ± 0.1^{b}	1.5 ± 0.0^{b}	1.4 ± 0.0^{b}	1.1 ± 0.1^{a}	<0.0001
Sulfur, mg	8.5 ± 0.4^{a}	9.2 ± 0.2^{ab}	9.3 ± 0.3^{ab}	9.7 ± 0.8^{ab}	9.9 ± 0.6^{b}	9.6 ± 0.6^{ab}	9.1 ± 1.1^{ab}	<0.0001
Zinc, mg	15.9 ± 1.8	17.5 ± 1.6	19.0 ± 3.5	17.8 ± 1.9	19.7 ± 2.2	18.4 ± 1.8	15.4 ± 2.0	0.0456
Copper, mg	1.8 ± 0.3	1.5 ± 0.3	2.2 ± 1.0	1.7 ± 0.3	2.1 ± 0.6	1.7 ± 0.2	1.8 ± 0.3	0.2065
Manganese, mg	0.6 ± 0.2^{a}	1.0 ± 0.3^{ab}	1.4 ± 0.8^{b}	1.0 ± 0.2^{ab}	1.3 ± 0.3^{ab}	0.8 ± 0.1^{ab}	1.0 ± 0.2^{ab}	0.0026
Iron, mg	12.4 ± 1.5	14.6 ± 3.7	19.5 ± 11.6	16.9 ± 7.8	21.5 ± 4.7	15.5 ± 1.9	23.1 ± 16.9	0.4378

FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal.
Data represent mean ± SD. One-way ANOVA is performed to test for significant differences between dietary treatments (Natural diet excluded). Tukey's HSD test is used for multiple comparisons of the means. Mean values in the same line with different superscripts are significantly different ($P < 0.05$).

Both whole fish samples and fillets were dried in an oven at 80 °C to determine DM. Dried samples were subsequently homogenized using a coffee grinder (Hamilton Beach, Glen Allen, VA, USA) and sent to the Clemson University Feed and Forage Laboratory (Clemson, SC) for proximate analysis by standard methods (http://www.clemson.edu/public/regulatory/ag_svc_lab/index.html).

Standard growth performance parameters, feed utilization, and morphological indices utilized in this feeding trial to compare treatments were calculated as follows:

Weight gain (%) = (final weight − initial weight)/initial weight × 100;

Specific growth rate (SGR) = (ln final weight − ln initial weight)/days × 100;

Protein efficiency ratio (PER) = (final weight − initial weight)/protein fed;

Feed conversion ratio (FCR) = dry feed intake/(final weight − initial weight);

Condition factor = (body weight, g) × 100/(body length, cm)^3;

Hepatosomatic index (HSI, %) = liver weight/body weight × 100.

2.5. Tissue metabolite extraction for NMR-based metabolomic analysis

Frozen liver and muscle tissue samples were homogenized using a cryogenic mill (Retsch, Inc., Newtown, PA, USA) and extracted using the chloroform-methanol–water extraction protocol by Bligh and Dyer (Bligh and Dyer, 1959; Lin et al., 2007; Viant, 2007) as described in detail elsewhere (Casu et al., 2017; 2019; Schock et al., 2012; 2013) (see Supplementary methods). Every step in the homogenization protocol was performed inside a Cryocart (Chart Industries, Inc., Garfield Heights, OH, USA) to prevent the tissue samples from thawing. The tissue sample homogenates were aliquoted by weighing 100 mg (±3 mg) per sample, transferred into ceramic bead tubes (2.8 mm) (Mo Bio Laboratories, Carlsbad, CA, USA), and subsequently stored at −80 °C until further processing. Details on the NMR spectra acquisition and processing are provided (see Supplementary methods).

2.6. Multivariate statistical analysis

The 1D NOESY ^{1}H spectra were initially binned (bin size: 0.005 ppm) in the spectral region 10.0 to 0.2 ppm. Spectral regions including formate (8.47 to 8.45 ppm), chloroform (7.69 to 7.67 ppm), water (4.90 to 4.70 ppm), acetate (1.93 to 1.91 ppm) were excluded prior to statistical analysis to remove residual water signal from solvent suppression and traces of contaminants found in blanks. Alignment and binning were performed using NMRProcFlow 1.3 software (www.nmrprocflow.org). The spectra were scaled to the sum of total spectral intensities and Pareto normalization was applied to the binned spectra using MetaboAnalyst 4.0 software (www.metaboanalyst.ca). Principal component analysis (PCA) was performed to assess clustering within the data and significant differences between different groups were determined based on Student's *t*-tests (two-tailed, unequal variance).

To assess experimental reproducibility, spectral relative standard deviation (RSD = standard deviation/mean × 100%) (Parsons et al., 2009) was measured for quality control samples (LCM, MCM, SRM1946).

2.7. Statistical analysis

A one-way analysis of variance (ANOVA) was performed to test the significance of differences among the dietary treatments. Post-hoc comparisons of the means were conducted using Tukey's multiple-comparison test. All data were presented as the means ± SD. Differences were considered statistically significant if the *P*-value was less than 0.05.

One-way ANOVA was also performed to test the significance of differences in metabolite levels among different treatments. All statistical calculations were performed using GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Growth performance, feed utilization and morphological indices

Growth performance and feed utilization of juvenile red drum fed the different experimental diets are shown in Table 5. As expected, the N diet outperformed all experimental diets under all performance metrics. The best-performing pelleted diet was the

Table 5
Growth performance and feed utilization of experimental diets for juvenile red drum.

Parameter	FM	SBM60	Fol	Met	B12	B12/Fol/Met	FWS	N	*P*-value
Feed consumption, g/fish	183.51 ± 23.82[a]	205.27 ± 20.14[a]	181.88 ± 25.59[a]	194.18 ± 6.31[a]	187.81 ± 11.73[a]	199.86 ± 14.41[a]	249.20 ± 1.92[b]	1364.01 ± 44.77	0.0038
Weight gain, g/fish	195.85 ± 21.81[a]	206.30 ± 33.50[a]	199.10 ± 47.83[a]	202.00 ± 6.53[a]	203.41 ± 34.94[a]	202.44 ± 17.73[a]	325.76 ± 13.24[b]	562.63 ± 24.24	0.0006
Weight gain, %	201.04 ± 20.01[a]	203.37 ± 29.50[a]	197.85 ± 41.55[a]	207.91 ± 10.00[a]	205.73 ± 38.29[a]	204.92 ± 17.01[a]	327.71 ± 26.57[b]	565.67 ± 33.80	0.0006
Final weight, g	293.19 ± 22.97[a]	307.56 ± 35.44[a]	299.33 ± 51.75[a]	299.24 ± 8.00[a]	302.47 ± 34.34[a]	301.20 ± 18.32[a]	425.39 ± 9.42[b]	662.16 ± 22.67	0.0008
Final length, mm	278.22 ± 21.25[a]	289.11 ± 20.97[a]	276.89 ± 22.09[a]	289.22 ± 8.77[a]	292.33 ± 14.14[a]	285.89 ± 18.54[a]	332.00 ± 11.03[b]	356.67 ± 20.05	<0.0001
FCR	0.94 ± 0.02[b]	1.00 ± 0.08[b]	0.93 ± 0.10[b]	0.96 ± 0.05[b]	0.94 ± 0.12[b]	0.99 ± 0.02[b]	0.77 ± 0.04[a]	2.43 ± 0.06	0.0227
PER	2.81 ± 0.07[a]	2.33 ± 0.18[a]	2.72 ± 0.33[a]	2.42 ± 0.13[a]	2.57 ± 0.31[a]	2.41 ± 0.06[a]	3.11 ± 0.15[b]	0.63 ± 0.02	0.0036
SGR	1.3 ± 0.08[a]	1.3 ± 0.12[a]	1.3 ± 0.16[a]	1.3 ± 0.04[a]	1.3 ± 0.15[a]	1.3 ± 0.07[a]	1.7 ± 0.07[b]	2.2 ± 0.06	0.0022
Condition factor	1.32 ± 0.08	1.25 ± 0.11	1.26 ± 0.09	1.29 ± 0.11	1.28 ± 0.03	1.24 ± 0.11	1.22 ± 0.11	1.39 ± 0.11	0.6677

FCR = feed conversion ratio; PER = protein efficiency ratio; SGR = specific growth rate.
FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.
Data represent mean ± SD. One-way ANOVA is performed to test for significant differences between dietary treatments (Natural diet excluded). Tukey's HSD test is used for multiple comparisons of the means. Mean values in the same line with different superscripts are significantly different ($P < 0.05$).

FWS diet. Feed consumption, weight gain, final weight, final length, PER, and SGR were all significantly higher in fish fed the FWS diet compared with those fed the other diets ($P < 0.05$). Additionally, FCR was significantly lower in fish fed the FWS diet compared with those fed the other diets ($P = 0.0227$). No significant differences in feed consumption, weight gain, final weight, final length, PER, SGR or condition factor were detected among fish fed any of the experimental treatments ($P > 0.05$). As shown in Table S1, HSI was significantly lower in fish fed the FWS diet compared with those fed the other diets ($P = 0.0374$), while the viscerosomatic index was not affected by the dietary treatments ($P > 0.05$). Although the N diet outperformed all experimental diets under all performance metrics measured, an important finding from this study is that the FWS diet, a fishmeal-free, 60% SBM diet performed second best, after the N diet under all measured performance parameters (feed consumption, weight gain, final weight, final length, PER, and SGR). No significant differences in performance were detected among the other experimental diets.

