

# High carbohydrate to protein ratio promotes changes in intestinal microbiota and host metabolism in rainbow trout (Oncorhynchus mykiss) fed plant-based diet

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Raphaël Defaix, Jep Lokesh, Mylène Ghislain, Mickael Le Béchec, Michaël Marchand, et al.. High carbohydrate to protein ratio promotes changes in intestinal microbiota and host metabolism in rainbow trout (Oncorhynchus mykiss) fed plant-based diet. Aquaculture, 2024, 578, pp.740049. 10.1016/j.aquaculture.2023.740049. hal-04211282

# HAL Id: hal-04211282 https://hal.inrae.fr/hal-04211282

Submitted on 26 Jan 2024

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#### 3

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19 **Running title**: Effect of carbohydrates on gut microbiota in rainbow trout

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## 23 Abstract

To ensure the sustainability of aquaculture, it is necessary to change the "menu" of carnivorous fish such as rainbow trout from a fish-based diet to one with plant-based ingredients. However, there is a major problem with the growth performance decrease of fish fed with a 100% plant-based diet due to the reduction in feed intake and feed efficiency. For the first time, we incorporated high levels of digestible carbohydrates (high-starch diet) in a 100% plant-based diet during a 12-week feeding trial in order to improve protein utilization for growth (protein sparing effect) and reduce nitrogen waste.

30 We measured the changes in the intestinal microbiota, Short-Chain Fatty Acid (SCFA) levels and 31 metabolic responses in liver. Dietary carbohydrates had a strong effect on alpha and beta diversity and 32 abundance of 12 genera, including Ralstonia and Bacillus in digesta associated microbiota whereas 33 mucosa associated microbiota was less affected. The change in microbial diversity might be linked to 34 the change observed in SCFA production. High levels of Mycoplasma were observed in the intestinal mucosa. Overall, hepatic gene expression was significantly altered by the CHO/protein ratio. Up-35 36 regulation of genes involved in glucose metabolism (*qcka*, *qckb*, *q6pcb2a*), down-regulation of genes 37 involved in lipid metabolism (hadh, acox3, srebp2a, and cyp51a) were associated with higher 38 enzymatic activities (such as glucokinase or pyruvate kinase) and higher glycogen levels in the liver, 39 suggesting adequate adaptation to diet. Interestingly, strong correlations were observed between 40 abundances of certain bacterial OTUs and gene expression in the liver.

The inclusion of digestible carbohydrates in combination with a 100% plant-based diet, could be a promising way to improve and reduce the use of plant proteins in rainbow trout. In addition, the relationship between intestinal microbiota and host metabolism needs further investigation to better understand fish nutrition.

45 Keywords: rainbow trout, gut microbiota, intermediary metabolism, aquaculture, fish nutrition

## 46 **1. Introduction**

47 Sustainable and efficient seafood production is urgently needed to meet the growth in world's 48 nutritional requirements (FAO, 2020). Seafood products for human consumption are provided at 46.8 % (2016) by aquaculture (FAO, 2018) which allows to reduce the depletion of fish stock in the oceans 49 50 caused by overfishing. Traditionally, Fish meal (FM) and fish oil (FO) coming from industrial fishing are 51 used to feed aquaculture fishes. Although these ingredients have been partially replaced in the aqua-52 diets by plant-based products, the use of FM and FO is not sustainable due to the constant shortage 53 and their continuous rising costs (Hua et al., 2019). A sustainable alternative is therefore necessary to feed the species under aquaculture instead of the FM and FO in commercial diets. The utilization of a 54 55 plant-based diet as an alternative has been widely studied for more than 25 years in carnivorous fish 56 such as rainbow trout (Oncorhynchus mykiss) (S.J. Kaushik et al., 1995). Although a 100% plant-based 57 diet cover the nutritional needs of fish, long-term feeding induces reduction of growth, feed intake, 58 and feed efficiency as well as activities of key liver enzymes that are involved in glycolysis (Véron et al., 59 2016). Moreover, the use of plant-protein and the presence of anti-nutritional factors lead to an unbalanced and lower amino-acid utilization (Deborde et al., 2021), has negative effects on 60 reproduction, survival of the offspring (Lazzarotto et al., 2015), and reduced levels of long-chain 61 62 polyunsaturated fatty acid in muscle (Nasopoulou and Zabetakis, 2012). Thus, it is necessary to 63 improve and optimize the use of 100% plant-based diets. Interestingly, one strategy to improve the 64 100% plant-based diets could be the use of digestible carbohydrates (CHO) (Kamalam et al., 2017). CHO are one of the most abundant sources of energy-yielding nutrients on earth and have never been 65 66 tested in association with 100% plant-based diet in fish. Increasing the CHO level in rainbow trout diets 67 will reduce the proportion of plant proteins and then decrease all the negative impact of plant ingredients. In association with FM and FO diets, an appropriate amount (less than 20%) of digestible 68 69 carbohydrates, such as starch, improve fish growth (Boonanuntanasarn et al., 2018), reduce 70 environmental nitrogen pollution, ameliorate feed adhesion, and have a strong protein-sparing effect

71 (Kamalam et al., 2012). However, carnivorous fish are metabolically adapted for high protein 72 catabolism as energy source. Indeed, when fed with more than 20% of carbohydrates, metabolic 73 disorders are observed such as a persistent post-prandial hyperglycemia explained (at least partially) 74 by a non-inhibition of gluconeogenesis pathway (Panserat et al., 2019; Polakof et al., 2012). 75 Nevertheless, these fish present mechanisms adapted to the use of glucose, such as the presence of 76 enzymes involved in starch digestion and glucose metabolism (Enes et al., 2009), the presence of 77 glucose transporter (Krasnov et al., 2001), and an inducible glucokinase (Panserat et al., 2000), 78 indicating that fish have a glucose homeostatic system (Kamalam et al., 2017). All these data have been 79 obtained with diets containing fish meal and fish oil. Many different factors can play a critical role in 80 fish nutrition and feed efficiency but among them, the gut microbiota plays an important role in several 81 large functions known to be involved in fish growth, such as energy production, nutrient metabolism 82 and fermentation of dietary non-digestible components (dietary fibers) (Bäckhed et al., 2004; Butt and Volkoff, 2019; Gomes et al., 2018). Plant based-diets strongly modify gut microbiota composition in 83 84 salmonids (Ingerslev et al., 2014; Pérez-Pascual et al., 2021). Few studies have examined the effect of 85 CHO on fish gut microbiome (Huang et al., 2021; Lin et al., 2018; Ortiz LT, Rebole A, 2013; T. Wang et 86 al., 2021; Zhang et al., 2020) but none of them with 100% plant-based diets. The functional role of the 87 gut microbiota on the host physiology and health remains to be explored in fish (Perry et al., 2020). 88 The key role of the gut microbiota on fish health and metabolism is being increasingly described 89 (Dvergedal et al., 2020; A. Wang et al., 2021), showing strong associations between gut microbiome 90 and lipid metabolism, fish growth, and feed efficiency. To partially explain the interaction between 91 microbiota and host, short-chain fatty acids (SCFAs) are relevant candidates: they are the products of 92 fermentation of non-digestible carbohydrates that become available to the gut microbiota and have 93 been already linked to the host glucose metabolism in mammals (Morrison and Preston, 2016) but their role remains to be investigated in fish. The objective of this study is to understand the effect of 94 the partial replacement of plant proteins by CHO (starch) in rainbow trout fed with a 100% plant-based 95 96 diet on the gut microbiota and host metabolism. For this purpose, juveniles rainbow trout were fed 97 with 100% plant-based diets with high or low CHO (starch) for 12 weeks. In particular, we evaluated 98 the effect of the high-starch (HS) diet on (I) the zootechnical, growth and feed parameters, (II) the 99 diversity and composition of the midgut microbiota (both digesta and mucosa associated microbiota), 100 (III) the short-chain fatty acid concentration in hindgut, (IV) and the host glucose metabolism i.e gene 101 expressions, liver enzymatic activities, glycogen contents, and plasmatic parameters. The results of this 102 study will allow to have new directions to optimize alternative 100% plant-based diet for fish nutrition 103 in the future.

## 104 2. Methods

#### 105 **2.1. Ethical approval**

The rearing experiment was conducted in accordance with the guidelines laid down by French and European legislation for the use and care of laboratory animals (Decree no. 2013-36, February 1st 2013 and Directive 2010/63/EU, respectively). The fish handling protocols and the sampling for the experiment were described by the INRAE ethics committee (INRAE, 2002-36, April 14, 2002). The INRAE experimental station (INRAE facilities of Donzacq, Landes, France) is certified for animal services under the license number A40-228.1 by the French veterinary service, which is a competent authority.

