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Original Research Article

# Dietary carbohydrate-to-protein ratio influences growth performance, hepatic health and dynamic of gut microbiota in atlantic salmon (*Salmo salar*)

Alejandro Villasante [a], Carolina Ramírez [b], Héctor Rodríguez [c], Patricio Dantagnan [d], Adrián Hernández [d], Elías Figueroa [d], Jaime Romero [b, *]

[a] Facultad de Medicina Veterinaria y Agronomía, Universidad de Las Américas, Santiago, Chile
[b] Laboratorio de Biotecnología de Alimentos, Unidad de Alimentos, Instituto de Nutrición y Tecnología de Los Alimentos (INTA), Universidad de Chile, Santiago, Chile
[c] Programa de Anatomía y Biología Del Desarrollo, Facultad de Medicina, Universidad de Chile, Santiago, Chile
[d] Núcleo de Investigación en Producción Alimentaria, Facultad de Recursos Naturales, Universidad Católica de Temuco, Temuco, Chile

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ABSTRACT

Atlantic salmon (*Salmo salar*) fed a carbohydrate-rich diet exhibit suboptimal growth performance, along with other metabolic disturbances. It is well known that gut microbes play a pivotal role in influencing metabolism of the host, and these microbes can be modified by the diet. The main goal of the present study was to determine the effect of feeding graded levels of digestible carbohydrates to Atlantic salmon on the distal intestine digesta microbiota at 3 sampling times (i.e., weeks 4, 8 and 12), during a 12-week trial. A low carbohydrate-to-high protein diet (LC/HP, 0% wheat starch), a medium carbohydrate-to-medium protein diet (MC/MP, 15% wheat starch) or a high carbohydrate-to-low protein diet (HC/LP, 30% wheat starch) was fed to triplicate fish tanks (27 to 28 fish per tank). We performed an in-depth characterization of the distal intestine digesta microbiota. Further, growth parameters, liver histology and the expression of genes involved in hepatic neolipogenesis in fish were measured. Fish fed a HC/LP diet showed greater hepatosomatic and viscerosomatic indexes ($P = 0.026$ and $P = 0.018$, respectively), lower final weight ($P = 0.005$), weight gain ($P = 0.003$), feed efficiency ($P = 0.033$) and growth rate ($P = 0.003$) compared with fish fed the LC/HP diet. Further, feeding salmon a high digestible carbohydrate diet caused greater lipid vacuolization, steatosis index ($P = 0.007$) and expression of fatty acid synthase (*fas*) and delta-6 fatty acyl desaturase (*d6fad*) ($P = 0.001$ and $P = 0.001$, respectively) in the liver compared with fish fed the LC/HP diet. Although, the major impact of feeding a carbohydrate-rich diet to Atlantic salmon in beta diversity of distal intestine digesta microbiota was observed at week 4 (HC/LP vs MC/MP and HC/LP vs LC/HP; $P = 0.007$ and $P = 0.008$, respectively) and week 8 (HC/LP vs MC/MP; $P = 0.04$), no differences between experimental groups were detected after 12 weeks of feeding. Finally, at the end of the trial, there was a negative correlation between lactic acid bacteria (LAB) members, including *Leuconostoc and Lactobacillus*, with hepatic steatosis level, the hepatosomatic and viscerosomatic indexes as well as the expression of *fas* and *d6fad*. *Weissella* showed negative correlation with hepatic steatosis level and the hepatosomatic index. Finally, further research to explore the potential use of LAB as probiotics to improve liver health in carnivorous fish fed fatty liver-induced diet is warranted.



* Corresponding author.
*E-mail address:* jromero@inta.uchile.cl (J. Romero).
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## 1. Introduction

Enhancing fish growth performance while reducing production costs is a major goal to optimize fish farming in aquaculture facilities. In fish intensive culture systems, feed represents the largest variable cost (>55%); hence, the search for less expensive aquafeed