3.2. Whole-body proximate composition

Whole-body composition is shown in Table 3. There were no significant differences in crude protein, crude lipid, ash or dry matter content among fish fed any of the experimental treatments. Additionally, there were no significant differences in phosphorus, potassium, sodium, calcium, magnesium, and manganese content among fish fed any of the experimental treatments ($P > 0.05$). Significantly higher sulfur content was detected in whole body of fish fed the FWS diet than those fed the other diets ($P < 0.05$), with the exception of B12 ($P > 0.05$). Significantly higher zinc content was detected in whole body of fish fed the B12 diet than those fed the FM and SBM60 diets ($P = 0.0005$). Significantly higher copper and iron content was detected in whole body of fish fed the B12 diet than those fed the other diets ($P < 0.05$).

3.3. Fillet proximate composition

Fillet composition is shown in Table 4. Crude protein was significantly higher in the fillet of fish fed SBM60 and B12/Fol/Met than those fed the FWS diet ($P = 0.0034$). Crude fat was significantly higher in the fillet of fish fed the FWS diet than those fed SBM60, Met, and B12/Fol/Met ($P = 0.0009$). Ash was significantly higher in the fillet of fish fed SBM60 and Fol than those fed FM and Met. There were no significant differences in dry matter content among fish fed any of the experimental treatments. Significantly lower phosphorus, potassium, sodium and magnesium content was detected in the fillet of fish fed the FWS diet than all other treatments ($P < 0.05$) with the exception of FM ($P > 0.05$). Significantly lower sodium and sulfur content was detected in the fillet of fish fed the FM diet than those fed the B12 diet ($P < 0.05$). There were no significant differences in calcium, zinc, copper, or iron content among fish fed any of the experimental treatments. Significantly lower manganese content was detected in the fillet of fish fed the FM diet than those fed the Fol diet ($P = 0.0026$).

3.4. Untargeted NMR-based metabolomics

In order to assess experiment repeatability, including sample processing and instrumental variability, spectral median relative standard deviation (%RSD) was calculated for each of the QC sample spectra: SRM 1946 (8.40%), LCM (8.17%), MCM (8.84%). All of the QC samples resulted in %RSDs of less than 10% suggesting overall good experimental repeatability.

3.5. NMR-metabolomic data analysis

To investigate the observed metabolic differences among the experimental treatments at the end of the 12-week feeding trial (T12), PCA score plots for both liver and muscle extracts were calculated (Fig. 1A and B). The score plots show a total explained variance in PC1 and PC2 of 49.9% and 43.3% for the liver and muscle, respectively. In these PCA plots, the N diet and the FWS groups separate from the other dietary treatments along PC1 with an explained variance along this principal component of 30.7% and 30.3% for liver and muscle, respectively. However, while there is no significant separation ($P = 0.078$) along PC1 between the SBM-based diet FWS group and the N group in liver extracts, the same groups show a clear separation ($P < 0.001$) along PC1 in muscle extracts. Furthermore, the N group and the FWS group show a significant separation ($P < 0.001$) along PC2 in the liver, but this is not observed in the muscle ($P = 0.472$). Additionally, a significant separation ($P < 0.001$) of the fishmeal (FM) group from the soy-based dietary treatments was also detected along PC2 (EV = 19.2%) in liver extracts, whereas these groups were not significantly different ($P > 0.05$) in the muscle tissue. Furthermore, no significant separation was apparent among the other dietary treatments from these PCA plots either in the liver or the muscle tissue. Overall, results from NMR-based metabolomics analysis showed significant differences both in liver and muscle metabolite profiles between the N diet and the experimental diets, but the FWS diet was also significantly different from all other dietary treatments, including other 60% SBM diets. Importantly, fish fed the

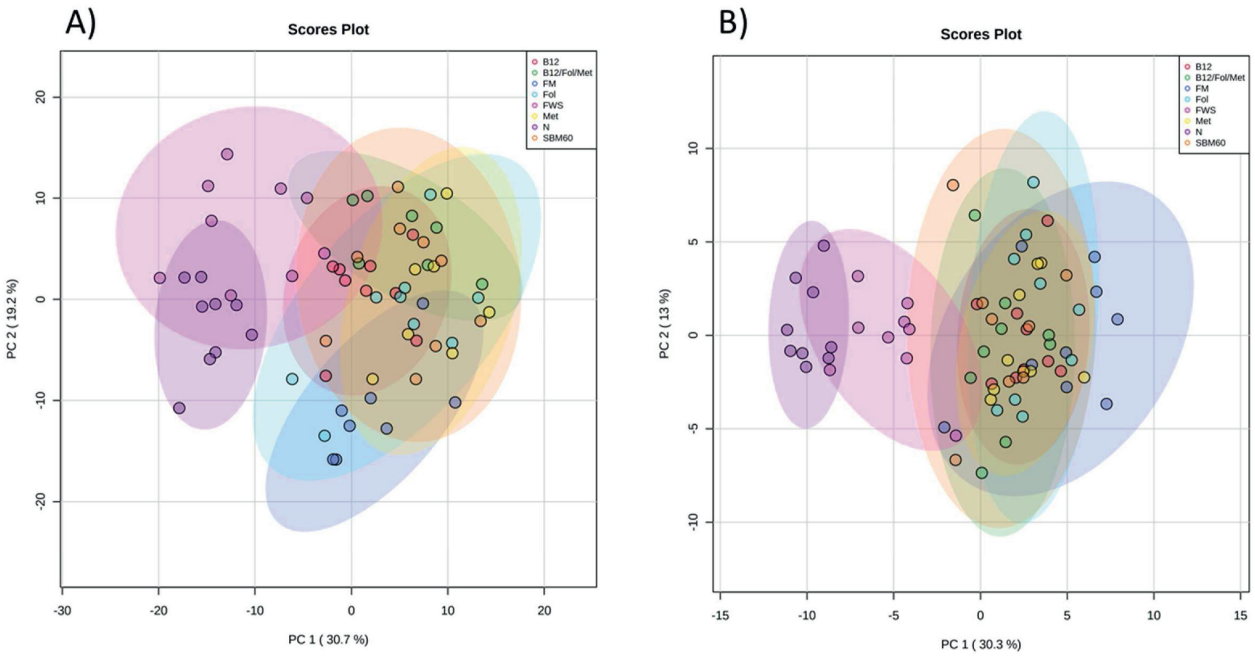

Fig. 1. Unsupervised PCA score plots from red drum tissue extracts for the five experimental diets and three reference treatments at the end of the 12-week feeding trial (T12). (A) Liver; N ($n = 9$), FM ($n = 8$), FWS ($n = 9$), SBM60 ($n = 9$), Fol ($n = 9$), Met ($n = 7$), B12 ($n = 9$), B12/Fol/Met ($n = 7$). (B) Muscle; N ($n = 9$), FM ($n = 9$), FWS ($n = 9$), SBM60 ($n = 8$), Fol ($n = 9$), Met ($n = 9$), B12 ($n = 9$), B12/Fol/Met ($n = 9$). The explained variances along PC1 and PC2 are shown in brackets. Shaded areas represent the 95% confidence regions. PCA = principal component analysis. FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

FWS diet displayed metabolite profiles that were more similar to those of fish fed the natural diet both in the liver and muscle tissue compared with the other SBM diets and the fishmeal-based diet.

To further evaluate the observed differences in metabolite profiles among the dietary treatments both in liver and muscle extracts, we generated supervised partial least square discriminant analysis (PLS-DA) score plots (Fig. 2A and B) for biomarker selection using variable importance in projection (VIP) to estimate the importance of the variables in the model. The PLS-DA models were constructed using NMR spectral data as the X-matrix and class information as the Y-matrix. In these plots, the separation of the FM group from the other experimental treatments is more pronounced both in liver and muscle extracts. Additionally, in the muscle tissue, the Met group shows a significant degree of separation from the other treatments, while this was not observed in the liver.

Next, we examined the PLS-DA component 1 loading plots to evaluate the metabolite differences that were associated with the dietary treatments. VIP score 1.2 was set as a threshold and variables with VIP scores >1.2 were selected. In the liver, a total of 20 metabolites and an unknown compound were found to be the most discriminating across the different dietary treatments at T12 (Fig. 3). Amino acids and derivatives included alanine, glutamine, glycine, and FIGLU. Metabolic intermediates of choline oxidation included betaine, and sarcosine. Carbohydrates included fructose 1-phosphate (Fru 1-P), glucose, glucose 6-phosphate (Glu 6-P), and *myo*-inositol. Osmolytes included various betaines such as butyrobetaine, proline-betaine (Pro betaine) and taurine-betaine (Tau betaine). Other metabolites included beta-alanine, creatine, glycerol 3-phosphate (Glycerol 3-P), lactate, taurine, choline and one unknown compound (unknown_105).

In the muscle, a total of 18 metabolites and 2 unknown compounds were found to be the most discriminating across the different dietary treatments at T12 (Fig. 4). Amino acids included asparagine, glutamine, glycine, histidine, lysine, serine and alanine. Carbohydrates included fructose 6-phosphate (Fru 6-P). Other metabolites included 2-aminobutyrate, 4-hydroxyproline (HyPro), betaine, malate, methylguanidine, ornithine, pantothenate, succinate, taurine, trimethylamine N-oxide (TMAO) and 2 unknown compounds (unknown_329 and unknown_365).