#### 112 2.2. Diet and experimental setup

113 Two experimental 100% plant-based diets were formulated for juvenile rainbow trout. The High-starch 114 diet (HS) corresponds to a high CHO/protein ratio, with 20% of dietary carbohydrates and 42% of 115 proteins. Regarding the Low-starch diet (LS) it is a low CHO/protein ratio, with 3% of dietary 116 carbohydrates and 51% of proteins. The proteins are issue from different plant ingredients. 117 Descriptions of the diets are given in table 1. These diets, adjusted to meet the nutritional requirement 118 of rainbow trout, were isolipidic (23.565 % crude fat) and isoenergetic (~24.905 KJ-g dry matter). These 119 diets have been produced as extruded pellets. The fish were manually fed twice a day (with an interval 120 of 8h) during 12 weeks. For both experimental groups, eighty-one female Trout (~60g) were randomly

distributed in three tanks (130 L). During the experimental period, the fish were kept under standard rearing conditions with water t 17°C, pH 7.5, water flow rate 0.3 L/s, and oxygen levels 9mg/L. The fish mortality was checked (if any) every day, and the tanks were weighted every 3 weeks to evaluate the growth and zootechnical parameters **(Figure1)**.

#### 125 2.3. Sampling

126 After 12 weeks of feeding, four fish from each tank (twelve fish per group) were randomly sampled. 127 The fish were anaesthetized with benzocaine (50 mg-L) before being euthanized with a benzocaine 128 overdose of 150 mg-L, followed by blood collection for plasma isolation. After the blood collection, fish 129 were aseptically dissected and the digestive tract was separated. Liver and midgut was dissected at 6h 130 after the last meal (the peak of the postprandial regulation of metabolism in trout (Polakof et al., 2012)) 131 and immediately frozen with liquid nitrogen and stored at -80°C for long-term storage. For microbiota 132 analysis, the midgut was separated from the rest of the digestive tract and was cut open using sterile instruments. The midgut was chosen because microbiota of this intestine part is involved in the 133 134 digestion of food in trout, moreover the existence of a glucosensing mechanism have already been 135 demonstrated in the midgut for these carnivorous fish species (Polakof et al., 2010). The digesta was 136 separated carefully and the mucosa samples were collected by scraping the intestinal epithelium using 137 a glass slide and then collected into sterile tubes, frozen in liquid nitrogen and stored at -80°C for DNA 138 extraction. Intestinal contents from hindgut were sampled 25h after last meal for SCFA analysis in 6 139 fish per group (2 per tanks) (Figure 1).

#### 140 **2.4. Diets and whole-body proximate composition**

141 Composition of diets and whole-body fish were determined by the following procedures. Dry matter 142 was obtained by drying the samples at 105°C for 24h. The weight of the post-dried samples was 143 subtracted from the pre-died samples. Ash content was measured by incinerating the samples at 550°C 144 for 16h. Protein content was measured by the Kjeldahl<sup>™</sup> method. Lipid content was determined by the 145 Soxtherm method. Gross energy was measured with an adiabatic bomb calorimeter (IKA, Heitersheim

Gribheimer, Germany). Starch content was determined using the Megazyme<sup>©</sup> (Bray, Ireland) total
starch assay procedure.

#### 148 **2.5. Measurement of the plasma biochemical parameters**

Blood was sampled for plasma collection from the caudal vein and then were directly centrifuged at 12,000g at 4°C for 5 min, and stored in heparinized tubes at -20°C until use. Commercial kits were used to determine the level of several plasma metabolites: glucose (Sobioda, Montbonnot-Saint-Martin, France), lactate (kit Randox, Crumlin, United Kingdom), triglycerides (Sobioda), and cholesterol (Sobioda). These kits were adapted to 96-well plates formats according to the manufacturer's instructions.

#### 155 2.6. Hepatic metabolites measurement

156 Liver glycogen was measured using a protocol previously described (Good et al., 1933). For glycogen 157 determination, 250mg of liver were homogenized with 1M HCL and divided in two aliquots. One part 158 was neutralized with 5M KOH, centrifuged and the supernatant was used to measure the free glucose 159 with a commercial kit (Sobioda). The second group of aliquots was hydrolyzed during a boiling step 160 (2h30 at 100°C) and then neutralized with 5M KOH. After centrifugation, the total glucose (free glucose 161 and glucose released by hydrolysis of glycogen) was measured in the supernatant. For hepatic 162 cholesterol determination 100mg of liver was homogenized in 5% Igepal (sigma Aldrich, Saint-Louis, 163 MO, USA). Samples are placed twice at 90°C during 5min and then centrifuged 2 min at 5,000g. 164 Supernatants were recovered and the cholesterol concentration measured in 96-well plates thanks to 165 a commercial kit (Sobioda).

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## 169 **2.7. Microbial composition analysis**

#### 170 2.7.1. DNA extraction and Purification

171 DNA from gut samples (digesta and mucosa) were extracted using the QIAamp Fast DNA Stool Mini kit 172 (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the following 173 modifications as suggested by (Lokesh et al., 2022). About 200mg of -80°C frozen samples were mixed 174 with the pre-heated Inhibitex buffer (1/7 ratio) and homogenized with 0.5mm and 1mm zirconia beads 175 in a bead beater for 30 sec on high speed (Beater, VWR, Radnor, USA). The homogenized samples were 176 placed in a heat block for 10 min at 70°C and then centrifuged for 2 min at 20,000g. DNA was assessed 177 for purity and integrity, and quantified using a microplate Spectrophotometer (Epoch2, BioTek, 178 France).

#### 179 2.7.2. Generation of the 16S rRNA gene libraries

180 The 460 base-pair (bp) V3-V4 regions of 16S rRNA genes were amplified by PCR (first stage of PCR) 181 using 12.5µL of 2X KAPA HiFi HotStart Ready Mix (Roche, France), 5µL of amplicon PCR Forward primer 182 1 μM (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG -3'), 5μL of amplicon 183 PCR 1 (5' Reverse primer μΜ 184 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC - 3'), and 2.5µL of DNA 185 (5ng/µL). Thermocycler conditions included 95°C for 3min (a pre-incubation), followed by 35 cycles of 186 95°C for 30sec, 55°C for 30sec, 72°C for 30sec, and a final elongation step at 72°C for 5min. Controls, 187 including water from fish tanks, diets samples, Escherichia coli, and ultra-pure water were also added 188 to the run. After confirmation of PCR amplification by agarose electrophoresis, the samples and 189 controls were transferred to the genomic platform of Bordeaux (PGTB, Bordeaux, France). Libraries 190 were prepared according to the standard protocol recommended by Illumina (Illumina, CA, USA). Index 191 PCR was used to add the unique dual indexes to the sequences, by using the Nextera XT index kit 192 according to the manufacturer's recommendation (Illumina). The thermocycling conditions were the 193 same than in step 1, except that PCR was performed for only 8 cycles. After PCR cleaning, libraries were 194 quantified using the KAPA library quantification kit for Illumina platforms (Roche). Libraries were 195 pooled at an equimolar concentration (4nM) and sequenced on a MiSeq platform using a 250 bp Paired 196 End Sequencing Kit v2 (Illumina).

#### 197 **2.7.3. Data analysis**

198 The initial data analysis was performed using the FROGS pipeline according to standard procedures 199 (Escudié et al., 2017). First, the forward and reverse reads of each sample are merged. Only amplicons 200 with size between 380 bp and 500 bp corresponding to the size of the V3-V4 region of the 16S rRNA 201 gene, without ambiguous bases and with the two primers were kept. The adapter sequences were 202 removed and the sequences with not expected lengths or ambiguous bases (N) were deleted. After 203 this step 7800687 sequences have been kept, which represents 79.42 % of initial input sequences. The 204 clustering swarm algorithm was used to group together amplicons with a maximum of one nucleotide 205 difference between two amplicons (Mahé et al., 2014), 476371 clusters were created. PCR-generated 206 chimeras, typically created when an aborted amplicon acts as a primer, are removed. Clusters present 207 in less than 4 samples, and having a minimum abundance of 0.005% are removed. After this step, 261 208 clusters have remained with 6716271 sequences. The PhiX database was used to removed 209 contaminants such as chloroplasmic or mitochondrial sequences (Mukherjee et al., 2015). Then, 210 taxonomic affiliations are carried out for each OTUs (Operational Taxonomic Unit), using the silva138.1 211 pintail100 16S reference database ("https://www.arb-silva.de/documentation/release-138/," n.d.). 212 However, some multi-affiliations can be generated when a cluster is composed by several amplicons 213 with not the exact same nucleotide sequence. Samples had an average of 61.729 +/- 18.210 sequences 214 (minimum: 17.009; maximum: 107.484 sequences) with 202 OTUs remaining (ranging from 56 to 119 215 OTUs per sample).

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### 218 **2.8. Short-chain fatty acid measurement**

#### 219 2.8.1. Sample preparation

Frozen intestinal samples were weighed and added into a 114 mL micro-chamber μCTE250 (Markes
international, Llantrisant, UK). The micro-chamber was heated at 100 °C with a flow rate of 60 mL min1 of dry nitrogen. The SIFT-MS sampling was carried out at the outlet of the micro-chamber at a flow
rate of 20 mL min-1.

