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Research article

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Highlights

1) Supplementation of a full plant-based diet (PB) with 5% and 15% spirulina (SPI-05 and SPI-15) maintained fish growth and feed efficiency but did not reach the performances of a commercial-like diet (COM) for trout feeding.

2) Specific compounds of the spirulina biomass were accumulated (quinone-like compound) or metabolized (β-hydroxybutyrate and glucosides) in the body of the fish, suggesting contrasting outcomes of spirulina as an ingredient.

3) The use of ^{1}H-NMR metabolomics revealed a progressive restoration of amino acids and glucose plasma profiles of SPI-05 and SPI-15 48 hours after last feeding, which were nevertheless associated with inverse higher amino acid, glucose, lactate and betaine levels in the liver.

4) A correlative analysis between metabolic data and physiological traits highlighted cross-dependent relationships of polar metabolites, free fatty acids, and lipoproteins with key performance indicators and quality traits.

Abstract

Nowadays in aquaculture, the nutrition of carnivorous fish species requires the development of sustainable plant-based diets. Supplementation with microorganisms such as spirulina could offer an alternative to full plant-based diets that are inappropriate for carnivorous species such as rainbow trout. However, the benefits and drawbacks of spirulina supplementation on fish performance and metabolism need to be assessed to substantiate spirulina as an appropriate ingredient. The objective of this work was to determine the metabolic utilization of a plant-based diet supplemented with two levels of spirulina (5% and 15%) and to compare it to a full plant-based (PB) and a commercial-like (COM) diet for trout. Spirulina supplementation induced both a

decrease in feed intake and an increase in feed efficiency while maintaining growth and a putative reorientation of lipid deposition from viscera to muscle. Fatty acid changes, including an increase in saturated fatty acids, a decrease in monounsaturated fatty acids, and color changes with an increase in b* index (yellow), suggested a slight alteration of the nutritional and visual quality of fish fed a spirulina diet. ^{1}H-NMR metabolomics profiling was used to measure metabolites in experimental feeds and in fish plasma, liver, and muscle. Two specific compounds of the spirulina ingredient were identified and determined to have opposed metabolic fates: i) a quinone-like compound was found to accumulate in tissue and ii) β-hydroxybutyrate was found to be completely metabolized. The main metabolites modulated by spirulina in plasma and tissues were involved in energy and amino acid metabolisms. Spirulina supplementation almost suppressed the accumulation of glucose and essential amino acids in fish plasma and muscle induced by the PB diet. However, these compounds, as well as betaine, were found to accumulate in the liver of fish fed spirulina-supplemented diets. An alteration of liver metabolism due to the specific fatty acid profile and quinone-like compound of spirulina is suspected. A correlative approach of fish performance, quality traits, and metabolomic data in plasma and liver suggests that the maintenance of growth rate and feed efficiency using spirulina supplementation of a PB diet requires not only the supply of a well-balanced protein but also the redirection of lipid metabolism. However, observed changes in fatty acid composition and color suggest further alterations of the phospholipid metabolism. This work thus provides a contrasted fate of spirulina supplementation of a PB diet with both expected positive effects and unexpected negative effects that must be investigated further.

Keywords: metabolomics; fish nutrition; plant-based diet, single-cell ingredient, spirulina, proton NMR

1. Introduction

The increasing demand for healthy and nutritious foods, such as aquatic food products, is closely associated with the requirement for sustainable production. Aquatic products, particularly fish products, are now supplied by aquaculture, which currently faces important sustainability challenges (Boyd et al., 2020). One of these challenges is related to the origin of feeds that have drastically switched from marine ingredients to plant feedstuff in the last two decades. The increase in plant ingredients incorporated in fish feed has not achieved sustainability objectives in terms of environmental impact (Boissy et al., 2011) and economic profitability (Egerton et al., 2020). There is thus a recent growing interest for new ingredients that could be used as substitutes for plant-based ingredients in fish feed.

Single-cell ingredients show possibility for human and animal nutrition, particularly due to their protein content (García-Garibay et al., 2014; Sharif et al., 2021), their fatty acid composition (Kothri et al., 2020; Roques et al., 2020c) and the presence of specific bioactive compounds such as polysaccharides, vitamins, and pigments (Gamboa-Delgado and Márquez-Reyes 2018; Roy and Pal 2015). Yeast and bacteria—both single-cell ingredients—have long been considered for human and animal nutrition as they are very easy to produce in controlled conditions and are readily available at an industrial scale due to their use in various fermentation processes (Maicas 2020; Ochsenreither et al., 2016). There is a growing interest in micro-algae in particular, due to their ability to synthesize long chain fatty acids, vitamins, and bioactive compounds (Camacho-Rodríguez et al., 2018). However, large-scale production of micro-algae is still limited because of the large variety of species needing to be tested and the current lack of experience in their culture (Alagawany et al., 2021; Linder 2019). These single-cell ingredients are particularly relevant in fish nutrition as alternative ingredients to plant-based diets, either for their protein content, regardless of the fish species (Alagawany et al., 2021; Glencross et al., 2020; Jones et al., 2020), or for their long-chain-polyunsaturated fatty acid content, especially for marine fish (Tocher et al., 2019; Velasco-Escudero and Gong 2010).

Among the single-cell ingredients, spirulina has a special status. It is classified as a cyanobacteria with a photosynthetic capacity. Large-scale controlled productions of spirulina strains exist, from pilot-plant cultures to outdoor cultures in brackish water lagoons. It is already one of the main complementary ingredients in human and animal feeds (Soni et al., 2017) and in fish feeds (Rosas et al., 2019; P. Singh et al., 2018). Furthermore, there are no known adverse effects of spirulina supplementation (ANSES 2017, Marles et al., 2011), except the risk of contamination by other toxic cyanobacteria taxa (Grosshagauer et al., 2020). Thus, spirulina is a good candidate to be tested as a substitute for plant-based ingredients in fish feeds.

There is a growing interest in metabolomic-based studies in fish nutrition (Jasour et al., 2017; Roques et al., 2018). Nuclear magnetic resonance (NMR) or mass spectrometry (MS) metabolomics have helped pinpoint metabolism alterations induced by plant-based diets compared to marine fish meal and fish oil diets in various fish species (Casu et al., 2019; Deborde et al., 2021; Gatesoupe et al., 2018; Gil-Solsona et al., 2019; Wei et al., 2017). Furthermore, NMR-based metabolomics have also begun to be implemented to test alternative sustainable ingredients as substitutes for plant-based ingredients (Jasour et al., 2017; Roques et al., 2020a; Roques et al., 2020b). This has paved the way to identify specific ingredient compounds and to reveal their unexpected effects on human and animal metabolism.

The present work aimed to assess the effects of using a plant-based diet supplemented with a spirulina ingredient on fish growth performance, fatty acid composition and fish metabolism, using ^{1}H-NMR metabolomics in fish plasma, liver and muscle.

2. Materials and Methods

2.1. Growth Trial and Sampling

The experiment was carried out at the INRAE experimental facilities (UMR1419 Nutrition, Métabolisme, Aquaculture, Donzacq, France), authorized for animal experimentation by the French veterinary service, which is the competent authority (A 64-495-1), as described in (Roques et al.,

2018). Growth trials and sampling were performed in strict accordance with (i) the EU legal frameworks on the protection of animals used for scientific purposes (Directive 2010/63/EU), (ii) the National Guidelines for Animal Care of the French Ministry of Agriculture (decree no 2013-118, February 1, 2013), and (iii) the local ethics committee "Comité d'Ethique en Expérimentation Animale Aquitaine Poissons Oiseaux" (CEEA-73). The trial did not require a specific ethics approval because it involved standard rearing practices and the diets were formulated to cover the nutritional requirements of rainbow trout. The entire staff of the experimental facility received training and personal authorization for fish rearing and manipulation (No. B64 10 005).

We tested four different diets a commercial-like diet (COM) containing fish meal and fish oil, a fully plant-based diet (PB) based on plant protein ingredients and vegetable oils complemented with biomass of DHA-enriched microalgae and two plant-based diets supplemented with spirulina biomass at 5% and 15% (SPI-05 and SPI-15 respectively) (Table 1, Roques et al., 2018). The spirulina ingredient tested is a dried biomass of *Arthrospira platensis* cultivated in autotrophic conditions (Spirulina solutions www.spirulinasolutions.fr). The feeds designed by Yann Marchand (Le Gouessant Aquaculture, Lamballe France) were produced in our experimental food manufacturing (UMR1419 Nutrition, Métabolisme, Aquaculture, INRAE, Donzacq France) using a double screw extruder (CLEXTRAL BC45, Firminy, France) as described previously (Roques et al., 2018). All diets were isoproteic, isolipidic and isoenergetic. Samples of experimental feeds were taken for proximate and fatty acid analyses and for metabolomics analyses.

At the beginning of the feeding experiment, 360 fish were separated randomly into 12 groups of 30 fish using three replicate tanks per diet. They were reared in 100 L tanks supplied with natural spring water at a constant temperature 17 ± 1°C and constant oxygen concentration >9.0 mg/L and fed manually twice a day for 84 days. Total amount of feed distributed and total fish biomass for each tank were measured every three weeks as described previously (Roques et al., 2020a). The growth rate was calculated from the total biomass gain per tank divided by the duration of the experiment and the feed conversion ratio (FCR) was calculated by the total biomass gain divided by

149 the total amount of feed distributed per tank (Roques et al., 2020a). At the end of the experiment,
150 nine juvenile immature fish were randomly sampled per diet 48 h after the last feeding first sedated
151 in a 10 mg/L benzocaine solution and then anesthesized by immersion in a lethal solution of
152 benzocaine (30 mg/L). Individual body weight and body length were measured. Fish plasma, liver
153 and muscle were sample as described previously (Roques et al., 2020a). Briefly blood samples were
154 collected with heparinized syringes, centrifuged (3000 × *g*, 5 min), and the plasma was collected and
155 immediately stored at 20°C until the end of sampling. Just after the blood sampling, fish were
156 euthanized by section of the spinal cord. The liver was collected, separated from the gallbladder and
157 rinsed in a saline solution (NaCl 9 g/L). A sample of deep dorsal white muscle was dissected. Liver
158 weight and whole visceral weight were measured. Plasma and tissues samples were stored at -80°C
159 before analysis. A sample of 15 fish (5 fish per triplicate) was also taken for whole body proximate
160 and fatty acid analyses.

161 *2.2. Fish Diet and Whole Body Composition Analysis*

162 The proximate analyses (protein, lipid, ash and energy content) of the experimental diets and
163 fish were performed as described in Roques et al. (2020a). Diet proximate composition is provided
164 in Table 1. Nitrogen intake and nitrogen gain were calculated accordingly by multiplying the total
165 amount of feed distributed and the whole biomass gain per tank by their respective nitrogen
166 content. Nitrogen efficiency ratio was calculated by dividing the nitrogen gain by total nitrogen
167 intake. Fatty acid composition in diets and whole fish was determined using gas chromatography
168 as previously described (Lazzarotto et al., 2015). Diet fatty acid composition is provided in Table S1.
169 Flesh color was determined at three different locations on the dorsal part of the fish fillet using a
170 CR-400/410 (Konica-Minolta, France) Chroma Meter to measure the luminance (L*), redness (a*)
171 and yellowness (b*) parameters according to the CIELAB color space.

172 *2.3. Feed and Spirulina Ingredient ^{1}H-NMR Analyses*

Freeze-dried feed and spirulina ingredient powders were extracted and the polar extracts were prepared for NMR spectra acquisition as previously described (Roques et al., 2018) using four or five replicates per feed and as detailed in Texts S1 and S2. Briefly, the dried pH-adjusted extracts were solubilized in D_2O with the addition of (trimethylsilyl)propionic-2,2,3,3-*d4* acid sodium salt (TSP), and transferred to a 5 mm NMR tube for NMR acquisition.