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formulations constitutes a determinant factor towards a sustainable aquaculture industry (Naylor et al., 2009; Rana et al., 2009; Overturf et al., 2016). Remarkable advances have been made to reduce the ratio of wild fisheries inputs to farmed fish output in nutrition and feeding, and thus decreasing aquaculture's pressure on forage fisheries (Naylor et al., 2009; Overturf et al., 2016; FAO 2018). The search for cost effective alternatives to pricey marine derived ingredients (i.e., fishmeal and fish oil) is a major challenge for aquaculture in order to become the main fish supply industry for the human population in future decades. In carnivorous fish diet formulation, great progress had been achieved replacing significant portions of fishmeal with more sustainable and environmentally friendly sources, notably plant proteins (Naylor et al., 2009; Overturf and Gaylord 2009; FAO 2018). Greater levels of plant feedstuff concomitantly increase the amounts of plant-derived carbohydrates, including digestible carbohydrates, in aquafeed. Feed manufacturing technical advantages such as improving pellet binding, stability and floatability during extrusion cooking are obtained by adding digestible carbohydrates (i.e., starch) to aquafeed (Kamalam et al., 2017). Although, digestible carbohydrates are not nutritionally essential, they constitute low-cost energy yielding substrate to spare protein in fish. However, the extent of protein sparing effect of carbohydrates is determined by the interaction of several factors, such as fish species trophic level (i.e., herbivorous, omnivorous and carnivorous) and environmental water temperature (i.e., cold water fish species or warm water fish species; Kamalam et al., 2017; Wilson 1994). In addition, fish genotype, feed manufacture technology, dietary formulation and culture system management exert an effect on dietary carbohydrate utilization efficiency and growth performance in farmed fish. Overall, carnivorous fish, such as salmonids species, display poor ability to metabolize energy from digestible carbohydrate sources, since evolution has shaped their physiology and metabolism to deal with rich protein/low carbohydrate feed due to their high trophic level (Polakof et al., 2012; Panserat et al., 2013; Kamalam et al., 2017). In the past, great effort has been addressed to improve our understanding regarding the cellular, metabolic and endocrine mechanisms involved in the regulation of glucose homeostasis in carnivorous fish (Hilton and Atkinson (1982); Kaushik et al., 1989; Panserat et al., 2001; Kirchner et al., 2005; Chapalamadugu et al., 2009; Enes et al., 2009; Polakof et al., 2011; Seiliez et al., 2011; Craig and Moon 2013; Dai et al., 2013; Kamalam et al., 2017). The reported evidence suggests factors including low adaptability in digestive and glucose transport capacities, complex hormonal regulation, inefficient regulation in hepatic intermediary metabolism and low glucose utilization in peripheral tissues, contribute to poor regulation of glucose homeostasis in carnivorous fish (Kamalam et al., 2017). This implies a narrow physiological and metabolic capacity to evoke a protein sparing effect of carbohydrates in carnivorous farmed fish. In Atlantic salmon (*Salmo salar*), a high-value carnivorous fish, research has been conducted to evaluate dietary carbohydrate utilization efficiency by determining the effect of either feeding graded levels or different forms of carbohydrates on tissue enzyme activity, nutrient digestibility and retention, blood chemistry parameters and growth performance in fish (Hemre et al., 1995, 2002; Lygren and Hemre (2001); Krogdahl et al., 2004, 2005). On the other hand, evaluating the effect of feeding digestible carbohydrates to Atlantic salmon on gut microbiota has received less attention, and requires further exploration since a vertebrate's gastrointestinal microbiota plays a role mastering the metabolism and physiology in host (Sonnenburg and Bäckhed, 2006). In the past, the majority of the research on dietary modulation of carnivorous fish gut microbiota has addressed the effect of replacing fishmeal by alternative protein rich ingredients (i.e., plant-derived feedstuff) (Ringø et al., 2016). Few studies have evaluated the effects of digestible carbohydrates rich diets on gut microbiota in carnivorous fish, such as sea bass (*Dicentrarchs labrax*) and rainbow trout (*Oncorhynchus mykiss*) (Gatesoupe et al., 2014; Geurden et al., 2014). These studies evaluated the effect of feeding high digestible carbohydrate diets to fish, using marine-derived protein as the main dietary protein source. Recently, in a previous article, we described the effect of feeding a high carbohydrate diet to Atlantic salmon on distal intestine digesta microbiota, when compared with fish fed a medium carbohydrate diet, during a 4-week feeding period (Villasante et al., 2019). We observed marginal effect on gut microbial composition, mostly affecting low-abundance bacteria. As part of the same study, we have further analyzed the effect of feeding 3 graded levels of digestible carbohydrate in the form of a low carbohydrate-to-high protein diet (LC/HP, 0% wheat starch), a medium carbohydrate-to-medium protein diet (MC/MP, 15% wheat starch) or a high carbohydrate-to-low protein diet (HC/LP, 30% wheat starch) to Atlantic salmon on distal intestine digesta microbiota communities over 3 different sampling times (i.e., weeks 4, 8 and 12), during a 12-week feeding trial. Secondary objectives were to determine whether the nutritional challenge exerts an effect in growth performance, hepatic steatosis, and hepatic lipid synthesis in fish, since previous works have described carnivorous fish fed carbohydrate rich meals exhibit reduced growth performance and/or “fatty liver” condition due to neolipogenesis, which appears to be consequence of a long-term effect of high-carbohydrate diet-associated persistent post-prandial hyperglycemia (Hemre et al., 2002; Panserat (2009); Enes et al., 2011; Gatesoupe et al., 2014).