#### 224 **2.8.2. Selected Ion Flow Tube – Mass Spectrometry (SIFT-MS) measurements**

225 A Voice 200 Ultra SIFT-MS (SYFT Technologies, Christchurch, New Zealand) equipped with a dual source 226 generating positive soft ionizing reagent ions (H3O+, O2+, NO+) with the nitrogen carrier gas (Air 227 Liquid, Alphagaz 2) was used in this study. Full-scan mass spectra were recorded for each positive 228 precursor ion (H3O+, O2+, NO+) in a range from 15 to 250 with an integration time of 60 s and 229 accumulated during 16h identification was based on specific ion-molecule reactions patterns of target 230 analytes with the three positive precursor ions described in the literature and in the database of the 231 LabSyft software (LabSyft 1.6.2, SYFT Technologies). Product ions from ion-molecule reactions of SCFA 232 are summarized in Supplementary table 1. Quantification of short-chain fatty acids was performed 233 using the NO+ precursor ion. In SIFT-MS analysis, quantification is straight forward and requires only 234 measurement of the count rate of the precursor ion [R] and product ions [P]. The analyte concentration 235 in the flow tube [A] can be determined according to the following calculation:

$$[A] = \frac{[P]}{t_r \ k \ [R]}$$

237 Where  $t_r$  is the reaction time in the flow tube and k is the apparent reaction rate constant.

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#### 241 **2.9. Gene expression analysis**

242 RNA from liver, were extracted using TRIzol<sup>™</sup> reagent (Invitrogen, Waltham, MA, USA) according to 243 the manufacturer's instruction, and quantified by spectrophotometry (absorbance at 260 nm). The 244 qualities of the RNA extracted were assessed using agarose gel electrophoresis. One µg of RNA was 245 transcribed into cDNA using the SuperScript III reverse transcriptase (Invitrogen, Waltham, MA, USA) 246 with random primer (Promega, Madison, WI, USA). After the reverse transcription, cDNA was diluted 247 76-fold for each tissue before it uses in quantitative real-time (g) RT-gPCR. RT-gPCR was performed on 248 a C1000 Touch<sup>tm</sup> thermal cycler (BioRad, Hercules, CA, USA) using PerfeCTa SYBR green (VWR, Radnor, 249 PA, USA). Reactions were performed on a 384 well plate. The total volume of reaction was with 2µL of 250 diluted cDNA mixed with 0.24µL of forward and reverse primer (10µM), 0.52 µL of RNase-free water, 251 and 3µL of SYBR green. Thermocycling conditions included a pre-incubation at 95°C for 10 min, 252 followed by 45 cycles of denaturation at 95°C for 15sec, annealing 60°C for 10sec, and extension at 253 72°C for 15sec. Melting curves were systematically monitored (95°C for 5sec, 65°C for 60sec, 40°C for 254 30sec) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. 255 Each RT-qPCR included replicate samples (duplicate of reverse transcription and PCR amplification), a 256 standard curve (a range of dilution of cDNA from a pool of all cDNA samples) in triplicate, and negative 257 controls (reverse transcriptase-free samples and RNA-free samples) in duplicate. The relative 258 quantification of gene expression was carried out by the Bio-Rad CFX Maestro software (Version 259 4.0.2325.0418). Cq (Quantification cycle) values were further converted to relative quantities.  $ef1\alpha$ gene was used as reference gene for liver (Song et al., 2018), samples. The list primer used to study 260 261 the genes of interest are present in **Supplementary table 2**.

262 **2.10. Hepatic enzymatic analysis** 

The hepatic enzymatic activity level of Glucokinase, Pyruvate kinase, Glucose-6-phosphatase, and Fatty acid synthase were determined according to the protocol described by (Véron et al., 2016). For the measurement of these enzymatic activities the livers were crushed in 10 volumes of ice-cold buffer at

pH 7.4 (50 mmol/l TRIS, 5 mmol/l EDTA, 2 mmol/l DTT and a protease inhibitor cocktail (P2714, 266 267 purchased from Sigma, St Louis, MO)). After homogenization, homogenates were then centrifuged at 268 4°C, and the supernatants were used immediately for enzyme assays. The enzymes assayed were: 269 glucokinase (GK; EC 2.7.1.2) (15), pyruvate kinase (PK; EC 2.7.1.40) as described by (Kirchner et al., 270 2003), glucose-6-phosphatase (G6Pase; EC 3.1.3.9) according to (Kirchner et al., 2003), fatty acid 271 synthase (FAS; EC 2.3.1.85) from (Chang et al., 1967). Enzyme activity was measured at 37 °C in 272 duplicate following variation of absorbance of NAD(P)(H) at 340 nm. The reactions were started by 273 adding the specific substrate; a Power Wave X (BioTek Instrument, Inc.) plate reader was used. For 274 each sample, a blank with water instead of the substrate was run. One unit of enzyme activity was 275 defined as the amount of enzyme that catalyzed the transformation of 1 µmol of substrate per min at 276 37°C, except for FAS which was expressed as the amount of enzyme oxidizing 1 µmol of NADPH at 277 37°C. Enzymatic activities was expressed per mg of soluble protein. Protein concentration was 278 measured in triplicate according to Bradford (Bradford, 1976), using a protein assay kit (Bio Rad, 279 München, Germany) with bovine serum albumin as a standard.

#### 280 2.11. Statistical analysis

281 Zootechnical parameters, including initial and final body weight, specific growth rate, feed intake, feed efficiency, and protein retention efficiency were calculated per tank (n=3). The hepatosomatic index 282 283 (liver weight\*100/fish weight) were obtained during the final sampling of the trial experimentation 284 (n=12). All data were presented as mean ± SEM. All statistical analysis were performed with R software 285 (version 4.0.3). Data were tested for normally distribution using the Shapiro-Wilk test and 286 homogeneity of variance using the Bartlett's test. All Data were analyzed using the one-way ANOVA. 287 These statistical tests were followed by Tukey's HSD as post hos test when the normal distribution is 288 respected, otherwise a Wilcoxon non-parametric test is carried out. Results with a P-value < 0.05 were 289 considered statistically significant. In the figures: \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001. The microbial 290 composition was analyzed using the phyloseq package. For alpha and beta diversity, data samples were rarified. Beta-diversity was analyzed with the Bray-Curtis distance using permutational multivariate
analysis of variance (PERMANOVA). The mixOmics package was used to perform a Partial Least Square
Discriminant Analysis (PLS-DA) to determine the most discriminant OTUs. The rCCA (regularized
canonical correlation analysis) function of the same package was used to understand the correlations
between the bacterial OTUs and different host parameters. The core microbiota visualization was
made using the phyloseq package on R and the Venn diagram using a website.

## 297 **3. Results**

#### **3.1.** Whole-body composition, zootechnical, hepatic and plasma metabolite levels

299 At the end of the feeding trial different zootechnical parameters such as final weight, specific growth 300 rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) were measured, showing no statistical 301 differences (Table 2). Plasma parameters such as triglycerides, glucose, lactate and cholesterol were 302 also measured. Statistical analysis did not reveal any change between the two experimental groups 303 (Table 3). Regarding feed utilization, protein efficiency ratio increased (p = 2.41e-4) in trout fed with 304 high starch diet (HS), whereas no significant differences in daily feed intake and feed efficiency were 305 measured (table 2). Whole-body composition (dry matter, gross energy, crude protein and lipid) of fish 306 for the two experimental groups were evaluated (Supplementary table 3). The two diets did not lead 307 to any significant differences of whole-body composition of fish. Moreover, plasma metabolites 308 (glucose, lactate, triglycerides, cholesterol) were also not affected by the diets (Table 3). Finally, 309 several significant changes were observed for hepatic parameters. The use of the high-starch diet 310 increased significantly the average liver weight (hepatosomatic index). The level of glycogen stored in 311 the liver was also significantly higher (p = 1.894e-8) in the group of fish fed with the high-starch diet. 312 Inversely, a decrease of the hepatic cholesterol was observed when trout were fed with the high-starch 313 diet.

#### 315 **3.2. Microbiota diversities and composition**

316 Sequence data were rarefied to 17000 sequences per sample. Alpha diversity measures including the 317 indexes Observed OTUs, Chao1, Shannon, Simpson, and InvSimpson were calculated in the digesta 318 (Figure 2A) and in mucosa (Figure 2B). A significant decrease (ANOVA, p < 0.05) of all the alpha 319 diversities indices only in the digesta associated microbiota was observed for the high-starch group. 320 Most of the alpha diversity indices did not differ between digesta and mucosa samples except for the 321 Shannon index which is significantly (p = 0.01) higher in digesta (data not shown). Beta diversities were 322 measured in the digesta (Figure 2C), and in the mucosa (figure 2D) of the midgut using the Weighted 323 UniFrac dissimilarity index and visualized using NMDS ordination. The pairwise PERMANOVA test was 324 used to compare both groups, showing a significant (p = 0.003) modification of the beta diversities only 325 in the digesta. At total of 202 OTUs (Operational Taxonomic Units) were detected in all samples (Figure 326 3A). Among them, 138 OTU i.e 68.3 % were common across the four experimental groups (Figure 3A). 327 The 10 most abundant common OTUs are Ralstonia pickettii (OTU 1), Mycoplasma sp. (OTU 2), 328 Sphingomonas sp. (OTU 4), Cutibacterium sp. (OTU 6), Sphingomonas sp. (OTU 7), Lactobacillus sp. (OTU 20), Ligilactobacillus sp. (OTU 11), Bacillus sp. (OTU 18), Weissella sp. (OTU 13), and 329 330 Enhydrobacter sp. (OTU 10) (Figure 3B). After performing taxonomic affiliations on OTUs, removing 331 OTUs found in less than 4 samples, and having a minimum abundance of 0.005 %, 7 phyla, 54 families, 332 and 85 genera were observed in the overall dataset. Results are presented at phylum (Figure 4A, 4B), 333 family (Figure 4D, 4E) and genera (Figure 4G, 4H) levels. Proteobacteria and Firmicutes were the most 334 abundant phyla regardless the sample origin. Independently of the diet, we observed significant 335 difference between mucosa associated microbiota and digesta associated microbiota. In digesta, 336 Proteobacteria was the dominant phylum (76.21 ± 11.22 %) (Figure 4B) which is not the case in the 337 mucosa associated microbiota where Firmicutes dominates (66.58 ± 14.10 %) (Figure 4A). In both 338 mucosa and digesta associated microbiota, Proteobacteria were dominated by the genus Ralstonia 339 belonging to the Burkholderiaceae family, whereas the genus Mycoplasma (Mycoplasmaceae family) 340 dominated in *Firmicutes* but significant differences occurred regarding their relative abundance. In