Quantitative 1D-^{1}H-NMR experiments of feed extracts were performed on a 500 MHz spectrometer (Avance III Bruker, Wissembourg, France) equipped with a 5 mm broadband inverse, z-gradient, ATMA probe flushed with nitrogen gas and with a sample changer (BACS-120, Bruker). The data were acquired using TOPSPIN 3.6 software. The NMR protocol is detailed in Text S1. Briefly, NMR spectra of pH-adjusted feed extracts were acquired with a single-pulse sequence (zg) and a 90° pulse angle in order to obtain quantitative data. The resulting spectra were processed using the NMRProcFlow tool (*https://nmrprocflow.org/*; (Jacob et al. 2017)). The assignments of 37 feed metabolites in the one-dimensional ^{1}H NMR spectra are based on a previous study (Roques et al. 2018), the in-house spectral library was used to select specific resonances, and intelligent binning was used to obtain the spectral area integration (Table S2). Sucrose solutions in the same solvent as the extracts were used for external calibration to quantify all compounds as detailed in Text S1.

Two-dimensional NMR spectra were recorded on a selected pH-adjusted feed extracts (SPI-15) to help with metabolite identification using a 500 MHZ spectrometer. Two-dimensional spectra were recorded on spirulina ingredient extracts to help with metabolite identification, using either a 1000 MHz or a 500 MHz spectrometer. For the 1000 MHz spectrometer experiments, one- and two-dimensionnal NMR spectra were obtained on a 1000 MHz spectrometer (Avance Neo Bruker, Wissembourg, France) equipped with a 5 mm CP-TCI-H-C/N-D ATMA cryoprobe. The spectra and data were acquired and processed using TOPSPIN 4.0 software (Bruker) as detailed in Text S2. Briefly, a one-dimensional ^{1}H-NMR spectrum was acquired using a classic presaturation (zgpr) pulse sequence and a 90° pulse angle, and the following two-dimensional spectra were acquired: ^{1}H,^{1}H total correlation spectroscopy (TOCSY), echo/antiecho ^{1}H,^{13}C heteronuclear single quantum

coherence (HSQC), and $^{1}H,^{13}C$ heteronuclear multiple bond correlation (HMBC). For the 500 MHz spectrometer experiments, 1D and 2D NMR spectra were acquired and processed using TOPSPIN 3.6 software as detailed in Text S2. Briefly, a one-dimensional ^{1}H-NMR spectrum was acquired with a single pulse sequence (zg) and a 90° pulse angle and the following NMR experiments were also performed: one-dimensional selective TOCSY, one-dimensional ^{13}C-NMR, two-dimensional echo/antiecho $^{1}H,^{13}C$-HSQC, and two-dimensional $^{1}H,^{13}C$-HMBC.

NMR data and metadata have been stored in the Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement Dataverse repository (*https://doi.org/10.15454/TBGLJC*) for spirulina ingredient analyses.

2.4. Plasma, Liver and Muscle ^{1}H-NMR Analyses

Plasma, liver and muscle samples were prepared as described previously (Roques et al., 2020a). Briefly, plasma samples were individually thawed, diluted with D_2O, no TSP was added and the diluted plasma was transferred to a 5 mm NMR tube for NMR acquisition performed immediately after each sample preparation. For liver and muscle samples, polar metabolites were extracted using phase separation based on methanol/dichloromethane/water using 0.01% butylhydroxytoluene as an antioxidant. The polar phase was collected and stored at -20°C until preparation for NMR acquisition. The volume of the polar phase collected for drying under a nitrogen flow was carefully adjusted to the weight of the frozen tissue extracted in order to obtain the same final dried matter in each polar extract. Dry extracts were solubilized in a deuterated potassium phosphate buffer solution containing deuterated ethylene diamine tetra-acetic acid (EDTA-*d12*, Sigma-Aldrich, Saint-Quentin-Fallavier, France) to chelate paramagnetic cations. The pH of the buffered extract was adjusted to an apparent pH of 6.00. Finally, the pH-adjusted extract was transferred to a 5 mm NMR tube containing TSP.

One-dimensional ^{1}H-NMR acquisition parameters have been described previously (Roques et al., 2020a) and are summerized in Text S3. Briefly, for plasma, one-dimensional

224 Carr-Purcell-Meiboom-Gill (CPMG) ^{1}H-NMR spectra with presaturation and a 90° pulse angle were
225 acquired. In addition, a water presaturation pulse sequence (zgpr) was used to analyze circulating
226 lipoproteins (Gatesoupe et al., 2018). The two corresponding datasets were combined as already
227 described (Gatesoupe et al., 2018). For liver and muscle, one-dimensional ^{1}H-NMR spectra of tissue
228 extracts with presaturation (zgpr) were acquired with a 90° pulse angle (Roques et al., 2020a). The
229 FID and spectra were processed using the NMRProcFlow tool. The spectra regions issued from
230 intelligent binning or bucketing were named according to the center of the corresponding region
231 (e.g., B4.4415 for a bucket at 4.4415 ppm). If they met the criterion of a signal-to-noise ratio greater
232 than 3, their area was integrated and standardised using constant sum normalization (CSN). The
233 assignments in the one-dimensional ^{1}H NMR spectra were based on (Roques et al., 2020a), an
234 in-house database, a public database (BMRB Metabolomics, https://bmrb.io/metabolomics/) and the
235 ChenomX NMR Suite library 8.3 (ChenomX Inc., Edmonton, Canada). Two-dimensional 500 MHz
236 spectra (COSY, HSQC and HMBC) were also recorded on selected pH-adjusted liver and muscle
237 extracts for metabolite identification. When annotated, each integrated spectral region was also
238 named after the compound/metabolite it corresponded to.

239 NMR data and metadata have been stored in the Institut National de la Recherche pour
240 l'Agriculture, l'Alimentation et l'Environnement Dataverse repository
241 (*https://doi.org/10.15454/TDIVWK*) for representative extracts (liver and muscle) and plasma of
242 rainbow trout fed the PB and SPI-15 diets.

243 *2.5. Statistical Analyses*

244 To study the effects of diet on fish performance and quality, we used a variance analysis
245 (ANOVA) and Student's t test. Each dataset of metabolomic profiles (diet, plasma, liver or muscle
246 dataset) was mean-centred and scaled to unit variance prior to multivariate analyses. A principal
247 component analysis (PCA) was individually performed on data obtained from the four diets, and
248 fish plasma, and liver and muscle extracts using the BioStatFlow tool based on R scripts (v2.9,

http://www.biostatflow.org). A double orthogonal signal correction partial least square discriminant analysis (OSC2-PLS-DA) was performed individually on fish plasma and liver using data obtained from three of the diets and the BioStatFlow tool. A 200-fold cross validation (K=7) was used to assess the significance (*P*-value) of the prediction and separation parameters (Q^2 and R^2Y). For muscle extracts, the PB and SPI-15 diets were compared using a volcano plot analysis (Kruskal Wallis test with $P<0.05$ after FDR correction, and 1.2 threshold between means) performed with the BioStatFlow tool. Fish performance and quality data for the PB, SPI-05 and SPI-15 diets were combined with the annotated variables of the plasma and liver metabolomic profiles using the mixOmics R package (Rohart et al., 2017) and the DIABLO application (Singh et al., 2019). We used default parameters for the sparse PLS-DA model on the data or their means, calculated for each tank. When several spectra regions corresponded to a given metabolite, the most intense one was kept in each dataset. This approach was used to retain nearly all the variables of fish performance and quality and to select an equivalent number of variables in the plasma and liver profile datasets. Consequently, we selected 20 annotated variables for each of the plasma and liver profiles and 20 variables of fish performance and quality (the three blocks of the multiblock analysis) that contributed to separate the diets and covary between blocks using a multiblock sparse PLS-DA. To visualize the relationships between the variables selected by the multiblock sparse PLS-DA, Pearson correlations were calculated and the corresponding correlation network was reconstructed using Cytoscape (Shannon et al., 2003) with a $P<0.01$ threshold for Pearson correlations. Only subnetworks comprising variables from at least two blocks were considered.

3. Results

3.1. Growth Performance

Growth performances are detailed in Table 2 ~~and Figure S1~~. The final body weight as well as the specific growth rate of fish fed the plant-based diet supplemented with 5% or 15% spirulina (SPI-05 and SPI-15 diet respectively), were not significantly different from those of the reference COM diet

and the control plant-based (PB) diet. The voluntary feed intake and the feed conversion ratio of fish on the PB diet were significantly higher than those of the fish on the COM diet. The voluntary feed intake of fish fed the SPI-05 and SPI-15 diets was intermediate between those of fish fed COM and PB diets and significantly different from each of these two diets. The feed conversion ratio of fish fed the SPI-05 and SPI-15 diets did not significantly differ from that of PB diet. The nitrogen efficiency ratios of fish fed the PB, SPI-05 and SPI-15 diets were not statistically different and were significantly lower than that of the fish fed the COM diet. The condition factors of fish fed the SPI-05, SPI-15 and COM diets were significantly higher than those of the fish fed the PB diet. Furthermore, the hepatosomatic indices of fish fed the PB, SPI-05 and SPI-15 diets were not statistically different and were significantly lower than those of the fish fed the COM diet. Finally, the viscerosomatic index of fish fed the SPI-15 diet was significantly lower than that of the fish fed the COM and PB diets, but not of the fish fed the SPI-05 diet, where the index was intermediate, and not significantly different from each of the other diets.

3.2. Proximate and Fatty Acid Composition of Fish

The proximate composition of fish (Table 3) was not significantly affected by the experimental diets. The fatty acid composition of whole fish (Table 4) was significantly affected by the experimental diets. The fish fed a PB diet had significantly lower monounsaturated fatty acids and significantly higher polyunsaturated fatty acids (PUFAs) – both the (n-3) and (n-6) series – than the fish fed a COM diet. The resulting ratios of saturated fatty acids to PUFAs and of (n-3)/(n-6) PUFAs were significantly lower in fish fed a PB diet than in those fed a COM diet. However, the detailed composition of PUFAs (Table S3) showed that long-chain PUFAs – [20:4(n-6)] arachidonic acid (AA) and [20:5(n-3)] eicosapentaenoic acid (EPA) – were significantly lower in fish fed the PB diet than in those fed the COM diet, while 18:2(n-6) linoleic acid, and 18:3(n-6) and 18:3(n-3) linolenic acid were significantly higher in fish fed a PB diet than in those fed a COM diet. The fatty acid composition of fish fed the SPI-05 diet was not significantly different from that of the fish fed a PB diet. However,

fish fed the SPI-15 diet were characterized by a significantly higher content in saturated fatty acids, particularly 16:0 (palmitic acid), and by a significantly lower content in monounsaturated fatty acids, especially 18:1 (oleic acid), compared to other diets. Furthermore, the (n-3) PUFA of fish fed the SPI-15 diet was intermediate between that of those fed the COM and the PB diets so that the (n-3)/(n-6) ratio was significantly lower in fish fed the SPI-15 diet than in those fed the PB and SPI-05 diets.

3.3. Fillet Color

Yellow and red luminance were significantly higher in the fillets of fish fed a PB diet than in the fish fed a COM diet (Table 5). The luminance of fish fillets on the SPI-05 and SPI-15 diets was intermediate but significantly different from those fed the COM and the PB diets. Red and yellow luminance was significantly higher in fish fed the SPI-05 and SPI-15 diets than in those fed the COM and PB diets except for the red luminance of the fish fed the SPI-05 diet, which was not significantly different from that of those on the PB diet.