3.6. Cluster analysis

Supervised hierarchical clustering of samples and metabolites was performed and the results were visualized as a heatmap. Similarity measurement for clustering were based on Euclidean distance, and the Ward's linkage algorithm was used. Clustering of liver samples at T12 using the 29 significantly regulated metabolites selected based on PLS-DA VIP scores >1.2 (Table S2) resulted in complete separation of the natural diet group and the FWS group from all other treatments, with almost complete separation of the fishmeal-based treatment. All of the other dietary SBM-based treatments did not show significant separation from each other, but clustered together (Fig. 3). Similar to the liver, clustering of muscle samples using the 18 significantly regulated metabolites selected based on PLS-DA VIP scores >1.2 (Table S3) resulted in a clear separation of samples of the natural diet group and the FWS groups from all other dietary treatments, with nearly complete separation of the fishmeal-based treatment (Fig. 4). Additionally, the Met group shows almost complete separation in the muscle, while this separation was not observed in the liver.

3.7. Orthogonal partial least square discriminant analysis (OPLS-DA)

OPLS-DA models were generated for all 28 pairwise comparisons of dietary treatments to investigate differences in metabolite profiles specifically associated with these treatments. The corresponding S-plots were used to select the metabolites responsible

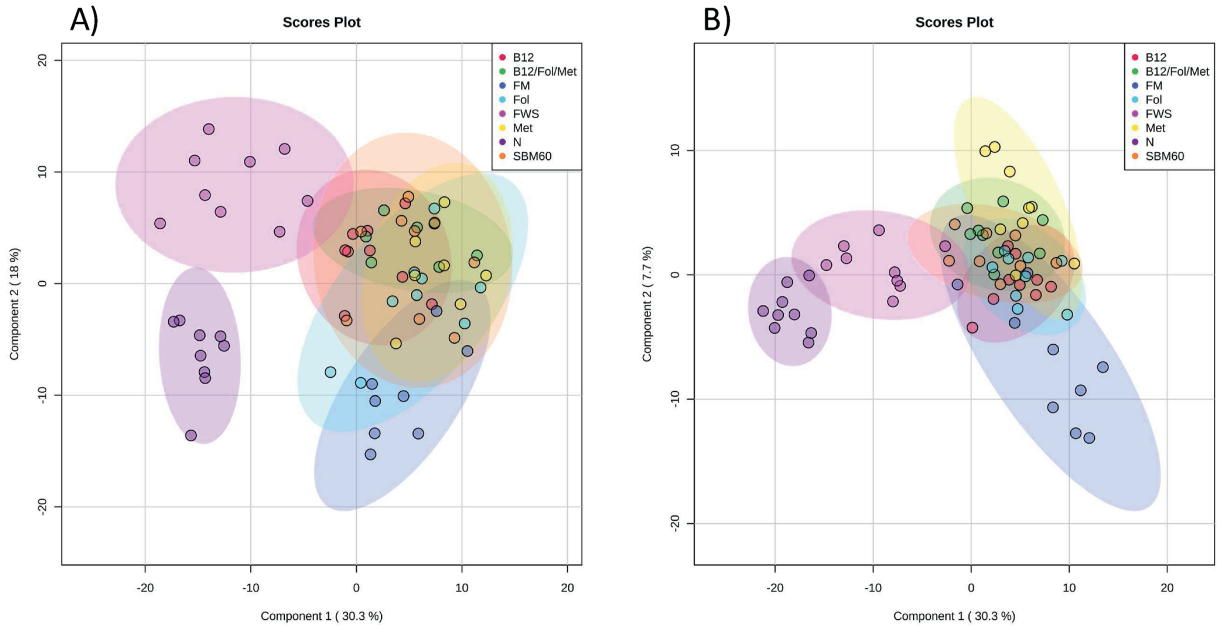

Fig. 2. Supervised PLS-DA score plot from red drum tissue extracts for the five experimental diets and three reference treatments at the end of the 12-week feeding trial (T12). (A) Liver; N ($n = 9$), FM ($n = 8$), FWS ($n = 9$), SBM60 ($n = 9$), Fol ($n = 9$), Met ($n = 7$), B12 ($n = 9$), B12/Fol/Met ($n = 7$). (B) Muscle; N ($n = 9$), FM ($n = 9$), FWS ($n = 9$), SBM60 ($n = 8$), Fol ($n = 9$), Met ($n = 9$), B12 ($n = 9$), B12/Fol/Met ($n = 9$). The explained variances along PC1 and PC2 are shown in brackets. Shaded areas represent the 95% confidence regions. PLS-DA = partial least square discriminant analysis. FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

for group discrimination. Specifically, metabolites with |p(corr)| > 0.5 were selected as the most discriminating metabolites between groups. The higher the absolute values of p(corr), the higher the discriminating power of that particular metabolite. Quality parameters for all OPLS-DA models are summarized in Tables S4 and S5 for liver and muscle extracts, respectively.

3.7.1. Liver analysis

3.7.1.1. N diet and FWS diet. PCA and OPLS-DA models were generated to compare liver extracts from red drum fed either the N diet or the FWS diet (Fig. 5 and Fig. S1A). The FWS group and the natural diet group showed complete separation indicating that fish fed these diets were characterized by significantly different metabolite fingerprints. The OPLS-DA model displayed good quality with a Q^2 of 0.773 and a R^2Y of 0.932. Our model clearly identified 19 metabolites and 5 unknown features that were significantly different in the two dietary groups. These metabolites include alanine, beta-alanine, betaine, butyrobetaine, choline, cystathionine, creatine, dimethylglycine (DMG), glucose, Glu 6-P, glutamine, glycine, inosine, lactate, malate, Pro betaine, taurine, Tau betaine, UDP-N-acetylglucosamine (UDP-GlcNAc), and 5 unknown compounds.

3.7.1.2. FM and FWS diet. PCA and OPLS-DA models were generated to compare liver extracts from red drum fed either the FM diet or the FWS diet (Fig. S2). The two dietary groups showed complete separation indicating significantly different metabolite profiles. The OPLS-DA model displayed good quality with a Q^2 of 0.773 and a R^2Y of 0.932. Our model identified 26 metabolites that were significantly discriminating between the two dietary groups. These metabolites include ADP, AMP, ATP, alanine, beta-alanine, 3-aminoisobutyrate, betaine, bile acids, choline, creatine, DMG, Fru 1-P, glucose, Glu 6-P, glutamate, glutamine, Glycerol 3-P, glycine, glycogen, lactate, maltose, *myo*-inositol, O-phosphocholine, Pro betaine, sarcosine, taurine.

3.7.1.3. SBM60 and FWS diet. PCA and OPLS-DA models were generated to compare liver extracts from red drum fed either the unsupplemented SBM60 reference diet or the FWS diet (Fig. S3). The two dietary groups showed almost complete separation in the PCA score plot, and complete separation in the corresponding OPLS-DA model indicating significant differences in metabolite profiles between the two groups. The OPLS-DA model displayed good quality with a Q^2 of 0.649 and a R^2Y of 0.813. Our model identified 11 metabolites that were significantly discriminating between the two dietary groups. These metabolites include alanine, beta-alanine, betaine, creatine, Fru 1-P, glucose, Glycerol 3-P, glycine, lactate, sarcosine, taurine.

3.7.1.4. N diet and SBM60 diet. PCA and OPLS-DA models were generated to compare liver extracts from red drum fed either the N diet or the SBM60 diet (Fig. 6 and Fig. S1B). The two dietary groups showed complete separation indicating significantly different metabolite profiles. The OPLS-DA model displayed good quality with a Q^2 of 0.831 and a R^2Y of 0.928. Our model identified 27 metabolites and 6 unknown features that were significantly discriminating between the two dietary groups. These metabolites include ATP, alanine, beta-alanine, 3-aminoisobutyrate, ascorbate, betaine, butyrobetaine, choline, creatine, cystathionine, DMG, FIGLU, Fru 1-P, glucose, Glu 6-P, glutamine, Glycerol 3-P, glycine, histidine, inosine, lactate, *myo*-inositol, O-phosphocholine, Pro betaine, sarcosine, taurine, Tau betaine.

3.7.2. Muscle analysis

3.7.2.1. N diet and FWS diet. PCA and OPLS-DA models comparing muscle extracts from red drum fed either the N diet or the FWS diet were generated (Fig. 7 and Fig. S1C). The FWS group and the natural diet group showed perfect separation. The OPLS-DA model displayed good quality with a Q^2 of 0.833 and a R^2Y of 0.899. Our model clearly identified 21 metabolites and 1 unknown compound that were significantly different in the two dietary groups. These