341 mucosa, no change in the relative abundance was observed at phylum, family, and genus levels (Figure 342 4A, 4D, 4G), except for three genera significantly lower in high-starch i.e Bacillus, Peptoniphilus, and 343 *Clostridium sensu stricto 1* (Table 4). Only in digesta associated microbiota, these three phyla were 344 significantly affected by the diet, resulting in an increase in the relative abundance of the 345 Proteobacteria (81.84  $\pm$  5.25 % in HS and 70.58  $\pm$  12.91 % in LS, p = 0.010) and the Actinobacteria (4.39 346  $\pm$  2.68 % in HS and 2.48  $\pm$  0.93 % in LS, p = 0.02987) in trout fed with the high-starch diet. The *Firmicutes* 347 proportion was lower in the high-starch group (13.68  $\pm$  4.32 % in HS and 26.64  $\pm$  13.49 % in LS, p = 348 0.0044). The Ralstonia genus was present a larger relative abundance, in the digesta associated 349 microbiota, with a significant higher genera level with the high-starch diet ( $0.74 \pm 0.06$  in HS and 0.64350  $\pm$  0.12 in LS, p = 0.019) (Figure 4C, 4F, 4I). The proportion of *Bacillus*, belonging to the *Bacillaceae* family 351 and *Firmicutes* phylum was significantly lower in the high-starch diet  $(0.0047 \pm 0.0085$  in HS and 0.033 352  $\pm$  0.014 in LS, p = 4.45e-06). Moreover, the proportion of *Cutibacterium*, belonging to the 353 Propionibacteriaceae family and Actinobacteria phylum was significantly higher in the high-starch 354 group ( $0.037 \pm 0.025$  in HS and  $0.020 \pm 0.0083$  in LS, p = 0.042). Furthermore, 12 bacterial genera were 355 significantly affected by the diet in the digesta i.e. the genera Ralstonia, Bacillus, Cutibacterium, 356 Ligilactobacillus, Weissella, Anaerobacillus, Limosilactobacillus, Rickettsiella, Peptoniphilus, 357 Lactococcus, Floricoccus, and Anoxybacillus (Table 4), whereas in mucosa only 3 genera were 358 impacted, all in the Firmicutes phylum: Bacillus, Peptoniphilus and Clostridium sensu stricto 1 (s.s. 1). 359 In order to evaluate whether some bacterial taxa could distinguish the two diet groups, a PLS-DA was 360 performed, keeping the OTU with abundance above 0.005% in at least one group. A clear separation 361 was observed for digesta (Figure 5A) and the top 30 most contributing OTUs were identified (Figure 362 5B). Indeed, one-way ANOVA allowed to indicate that all of these OTUs were significantly different 363 between the diets (Supplementary table 4). OTUs belonging to Ralstonia pickettii, Peptoniphilus 364 Koenoeneniae, Anoxybacillus flavithermus, Enterococcus cecorum, Mycoplasma sp., Rickettsiella sp., Streptococcus sp., Veillonella sp., and Bacillus licheniformis, cytotoxicus, and amyloliquefaciens 365 366 decrease in fish fed with the high starch diet. One OTU belonging to Ralstonia pickettii, and several

OTUs from *Weissella cibaria*, *Cutibacterium namnetense*, *Sphingomonas* sp., and *Streptococcus* sp., were higher in the high-starch group. Regarding mucosa associated microbiota, the separation was not clear and only 6 OTUs were significantly different between the diet groups: 4 OTUs belonging to *Bacillus* genus, 1 from *Peptoniphilus* and the last one from *Clostridium s.s 1* as previously described (data not shown).

#### 372 **3.3. Short-chain fatty acid**

373 Short-chain fatty acid concentrations, including acetic, propionic, butyric, and valeric acid were 374 measured in samples collected from the fish hindgut. A significant increase of the valeric acid 375 concentration, going from 5.23  $\pm$  1.27 µg/g for the low-starch group to 17.96  $\pm$  14.00 µg/g for the high-376 starch group were obtained according to a Wilcoxon non-parametric test. The level of acetic, butyric, 377 and propionic acid did not differ between the diets (Figure 6A) but show the same trend as valeric acid. 378 Clustering of the samples based on the count rates of the product ions derived from the SCFAs using 379 PLS-DA showing a clear separation between the two diet groups (Figure 6B) and the 15 most 380 discriminating ions were identified and showed significant differences (Figure 6C). Lactate fragmentation induce the production of multiple ions including H3O+ 45, and H3O+ 81 (Figure 6D). A 381 382 significant higher proportion of lactate (H3O+ 81 signal) was measured in the hindgut sample in trout 383 fed with the high-starch diet.

#### 384 3.4. Gene expression in liver

We evaluated the expression of different genes involved in gluconeogenesis, glycolysis, lipogenesis and fatty acid oxidation in the liver, the center of the intermediary metabolism. ANOVA revealed that there was not a significant effect of CHO on the expression of *glut2a* and *glut2b*, two genes involved in glucose transport **(Table 5)**. The mRNA level of genes implicated in the first (*gcka*, *gckb*), the third (*pfkla*, *pfklb*) and last (*pk*) glycolysis steps was measured. The expression of genes involved in the first steps of glycolysis (*gcka*: *p*<0.001 \*\*\*; *gckb*: *p*<0.01 \*\*) increased whereas the expression of the gene implicated in the last-step (*pk*: *p*<0.001 \*\*\*) decreased in the high-starch group. In the same way, the 392 pck1 (p<0.05 \*) and fbp1b1 (p<0.05 \*) mRNA gene expression involved in gluconeogenesis were 393 significantly lower in the high-starch group, while in this group the mRNA level of g6pcb2a (p<0.05 \*) 394 was increased. High-starch diet did not affect the expression of genes involved in lipogenesis. By 395 contrast, the hadh (p<0.01 \*\*) and acox3 (p<0.05 \*) expression implicated in beta oxidation of lipids 396 decreased in trout fed with the high-starch diet. Regarding cholesterol biosynthesis gene, the 397 expression of the *srebp2a*, *hmgcs*, and *cyp51a* genes decreased significantly in high-starch diet group.

#### 398 **3.5. Hepatic enzymatic activities**

399 To validate the gene expression at functional level, the specific activities of glucokinase, pyruvate 400 kinase, glucose-6-phosphatase, and fatty acid synthase were measured in the liver. The enzymes 401 involved in the first (glucokinase) and in the last (PK) steps of glycolysis were both significantly 402 increased in trout fed with high starch diet (Figure 7A, B). Indeed, the average activity of glucokinase 403 was higher in the high-starch group (+ 0.023 mU/mg of protein, p<0.001 \*\*\*). Glucose-6-phosphate, 404 implicated in gluconeogenesis presented a significant (p < 0.05 \*) higher specific activity in high-starch 405 group,  $0.36 \pm 0.098$  mU/mg of protein, than low-starch group,  $0.26 \pm 0.11$  mU/mg of protein (Figure 406 7C). Then, for lipogenesis the key enzyme, Fatty Acid Synthase (FAS), did not show significant 407 difference between the two groups (Figure 7D).

# 3.6. Correlations between the OTUs and the hepatic gene expression, zootechnical, liver, plasma parameters and enzymatic activities

The correlations between discriminatory bacterial genera and hepatic gene expression was firstly evaluated using regularized canonical correlation analysis (rCCA) (Figure 8A). *Anoxybacillus*, *Ligilactobacillus*, *Bacillus*, *Lactobacillus*, *Lentilactobacillus*, *Rickettsiella*, *Mycoplasma*, *Brevundimonas*, and *Fibrella* were positively correlated with *fbp1b1*, and *pck1* genes evolved in gluconeogenesis, as well as *pk* involved in glycolysis. These genera were all negatively correlated with *pck2* (gluconeogenesis) and *g6pcb2a* (glycolysis). *Sphingomonas*, *Undibacterium*, *Acinetobacter*, *Veillonella*, and *Salmonella*, were positively correlated with *g6pca*, *g6pcb2a*, *g6pcb2b*, *fbp1b1*, *pck2* (implicated in 417 gluconeogenesis), and negatively with pk and pck1. Ralstonia was negatively correlated with all of 418 these gene expressions. Secondly, OTUs abundances were correlated with zootechnical, liver, and 419 plasmatic parameters as well as enzymatic activities (Figure 8B). The first 9 OTUs i.e. Lactococcus, 420 Floricoccus, Ligilactobacillus, Lactobacillus, Peptoniphilus, Anoxybacillus, Bacillus, Rickettsiella and 421 Mycoplasma were positively correlated with FBM (Final Body Mass), DGI (Daily Growth Intake), SGR, 422 DFI, FE and the level of liver cholesterol. These 9 OTUs were negatively correlated with the PER, HSI, 423 as well as the level of glycogen and all the enzymatic activities and the plasma parameters. Inversely, 424 the 9 OTUs, Ralstonia, Burkholderia, Weissella, Cutibacterium, Limosilactobacillus, Acinetobacter, 425 Fusobacterium, Macrococcus, and Anaerobacillus, were positively correlated with PER, HSI, as well as 426 the enzymatic activities and the level of glucose and plasmatic lactate. These OTUs were negatively 427 correlated with the zootechnical parameters implicated in growth parameters as well as the DFI and 428 the FE and the level of liver cholesterol.