3.4. ^{1}H-NMR Characterization of the Spirulina Ingredient and Experimental Feeds

Fish feeds and the spirulina ingredient were characterized using NMR experiments at 500 and high-field 1000 MHz (Figure 1) to identify the specific compounds of spirulina. A compound detected in the spirulina ingredient exhibited a broad singlet resonance at 6.8 ppm (TOCSY, Figure 1A) with no cross peak, a single cross peak at 115.26 ppm on HSQC (Figures 1A-B) and another one at 160.3 ppm on the HMBC (Figure 1C) spectra recorded at 1000 MHz. This compound was below detection limit in the feeds. Based on these TOCSY, HSQC and HMBC spectral analyses, we made an a priori assignment of a quinone-like compound. In addition to these quinone-like resonances, three doublet resonances at 5.2, 5.13 and 4.42 ppm were further examined by means of a selective gradient TOCSY at 500 MHz to identify the corresponding compounds (Figure S1). (i) The first resonance doublet at 5.2 ppm (J 3.8 Hz) is correlated with a triplet at 3.86 ppm, a doublet of doublet at 3.65 ppm and a triplet at 3.46 ppm. Comparison of these proton resonances and the ^{13}C resonances with those

of trehalose recorded in the same solvent and pH conditions confirmed the assignments of these signals as trehalose in our sample (Figure S1). (ii) The second resonance doublet at 5.13 ppm (J 3.9 Hz) is correlated with a triplet at 3.76 ppm, a doublet of doublet at 3.57 ppm and a triplet at 3.44 ppm. Based on this spectral and coupling constant data, we made an a priori assignment of an α-glucosyl-containing compound. The cross peaks of HSQC and the nine resonances observed by direct ^{13}C NMR (100.4, 81.63, 75.77, 74.84, 74.39, 72.42, 64.3, 63.34, 63.19 ppm) are consistent with the chemical shifts of α-1,2-glucosylglycerol previously detected in *S. platensis* (Nihira et al., 2014; Warr et al., 1985). (iii) The third resonance doublet at 4.42 ppm (J 7.85 Hz) is correlated with three doublets of doublet at 3.94, 3.68 and 3.56 ppm. Based on this spectral and coupling constant data, we made an a priori assignment of a β-glucosyl-containing compound. Spectral data obtained from HSQC and HMBC with the ^{13}C chemical shifts (96.2 to 105 ppm) of these anomeric protons also indicate glucosides (Figure S1). The less intense patterns of doublets at 5.02 and 4.9 ppm are correlated respectively with two triplets at 3.75 and 3.28 ppm and a doublet of doublet at 3.6 ppm, and with two doublets of doublet at 3.55 and 3.44 ppm and a triplet at 3.83 ppm. Based on this spectral and coupling constant data, we made a priori assignments of an α-glucosyl-containing compound. The β-glucosyl-containing compound may come from β-1,2-glucan – the polysaccharide from the cell wall of *S. platensis* (van Eykelenburg 1978) – and the α-glucosyl-containing compound from α-glucan (polysaccharide composed with linked α-D-glucose (1→3)(1→4)- or (1→3)(1→2)) (Liu et al., 2019). These compounds were also detected in the SPI feeds.

The ^{1}H-NMR profiles of soluble compounds largely differed between the experimental feeds. A PCA of the quantitative profiling data of 37 metabolites (Table S2, Figure 2) demonstrated that the plant-based feeds (PB and SPI) on the positive side of PC1 (explaining over 59% of total variance) differed from the reference COM feed (on the negative side of PC1) (PCA scores, Figure 2A). The SPI-15 supplemented feed was intermediate between COM and PB feeds along PC1. The compounds that contributed most to the difference observed between COM and all plant-based feeds (PCA loadings, Figure 2B) were trigonelline, choline, betaine, stachyose plus raffinose and citrate on the

positive side of PC1, and uracil, glycine, xanthine, lactate and valine on the negative side of PC1. A trend differing from that of the PB and COM feeds was observed with the increased supplementation level along PC2 (explaining 31% of total variance, Figure 2A), suggesting that there were specific compounds in the SPI-05 and SPI-15 feeds. The compounds that contributed most to this trend (Figure 2B) were sucrose on the positive side of PC2 (higher in the PB feed), and glutamate, AMP, α-glucosyl-glycerol, β-hydroxybutyrate (or 3-hydroxybutyrate) and α- and β-glucosides, lysine and trehalose on the negative side of PC2 (higher in the SPI-15 feed).

3.5. Overview of Plasma and Liver Polar Extract ^{1}H-NMR Profiles for the Four Diets

PCAs were first used to acquire an overview of the plasma (Figure S2) and liver polar extract (Figure S3) ^{1}H-NMR profiles of trout fed the four diets (Figure S4). Overall, the second principal component (PC2) tended to separate at least one diet from the other ones (Figure S4). For the plasma profiles combining signals of the NMR presaturation pulse sequence and the cpmg sequence, it explained 10% of total variance and allowed to separate the COM diet from the other three diets. For the liver profiles, it explained 13% of total variance and allowed to separate the COM and PB diets from the SPI-05 and SPI-15 diets. The analysis of plasma and liver ^{1}H-NMR profiles was thus focused on the comparison between the spirulina-supplemented diets and the PB diet.

3.6. Effects of Spirulina Supplementation on Plasma, Liver and Muscle Extract ^{1}H-NMR Profiles

A double orthogonal signal correction partial least square discriminant analysis (OSC2-PLS-DA) was performed to study the effects of spirulina supplementation (Figure 3). For plasma, the model based on the first two latent variables explained 43% of total variance with Q^2=0.87 and P=0.043 (Figure 3A). The three experimental groups were separated on the first latent variable (LV1) with trout fed a PB diet on the negative side and trout fed a SPI-15 diet on the positive side of LV1. Seventy-seven variables had VIP scores higher than 1 and the following annotated ones were involved in the discrimination along LV1 (Figure 3B). The plasma of trout fed a PB diet tended to have higher contents of creatine, glucose, glutamine, methionine, valine, serine, phenylalanine,

375 betaine and a mannose-like compound. The plasma of trout fed a SPI-15 diet tended to have higher
376 contents of a quinone-like compound and lipids with a CH_2-CH_2-CO signature.

377 The same approach was used for the liver polar extract ^{1}H-NMR profiles (Figure 4). For liver,
378 the LV1 x LV2 model explained 29 % of total variance with Q^2=0.81 and *P*=0.037 (Figure 4A). The
379 three experimental groups were separated on LV1 with trout fed a PB diet on the negative side, trout
380 fed a SPI-15 diet on the positive side, and trout fed a SPI-05 diet between the other two. One hundred
381 and twenty variables had VIP scores higher than 1 and the following annotated ones were involved
382 in the discrimination along LV1 (Figure 4B). The liver of trout fed a PB diet tended to have higher
383 contents of ethanol, betaine, taurine, acetate and formate. The liver of trout fed a SPI-15 diet tended
384 to have higher contents of inosine, threonine, lactate, glycerol, uridine, valine, glucose, β-alanine,
385 phenylalanine and methionine.

386 To draw a comprehensive scheme of the metabolic effects of spirulina supplementation, we
387 analyzed muscle ^{1}H-NMR profiles (Figure S5) in trout fed the SPI-15 diet and compared them to
388 those on the PB diet. A volcano plot analysis highlighted nine spectra regions increasing in the PB
389 diet compared to the SPI-15 diet and only two spectra regions increasing in the SPI-15 diet compared
390 to the PB diet (Figure 5A). These significantly modified spectra regions were tentatively annotated in
391 the muscle extract ^{1}H-NMR spectra: six regions corresponded to histidine and two to
392 1,2-propanediol. Two different and significant patterns were observed: (i) metabolites such as
393 histidine and 1-2 propanediol were lower in the fish fed the SPI-15 and the COM diets than in those
394 fed the PB diet and (ii) metabolites such as unkM2.83 and unkS2.73 were significantly higher in the
395 fish fed the SPI-15 and COM diets than in those fed the PB diet (Figure 5B). Furthermore, no
396 significant difference in glucose or essential amino acids was observed in the muscle of fish fed the
397 SPI-15 diet compared to those fed the PB diet (Figure 5A).

398 The liver and muscle polar extract ^{1}H-NMR profiles were also visually compared to a raw
399 plasma ^{1}H-NMR profile to locate compounds that are specific to spirulina diets (Figure 6). The
400 quinone-like compound previously detected in the spirulina ingredient was found specifically in the

plasma and liver of fish fed the SPI-05 and SPI-15 diets compared to those fed the PB and COM diets. This compound was not detected in fish muscle.

3.7. Combining the Metabolic Profiles of Plasma and Liver Extracts with Fish Phenotypic Data

To highlight co-regulations between metabolism and physiology, a multiblock sparse PLS-DA was used to combine fish phenotype data and the corresponding means per tank of the plasma and liver extracts ^{1}H-NMR profiles for trout fed the PB, SPI-05 and SPI-15 diets (Figure S6, Figure 7). This global analysis of metabolome data and fish performance and quality data collected made it possible to select 20 variables to discriminate between the diets and that were covarying between the three datasets (Figure S6). The correlation network between the variables selected in each dataset showed three networks, one large one and two small ones, each of them comprising variables from at least two datasets (Figure 7). The large network was clustered into three subnetworks. Within the large network, a first cluster (CL1) comprised six fish quality variables and two plasma metabolites. This cluster showed connections between the fatty acid composition of fish, especially saturated FA (saturated FA, saturated FA over PUFA ratio, 16:0), oleic acid (18:1) and alpha-linolenic [18:3(n-3)], with a valine-inositol variable in plasma. A second cluster (CL2) comprised three fish quality variables, six plasma metabolites and three liver metabolites. This cluster showed connections between fillet color (luminance L*, yellow index b*) and [20:3(n-6)] FA with the quinone-like compound, betaine, glucose, creatine, methionine and tyrosine in the plasma, and the quinone-like compound, betaine and ethanol in the liver. A third cluster (CL3) comprised five fish performance variables, seven plasma metabolites or macromolecules, and one liver metabolite. This cluster demonstrated connections between growth (final body weight, specific growth rate) and feed efficiency (feed intake, FCR and nitrogen efficiency ratio) with circulating lipids (-CH_2-CH_2-CO-, -$(CH)_2$-CH_2-$(CH)_2$, -CO-CH_2-CH(CO)-CH2-CO, -$(CH)_2$-CH_2-$(CH)_2$-), lipoproteins (VLDL/LDL-$(CH_2)_n$), mannose, and adenosine in plasma and lactate in the liver. For the two small networks, a fourth cluster (CL4) comprised only whole fish lipid and protein contents and inositol in

426 plasma. In addition, VLDL/LD-CH_3 lipoproteins in the plasma were connected to propionate in the
427 liver.

428 **4. Discussion**

429 *4.1. Contrasted Effects of Spirulina Supplementation*

430 This work, based on well-conceived experimental diets, clearly reproduced the expected
431 performance of a plant-based PB diet, with fish showing less growth compared to that of fish on a
432 commercial-like (COM) diet (Deborde et al., 2021; Lazzarotto et al., 2018) and a progressive
433 restoration of growth with the supplementation of a given ingredient (Roques et al., 2020a; Roques et
434 al., 2020b; Wei et al., 2017) – in this instance, a spirulina ingredient. It demonstrated that spirulina
435 proteins could also be used as a substitute for plant-based protein ingredients, at least up to 15%,
436 without altering growth performances and not only as a substitute for fish meal, as is usually
437 observed (Glencross et al., 2020; Jones et al., 2020; Sharif et al., 2021). The nutritional and somatic
438 indices, the fatty acid composition and the color parameters of the fillet thus made it possible to
439 draw a contrasted picture of the pros and cons of using spirulina as a substitute ingredient in a
440 plant-based diet.