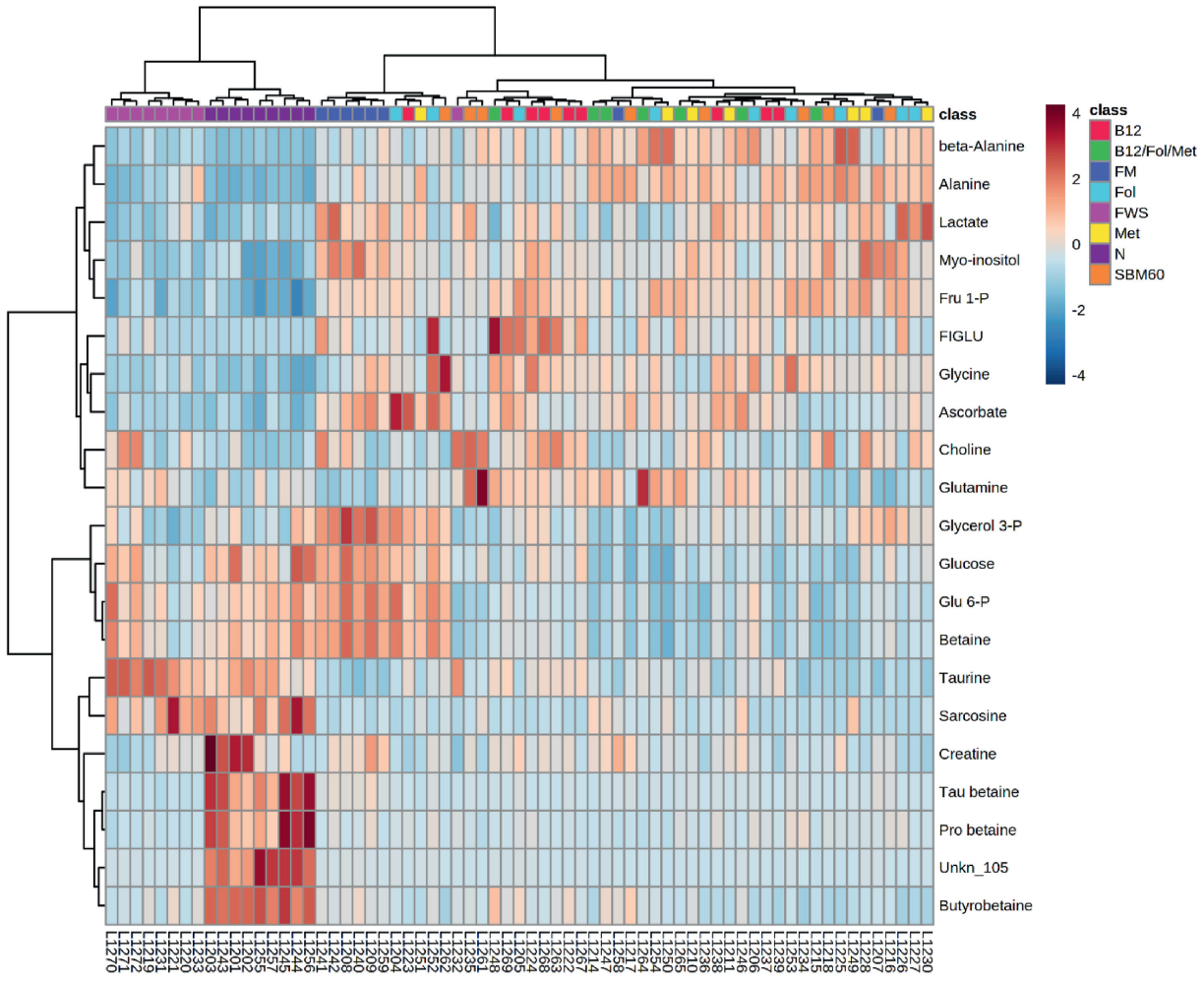

Fig. 3. Supervised clustering of liver samples from all dietary treatments at the end of the 12-week feeding trial (T12) with respect to 21 differentially regulated metabolites selected by the PLS-DA VIP score >1.2 visualized as a heat map. Each column corresponds to a specific liver sample and each row represents the relative abundance of a specific metabolite based on normalized bin intensity. Changes in the abundance of metabolites from the overall mean bin intensity are color-coded. A red color indicates an increase, while a blue color indicates a decrease. PLS-DA = partial least squares discriminant analysis; VIP = variable importance in projection; Fru 1-P = fructose 1-phosphate; FIGLU = N-formimino-L-glutamate; Glycerol 3-P = glycerol 3-phosphate; Glu 6-P = glucose 6-phosphate; Tau betaine = taurine-betaine; Pro betaine = proline-betaine. FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

metabolites include ATP, alanine, beta-alanine, asparagine, betaine, butyrobetaine, choline, creatine, creatine phosphate, Fru 6-P, Glu 6-P, Glycerol 3-P, glycine, lysine, malate, ornithine, pantothenate, proline, succinate, taurine, TMAO.

3.7.2.2. FM and FWS diet. PCA and OPLS-DA models comparing muscle extracts from red drum fed either the FM diet or the FWS diet were generated (Fig. S4). The two groups showed perfect separation. The OPLS-DA model displayed good quality with a Q^2 of 0.774 and a R^2Y of 0.863. Our model clearly identified 16 metabolites and 3 unknown features that were significantly different in the two dietary groups. These metabolites include alanine, betaine, choline, creatine, Fru 6-P, fructose 1,6-bisphosphate (Fru 1,6-bisphosphate), glycine, histidine, Hypro, IMP, lactate, lysine, serine, succinate, taurine, TMAO.

3.7.2.3. SBM60 and FWS diet. PCA and OPLS-DA models were generated to compare muscle extracts from red drum fed either the unsupplemented SBM60 reference diet or the FWS diet (Fig. S5). The two groups showed perfect separation. The OPLS-DA model displayed good quality with a Q^2 of 0.711 and a R^2Y of 0.849. Our model clearly identified 13 metabolites that were significantly different in the two dietary groups. These metabolites include betaine, choline, creatine, Fru 6-P, Glu 6-P, glycine, histidine, IMP, lactate, lysine, serine, taurine, TMAO.

3.7.2.4. N diet and SBM60 diet. PCA and OPLS-DA models were generated to compare muscle extracts from red drum fed either the N diet or the SBM60 diet (Fig. 8 and Fig. S1D). The two groups showed perfect separation. The OPLS-DA model displayed good quality with a Q^2 of 0.902 and a R^2Y of 0.940. Our model clearly identified 21 metabolites and 4 unknown features that were significantly different in the two dietary groups. These metabolites include ATP, alanine, betaine, butyrobetaine, choline, creatine, creatine phosphate, Fru 6-P, Glu 6-P, glycine, histidine, Hypro, lysine, malate, *myo*-inositol, ornithine, pantothenate, serine, succinate, taurine, TMAO.

3.7.2.5. N diet and Met diet. PCA and OPLS-DA models were generated to compare muscle extracts from red drum fed either the N diet or the Met diet (Fig. S6). The two groups showed perfect separation. The OPLS-DA model displayed good quality with a Q^2 of 0.963 and a R^2Y of 0.974. Our model clearly identified 19

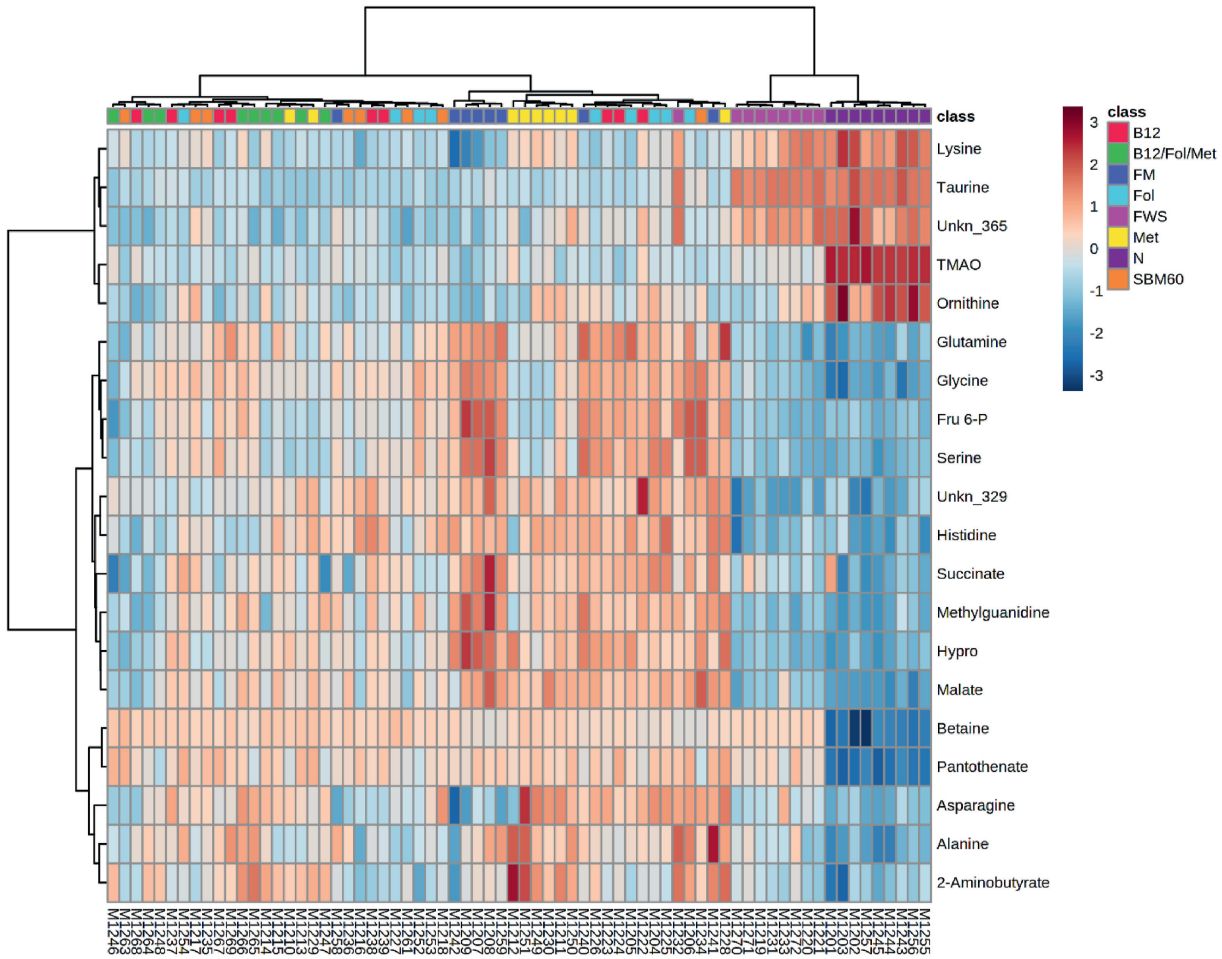

Fig. 4. Supervised clustering of muscle samples from all dietary treatments at the end of the 12-week feeding trial (T12) with respect to 20 differentially regulated metabolites selected by the PLS-DA VIP score >1.2 visualized as a heat map. Each column corresponds to a specific muscle sample and each row represents the relative abundance of a specific metabolite based on normalized bin intensity. Changes in the abundance of metabolites from the overall mean bin intensity are color-coded. A red color indicates an increase, while a blue color indicates a decrease. PLS-DA = partial least squares discriminant analysis; VIP = variable importance in projection; TMAO = trimethylamine N-oxide; Fru 6-P = fructose 6-phosphate; Hypro = 4-hydroxyproline. FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