## 429 **4. Discussion**

430 It is widely known that the vertebrate gut microbiome plays a critical role in digestive system by 431 breaking down nutrients. Bacteria can provide vitamins, fatty acids, as well as nutrients to the host 432 (Dhanasiri et al., 2011), and can also affect the gut morphology (Taschuk and Griebel, 2012). Diet 433 composition is an important factor that could modify both intestinal microbiome (its structure and its 434 metabolic function) and host metabolic response in fish (Ingerslev et al., 2014; Naya-Català et al., 2021; 435 Yang et al., 2021). Although the gut microbiota diversity in fish has been characterized in the last 436 decade (Mansfield et al., 2010), the influence of changes in nutrient composition linked to 437 carbohydrates and proteins levels needs to be investigated in order to improve the use of all plant-438 based diet for farmed carnivorous fish. Therefore, the goal of this study was to evaluate the effect of 439 dietary carbohydrates in rainbow trout fed with 100% plant-based diet. The use of high-starch diet have already been studied in diets containing FM and/or FO showing strong changes in the fish 440

441 microbiota and metabolism (Huang et al., 2021; T. Wang et al., 2021; Zhang et al., 2020) but has never

been studied with a 100% plant-based diet. We used a similar approach in this study.

#### 443 **4.1.** The high-starch diet strongly affects the composition and biodiversity of the gut microbiota

444 Firstly, Proteobacteria and Firmicutes phyla were the most abundant phyla regardless the location and 445 the diet according to previous studies in salmon and rainbow trout (Gajardo et al., 2017, 2016; Li et al., 446 2021; Villasante et al., 2022, 2019). Several studies have already shown that a high-starch diet 447 decreases the intestinal microbiota diversity on the fish intestinal microbiota when combined with 448 diets based on FM and FO in rainbow trout (Geurden et al., 2014), largemouth bass (Huang et al., 2021; 449 Zhou et al., 2021), Chinese perch (Zhang et al., 2020), and Nile tilapia (Xu et al., 2022). Moreover, the 450 comparison between a plant-based diet (but not 100% plant based-diet) and a FM/FO diet have been 451 studied in rainbow trout, revealing that the Firmicutes phylum were dominant with the plant-based 452 diet, while with the FM / FO diet, the Proteobacteria were dominant (Ingerslev et al., 2014). We 453 sampled the microbiota in the midgut region by collecting the digesta, and by scrapping the mucus 454 layer (mucosa associated microbiota) according to (Li et al., 2021). The results obtained in this study 455 allows us to demonstrate that regardless of the diet, a significant difference between digesta and 456 mucosa associated microbiota were observed according to previous works in mammals and fish (Bruni 457 et al., 2022; Gajardo et al., 2017, 2016; Li et al., 2021). Most studies investigating diet effect on gut 458 microbiota in fish were focused only on digesta or on both mucosa and content mixed together. 459 Investigating only one or a mix could allow us to misinterpret the response of intestinal microbiota to 460 dietary changes. Interestingly, in our study, Ralstonia dominates in digesta microbiota when 461 Mycoplasma is highly abundant in mucus samples (Lokesh et al., 2023; Rasmussen et al., 2021). In the 462 last version of silva database (n°138.1) used in this study ("https://www.arb-463 silva.de/documentation/release-138/," n.d.), Mycoplasma is now part of the Bacilli class explaining the 464 high abundance of Firmicutes in mucosa associated microbiota. The mucosa associated microbiota in 465 salmonids is well known to be dominated by Mycoplasma, a potential intracellular bacterium (Cheaib 466 et al., 2021). Previous studies have shown a lack of pathogenicity genes in Mycoplasma strains 467 identified in salmon suggesting a symbiotic relationship by enhancing host defense (Bozzi et al., 2021; 468 Lian et al., 2020) and providing biotin in a nutrient poor environment (Lian et al., 2020). Ralstonia is 469 already described in high abundance in the gut of marine fish (Huang et al., 2020) and in rainbow trout 470 occasionally (Kim et al., 2007). But it is not the case in most studies on gut microbiota of salmonids. 471 Interestingly, a study in yellowtail kingfish (Seriola lalandi) using a protocol allowing the authors to 472 identify both active and inactive bacteria, showed that Ralstonia was more abundant after depletion 473 of dead bacteria (Legrand et al., 2021). Regarding the effect of diet on gut microbiota diversity, we 474 observed that it was dependent on the sample origin. Digesta associated microbiota was more affected 475 by the diet than the mucosa associated microbiota. A significant decrease of the microbial diversity 476 and richness (both alpha and beta diversity) was observed in the digesta for trout fed with the high-477 starch diet as previously described in fish fed with high CHO associated with FM/FO (Gajardo et al., 478 2017; Huang et al., 2021; Morrison and Preston, 2016). Inversely, no change in the alpha and beta 479 diversities were observed in the mucosa associated microbiota, suggesting that the mucosa associated 480 microbiota is more resilient to diet changes. In addition, it has already been shown that transient 481 microbes in the digesta are influenced by multiple environmental factors such as diet, while mucosa 482 contains more resident microbes that are more influenced by the host (Legrand et al., 2021). While in 483 mammals the Firmicutes/Bacteroidetes ratio is helpful to understand the link with diet, in fish the ratio 484 Firmicutes/Proteobacteria is more relevant regarding the low abundance of Bacteroidetes in fish 485 microbiota (Desai et al., 2012). In our study, the gut microbiota of rainbow trout in the midgut digesta 486 were mainly colonized by bacteria belonging to the Proteobacteria and Firmicutes phyla. The inclusion 487 of high-starch diet (corresponding to a high CHO/Protein ratio) led to an increase to the abundance of 488 Proteobacteria and Actinobacteria with a decrease in the Firmicutes. The Firmicutes/Proteobacteria 489 ratio is more relevant in fish and the ratio decreased when rainbow trout are fed with the high-starch 490 diet. This has been previously described in salmonids fed with FM/FO diets (Gajardo et al., 2017; Huang 491 et al., 2021; Morrison and Preston, 2016). More specifically at genus level, the digesta is dominated by

492 Ralstonia which increased with the high-starch diet. Interestingly, we observed in the digesta 493 significant higher proportion of Gram positive Limosilactobacillus and Weissella which are members of 494 the Lactic Acid Bacteria (LAB). These bacteria are known to produce lactic acid through glucose 495 metabolism and Weissella has been already identified at higher abundance in salmonids fed with high 496 content of plant ingredients (Desai et al., 2012; Gajardo et al., 2016; Villasante et al., 2019; Zarkasi et 497 al., 2014). Finally, 12 genera of bacteria were significantly affected by the high-starch diet in the digesta 498 of the midgut. Among these 12 bacterial genera, other lactic acid bacteria were observed in lower 499 abundance in the high-starch group, i.e. Ligilactobacillus showing all the complexity of gut microbiota 500 analysis. To go further, the PLS-DA analysis, showing clear separation of the microbial composition, 501 allowed us to identify the most divergent OTUs between the two groups. Most of these OTUs are part 502 of the genera already identified as affected by the diet such as Weissella, Ralstonia or Ligilactobacillus. 503 Regarding Ralstonia pickettii, in particular the OTU1 which increases in the high-starch group whereas 504 other OTUs belonging to R. pickettii were significantly in lower proportion showing we need to go 505 further the species level by using shotgun metagenomics to fully understand the role of these bacteria. 506 Interestingly, in our study, Ralstonia pickettii was dominant in the digesta microbiota, regardless of the 507 diet, and has been found in several environment such as soil, rivers or lakes. In mammals, Ralstonia 508 pickettii has been linked to the development of obesity and type 2 diabetes (Udayappan et al., 2017). 509 In this, the increase of *Ralstonia* was not associated to any negative effect of fish physiology. OTUs 510 belonging to genus Bacillus: Bacillus licheniformis, B. cytotoxicus, and B. amyloliquefaciens as 511 previously described, have been observed in lower abundance in the high-starch group (Burtscher et 512 al., 2021; Cao et al., 2019; Xu et al., 2022). In the high-starch group we can notify the higher proportion 513 of 4 OTUs corresponding to Weissella cibaria. The presence of these lactic acid-producing bacteria 514 could have beneficial effects on the immune system and may protect against pathogen invasion through the intestinal surface (Nayak, 2010; Salinas et al., 2008). 515