441 On the one hand, spirulina supplementation maintained growth and feed efficiency compared
442 to PB diet despite a decrease in feed intake, which could account for a positive effect of adding
443 spirulina to the diet. The same effect of spirulina supplementation was observed with respect to the
444 increased condition factor in the SPI-05 and SPI-15 diets and the decreased viscerosomatic index in
445 the SPI-15 diet. This result could be related to the reorientation of lipid deposition from viscera to
446 muscle. Such an effect could be related to the spirulina lipid fraction, as suggested by the specific
447 effects of spirulina observed in an obese mice model (Yang et al., 2020). This is also coherent with the
448 color of the fillets that showed an increase of the red color index given that the diets were not
449 supplemented with any liposoluble carotenoid pigments. This is in agreement with previous data

450 (Teimouri et al., 2013), and with the fact that spirulina provides not only well-balanced proteins but
451 also specific pigments and vitamins (Soni et al., 2017).

452 On the other hand, the fish fatty acid composition, largely altered by the PB diet in contrast to
453 the COM diet, was further modified by spirulina supplementation. The increase in saturated fatty
454 acids and decrease in monounsaturated fatty acids, together with the lower (n-3)/(n-6) ratio in fish
455 fed SPI-05 and SPI-15 diets compared to those fed the PB diet, is in accordance with the differences
456 observed in the fatty acid composition of the SPI-05 and SPI-15 diets (increase in 16:0 palmitic acid,
457 18:3(n-6) linolenic acid and 20:3(n-6) dihomo-gamma-linolenic acid). The same changes have already
458 been observed in steelhead fed diets supplemented with spirulina (Twibell et al., 2020). The
459 increased color of the fillet was also associated with a higher yellow index which is the specific
460 signature of spirulina phycocyanin, chlorophyll and carotenoid pigment spectra (Paramonov 2018;
461 Park et al., 2018). This may affect the acceptability of the product. Our results therefore suggest a
462 slight alteration of the nutritional quality of fish fed a spirulina diet. This is the first observation of an
463 adverse effect of the spirulina ingredient that is generally tested as a substitute of fish meal in fish
464 nutrition and not as a substitute of a plant-based ingredient.

465 This work provides interesting material for the use of ^{1}H-NMR metabolomics to characterize
466 the metabolic pathways that could be either stimulated or depressed by spirulina and to build a
467 comprehensive fate of the pros and cons of spirulina (Table 6).

468 *4.2. Status of a Quinone-Like Compound Specific to the Spirulina Ingredient*

469 A quinone-like compound was specifically detected in the spirulina ingredient but was
470 probably under the detection limit in the 5% and 15% spirulina-supplemented feeds. Spirulina is a
471 cyanobacteria with a photosynthetic capacity which involves specific electron transporters including
472 quinone and plastoquinone (Müh et al., 2012). Furthermore, specific quinone compounds have been
473 detected in aqueous spirulina extracts (Mane et al., 2019). The quinone-like compound was
474 specifically found in the plasma and liver but not in the muscle of fish fed spirulina-supplemented

475 diets and its content in the plasma and liver varied according to the spirulina ingredient's level of
476 inclusion. The quinone-like compound could thus be considered as a biomarker of spirulina as it
477 was absorbed and specifically transported in the plasma ending accumulated in the liver.
478 However, although in human quinone plays interesting antioxidant functions (Ježek et al., 2020) it
479 could also induce ROS formation through futile redox cycling (Cohen & d'Arcy Doherty, 1987). In
480 trout, the ROS detoxification are handled through glutathione reductase and related enzymes
481 (Pérez-Gálvez et al., 2020, Stephensen et al., 2002). Thus, the measurement of glutathione reductase
482 and enzymatic antioxidant system in the liver of fish fed spirulina would help to understand the
483 potential drawbacks and find ways to strengthen the ingredient spirulina.

484 *4.3. Suspected Effects of β–Hydroxybutyrate and Glucan-Related Glucosides from Spirulina Ingredients*

485 The spirulina ingredient comprises specific compounds related to the vital functions of the
486 cyanobacteria, such as β-hydroxybutyrate, and α-glucoside or β-glucoside-containing compounds.
487 The latest derived from glucan cell walls have been specifically detected in the spirulina ingredient
488 and in spirulina supplemented feeds. β-hydroxybutyrate is the product of the degradation of a
489 poly-β-hydroxybutyrate polymer (PHB) that constitutes a specific energy store of microorganisms
490 and can be found in spirulina at concentrations as high as a few milligrams per gram of dry weight
491 (Ansari and Fatma 2016; Jau et al., 2005). This polymer is known to have beneficial effects on the
492 digestive function and immunity of various fish species including salmonids. However, these
493 direct effects are not mediated by β-hydroxybutyrate (Franke et al., 2017; Najdegerami et al., 2017),
494 which is only a marker of the presence of the polymer. Glucans from the microorganism's cell wall
495 could induce similar effects on fish gut integrity and immune functions. This is observed in fish with
496 β-glucan originating in yeast cell walls (Glencross et al., 2020) but also with glucans originating in
497 spirulina cell walls (Alagawany et al., 2021, Mahmoud et al., 2018). Thus, the altered digestive and
498 immune functions of intestines in fish fed a plant-based diet could be restored by combining both
499 PHBs and glucans found in the spirulina ingredient (Table 6).

500 The PHB monomer (β-hydroxybutyrate) is an energy substrate that could be used directly by
501 fish microbiota. In the human body, microbiota is known to have the ability to use dietary energy
502 substrates to then modify the host's metabolism (Selkrig et al., 2014). Microbiota has been shown to
503 be strongly altered by plant-based diets with related effects on fish metabolism (Gatesoupe et al.,
504 2018). It has been demonstrated that the supply of butyrate, a compound of close structure, helps to
505 restore the intestinal condition of fish fed a plant-based diet (Estensoro et al., 2016) and this
506 strengthen the hypothesis of positive effects of β-hydroxybutyrate on microbiota and the intestinal
507 condition of fish. β-hydroxybutyrate is also a ketone body used as energy source by mammals and
508 fish during periods of food deprivation (Comesaña et al., 2019, Mierziak et al., 2021) where it is
509 suspected to modify liver metabolism and even alter feed intake. In our case, it was not detected or
510 only at low levels in the plasma, liver and muscle of trout fed a spirulina diet. This suggests that it
511 was either fully consumed by microbiota, as suggested previously, or that the fish can also efficiently
512 use it as an energy substrate. Thus, β-hydroxybutyrate from spirulina ingredient could constitute a
513 back-up energy source in case of limiting nutrient supply that has to be considered further.

514 *4.4 Restoring Cell Functions with Spirulina Supplementation*

515 The plant-based diet has been demonstrated to induce high tissue levels of specific metabolites
516 such as taurine, betaine and histidine involved in key cell homeostasis functions such as ionic
517 strength, osmotic strength and buffering capacity (Casu et al., 2019; Deborde et al., 2021; Wei et al.,
518 2017). The decreased concentrations of taurine and betaine in the liver and of histidine in the muscle
519 of fish fed a spirulina-supplemented diet confirm that supplementing a plant-based diet with
520 adequate protein ingredients restore essential cell functions (Roques et al., 2020a; Roques et al.,
521 2020b; Wei et al., 2017).

522 *4.5. Metabolic Imprinting of the Spirulina Ingredient*

523 Feeding fish a plant-based diet is known to maintain high levels of glucose and amino acid in
524 the plasma throughout the post-prandial phase (Deborde et al., 2021; Larsen et al., 2012). The

accumulation of these essential metabolites in tissues is usually related to an imbalanced amino acid supply and to a non-concomitant supply of energy substrate (Deborde et al., 2021; Rolland et al., 2015; Roques et al., 2020a). The supplementation with an SPI ingredient clearly reduced glucose and essential amino acid concentrations in plasma, namely methionine, phenylalanine and valine compared to PB diet. Thus, these nutrients should be better used respectively for energy production and protein synthesis in fish fed the SPI-05 and SPI-15 diets than in fish fed the PB diet (Table 6). However, the plasma and liver metabolite profiles showed an unexpected opposing pattern with respect to energy substrates and essential amino acids. The liver of fish fed the SPI ingredient clearly accumulated glucose and essential amino acids such as methionine, phenylalanine, threonine, valine as well as inosine compared to the liver of those fed the PB diet. The remarkable accumulation of these nutrients in the liver first suggests that these substrates are not fully used for energy metabolism and protein synthesis (Table 6). We examined the muscle of trout fed the SPI-15 diet compared to the PB and COM ones and in accordance to what was observed in plasma we did not observe any accumulation of essential amino acids in the muscle of fish fed the SPI-15 diet compared to those fed the PB. Thus, we did not confirm the hypothesis of an overall imbalanced supply of essential nutrients in fish fed SPI diets. The pattern of threonine in liver advocates to test the hypothesis of a sparing of this amino acid promoted by the positive effects of PHB, glucans and β-hydroxybutyrate on gut integrity knowing that dietary threonine is specifically utilized for the synthesis of intestinal mucosal-protein (Feng et al., 2013, Mao et al., 2011).

4.6. Contribution of the Observed Changes to Fish Phenotype

The overall analysis, combining metabolome data together with fish performance and quality traits data, made it possible to select variables discriminating the diets and covarying between the three datasets. This revealed specific interesting correlation clusters. First, the presence of the quinone-like compound in fish plasma and liver was revealed to be a relevant biomarker of spirulina inclusion. Indeed, it was related to the specific effects of spirulina: on one hand, a fatty acid

550 composition trending toward more saturated and less oleic and α-linolenic fatty acids in the whole
551 fish, and on the other hand, fillet color indices trending toward a lower luminance and a higher
552 yellow flesh color in fish fed SPI diets.

553 The differences in fatty acid composition of whole fish, specifically the increase in saturated
554 fatty acids and the decrease in oleic and α-linolenic acids, were surprisingly related to amino acid
555 levels - tyrosine and valine - in plasma. The decrease in plasma of valine and tyrosine, an essential
556 and a semi-essential amino acid, respectively, suggests a sparing effect of spirulina lipids on these
557 two amino acids but this requires further investigation. Furthermore, the valine-containing variable
558 was a composite one, combining the valine signal with that of inositol, a structural component of cell
559 membrane phospholipids (Gonzalez-Uarquin et al., 2020; Shirmohammad et al., 2016). This suggests
560 that the differences in lipid composition of the SPI-05 and SPI-15 diets could induce further
561 alteration of fish phospholipid composition, which had already been altered by the plant-based diet
562 (Caballero-Solares et al., 2020). These metabolic alterations related to fatty acid composition of
563 spirulina ingredient could be compensated by selecting strains of spirulina with higher contents of
564 poly-unsaturated fatty acids.

565 The differences in fillet color, showing a higher yellow index and a concomitant lower
566 luminance, are induced by specific spirulina pigments, particularly carotenoids (Marzorati et al.,
567 2020). These differences were positively related to the quinone-like compound in plasma and liver,
568 but the absence of a quinone-signal in muscle deserves to confirm the role of this spirulina
569 compound in muscle coloration. Nevertheless, the yellow index was negatively related to the
570 presence of methionine in plasma and of betaine both in plasma and liver. Methionine and betaine
571 are also known to be key intermediates of the one-carbon metabolism (Arumugam et al., 2021)
572 which is involved in redox defense (Ducker and Rabinowitz 2017). As quinones are extremely
573 reactive to oxido-reduction reactions, it can be hypothesized that betaine and methionine are
574 witnesses of an activated one-carbon metabolism toward redox defense. This suggests that the

575 supplementation with spirulina ingredients has to be associated with an adequate supply of not only
576 antioxidant compounds but also nutrients related to one-carbon metabolism.