metabolites and 1 unknown feature that were significantly different in the two dietary groups. These metabolites include ATP, alanine, asparagine, betaine, butyrobetaine, choline, creatine, creatine phosphate, Fru 6-P, glycine, histidine, Hypro, IMP, lactate, lysine, ornithine, succinate, taurine, TMAO.

3.7.2.6. FM and Met diet. PCA and OPLS-DA models comparing muscle extracts from red drum fed either the FM diet or the Met diet were generated (Fig. S7). The two groups showed partial separation in the PCA score plot, and complete separation in the corresponding OPLS-DA model indicating significant differences in metabolite profiles between the two groups. The OPLS-DA model displayed good quality with a Q^2 of 0.583 and a R^2Y of 0.744. Our model clearly identified 12 metabolites and 1 unknown feature that were significantly different in the two dietary groups. These metabolites include ATP, betaine, creatine, creatine phosphate, Fru 6-P, Glu 6-P, glycine, histidine, lysine, serine, taurine, TMAO.

3.8. Hepatic FIGLU levels

FIGLU levels were derived from integration of both FIGLU peaks at 7.83 and 7.80 ppm using Topspin 3.2 (Bruker Biospin). Peak integrals were normalized to TMSP (1.0 mmol/L, at 0 ppm) and the resulting peak areas were exported to Excel. FIGLU levels as a function of dietary treatment are displayed in Fig. 9. Significant differences (one-way ANOVA, Tukey post-hoc test, $P < 0.05$) were found between the B12, Fol and B12/Fol/Met treatments, which displayed the highest levels of FIGLU, and the N reference diet, which had undetectable levels of FIGLU. Furthermore, significant differences were detected between the B12 and Fol treatments with the highest levels of FIGLU, and the FWS and Met dietary treatments which displayed the lowest levels of FIGLU among the pelleted diets. Additionally, the B12 treatment had significantly higher levels of FIGLU compared with the Met diet.

4. Discussion

In the present study, a proton NMR-based metabolomics approach was used to investigate a putative biomarker of nutritional imbalance, FIGLU, by testing potential mitigation approaches aimed at reducing hepatic FIGLU levels in juvenile red drum as well as evaluating additional changes in tissue metabolite profiles and overall growth performance over the course of a 12-week feeding trial.

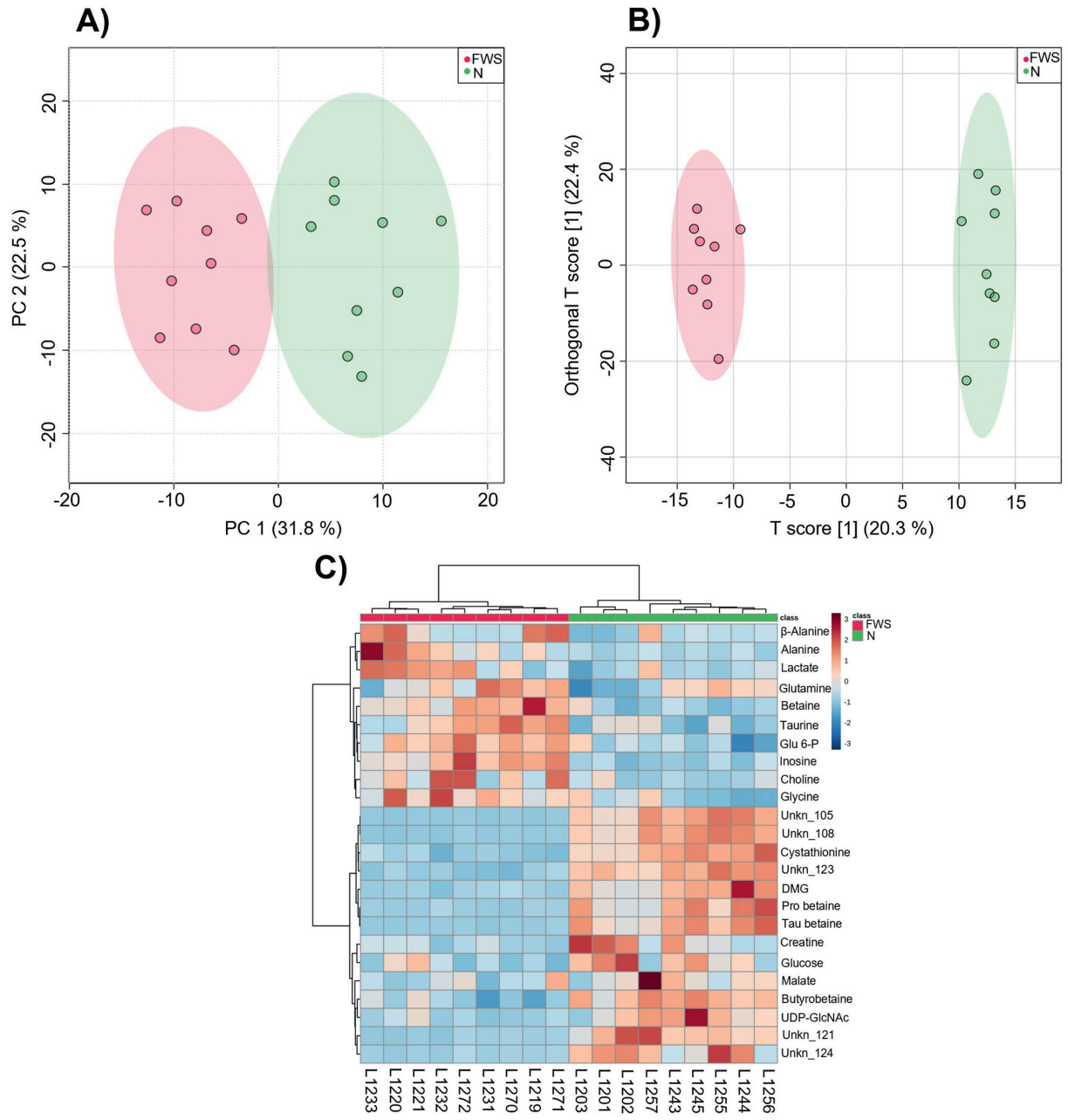

Fig. 5. The liver (A) PCA score plot, (B) OPLS-DA score plot, and (C) heat map for the FWS group and N reference group. A total of 24 metabolites were found to be significantly discriminatory between the two dietary groups. PCA = principal component analysis; OPLS-DA = orthogonal partial least square discriminant analysis; Glu 6-P = glucose 6-phosphate; DMG = dimethylglycine; Pro betaine = proline-betaine; Tau betaine = taurine-betaine; UDP-GlcNAc = UDP-N-acetylglucosamine. FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

4.1. Effects of dietary treatments on tissue metabolite levels

Differences in metabolite profiles were detected which included compounds related to energy metabolism, amino acids, trimethylamines and vitamins.