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#### 517 4.2. Modification in gut microbiota potentially mediates changes in SCFA concentrations in

#### rainbow trout fed with the high-starch diet 518

519 In humans, the metabolic changes in the liver in response to carbohydrates are known to be due to 520 the activity of SCFA such as acetate and propionate, since butyrate is generally preferentially absorbed 521 by intestinal cells (Den Besten et al., 2013; Frampton et al., 2020). Indeed, SCFA are good candidates 522 to explain the crosstalk between diet, microbiota, host physiology and health (van der Hee and Wells, 523 2021). SCFA i.e. acetate, butyrate, propionate, valerate, caproate, are produced mainly in the colon 524 after saccharolytic fermentation (Cummings et al., 1987), mostly by the Firmicutes and Actinobacteria 525 phyla (Louis and Flint, 2017; Zhu et al., 2017). In fish, the production of SCFA have already been 526 described (Pardesi et al., 2022) as their beneficial effects on glucose tolerance and immune function 527 were identified in tilapia (T. Wang et al., 2021). Moreover, it has been recently demonstrated that 528 Bacillus amyloliquefaciens, could alleviate the metabolic phenotypes caused by a high-carbohydrate 529 diet by enriching the acetate-producing bacteria in Nile tilapia intestines (Xu et al., 2022). Furthermore, 530 a study in zebrafish has revealed that *Cetobacterium* improves glucose homeostasis, mediated by a 531 potential effect of acetate (A. Wang et al., 2021). In our study, a significant increase of the valerate 532 concentration was observed in the microbiota of trout fed with the high-starch diet whereas acetate, 533 butyrate and propionate showed the same trend. Regarding SCFA levels, their increase observed in the 534 high-starch group could at least partially explain differences in gene expression in the liver (Morrison 535 and Preston, 2016). In addition, our data suggested a high production of lactate probably linked to 536 lactate-producing bacteria, Weissella and Limosilactobacillus, in the high-starch group microbiota.

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#### 4.3. The high-starch diet did not affect growth performance and glucose homeostasis but have

#### 538 expected effect on glucose and lipid metabolism in liver

539 Overall, the incorporation of 20% of dietary starch to a 100% plan-based diet has resulted in strong 540 change in the digesta associated microbiota of the midgut. Indeed, we observed a decrease of 541 microbiota diversity, in particular in contents as well as changes in several bacterial groups abundance

542 without affecting the growth performance. Interestingly, while rainbow trout use high levels of protein 543 for growth (Cleveland and Radler, 2019; Seiliez et al., 2008), decreasing the proportion of plant protein 544 in the high-starch diet did not affect the trout final weight and even increase the protein efficiency 545 ratio suggesting that increasing the CHO/protein ratio could prevent protein catabolism for energy 546 needs as shown previously in fish fed with marine resources (Kamalam et al., 2017). For the first time, 547 we showed that it is possible to incorporate high levels of digestible carbohydrates (20%) without any 548 negative effects on zootechnical parameters and whole-body composition even in fish fed with a 100% 549 plant-based diet. While trout are often described as poor users of glucose caused by a persistent post-550 prandial hyperglycemia when fed a diet containing more than 20% of carbohydrates (Polakof et al., 551 2012), this metabolic disorder has not been observed with the high-starch diet in our study. Other 552 studies have also shown low blood glucose levels in mature brood stock trout fed with high levels of 553 CHO in their diets (Callet et al., 2020). suggesting that CHO can be efficiently metabolized and/or stored 554 as glycogen in the liver at least at later stage of development. Indeed, in rainbow trout fed with the 555 high-starch diet, the glycogen level is higher in the liver resulting in a higher hepatosomatic index. 556 Regarding the metabolism of glucose through glycolysis, our results reveal that the hepatic glucokinase 557 mRNA gene expression (gcka and gckb) and their enzymatic activities were significantly higher in fish 558 with the high-starch diet. The increase of glucokinase activity and glycogen level in trout fed with 559 carbohydrates suggests that the rainbow trout can adapt at a metabolic level to the carbohydrate 560 intake in fish fed with a 100% plant-based diet, as previously observed in fish fed with marine resources 561 (Capilla et al., 2003; Pereira et al., 1995). We observed a significant lower expression of the pk gene 562 (coding for the pyruvate kinase enzyme) involved in the last step of the glycolysis which is different to 563 what is found in mammals (Yamada and Noguchi, 1999). However, this result is consistent in rainbow 564 trout where it has been shown that the expression of the *pk* gene was poorly controlled with high 565 levels of dietary carbohydrates, linked to a strong and constant expression of this gene (Enes et al., 2009; Panserat et al., 2001; Skiba-Cassy et al., 2013). The production of glucose in the liver through 566 567 gluconeogenesis occurs from amino acids, glycerol, or lactate in mammals and fish (Kamalam et al.,

2017; Polakof et al., 2010) and is inhibited when the animals are fed with carbohydrates (Metzger et 568 569 al., 2004). In fish fed with the high-starch diet, we observed as expected a significant diminution of the 570 pck1, and fbp1b1, gene expression involved in different steps of gluconeogenesis pathway. By contrast, 571 the enzymatic activity of the glucose-6-phosphatase (allowing the hydrolysis of the glucose-6-572 phosphate to D-glucose) is higher in the high-starch diet with higher *q6pcb2a* gene expression 573 according to previous studies that the liver glucose-6-phosphatase (g6p) is not well regulated in 574 rainbow trout in fish fed with FM and FO (Kamalam et al., 2012; Marandel et al., 2015; Skiba-Cassy et 575 al., 2013). Indeed, the duplication of genes implicated in gluconeogenesis such as g6pcb was suggested 576 to contribute to the glucose intolerance and poor use of dietary carbohydrates in rainbow trout 577 (Marandel et al., 2015). In our study, we confirmed this atypical regulation of glucose-6-phosphatase 578 in fish fed without fish meal and fish oil, although no hyperglycemia was observed; this was observed 579 for the first time, suggesting that the deregulation of the *q6pcb2* gene expression by carbohydrates is 580 not sufficient to explain the low carbohydrate use in trout. The absence of differences in lipid contents 581 in whole body composition as well as triglycerides levels in plasma in fish fed carbohydrates suggests 582 that de novo lipogenesis is not induced in fish fed with CHO. According to lipid contents, all lipogenesis 583 genes were not regulated by CHO which was consistent with the FAS activity which is not modified in 584 the high starch group confirming that in rainbow trout fed with CHO (Sam et al., 2014), dietary glucose 585 is not a strong inducer of hepatic lipogenesis. Finally, we observed as expected a decrease of the beta 586 oxidation capacity for lipid catabolism (through hadh and acox3 genes expression) as it was 587 demonstrated in rainbow trout fed with a high-starch diet containing fish meal and fish oil (Song et al., 588 2018). We then investigated cholesterol metabolism, which is known to play an essential role in 589 modulating membrane fluidity and essentially synthetized in liver of all animals (Dietschy et al., 1993) 590 but can also be provided by the diet. Interestingly, we observed a significant decrease of the 591 cholesterol concentration in the liver of high starch group associated with a decrease in the expression 592 of several genes (*srebp2a*, *hmqcs*, *cyp51a*) involved in cholesterol biosynthesis (Khare and Gaur, 2020).

593	Lactobacillus species have been previously identified to have cholesterol lowering effect on the hosts,
594	in particular on hepatic cholesterol synthesis (Khare and Gaur, 2020; Martínez Cruz et al., 2012).
595	4.4. Strong interactions between trout microbiota and host metabolism were observed in trout fed
596	with high-starch diet
597	There were positive correlations between genes glycolysis, and gluconeogenesis as well as with, the
598	growth parameters and OTUS belonging to Bacillus, Mycoplasma, Rickettsiella, Anoxybacillus,
599	Ligilactobacillus, and Lactobacillus whereas Ralstonia is negatively correlated with these pathways.
600	correlated. A positive correlation with <i>Bacillus</i> groups with these metabolic pathways were previously
601	observed in rainbow trout (Lokesh et al., 2022). Interestingly, Bacillus and Lactobacillus are already
602	used as probiotics in teleost aquaculture showing different beneficial effect on fish (Martínez Cruz et
603	al., 2012). However, we need further investigation, especially, information on the genomes
604	Lactobacillus and Bacillus to fully understand the role of these bacteria.

## 605 5. Conclusions

606 The present work allows us to show clear differences between the digesta associated microbiota and 607 mucosa associated microbiota. Phyla from Proteobacteria and Firmicutes are dominant in both 608 contents and mucosa associated microbiota. CHO/protein ratio strongly modify the bacterial 609 community and diversity especially in digesta-associated microbiota, associated with differences in 610 concentration of SCFA. Nevertheless, we cannot discard that some of these effects can also be related 611 to the different plant protein sources. We also identified for the first time a good utilization of dietary 612 carbohydrates in carnivorous rainbow trout when associated with a 100% plant-based diet, as 613 reflected by the growth performance and the metabolic analysis. Finally, important correlations have 614 been highlighted between several OTUs and the trout zootechnical and metabolic parameters. Further 615 investigations through the use of meta-omics approaches in future studies could provide a better 616 understanding of the nutrient and carbohydrate utilization by the host microbiome in order to improve the use of a 100% plant-based diet and be a sustainable alternative to FM and FO for carnivorous fishin aquaculture.