577 It should be noted that the changes induced by spirulina supplementation on whole fish fatty
578 acid composition and fillet color were not directly related to the differences in circulating
579 lipoproteins (VLDL/LDL) and other circulating lipids, including unsaturated fatty acids. At 48 hours
580 after the last feeding, the circulating plasma lipoproteins are more a reflection of fish intrinsic lipid
581 metabolism than those induced directly by food during the post-prandial period. Moreover, the
582 differences in circulating lipoproteins, especially VLDL/LDL and -CH_2-CH_2-CO circulating lipids,
583 that tended to be higher in trout fed the SPI-05 and SPI-15 diets than in those fed the COM and PB
584 diets, were related to the maintenance of growth performance (final body weight, specific growth
585 rate) and feed efficiency (feed intake, feed conversion ratio, N-efficiency ratio) in fish fed spirulina
586 diets. Our results demonstrated that the restoration of growth rate and feed efficiency to what is
587 observed in fish fed the COM diet requires not only the restoration of amino acid metabolism
588 through the supply of well-balanced proteins but also a redirection of lipid metabolism.

589 **5 Conclusion**

590 This work demonstrated the contrasted effect of spirulina supplementation of a full plant-based
591 diet on both production traits and fish metabolism (Table 6). The spirulina inclusion of up to 15%
592 maintained both growth performance and feed efficiency compared to the full plant-based diet.
593 These effects were associated with an efficient use of amino acids and energy substrates. This
594 confirms the advantage of spirulina as a protein ingredient for fish nutrition in the context of
595 sustainable diet development. Moreover, the supply of specific higher saturated lipids and lower
596 (n-3)/(n-6) ratios induced a slight alteration in whole fish lipid composition, and likely including that
597 of phospholipids, but also an interesting reorientation of lipid deposition. This seems to be
598 associated to a sparing effect of the amino acid metabolism in liver as well as a redirection of lipid
599 metabolism.

600 Furthermore, the crude spirulina ingredient comprised other compounds that are suspected to
601 induce either positive changes in microbiota and fish energy metabolism due to the presence of
602 β-hydroxybutyrate and glucans, which needs to be preserved or negative changes due to xenobiotic
603 compounds such as quinone-like compounds and pigments. The latter redirect liver metabolism
604 toward their elimination with detrimental effects on other liver essential functions which could be
605 compensated by combining spirulina with other ingredients and supplementation with antioxidant
606 compounds and related nutrients.

607

608 **Tables**

609 Table 1 Formulation and proximate composition of commercial (COM), plant-based (PB) and

610 experimental feeds supplemented with spirulina (SPI-05 and SPI-15) for rainbow trout feeding.

611

Ingredients (g.100g^{-1} FW)	COM	PB	SPI-05	SPI-15 %
Fish meal	21.03			
Fish oil	4.88			
Rich DHA algae meal		6.84	6.84	6.84
Spirulina biomass			5.00	15.00
Processed animal proteins [1]	15.00			
Vegetal oils [2]	14.65	18.10	14.95	18.15
Plant proteins [3]	42.7	70.40	58.79	55.30
Rapeseed lecithin		1.00	1.00	1.00
Monocalcium phosphate		1.20	1.00	1.00
Phytase		0.02	0.02	0.02
Lysine 78%	0.39	0.50	0.50	0.84
DL-methionine 98%	0.44	0.65	0.61	0.56
Threonine 98%	0.20	0.20	0.20	0.20
Vitamin premix [4]	0.25	0.30	0.30	0.30
Vitamin C monophosphate 35 %	0.04	0.04	0.04	0.04
Mineral premix [5]	0.25	0.30	0.30	0.30
Liquid choline	0.15	0.15	0.15	0.15
Antioxidant	0.15	0.15	0.15	0.15
Antifungal	0.15	0.15	0.15	0.15
Proximate composition [6]				
Dry Matter (% FW)	95.8	96.9	97.0	96.2
Proteins (% DM)	43.0	44.7	45.0	45.5
Lipids (% DM)	21.0	21.6	21.7	20.7
Ash (% DM)	7.3	5.4	6.0	7.0
Energy (kJ.g^{-1} DM)	24.1	24.0	24.1	23.9

612 [1] Processed animal protein (feather meal protein, blood product, poultry meal, 5/3/7 g.kg^{-1})

[2] Vegetable oils: rapeseed oil and linseed oil.

[3] Plant proteins: wheat gluten, hydrolysed wheat gluten, pea protein, faba bean protein concentrate, soy concentrate, soybean meal, rapeseed meal, peeled faba bean and wheat. A fraction of wheat gluten, pea protein, faba bean protein, soy concentrate, soybean meal and wheat as well as a fraction of rapeseed oil were substituted by the spirulina fraction in SPI experimental feeds.

[4] Vitamin premix composition: vitamin A (retinyl acetate / 3a672a) 4 000 000 UI/kg; vitamin D (cholecalciferol / 3a671) 700 000 UI/kg; vitamin E (alpha-tocopheryl acetate / 3a700) 80 000 UI/kg; vitamin K3 (menadione / 3a711) 4 g/kg; vitamin B1 (thiamine mononitrate / 3a821) 4 g/kg; vitamin B2 (riboflavin) 6 g/kg; vitamin B6 (pyridoxine hydrochloride / 3a831) 6 g/kg; vitamin B12 (cyanocobalamin) 20 mg/kg; vitamin B5 (D-calcium pantothenate / 3a841) 12 g/kg; nicotinic acid (vitamin PP - B3 - niacin 3a314 / niacinamide 3a315) 12 g/kg; folic acid (vitamin B9 / 3a316) 3.6 g/kg; biotin (vitamin B8 / 3a880) 0.4 g/kg.

[5] Mineral premix composition: iodine (calcium iodide anhydrous / 3b202) 0.4 g/kg; manganese (manganese oxide II /3b502) 20 g/kg; zinc (zinc oxide/ 3b603); 40 g/kg; iron (iron II sulfate monohydrate / 3b103) 32 g/kg; copper (copper II sulfate pentahydrate /3b405) 1.2 g/kg.

[6] FW, fresh weight; DM, dry matter

630 **Table 2** Zootechnical performance of rainbow trout (initial body weight 49.0 g) fed a plant-based
631 diet (PB) for 84 days, supplemented with 5% or 15% spirulina (SPI-05 and SPI-15, respectively), and
632 a reference commercial-like diet (COM). For each variable, means with the same subscript letters are
633 not significantly different according to Student's t-test (P<0.05).
634

	COM	PB	SPI-05	SPI-15	ANOVA [4]
Final body weight[1](g)	279.1 ± 16.9	247.1 ± 14.0	258.0 ± 17.0	273.3 ± 27.8	NS
Specific growth rate[1,2] *(%.d^{-1})*	2.05 ± 0.05	1.93 ± 0.08	1.99 ± 0.06	2.04 ± 0.12	NS
Voluntary feed intake[1] *(% body weight.d^{-1})*	1.15 ^{c} ± 0.02	1.32 ^{a} ± 0.02	1.25 ^{b} ± 0.01	1.28 ^{ab} ± 0.05	4.5. 10^{-3}
Feed conversion ratio[1] *(g feed intake / g body weight growth))*	0.82 ^{b} ± 0.01	0.98 ^{a} ± 0.03	0.92 ^{a} ± 0.03	0.92 ^{a} ± 0.05	2. 10^{-2}
Nitrogen Efficiency Ratio[1] *(N gain g / N intake g)*	0.49 ^{a} ± 0.01	0.39 ^{b} ± 0.01	0.41 ^{b} ± 0.01	0.41 ^{b} ± 0.03	3. 10^{-3}
Condition factor[3] *(body weight / body length)*	1.02 ^{a} ± 0.01	0.99 ^{c} ± 0.01	1.00 ^{b} ± 0.01	1.01 ^{a} ± 0.01	5. 10^{-2}
Hepatosomatic index[3] *(liver weight as a % of body weight)*	1.06 ^{a} ± 0.19	0.77 ^{b} ± 0.12	0.75 ^{b} ± 0.06	0.80 ^{b} ± 0.10	3. 10^{-3}

Viscerosomatic index[3] (*viscera weight as a % of body weight)*	10.43 [a] ± 1.52	8.95 [ab] ± 1.37	9.89 [ab] ± 1.08	8.61 [b] ± 1.36	4. 10^{-2}

[1] mean ± SD of triplicate tanks for growth, feed intake and efficiency ratios.

[2] specific growth rate calculated as final body weight over initial body weight (%. d^{-1})

[3] mean ± SD of 9 fish in each diet for condition factors and indices

[4] *P*-value of one-way ANOVA, NS, *P*>0.05

640 **Table 3** Initial and final whole body proximate composition of rainbow trout fed a plant-based diet
641 (PB) for 84 days, supplemented with either 5% or 15% spirulina (SPI-05 and SPI-15, respectively), or
642 a reference commercial-like diet (COM). Mean ± SD of triplicate tanks. DM: Dry Matter.
643

Proximate	Initial	After 84 days on the diet				
		COM	PB	SPI-05	SPI-15	ANOVA [1]
Dry matter (% fresh weight)	27.6	31.7 ± 1.0	32.1 ± 0.8	32.0 ± 0.5	31.7 ± 0.8	NS
Proteins (% DM)	53.0	51.6 ± 1.6	52.2 ± 1.7	50.9 ± 1.5	54.0 ± 0.9	NS
Lipids (% DM)	38.6	41.6 ± 2.5	41.1 ± 1.5	42.9 ± 1.5	39.6 ± 0.4	NS
Ash (% DM)	6.3	5.3 ± 0.5	5.7 ± 0.1	5.8 ± 0.3	5.7 ± 0.2	NS
Energy ($kJ.g^{-1}$ DM)	28.4	28.9 ± 0.7	29.0 ± 0.2	28.9 ± 0.3	28.4 ± 0.2	NS

644 [1] NS, $P>0.05$ for one-way ANOVA
645

646 **Table 4** Initial and final fatty acid composition of rainbow trout fed a plant-based diet (PB) for 84
647 days, supplemented with 5% or 15% spirulina (SPI-05 and SPI-15, respectively), or a reference
648 commercial-like diet COM. Mean of triplicate tanks. *P*-value of one-way ANOVA. Data with
649 different superscript letters are significantly different according to Student's t-test ($P<0.05$). More
650 detailed data are provided in Table S3.
651

Fatty Acids	Initial	After 84 days on diet				
(% total lipids)		COM	PB	SPI-05	SPI-15	ANOVA *P*-value
16:0	19.54	16.03 ^{c}	18.40 ^{b}	18.15 ^{b}	20.23 ^{a}	$2.\ 10^{-4}$
Σ Saturated	28.58	23.20 ^{b}	22.89 ^{b}	22.35 ^{b}	24.86 ^{a}	$2.\ 10^{-2}$
18:1	40.50	46.70 ^{a}	44.48 ^{b}	44.61 ^{b}	42.75 ^{c}	$1.\ 10^{-3}$
Σ Monounsaturated	47.32	52.11 ^{a}	46.92 ^{b}	47.43 ^{b}	45.60 ^{c}	$9.\ 10^{-6}$
18:2 n-6	13.91	14.00 ^{b}	18.74 ^{a}	18.45 ^{a}	18.10 ^{a}	$3.\ 10^{-6}$
20:4 n-6	0.00	0.38 ^{a}	0.05 ^{b}	0.00 ^{b}	0.15 ^{b}	$2.\ 10^{-2}$
Σ PUFA n-6	14.27	15.25 ^{b}	19.74 ^{a}	19.63 ^{a}	19.60 ^{a}	$3.\ 10^{-6}$
18:3 n-3	4.17	4.75 ^{c}	6.59 ^{a}	6.55 ^{a}	6.10 ^{b}	$3.\ 10^{-6}$
20:5 n-3	1.48	0.97 ^{a}	0.35 ^{b}	0.31 ^{b}	0.32 ^{b}	$4.\ 10^{-3}$
22:6 n-3	2.34	2.69	2.78	2.70	2.45	NS
Σ n-3	8.85	9.19 ^{b}	10.45 ^{a}	10.40 ^{a}	9.60 ^{ab}	$5.\ 10^{-2}$
Total Fatty Acids	99.75	100.00	100.00	99.82	99.66	

Σ Saturated / Σ PUFA	1.20	0.94 [a]	0.76 [c]	0.74 [c]	0.85 [b]	$2.\ 10^{-3}$
Σ n-3 / Σ n-6	0.62	0.60 [a]	0.53 [b]	0.53 [b]	0.49 [b]	$2.\ 10^{-2}$

652

653 **Table 5** Coloration parameters L*, a*, b* of the fillet of rainbow trout fed a plant-based diet (PB) for
654 84 days, supplemented with 5% or 15% spirulina (SPI-05 and SPI-15, respectively), or a reference
655 commercial-like diet COM. Mean ± SD of 9 fish for each diet and 3 different locations in each fillet.
656

	COM	PB	SPI-05	SPI-15	MANOVA [2]
Luminance L*[1]	45.8 [b] ± 2.8	48.1 [a] ± 4.3	42.8 [c] ± 2.0	42.0 [c] ± 2.6	$5.\ 10^{-12}$
Red color a*	-0.56 [c] ± 0.70	0.18 [b] ± 1.55	0.12 [b] ± 0.64	1.03 [a] ± 0.87	$2.\ 10^{-7}$
Yellow color b*	0.48 [d] ± 1.46	2.88 [c] ± 1.88	11.94 [b] ± 2.76	13.96 [a] ± 2.46	$6.\ 10^{-46}$

657 [1] L*, a*, b* according to CIELAB color space

658 [2] MANOVA diet effect (location effect $P = 7.\ 10^{-4}$)

659

660

661 **Table 6** Pros and cons of spirulina supplementation in rainbow trout fed a plant-based diet. Related

662 differences in spirulina and fish metabolome compared to PB diet.