4.1.1. Effects on energy metabolism

The tricarboxylic acid (TCA) cycle has a prominent role in energy metabolism and homeostasis since it provides energy supply in the form of ATP, which can be generated from different substrates including glucose, certain amino acids and fatty acids (Bender, 2003). In our study, energy metabolism was altered by the dietary treatments with the TCA cycle intermediates succinate and malate found to be present at lower levels in the muscle of fish fed the natural diet, followed by fish fed the FWS diet compared with fish fed the SBM-based diets and the fishmeal-based diet. Our results suggest that the TCA cycle was upregulated in fish fed the SBM-based diets and the fishmeal-based diet compared with those fed the natural diet and the FWS diet in agreement with a previous study conducted in our laboratory on juvenile red drum as well as studies conducted by other groups on carnivorous fish species fed plant-based diets (Casu et al., 2017; Deborde et al., 2021; Li et al., 2009; Schock et al., 2012; Wei et al., 2016). Additionally, changes in the levels of energy-related metabolites such as creatine and creatine derivatives in the muscle tissue can also be indicative of altered energy metabolism. Methylguanidine (MG) is a metabolite generated from creatinine and from protein catabolism. MG was detected at higher levels in the muscle of red drum fed the SBM-

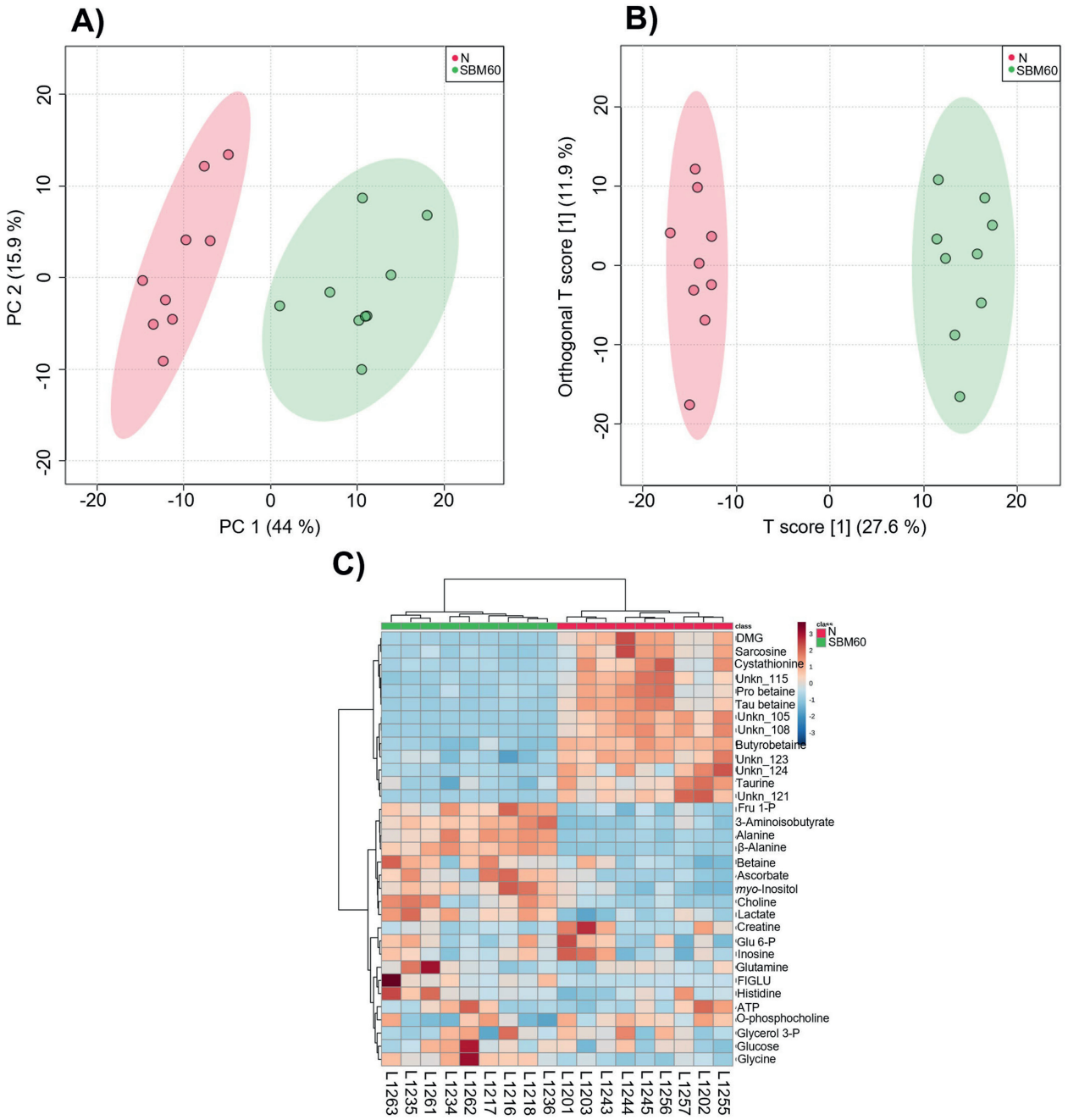

Fig. 6. The liver (A) PCA score plot, (B) OPLS-DA score plot, and (C) heat map for the SBM60 group and N reference group. A total of 11 metabolites were found to be significantly discriminatory between the two dietary groups. PCA = principal component analysis; OPLS-DA = orthogonal partial least square discriminant analysis; DMG = dimethylglycine; Pro betaine = proline-betaine; Tau betaine = taurine-betaine; Fru 1-P = fructose 1-phosphate; Glu 6-P = glucose 6-phosphate; FIGLU = N-formimino-L-glutamate; Glycerol 3-P = glycerol 3-phosphate. SBM60: 60% soybean meal based diet; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

based diets and the fishmeal-based diet compared with those fed the natural diet and the FWS diet. Differences in MG levels could be due to enhanced protein catabolism due to increased energy demand in fish fed the SBM-based diets and the fishmeal-based diet compared with those fed the natural diet and the FWS diet.

4.1.2. Effects on amino acids metabolism

Amino acid metabolism was significantly affected by the dietary treatments, with asparagine, glutamine, glycine, FIGLU (a histidine metabolite), lysine, and serine showing significant differences in relative levels among different diets. Lysine is an essential amino acid that along with methionine is naturally deficient in SBM and other plant-based feedstuffs, thus requiring adequate supplementation in plant-based aquafeeds (Craig and Gatlin, 1992; Gatlin et al., 2007; Moon and Gatlin, 1991). Lysine levels were significantly higher in the muscle of red drum fed the natural diet and the FWS diet compared with those fed the other dietary treatments. Among these, fish fed the fishmeal-based diet showed the lowest levels of lysine in the muscle tissue. These results are consistent with higher lysine supplementation levels in the FWS diet compared with the other SBM diets and the FM diet.

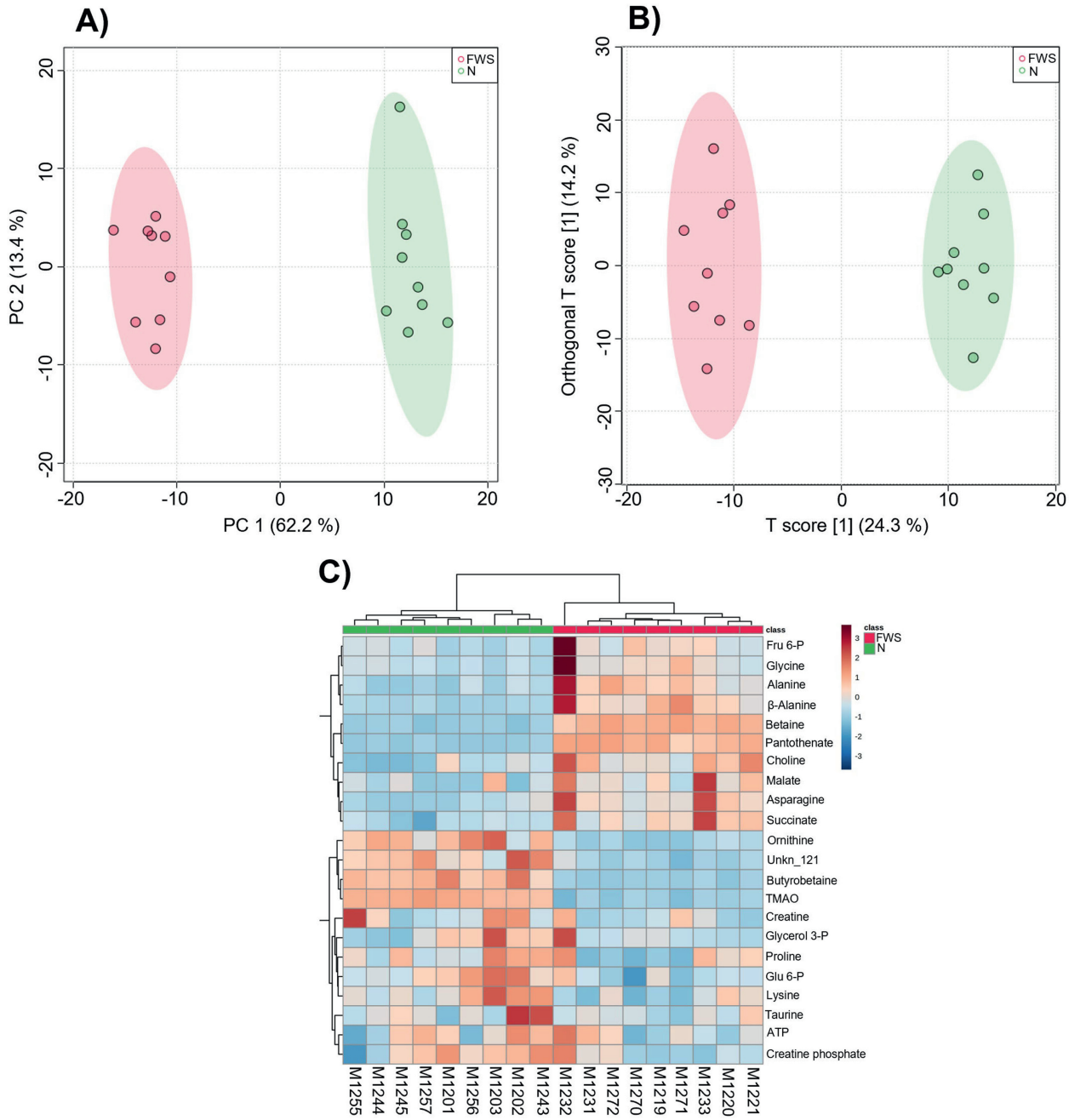

Fig. 7. The muscle (A) PCA score plot, (B) OPLS-DA score plot, and (C) heat map for the FWS group and N reference group. A total of 22 metabolites were found to be significantly discriminatory between the two dietary groups. PCA = principal component analysis; OPLS-DA = orthogonal partial least square discriminant analysis; Fru 6-P = fructose 6-phosphate; TMAO = trimethylamine N-oxide; Glycerol 3-P = glycerol 3-phosphate; Glu 6-P = glucose 6-phosphate. FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

Asparagine and glutamine are nonessential amino acids that constitute the main transport and storage forms of nitrogen. Asparagine is particularly abundant in plants (Gao et al., 2016) as a nitrogen storage compound. Asparagine can be converted to aspartate, a major glucogenic precursor and important energy substrate for fish, since it can feed into the TCA cycle via conversion to oxaloacetate (Inigo et al., 2021). Asparagine levels were found to be significantly higher in the muscle of red drum fed the SBM-based diets and the fishmeal-based diet compared with those fed the natural diet. Glutamine is important for the synthesis of purine and pyrimidine nucleotides, regulation of the body acid-base balance, and modulation of fish immune responses (Buentello and Gatlin, 1999; Cory and Cory, 2006; Taylor and Curthoys, 2004; Yan and Qiu-Zhou, 2006). Glutamine levels were significantly higher in the muscle of red drum fed the SBM-based diets and the fishmeal-based diet compared with those fed the natural diet.