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#### 620 Supplementary Information

Supplementary table 1: Product ions from the reaction of short-chain fatty acids and Lactic acid with H<sub>3</sub>O<sup>+</sup>, NO<sup>+</sup> and O<sub>2</sub><sup>+</sup> precursor ions in Selected Ion Flow Tube - Mass Spectrometry (from LabSyft software). Supplementary table 2: Primer sequences and accession numbers for qPCR analysis. Supplementary table 3. Whole-body composition in rainbow trout fed with 100% plant-based diet with high or low levels of dietary carbohydrates. Supplementary table 4. List of the top 30 most contributing OTU, discriminated by the diet on the Midgut digesta samples, with the one-way ANOVA results.

628

## 629 List of abbreviations

630 CHO: carbohydrates; HS: high-starch; LS: low-starch; SCFA: Short-Chain Fatty Acid; FM: Fish meal; FO:
631 Fish Oil; FBM: Final body mass; SGR: Specific growth rate; FE: Feed efficiency; PER: Protein efficiency

ratio; DGI: Daily growth intake; SD: Standard deviation; OTU: Operational taxonomic unit; PLS-DA:

- 633 Partial Least Squares Discriminant Analysis; bp: base-pair; 16S rRNA: 16S ribosomal Ribonucleic
- acid; PCR: Polymerase Chain Reaction; PERMANOVA: Permutational Analysis of Variance; FROGS:
- Find, Rapidly, OTUs with Galaxy Solution; LAB: Lactic-Acid bacteria; Cq: quantification cycle.

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## 639 **Declarations**

#### 640 Ethics approval and consent to participate.

- No submission to the bioethics committee has been made since the energy and nutritional needs of
- the animals have been covered. Moreover, all the samples were taken post mortem.

#### 643 Consent for publication

644 Not applicable

#### 645 Availability of data and material

- 646 All sequence data are available at the NCBI sequence read archive under accession numbers
- 647 PRJNA827991, https://www.ncbi.nlm.nih.gov/bioproject/827991.

#### 648 Competing interests

- 649 We declare no conflicts of interest
- 650 Funding
- 651 Funding was provided by the CD40 (Conseil Départemental des Landes) and the "Université de Pau et
- 652 Pays de l'Adour "(UPPA).

#### 653 Authors' contributions

RD designed and performed the wet lab experiments and data analysis, and drafted the first version of the paper with subsequent editing by coauthors. KR and SP designed, conceived and coordinated the study. JL contributed to the microbiota analysis and made the first correction of the first version. MG, MLB, and TP performed the SCFA analysis. FT formulated the diets and overlooked the experiment. MM, VV, SB, AS collected samples and performed several lab manipulations. All authors contributed to the review of the manuscript. All authors read and approved the final manuscript.

#### 660

## 661 Acknowledgements

- 662 We are grateful to the genotoul bioinformatics platform Toulouse Occitanie (Bioinfo Genotoul, doi:
- 663 10.15454/1.5572369328961167E12) and Sigenae group for providing help and/or computing and/or
- storage ressources thanks to Galaxy instance <u>http://sigenae-workbench.toulouse.inra.fr</u>. We thank
- 665 Frédéric Terrier, Franck Sandres and Anthony Lanuque for the fish rearing in the Donzacq (France)
- 666 INRAE experimental fish farm. Figure 1 created with BioRender.com.

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#### 983 Figure Legends:

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**Figure 1:** Experimental design. Two experimental diets containing a 100% plant-based diet with 20% of digestible starch (high-starch diet) or without digestible starch (low-starch diet) were produced as extruded pellets. The experimental diets were given manually, twice a day, during 12 weeks to 162 females rainbow trout (50 – 70g) distributed in 6 tanks. The trout tanks were weight every 3 weeks for zootechnical parameters (n=3). At the end of the rearing trial, 4 fish per tanks (n=12) were randomly sampled for microbiota and metabolism studies. Additional trout were recovered for whole-body composition. Figure 2: Bacterial alpha diversity represented in terms of observed OTUs, Chao1, Shannon Simpson,
InvSimpson, in digesta (A) and mucosa (B) according to the experimental diversity. Alpha diversity
between diet groups was compared using one-way ANOVA and p<0.05 was considered significant. Beta</li>
diversity is presented by a nMDS representation (Bray-Curtis distance, Weighted-Unifra analysis) in
digesta (C) and in mucosa (D). Beta diversity was compared using pairwise PERMANOVA and p<0.05</li>
was considered significant and indicated with asterisk. n=12

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Figure 3: Venn diagram showing compartmental core microbiota OTUs distribution (A) in digesta,
mucosa of the fish fed with high-starch (HS) or low-starch (LS) diet. Heatmap of the core microbiota
with the 12 most abundant OTUs among all groups (B).

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Figure 4: Microbial composition of the experimental groups in mucosa (A, D, G), and digesta (B, E, H) at phylum, family, and genus level. In panel D, E, G and H only the 10, and 11 most abundant Family and genus, respectively, were presented. Means are presented as black points and SD as horizontal error lines. Only some significant differences were showed on panels C, F, I. Significative differences were measured with one-way ANOVA (P<0.05), represented by asterisk. P<0.05 \*, P<0.01 \*\*, P<0,001 \*\*\*. n=12

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**Figure 5:** PLS-DA analysis on high-starch and low-starch groups based on OTU abundance **(A)**. Each red points or blue triangles represent a fish. Fish can be discriminated according to experimental group on component 1. Contribution level of the top 30 OTUs are presented **(B)**. The bar length represents the importance of the variable in the multivariable model. Red bars correspond to the high-starch group and blue bars to the low-starch group. Significative differences for each OTUs were calculated with one-way ANOVA. P<0.05 \*, P<0.01 \*\*, P<0,001 \*\*\*. n=12

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1017	Figure 6: SCFA levels of Acetic acid, Butyric acid, Propionic acid, and Valeric Acid in the digesta of
1018	different diets groups (A) were measured in function of the mass of the digestive tract (in gram) with
1019	SIFT-MS mass spectrometry. A PLS-DA based on the product ions on the full scan spectrum were made
1020	(B) using the H3O+, NO+, and O2+ precursor ions. Each red points or blue triangles represent a fish.
1021	The top 15 ions discriminated in SIFT-MS were presented <b>(C)</b> . The bar length represents the importance
1022	of variation contributed by each ions (variable). Red bars correspond to the high-starch group and blue
1023	bars to the low-starch group. The proportion of ion H3O+ 45 and H3O+ 81 corresponding to the lactate
1024	fragmentation are presented (D). All significative differences (P<0.05) were calculated according to a
1025	Wilcoxon non-parametric test. P<0.05 *, P<0.01 **, P<0,001 ***. n=5
1026	
1027	Figure 7: The enzymatic activities of Glucokinase (A), Pyruvate kinase (B), Glucose-6-phosphatase (C),
1028	and fatty-acid synthase (D) were measured in liver samples in function of the average of milligram of
1029	proteins. Significative differences were calculated with one-way ANOVA. P<0.05 *, P<0.01 **, P<0.001
1030	***. n=12
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1032	Figure 8: Correlations between OTU abundances and zootechnical, liver, plasma parameters, and
1033	enzymatic activities (A) or with several liver gene expressions (B), presented as heatmaps, were
1034	calculated using regularized canonical correlation analysis (rCCA). The red stars correspond to the
1035	parameters significantly affected by the diets (P<0.05). n=12
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## **Tables**:

### **Table 1:** Formulation and proximate composition of two experimental diets.

	High-Starch	Low-Starch
Ingredients (%)		
Wheat gluten	20	15
Lysamine pea protein	20	16
concentrate		
Corn gluten	5.44	7.24
Whole wheat	7.09	-
Faba Protein Concentrate	5	-
Pregelatinized wheat starch	15.02	-
Lupin flour	-	4.53
Soybean meal	-	15
Soybean protein concentrate	-	12
Rapeseed oil	12	10.74
Linseed oil	5.29	6
Palm oil	2	2
Dicalcium Phosphate	2	2
L-Lysine	0.5	1
L-Methionine	0.5	1
Soy Lecithin powder	2.5	2.5
Premix Vitamins	1.16	1.5
Premix minerals	1.5	1.5
Cellulose	-	2
Proximate composition (% DM)		
Dry Mass (DM)	97.20	96.90
Starch (% DM)	19.73	3.11
Proteins (% DM)	42.23	51.39
Lipids (% DM)	23.25	23.88
Energy (KJ g-1 DM)	24.54	25.27
Ash (% DM)	5.12	6.05

#### 1047 **Table 2**: Growth performance, and feed utilization, in rainbow trout fed with high-

#### 1048 starch diet and low-starch diet

Zootechnical parameters	High-Starch	Low-Starch	P value
Initial body weight (g)	53.33	53.33	-
Final body weight (g)	237.80 ± 20.14	$261.90 \pm 9.80$	NS
Daily growth index <sup>1</sup>	2.89 ± 0.17	3.13 ± 0.07	NS
Specific growth rate <sup>2</sup> (%/day)	1.78 ± 0.07	1.89 ± 0.03	NS
Daily feed intake <sup>3</sup> (gram/fish/day)	2.16 ± 0.18	2.38 ± 0.11	NS
Feed efficiency <sup>4</sup>	1.02 ± 0.02	$1.04 \pm 0.01$	NS
Protein efficiency ratio <sup>5</sup>	2.61 ± 0.05	2.21 ± 0.02	***

<sup>1049</sup> 

Data are presented as mean  $\pm$  SD (n=3 tanks). Statistical differences were calculated by one-way ANOVA (*P*<0.05). NS not significant. Daily growth index<sup>1</sup> = 100x (Mean final weight <sup>1/3</sup>. g - Mean initial weight<sup>1/3</sup>. g)/Experiment duration (days). Specific growth rate (%/day) = 100x [Ln (final body weight. g)-Ln (initial body weight. g)]/ Experiment duration (days). Daily feed intake<sup>3</sup> (gram/fish/day) = (Total feed consumed. g/Number of fish/Experiment duration (days)). Feed efficiency<sup>4</sup> = (Final biomass. g – Initial biomass. g)/Total feed consumed (g DM). Protein efficiency ratio<sup>5</sup> = (Final biomass – Initial biomass)/ Total protein consumed (g).