Traits	PRO	CON
Fish growth performances & quality traits		
Growth performance	Maintained growth	
Feed intake and conversion ratio	lower feed intake maintained feed conversion ratio	
Conformation	higher condition factor	
Somatic indices	lower viscerosomatic index	
Flesh coloration	higher red color	higher yellow color
Fatty acid composition		higher saturated fatty acid content lower n-3/n-6 ratio
NMR-based metabolome & complementary analyses		
Spirulina ingredient or feed	β-hydroxybutyrate witness of PHB[1] and intestine integrity β-hydroxybutyrate as energy substrate glucan residues and intestine integrity	higher saturated fatty acid first evidence of quinone-like compound
Fish metabolism	improvement of glucose & amino acid metabolism in plasma and muscle improvement of one-carbon metabolism in liver	accumulation of glucose & amino acids in liver accumulation of quinone-like xenobiotic in liver

663 [1] polyhydroxybutyrate (Ansari & Fatma 2016)

664

665 **Figure legends**

666 **Figure 1** High-field NMR experiments to characterize spirulina compounds. 1000 MHz TOCSY (A),
667 HSQC (B) and HMBC (C) spectra of the spirulina ingredient.

668

669 **Figure 2** PCA of quantitative ^{1}H-NMR profiles of polar extracts of a plant-based feed (PB, green
670 triangles) supplemented with 5% (SPI-05, light-blue squares) and 15% spirulina (SPI-15, dark-blue
671 diamonds) compared to a reference commercial like feed (COM, red circles). Thirty-seven annotated
672 metabolites were quantified (Table S2). (A) Scores plot on the PC1 x PC2 plan. (B) Loadings plot.

673

674 **Figure 3** OSC2-PLS-DA analysis of ^{1}H-NMR profiles of plasma of rainbow trout fed a plant-based
675 diet (PB, green triangles) supplemented with 5% and 15% spirulina (SPI-05, light blue squares and
676 SPI-15, dark-blue diamonds, respectively). Q^2=0.866, *P*-value=0.043. (A) Scores plot. (B) Loadings
677 plot. Annotated variables with VIP scores higher than 1 are indicated.

678

679 **Figure 4** OSC2-PLS-DA analysis of ^{1}H-NMR profiles of liver extract of rainbow trout fed a
680 plant-based diet (PB, green triangles) supplemented with 5% and 15% spirulina (SPI-05, light-blue
681 squares and SPI-15 dark-blue squares, respectively). Q2=0.812, P-value=0.037. (A) Scores plot. (B)
682 Loadings plot. Annotated variables with VIP scores higher than 1 are indicated.

683

684 **Figure 5** Differential effect of the SPI-15 diet compared to the PB diet and the COM diet for ^{1}H-NMR
685 profiles of muscle extracts. (A) Volcano plots with Kruskal Wallis (P<0.05 after FDR correction) and a
686 threshold of 1.2 for the ratio between means for the PB and SPI-15 diets. (B) Box plots for a selection
687 of variables highlighted in (A) for the PB, SPI-15 and COM diets.

688

689 **Figure 6** Quinone-like compound resonances in the ^{1}H-NMR profile of plasma, liver and muscle of
690 rainbow trout fed a commercial-like diet (COM), a plant-based diet (PB) and two diets
691 supplemented with 5% (SPI-05) and 15% (SPI-15) spirulina.
692
693 **Figure 7** Correlation network of phenotypical data—growth performance, fish proximate and fatty
694 acid composition, fillet color—with ^{1}H-NMR variables in plasma and liver of fish fed spirulina
695 supplemented diet SPI-05 and SPI-15 and a control plant-based (PB) diet, after a multiblock
696 sparse-PLS-DA. This network is based on the data measured or calculated per tank. Variables
697 selected using multiblock sparse PLS-DA (Figure S6) and Pearson correlations with $P<0.01$ are
698 shown in a network built with Cytoscape. Only subnetworks comprising variables of at least two out
699 of the three datasets are shown. Nodes are colored according to their block: plasma ^{1}H-NMRs are
700 shown as red circles; liver ^{1}H-NMRs as brown triangles; and fish phenotypical data and growth
701 performance are shown as grey squares. The node size is proportional to the number of connections.
702 For edges, a solid line means a positive correlation; a dashed line means a negative correlation.
703
704 **Supplementary Materials:**
705 **Figure S1**: 500 MHz NMR experiments to analyze the nature of glucosides present in the spirulina
706 ingredient. (a) Selective TOCSY for glucosides. (b) ^{13}C spectra for trehalose and α-glucosyl-glycerol
707 annotation. (c) Assignments of ^{13}C NMR signals of trehalose and α-glucosyl-glycerol.
708 **Figure S2**: Representative 500 MHz ^{1}H-NMR (cpmg) spectra (δ⊚0.9-8.5 ppm) of plasma of rainbow
709 trout fed a plant-based diet supplemented with 0% (PB) and 15% spirulina (SPI-15).
710 **Figure S3**: Representative 500 MHz ^{1}H-NMR (zgpr) spectra of polar extracts of liver of rainbow trout
711 fed a plant-based diet supplemented with 0% (PB) and 15% spirulina (SPI-15).
712 **Figure S4**: PCA of ^{1}H-NMR profiles of plasma and liver extracts of trout fed a plant-based diet
713 supplemented with spirulina compared to plant-based diet and commercial-like diet.

Figure S5: Representative 500 MHz ^{1}H-NMR (zgpr) spectra of polar extracts of muscle of rainbow trout fed a plant-based diet supplemented with 0% (PB) and 15% spirulina (SPI-15, dark blue).

Figure S6: Multiblock analysis of plasma and liver ^{1}H-NMR profiles and fish phenotypic data.

Table S1: Fatty acid composition of the four trout feeds.

Table S2: List of the metabolites quantified in the feed extracts using ^{1}H-NMR profiling.

Table S3: Detailed initial and final fatty acid composition of trout.

Text S1: Detailed materials and methods for spirulina feed extraction and NMR analysis.

Text S2: Detailed materials and methods for spirulina ingredient extraction and NMR analysis.

Text S3: Detailed materials and method for trout plasma and tissue extraction and NMR analyses.

Author Contributions: S.S.-C., B.F., and A.M. conceptualized and designed the experiments; Y.M. designed feeds and supplied the ingredients; S.R. and C.D. performed the NMR analysis experiment and validated the data. O.C. and C.D. performed the high field NMR experiments; A.M. performed the statistical analyses; S.R., B.F., and S.S.-C. interpreted the data; B.F. and A.M. prepared and wrote the original draft; S.R., B.F., A.M., C.D., S.S.-C. wrote, reviewed and edited the manuscript. All other authors reviewed and edited the manuscript.

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Declaration of Competing Interest