Glycine is one of the most common amino acids found in proteins, and it is synthesized from other metabolites namely serine, threonine, choline and hydroxyproline (Wang et al., 2013). Glycine participates in several metabolic processes in addition to being an important inhibitory neurotransmitter in the central nervous system (Gundersen et al., 2005). Glycine is also a constituent of the tripeptide glutathione, a natural antioxidant (Wang et al., 2013). Both glycine and serine participate in gluconeogenesis and are

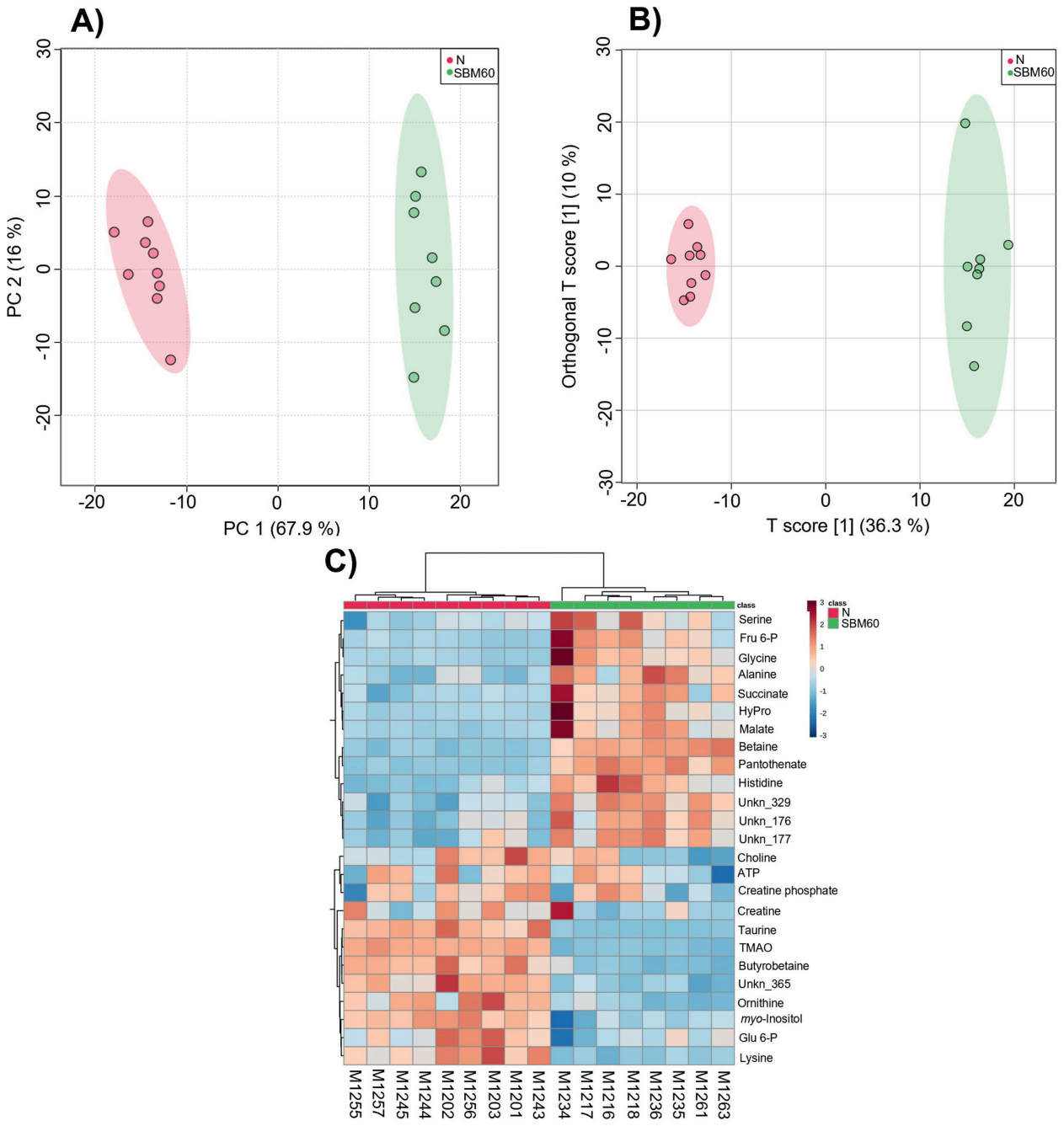

Fig. 8. The muscle (A) PCA score plot, (B) OPLS-DA score plot, and (C) heat map for the SBM60 group and N reference group. A total of 25 metabolites were found to be significantly discriminatory between the two dietary groups. PCA = principal component analysis; OPLS-DA = orthogonal partial least square discriminant analysis; Fru 6-P = fructose 6-phosphate; Hypro = 4-hydroxyproline; TMAO = trimethylamine N-oxide; Glu 6-P = glucose 6-phosphate. SBM60: 60% soybean meal based diet; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp.

important in energy metabolism (Wang et al., 2013). Fish fed the natural diet and the FWS diet had lower glycine levels than all other dietary treatments both in the liver and in the muscle tissue, in addition to lower serine levels in the muscle tissue. These results are consistent with an altered energy status in fish fed the plant-based diets compared with those fed the natural diet with the exception of the FWS diet.

Different types of meat, including poultry contain histidine-containing dipeptides such as anserine, which is formed by β-alanine and 3-methylhistidine (Mori et al., 2018). Anserine, as well as its catabolite β-alanine are known biomarkers of meat intake, especially chicken (Cuparencu et al., 2019). Fish fed the SBM-based diets, with the exception of the FWS diet showed higher levels of β-alanine compared with those fed the natural diet.

4-Hydroxyproline is a hydroxylated derivative of the non-essential amino acid proline that can be derived from dietary sources containing collagen such as foods rich in connective tissue (Cuparencu et al., 2019). Fish fed the natural diet and the FWS diet had lower HyPro levels in the muscle tissue than all other dietary treatments, while the fishmeal-based diet displayed the highest hydroxyproline levels among all dietary treatments. This is consistent with high levels of collagen found in fish and fish-derived products such as fishmeal (Castro et al., 2021).

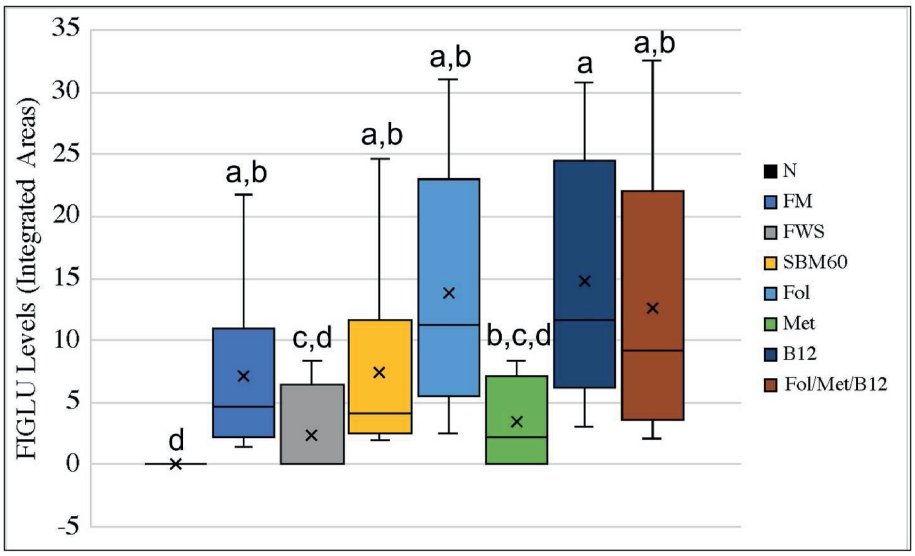

Fig. 9. FIGLU levels box plots. The two peaks at 7.83 ppm and 7.80 ppm were integrated and the sum of the two integrals was calculated for each ^{1}H NMR spectrum. Integrals were normalized to TMSP (1.0 mmol/L, at 0 ppm). FIGLU = N-formimino-L-glutamate; NMR = nuclear magnetic resonance. FM: fishmeal-based diet; SBM60: 60% soybean meal based diet; Fol: SBM60 diet supplemented with folate at 10 mg/kg; Met: SBM60 diet supplemented with methionine at 1 g/kg; B12: SBM60 diet supplemented with vitamin B_{12} at 0.012 mg/kg; B12/Fol/Met: SBM60 diet supplemented with a combination of vitamin B_{12}, folate and methionine at 0.012 mg/kg, 10 mg/kg and 1 g/kg, respectively; FWS: U.S. Fish and Wildlife Service diet consisting of 60% soybean meal; N: natural diet made up of equal parts fish (cigar minnows), squid and shrimp. One-way ANOVA was performed to test for significant differences between dietary treatments (Natural diet excluded). Tukey's HSD test was used for multiple comparisons of the means. Different letters indicate significantly different means ($P < 0.05$).