## 2. Materials and methods

### 2.1. Animal ethics

The study was conducted in accordance with the guidelines of the Bioethics and Biosecurity committee of the Instituto de Nutrición y Tecnología de los Alimentos (INTA) at Universidad de Chile.

### 2.2. Fish and rearing conditions

The study was conducted in a freshwater recirculation system at the Instituto de Nutrición y Tecnología de los Alimentos (INTA) of Universidad de Chile. Fish management protocol was in line with the recommendations of Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the Committee on the Ethics of Animal Experiments of INTA, Universidad de Chile. A total of 255 fish (*S. salar*; 82.5 ± 1 g) were obtained from a commercial freshwater fish farm (Cermaq, Puerto Montt, Región de Los Lagos, Chile). Fish were randomly distributed in nine 150-L fiberglass tanks (27 to 28 fish per tank) supplied with well-aerated fresh water at a constant temperature (15.5 ± 0.5 °C; 8 L/min; >90% oxygen saturation). Fish were acclimated to experimental conditions under 12 h light to 12 h dark photoperiod and fed a reference diet formulated with 15% of wheat starch (as a source of digestible carbohydrate) for 2 weeks. The daily freshwater replacement rate was close to 50% in the recirculation system. Water quality physiochemical parameters (i.e. oxygen, temperature, pH and nitrate concentration) were checked on a daily basis.

### 2.3. Feeding trial

Three experimental diets fulfilling the National Research Council nutritional requirements of *S. salar* (National Research Council, 2011) were manufactured at the Animal Feed Pilot Plant at the Department of Agricultural and Aquaculture Sciences,

Universidad Católica de Temuco, Chile. Ingredients were grinded and thoroughly mixed before the extrusion cooking process with a laboratory twin-screw extruder (Clextral BC-21, Firminy, France). Diets were dried at 50 °C in a hot air oven until they reached less than 10% moisture. Oil was added to each experimental diet according to the specific formulations with a vacuum coater (DinnissenVC10, Sevenum, Netherlands). Diets were stored in a −20 °C freezer until use. The experimental diets were formulated to contain close levels of lipids and energy density (Table 1). Briefly, experimental diets were formulated on a protein background base of fishmeal/soy protein concentrate-to-wheat gluten blend ratio between 0.7 and 0.8, with a gradual increment in the carbohydrate-to-protein ratio (as nitrogen free extract-to-crude protein ratio; 0.07, 0.35, 0.62 for low carbohydrate-to-high protein [LC/HP], medium carbohydrate-to-medium protein [MC/MP] and high carbohydrate-to-low protein [HC/LP], respectively). Either experimental diet was fed to triplicate tanks for a trial period of 12 weeks. Fish were fed by hand 3 times per day to apparent satiation, 6 d per week, during the experiment. In each tank, after 48 h of feed deprivation, fish were group weighed at the beginning of the trial and every 4 weeks until the end of the experiment (weeks 4, 8 and 12).

### 2.4. Chemical composition of diets and fish

Chemical analyses of diets and fish proximate composition were conducted in duplicate following AOAC methods (AOAC 1990). Briefly, dry matter was determined by drying samples overnight (12 h) in an oven (105 °C) to a constant weight. Crude protein content was determined (total nitrogen × 6.25) using a nitrogen analyzer (TruSpec N, LECO Instruments, St. Joseph, MI, USA). Crude fat content was determined with an ANKOM XT 15 extractor (ANKOM Technology, Macedon, NY, USA) using petroleum ether as the extracting solvent. Ash content was determined by incineration (600 °C) for 4 h. Nitrogen free extract (NFE) was estimated as follows % NFE = % DM - (% EE + % CP + % ash + % CF). Total energy content was determined using an adiabatic bomb calorimeter (Parr, Instrument Co., Moline, IL, USA).