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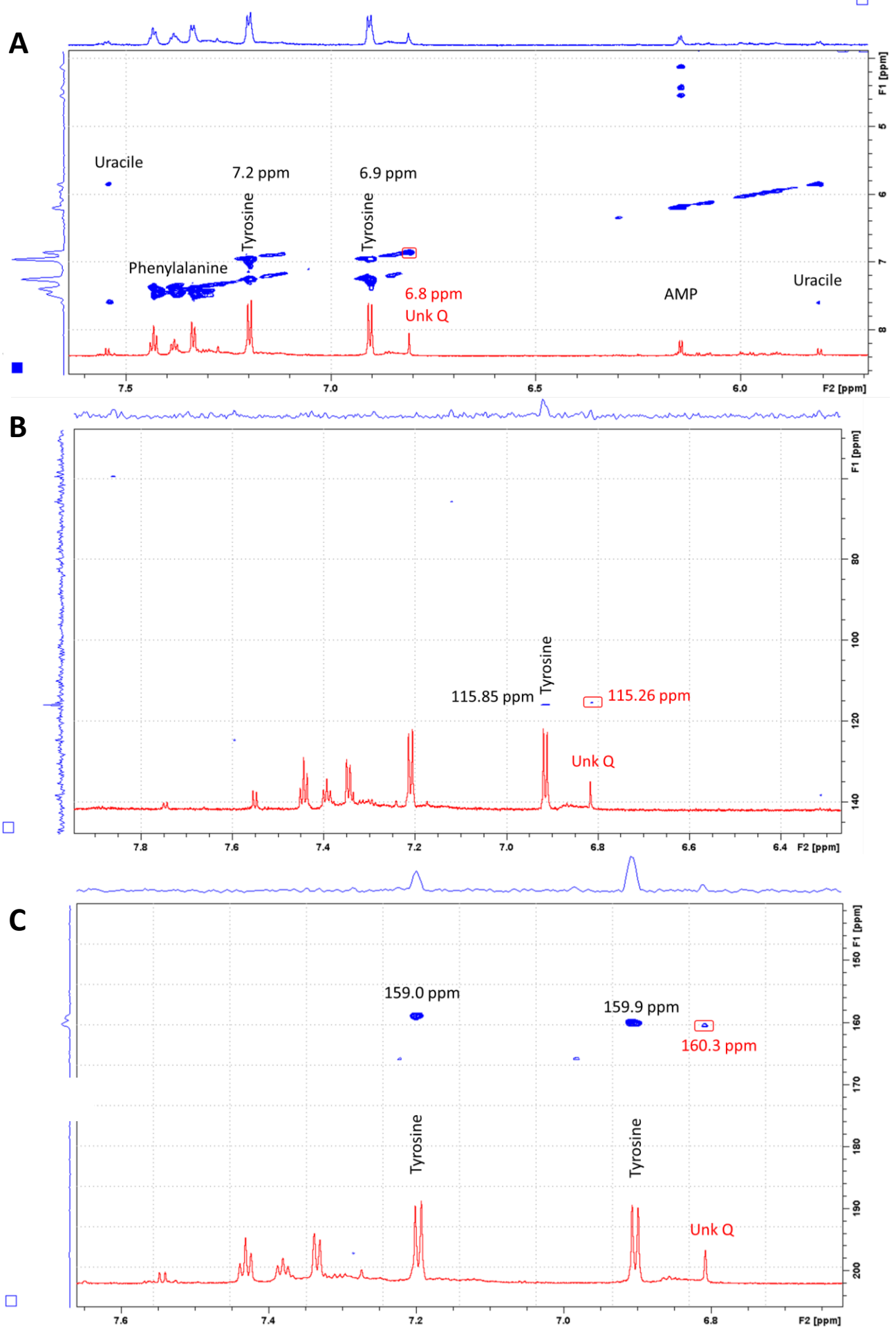
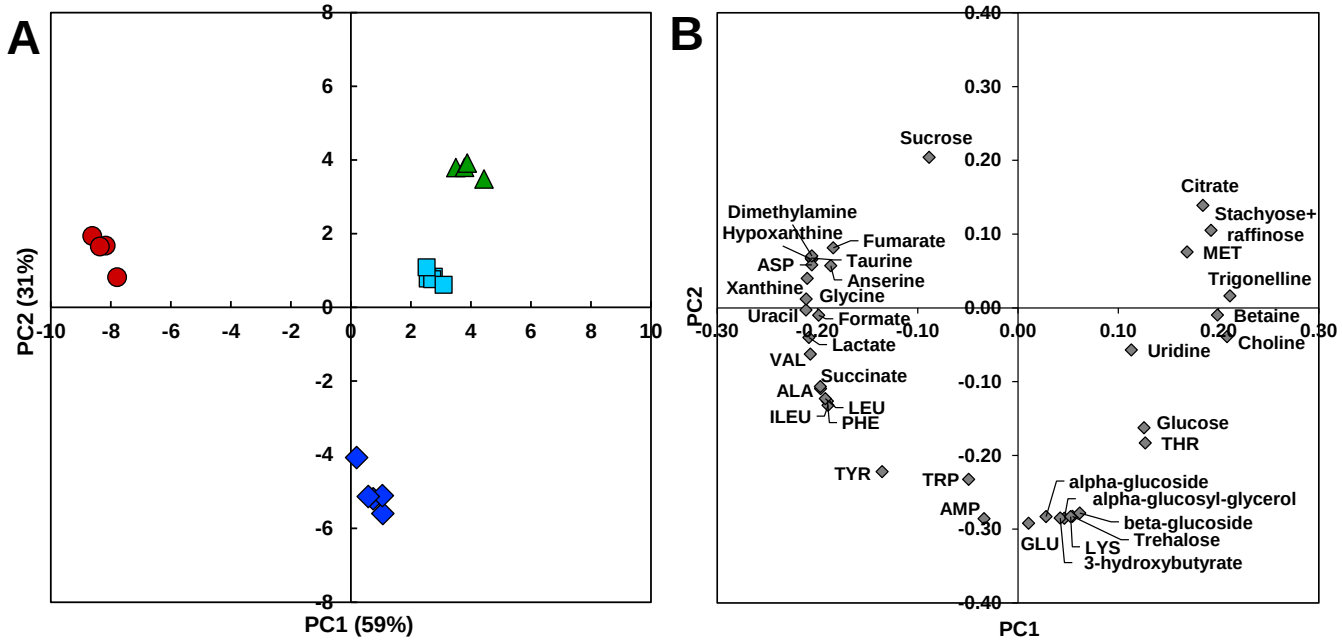
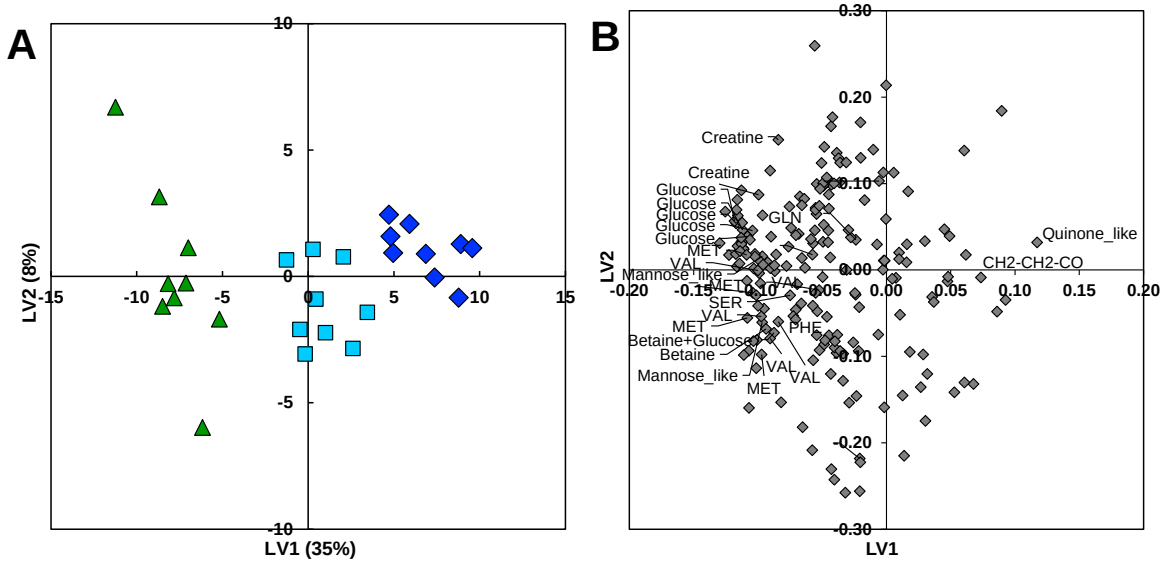
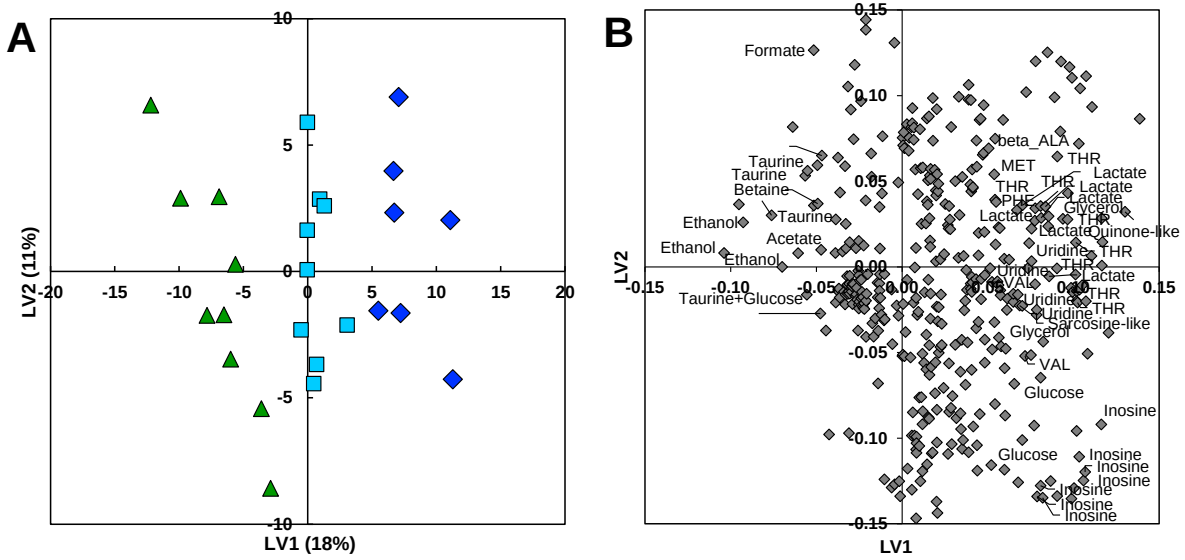
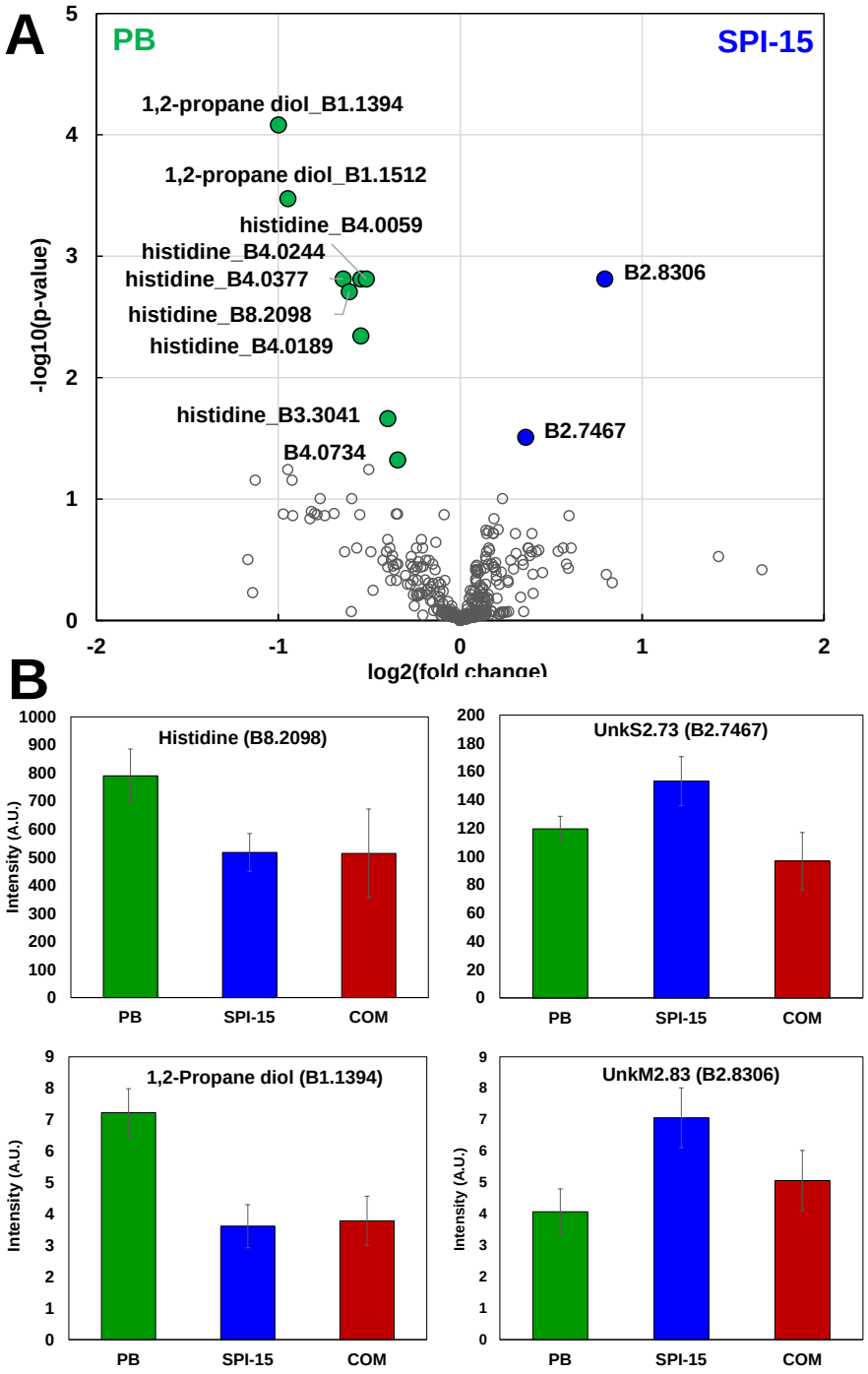
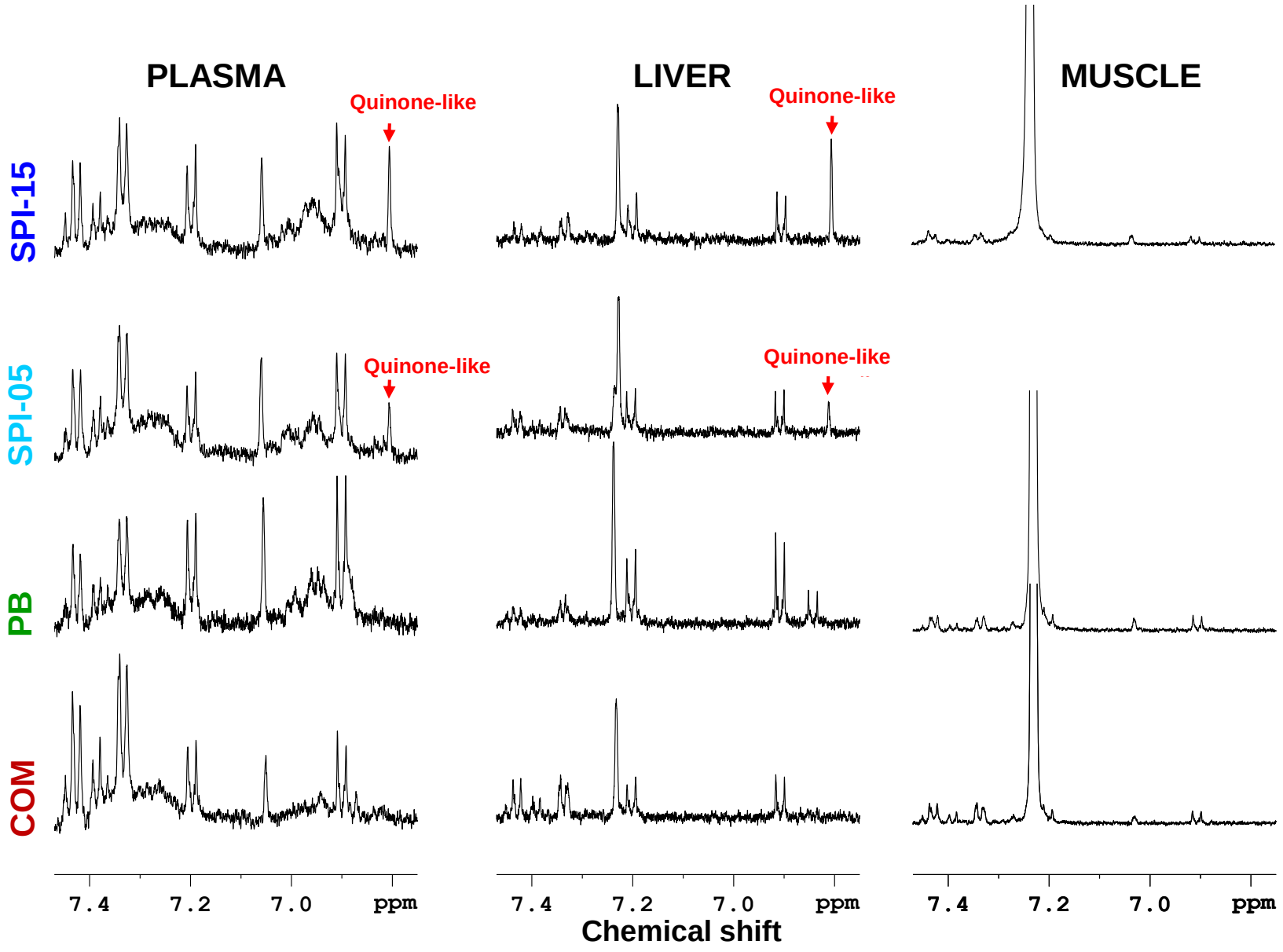