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#### **Table 3:** Hepatic and plasmatic parameters in rainbow trout fed with the high or low-starch

#### 1064 diet

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	High-Starch	Low-Starch	P value
Hepatic parameters			
Hepatosomatic index (%)	1.28 ± 0.13	$1.09 \pm 0.14$	**
Glycogen (mg/g)	32.65 ± 3.99	21.77 ± 1.86	***
Cholesterol (g/L)	5.30 ± 0.48	5.72 ± 0.36	*
Free-Glucose (mg/g)	0.45 ± 0.04	$0.42 \pm 0.03$	NS
Plasmatic parameters			
Glucose (g/L)	$1.11 \pm 0.40$	$1.01 \pm 0.38$	NS
Triglycerides (g/L)	$3.49 \pm 1.04$	$3.17 \pm 0.80$	NS
Lactate (g/L)	3.59 ± 0.93	3.37 ± 0.89	NS
Cholesterol (g/L)	1.87 ± 0.36	$1.84 \pm 0.44$	NS

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1068 Data are presented as mean ± SD (n=12 fish). statistical differences are calculated by one-ways ANOVA

1069 (P<0.05). NS not significant. P<0.05 \*. P<0.01 \*\*. P<0.001 \*\*\*.

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#### **Table 4**: Genera abundances significantly affected by diets

Location	Phylum	Family	Genus	High-starch	Low- starch	P value
Digesta	Proteobacteria	Burkholderiaceae	Ralstonia	0.74 ± 0.06	0.64 ±	0.019 *
					0.12	
Digesta	Firmicutes	Bacilliaceae	Bacillus	0.0047 ±	0.033 ±	4.45e-06
				0.0085	0.014	***
Digesta	Actinobacteria	Propionibacteriaceae	Cutibacterium	0.037 ±	0.020 ±	0.042 *
				0.025	0.0083	
Digesta	Firmicutes	Lactobacillaceae	Ligilactobacillus	0.014 ±	0.025 ±	0.035 *
				0.0075	0.016	
Digesta	Firmicutes	Lactobacillaceae	Weissella	0.017 ±	0.0075 ±	0.0014 **
				0.0080	0.0043	
Digesta	Firmicutes	Bacillaceae	Anaerobacillus	0.0045 ±	0.0026 ±	0.00019
				0.0027	0.0014	***
Digesta	Firmicutes	Lactobacillaceae	Limosilactobacillus	0.0044 ±	0.0017 ±	0.012 *
				0.0033	0.0011	
Digesta	Proteobacteria	Coxiellaceae	Rickettsiella	9.7E-06 ±	0.0049 ±	0.00076
Discoto	Einer in der	Denteniskilaans	Denteniskilus	2.2E-05	0.0044	~~~
Digesta	Firmicutes	Peptoniphilaceae	Peptoniphilus	$0.00052 \pm$	0.0022 ±	0.0052 **
Discoto	Einer in der	Characteristic	1	0.0010	0.0016	0.012 *
Digesta	Firmicutes	Streptococcaceae	Lactococcus	$0.00048 \pm$	$0.0013 \pm$	0.012 *
Diaasta	Firmieutos	Strantagagagaga	Floricoccus	0.00053	0.00093	0.00015
Digesta	Firmicutes	Streptococcaceae	FIOFICOCCUS	0±0	$0.0013 \pm$	0.00015
Diaasta	Firmieutos	Dacillacoac	Anovybacillus	0 ± 0	0.00099	0.040 *
Digesta	Firmicules	Вистисеце	Anoxybucillus	0±0	0.00080 +	0.040
					÷ 0.00060	
Mucosa	Firmicutes	Bacilliaceae	Bacillus	0.0049 +	0.018 +	0.0/1.*
Wideosu	1 mmcates	Bucimuccuc	Ducinus	0.0045 ±	0.010 ±	0.041
Mucosa	Firmicutes	Pentoninhilaceae	Pentoninhilus	0.00011+	0.0017 +	0 00078
	·······································	. cptomprinaceae	. ep comprindo	0.00032	0.0014	***
Mucosa	Firmicutes	Clostridiaceae	Clostridium s.s. 1	$0 \pm 0$	0.00020	0.00321 **
					±	
					0.00021	

1082 Data are presented as mean ± SD (n=12 fish). statistical differences were calculated by one-ways

1083 ANOVA (P<0.05). NS not significant. P<0.05 \*. P<0.01 \*\*. P<0.001 \*\*\*.

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#### **Table 5**: mRNA levels of liver genes of rainbow trout fed with the high or low-starch diet

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	High-starch	Low-starch	P value
Glucose transporter			
glut2a	1.31 ± 0.23	0.94 ± 1.02	NS
glut2b	$1.09 \pm 0.38$	$1.01 \pm 0.35$	NS
Glycolysis			
gcka	$5.0 \pm 4.07$	0.39 ± 0.29	***
gckb	$1.76 \pm 0.79$	0.74 ± 0.54	**
pfkla	$1.15 \pm 0.62$	$1.19 \pm 0.56$	NS
pfklb	$1.10 \pm 0.44$	$1.2 \pm 0.45$	NS
pk	$0.90 \pm 0.28$	1.42 ± 0.33	***
Gluconeogenesis			
pck1	$0.59 \pm 0.36$	4.76 ± 5.95	*
pck2	$1.28 \pm 0.96$	$1.21 \pm 0.52$	NS
fbp1b1	$0.93 \pm 0.41$	$1.48 \pm 0.62$	*
д6рса	$1.01 \pm 0.28$	$1.12 \pm 0.48$	NS
g6pcb2a	2.26 ± 1.91	0.79 ± 0.51	*
Lipogenesis			
aclya	$1.03 \pm 0.74$	$1.82 \pm 1.57$	NS
αcα-αα	$1.76 \pm 2.00$	$1.50 \pm 1.45$	NS
fasna	2.25 ± 2.08	1.45 ± 0.94	NS
fasnb	1.47 ± 1.53	$1.34 \pm 0.65$	NS
Beta oxidation			
hadh	$0.91 \pm 0.31$	1.47 ± 0.56	**
cpt1a	$1.08 \pm 0.52$	1.23 ± 0.57	NS
acox3	$0.91 \pm 0.35$	$1.48 \pm 0.68$	*
Cholesterol synthesis			
srebp2a	$0.89 \pm 0.48$	1.51 ± 0.83	**
hmgcs	$0.85 \pm 0.51$	1.70 ± 0.77	**
cyp51a	$0.86 \pm 0.47$	1.66 ± 0.73	**

1096	Data are presented as mean ± SD (n=12 fish). statistical differences are calculated by one-ways ANOVA
1097	(P<0.05). NS not significant. P<0.05 *. P<0.01 **. P<0.001 ***. glut2a and glut2b: glucose transporter
1098	2 paralogs. <i>gcka</i> and <i>gckb</i> : glucokinase paralogs. <i>pfkla</i> and <i>pfklb</i> : 6-phosphofructokinase. <i>pk</i> : pyruvate
1099	kinase. pck1 and pck2: phosphoenolpyruvate carboxykinase paralogs. fbp1b1: fructose 1.6-
1100	bisphosphatase. g6pca. g6pcb2a: glucose-6-phosphatase paralogs. aclya: adenosine triphosphate
1101	citrate lyase. <i>aca-αa</i> : acetylcoA carboxylase alpha. <i>fasna</i> and <i>fasnb</i> : fatty acid synthase paralogs. <i>hadh</i> :
1102	3-hydroxyacyl-CoA dehydrogenase. <i>cpt1a</i> : carnitine palmitoyl transferase 1. <i>acox3</i> : acylcoA oxidase.
1103	srebp-2: sterol regulatory element-binding protein 2. hmgcs: hydroxymethylglutaryl-CoA synthase.
1104	cyp51a: Lanosterol 14-alpha demethylase. abca1a: ATP-binding cassette transporter A1
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1107	









Prevalence 100.0% 90.0% 80.0% 70.0% 60.0% 40.0% 40.0% 20.0% 10.0% 0.0%









0.00

0.05

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