Taurine is a non-proteinogenic sulfur amino acid abundant in fish and other animal products, especially those derived from marine invertebrates, but deficient in plants that has been shown to promote fish growth particularly in carnivorous fish species (Gibson Gaylord et al., 2007; Kim et al., 2003; Watson et al., 2013). Most fish species are not capable of synthesizing taurine endogenously, and therefore taurine is introduced with the diet with adequate supplementation shown to be required in aquafeeds containing plant-based protein for several fish species (Kotzamanis et al., 2020). Our experimental diets did not include taurine supplementation with the exception of the FWS diet, that was supplemented with 5 g/kg taurine. Nevertheless, the other experimental diets contained lower levels of taurine provided by other dietary components, in particular squid meal that was part of our formulations. This was reflected in significantly higher taurine levels found in fish fed the FWS diet compared to fish fed all other SBM-based diets and the FM diet both in the liver and in the muscle tissue. Importantly, FWS showed taurine levels that were more similar to the fish fed the natural diet, which could explain, at least in part, the superior growth performance observed with this fishmeal-free, SBM-based diet. Metabolomic analysis identified taurine as one of the most significant metabolites in the discrimination among different treatment groups in both the liver and muscle tissue, while threonine levels were not found to be significantly different, and lysine levels were found to be different in the muscle tissue but not in the liver. Given the fact that taurine levels in fish fed the FWS diet were more similar to those fed the natural diet compared with the other experimental treatments, it is likely that the additional taurine supplementation is at least partially responsible for the superior growth performance of this diet compared with all other diets evaluated in this study.

4.1.3. Effects on osmolytes

Our results show that among the biomarkers of a natural diet fed to juvenile red drum there are a number of common osmolytes, among which we identified TMAO and the betaines (Pro betaine, Tau betaine and butyrobetaine). TMAO was found to be significantly higher in the muscle tissue of fish fed the natural diet compared with those fed the SBM-based diets and the fishmeal-based diet consistent with the high content of TMAO especially in marine invertebrates (Kelly and Yancey, 1999). A number of betaines are produced from different amino acids and different betaines have been detected in marine organisms such as algae (Blunden et al., 1992). Among these, glycine-betaine (betaine) is the most common betaine and the first one in this class of compounds to be discovered. Among its biological functions, betaine acts as an osmoprotectant, by protecting the cells from dehydration, high salinity, temperatures and osmotic stress (Ueland et al., 2005). As opposed to TMAO, betaine levels were found to be significantly higher in the muscle of fish fed the SBM-based diets and the fishmeal-based diet compared with those fed the natural diet. Another betaine, butyrobetaine (γ-butyrobetaine or 4-butyrobetaine) was found to be significantly higher in the liver of fish fed the natural diet compared with those fed the SBM-based diets and the fishmeal-based diet. Butyrobetaine is a derivative of γ-aminobutyric acid (GABA) and it is the biosynthetic precursor to carnitine, a compound which is essential for the transport of fatty acids into the mitochondria for fatty acid beta-oxidation and therefore energy production (Fujita et al., 2009).

4.1.4. Effects on vitamins

Ascorbate (vitamin C), an important antioxidant was supplemented in all our experimental diets as part of a multivitamin mixture. We found ascorbate levels to be significantly lower in the liver of fish fed the natural diet and the FWS diet compared to those fed all other dietary treatments. Pantothenate (vitamin B_5) was also supplemented in all our experimental diets. Pantothenate is important in the metabolism of carbohydrates, lipids (e.g., fatty acids, cholesterol, steroid hormones), and also protein (National Research Council, 2011). We found pantothenate levels to be significantly lower in the muscle of fish fed the natural diet compared to those fed all other dietary treatments.

4.2. Effects of dietary treatments on hepatic FIGLU levels

Our study confirmed the presence of FIGLU, a known marker of nutritional deficiency in mammals (vitamin B_{12}, folate or methionine), which had been detected in previous juvenile red drum feeding trials conducted in our laboratory using high-SBM diets (up to 60% SBM) (Tabor and Mehler, 1954; Brown et al., 1960; Rabinowitz and Tabor, 1958). Specifically, FIGLU, an intermediate of histidine catabolism had been detected in the liver tissue of fish fed the high-SBM diets as well as those fed the fishmeal-based diet (FM) but were not detected or were present only at low concentrations in fish fed the natural diet, thus ruling out the possibility that FIGLU could simply be a marker of SBM consumption in fish. In this study, FIGLU, was detected at significantly lower levels in the liver of fish fed the natural diet and the FWS diet compared to all other dietary treatments, suggesting that the nutritional deficiency associated with the other SBM-based diets was at least partially mitigated by additional supplementation of amino acids including methionine. Compared with the other SBM-based supplemented diets, red drum fed the Met diet as well as the FWS diet showed significantly lower levels of FIGLU in the liver tissue (Fig. 9), which would suggest that an imbalance in methionine levels developed over the course of the 12-week trial, rather than a deficiency in vitamin B_{12} or folate could have induced hepatic accumulation of FIGLU in juvenile red drum. However, despite the fact that the Fol/Met/B12 diet contained the same methionine supplementation levels (8.5 g/kg) as diets FWS and Met, fish fed the Fol/Met/B12 diet showed hepatic levels of FIGLU similar to those fed diets Fol and B12, and a decrease in FIGLU levels was not observed. This apparent inconsistency is in agreement with previous reports showing different physiological responses of methionine supplementation in animals with a pre-established vitamin B_{12} or folate deficiency compared with animals with an adequate supply of these nutrients (Batra et al., 1974; Brown et al., 1960). Paired combinations of these nutrients were not evaluated in this study; however, they should be investigated in future studies to further elucidate specific synergistic effects.

During our 12-week feeding trial the suggested dietary imbalance did not induce significant effects on juvenile red drum growth performance; however, metabolomics is sensitive to early changes in the metabolic state of an organism and the relatively short duration of this trial (12 weeks) might not have been long enough for these changes to significantly affect fish red drum growth. Future studies are warrantied to investigate the long-term effects of these dietary imbalances, specifically in association with dietary-induced hepatic FIGLU accumulation.

Lastly, based on superior growth performance compared to all other SBM-based diets and the FM-based diet evaluated in this study as well as based on the higher degree of similarity of the liver metabolite profile of fish fed this diet with those fed the natural (N) diet, the FWS diet, a fishmeal-free, 60% SBM diet constitutes a promising SBM-based alternative diet to fishmeal-based feeds for juvenile red drum.

Overall, the results from this study suggest that general requirements for nutrients such as methionine in some fish species might still be underestimated, in particular for fish fed diets containing high levels of plant-based ingredients, such as SBM, replacing fishmeal. Additionally, sensitive molecular technologies such as metabolomics may be able to detect nutritional imbalances at early stages in the aquaculture grow-out process, when appropriate mitigation approaches can be adopted to prevent detrimental effects on growth performance.

This study, while directed at a single fish species, provides novel information that can improve our understanding of the effects of soy-based diets in other important marine carnivorous aquaculture species and showcases an application of NMR-based metabolomics in aquaculture nutritional studies and biomarker research. The results from this study suggest that FIGLU should be a targeted biomarker for specific nutritional imbalances.

5. Conclusions

The objective of this study was to investigate a putative biomarker of nutritional imbalance, FIGLU by testing specific mitigation approaches aimed at lowering FIGLU hepatic levels in juvenile red drum fed high-soybean meal (60% SBM) diets. Therefore, we conducted a 12-week feeding trial with juvenile red drum fed experimental diets enriched in folate, vitamin B_{12}, or methionine.

Our results show that the FWS diet outperformed all other SBM diets and the FM diet under all performance metrics. Additionally, fish fed the FWS diet and the Met diet showed lower hepatic levels of FIGLU compared with the other SBM-based diets, suggesting that among the different supplementation regimes, methionine supplementation (but not in combination with vitamin B_{12} and folate supplementation) was associated with decreased FIGLU levels. Importantly, the FWS diet produced tissue (liver and muscle) metabolite profiles that were more similar to those of fish fed the N diet. Overall, our results suggest that the FWS diet might constitute a promising SBM-based alternative diet to fishmeal for red drum.

Author contributions

Fabio Casu: Conceptualization; Methodology; Investigation; Writing – Original Draft; Writing – Review & Editing; Project Administration; Funding acquisition. **Aaron M. Watson:** Conceptualization; Investigation; Writing – Review & Editing. **Justin Yost:** Investigation; Writing – Review & Editing. **T. Gibson Gaylord:** Conceptualization; Resources; Writing – Review & Editing. **Daniel W. Bearden:** Conceptualization; Methodology; Writing – Review & Editing. **Michael R. Denson:** Conceptualization; Writing – Review & Editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.03.014.

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