A
Uracile
7.2 ppm
6.9 ppm
Tyrosine
Tyrosine
Phenylalanine
6.8 ppm
Unk Q
AMP
Uracile
F1 [ppm]
F2 [ppm]
B
115.85 ppm
Tyrosine
115.26 ppm
Unk Q
F1 [ppm]
F2 [ppm]
C
159.0 ppm
159.9 ppm
160.3 ppm
Tyrosine
Tyrosine
Unk Q
F1 [ppm]
F2 [ppm]

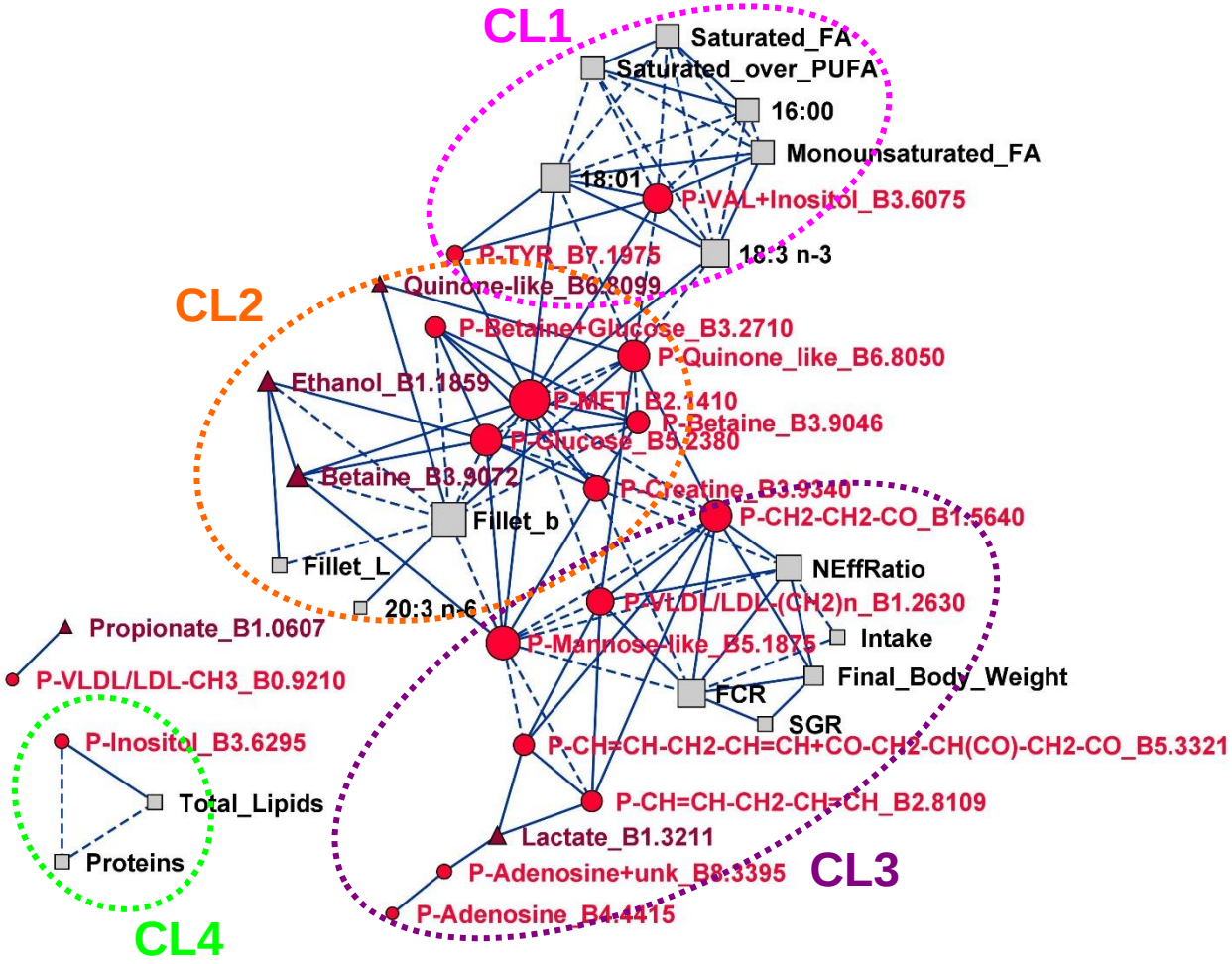

A
PC2 (31%)
PC1 (59%)
8
6
4
2
0
-2
-4
-6
-8
-10
-8
-6
-4
-2
0
2
4
6
8
10
B
PC2
PC1
0.40
0.30
0.20
0.10
0.00
-0.10
-0.20
-0.30
-0.40
-0.30
-0.20
-0.10
0.00
0.10
0.20
0.30
Sucrose
Citrate
Stachyose+
raffinose
MET
Trigonelline
Betaine
Choline
Uridine
Dimethylamine
Hypoxanthine
Fumarate
ASP
Taurine
Anserine
Xanthine
Glycine
Uracil
Formate
Lactate
VAL
Succinate
ALA
LEU
ILEU
PHE
Glucose
THR
TYR
TRP
AMP
alpha-glucoside
alpha-glucosyl-glycerol
beta-glucoside
Trehalose
GLU
LYS
3-hydroxybutyrate


A
10
5
0
-5
-10
-15
-10
-5
0
5
10
15
LV2 (8%)
LV1 (35%)
B
0.30
0.20
0.10
0.00
-0.10
-0.20
-0.30
-0.20
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
0.20
LV2
LV1
Creatine
Creatine
Glucose
Glucose
Glucose
Glucose
Glucose
GLN
MET
VAL
Mannose_like
MET
VAL
SER
VAL
MET
PHE
Betaine+Glucose
Betaine
VAL
Mannose_like
VAL
MET
Quinone_like
CH2-CH2-CO


A
10
5
0
-5
-10
-20
-15
-10
-5
0
5
10
15
20
LV2 (11%)
LV1 (18%)
B
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
LV2
LV1
Formate
beta_ALA
Taurine
THR
Taurine
MET
Lactate
Betaine
THR
THR
Lactate
PHE
Lactate
Glycerol
Ethanol
Taurine
Lactate
THR
Lactate
Quinone-like
Ethanol
Acetate
Uridine
THR
Ethanol
Uridine
THR
Lactate
VAL
THR
Taurine+Glucose
Uridine
Uridine
THR
Sarcosine-like
Glycerol
VAL
Glucose
Inosine
Glucose
Inosine
Inosine
Inosine
Inosine
Inosine


A
PB
SPI-15
1,2-propane diol_B1.1394
1,2-propane diol_B1.1512
histidine_B4.0059
histidine_B4.0244
histidine_B4.0377
histidine_B8.2098
histidine_B4.0189
histidine_B3.3041
B4.0734
B2.8306
B2.7467
-log10(p-value)
log2(fold change)
B
Histidine (B8.2098)
UnkS2.73 (B2.7467)
1,2-Propane diol (B1.1394)
UnkM2.83 (B2.8306)
Intensity (A.U.)
PB
SPI-15
COM


PLASMA
LIVER
MUSCLE
SPI-15
SPI-05
PB
COM
Quinone-like
Quinone-like
Quinone-like
Quinone-like
7.4
7.2
7.0
ppm
7.4
7.2
7.0
ppm
7.4
7.2
7.0
ppm
Chemical shift


CL1
Saturated_FA
Saturated_over_PUFA
16:00
Monounsaturated_FA
18:01
P-VAL+Inositol_B3.6075
18:3 n-3
P-TYR_B7.1975
Quinone-like_B6.8099
CL2
P-Betaine+Glucose_B3.2710
P-Quinone_like_B6.8050
Ethanol_B1.1859
P-MET_B2.1410
P-Betaine_B3.9046
P-Glucose_B5.2380
Betaine_B3.9072
P-Creatine_B3.9340
P-CH2-CH2-CO_B1.5640
Fillet_b
Fillet_L
NEffRatio
20:3 n-6
P-VLDL/LDL-(CH2)n_B1.2630
Propionate_B1.0607
P-Mannose-like_B5.1875
Intake
P-VLDL/LDL-CH3_B0.9210
Final_Body_Weight
FCR
SGR
P-Inositol_B3.6295
P-CH=CH-CH2-CH=CH+CO-CH2-CH(CO)-CH2-CO_B5.3321
Total_Lipids
P-CH=CH-CH2-CH=CH_B2.8109
Lactate_B1.3211
Proteins
P-Adenosine+unk_B8.3395
CL3
P-Adenosine_B4.4415
CL4