**Table 1**
Ingredients and chemical composition of the experimental diets fed to Atlantic salmon for 12 weeks.

| Item | Diet | | |
|---|---|---|---|
| | LC/HP | MC/MP | HC/LP |
| Ingredients, % as-fed basis | | | |
| Fish meal[1] | 37.0 | 30.0 | 21.0 |
| Soy protein concentrate[1] | 38.0 | 30.0 | 23.0 |
| Wheat gluten[1] | 7.5 | 7.5 | 7.5 |
| Wheat starch [1] | 0.0 | 15.0 | 30.0 |
| Fish oil[1] | 14.0 | 14.0 | 15.0 |
| Vitamin C (35%)[2] | 0.2 | 0.2 | 0.2 |
| Vitamin premix[2,3] | 0.8 | 0.8 | 0.8 |
| Mineral premix[2,4] | 0.2 | 0.2 | 0.2 |
| Choline chloride[2] | 0.3 | 0.3 | 0.3 |
| Dicalcium phosphate[2] | 2.0 | 2.0 | 1.7 |
| L-Methionine[5] | 0.0 | 0.0 | 0.2 |
| Lysine[6] | 0.0 | 0.0 | 0.1 |
| Chemical composition, % DM | | | |
| Moisture | 5.7 | 4.7 | 7.0 |
| Crude protein | 58.6 | 50.1 | 41.5 |
| Fat | 18.7 | 16.2 | 16.6 |
| Ash | 10.8 | 9.4 | 7.4 |
| Fiber | 1.8 | 1.9 | 1.6 |
| NFE | 4.4 | 17.7 | 25.9 |
| Gross energy, MJ/kg | 21.0 | 20.7 | 20.5 |

LC/HP = low carbohydrate-to-high protein, MC/MP = medium carbohydrate-to-medium protein; HC/LP = high carbohydrate-to-low protein; NFE = nitrogen free extract.

[1] Cargill Chile Ltda. Coronel, VIII Región, Chile.

[2] Veterquímica S.A. Santiago, RM, Chile.

[3] Provided the following per kilogram dry diet: thiamine mononitrate, 62 mg; riboflavin, 71 mg; niacin, 294 mg; calcium pantothenate, 153 mg; pyridoxine hydrochloride, 50 mg; folic acid, 22 mg; vitamin $B_{12}$, 0.08 mg; d-biotin, 0.8 mg; myoinositol, 176 mg; retinal acetate, 8,818 IU; vitamin $D_3$, 588 mg; α-tocopherol acetate, 670 mg; menadione sodium bisulfite complex, 37 mg.

[4] Provided the following per kilogram dry diet: KI, 1.9 mg; $MnSO_4.H_2O$, 75.8 mg; $ZnSo_4.7H_2O$, 132.0 mg; $Na_2SeO_3$, 0.88 mg; $CoCl_3.6H_2O$, 4.0 mg; $CuSO_4.H_2O$, 11.8 mg; $FeSO_4.H_2O$, 298.5 mg.

[5] M9625, Sigma–Aldrich, Santiago, RM, Chile.

[6] L5501, Sigma–Aldrich, Santiago, RM, Chile.

### 2.5. Sampling procedure

During the study, all sampled fish were euthanized with an overdose (200 mg/L) of tricaine methanesulfonate (MS222; Argent Chemical Laboratories, Redmond, WA, USA). The sampling scheme (Fig. S1) was as follows; for initial whole-body proximate determination, a total of 9 fish (from the initial population), following 48 h of feed deprivation, were sampled at the beginning of the study. For final whole-body proximate analysis, 2 fish per tank (6/treatment), following 48-h feed deprivation, were collected at the end of the study (at the end of week 12). For hepatosomatic and viscerosomatic index (HSI and VSI, respectively), 2 fish per tank (6/treatment), following 48-h feed deprivation, were sampled to collect liver and viscera at the end of the trial (at the end of week 12). For microbiota analysis, 2 fish per tank (6/treatment), were randomly sampled 6 h postprandially to collect distal intestine digesta at weeks 4, 8 and 12. For this purpose, distal intestine segment was aseptically dissected and digesta collected in a 1.5-mL Eppendorf tube. The digesta samples were flash frozen in liquid nitrogen and stored at −80 °C until DNA extraction for distal intestine digesta microbiota analysis. Although all tanks were represented by dietary treatment, not all sampled fish yield enough feces for microbiota analyses in each sampling time. Fish fed the LC/HP; $n = 4$, 6 and 3 for 4, 8 and 12 weeks, respectively. Fish fed the MC/MP; $n = 4$, 6 and 3 for 4, 8 and 12 weeks, respectively, and fish fed the HC/LP; $n = 5$, 4 and 6 for 4, 8 and 12 weeks, respectively. For hepatic gene expression analysis and hepatic histology, 2 fish per tank (6/treatment), were randomly sampled 6 h postprandially to collect liver samples at week 12. For hepatic gene expression analysis, liver samples (80 to 100 mg of tissue) were collected in criovials containing of 1 mL of TRIzol (Life technologies, Carlsbad, CA, USA), snap frozen in liquid nitrogen and stored at − 80 °C until further processing. For hepatic histology, liver samples were fixed with formalin solution, neutral buffered 10% (Sigma–Aldrich, St. Louis, MO, USA) during 48 h. Samples were stored at room temperature and protected from light until further analyses.

### 2.6. DNA extraction and sequencing

DNA was extracted from distal intestinal digesta lysed homogenates (approximately 0.25 g) using the MOBIO PowerFecalDNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA concentrations were measured using the Qubit dsDNA HS Assay kit (Life Technologies, Grand Island, NY, USA). The V4 region of the 16 S rRNA gene was amplified following the fusion primer method using the primers 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) as described by Caporaso et al. (2010). Variable region 4 was selected because of its high coverage, low error rate and its minimal loss of taxonomic resolution (Kuczynski et al., 2012; Lokesh and Kiron (2016)). The resulting amplicons were of suitable length to be used in the Illumina Inc. Sequencing platform. All PCR reactions were performed in duplicates in a 30-μL

reaction mixture containing 1.5 U (5 U/μL) GoTaq G2 Flexi DNA Polymerase (Promega, Madison, WI, USA), 6 μL of Buffer (5 ×), 2.4 μL of Mg (25 mmol/L), 1.2 μL of nucleotides mix (5 mmol/L each), 0.3 μL of primers (20 μmol/L) and 18.5 μL of nuclease free water. In addition, a negative PCR control without the DNA template was run. The PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 68 °C for 45 s. After the cycling procedure, the amplicons from each sample were pooled and run on a 1% agarose gel. Subsequently, the amplicons were purified with the QIAquickPCR Purification kit (Qiagen, Germantown, MD, USA). Libraries were sequenced on the paired end Illumina platform Hiseq PE250 adapted for 300-bp paired-end reads at the CD Genomics (http://www.cd-genomics.com). Sequence files associated with individual samples were deposited in the Sequencing Read Archive (SRA) of the National Centre for Biotechnology Information under Bioproject accession PRJNA498084 and PRJNA521253.

### 2.7. Bioinformatics analysis of microbial communities

In order to detect the presence and quantify the abundance of different microbial taxa based on the assembly of the 16 S rRNA sequence reads, quality-filtered reads were assembled into error-corrected amplicon sequence variants (ASVs) using Devisive Amplicon Denoising Algorithm (DADA2) v1.6.0 microbiome pipeline (available at https://github.com/benjjneb/dada2). Forward and reverse reads were truncated at 285 and 275 bp, respectively, by using read quality scores for the dataset via the filterAndTrim function set with standard parameters (max$N = 0$, truncQ $= 2$, and maxEE $= 2$). Singletons sequences were automatically removed by DADA2's error model, followed by sample inference step using the inferred error model. In addition, chimeric sequences were removed by using read quality scores for the dataset via the filter and trim function using standard parameters (max$N = 0$, truncQ $= 2$, and maxEE $= 2$). Assembled ASVs were assigned into the corresponding taxonomy (phylum to genus) level using the Ribosomal Database Project (RDP) naïve Bayesian classifier (implemented in DADA2) and the "RDP training set 14" (Wang et al., 2007). Using the R package Phyloseq (McMurdie (2013)), we eliminated any ASV without a bacterial phylum assignment, and also those assigned to Cyanobacteria/Chloroplast. Using DADA2, no rarefying of sequence reads was necessary.

### 2.8. RNA extraction, cDNA synthesis and quantification of gene expression by RT-qPCR

Liver total RNA isolation was carried out using TRizol according to the manufacturer's recommendations (Invitrogen, Rockville, MD, USA). Total RNA concentration was quantified spectrophotometrically using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA USA). RNA (5 μg) from each sampled fish was reverse transcribed using Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase and oligodT as primer, following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Primers sequences for genes of interest and reference genes were identified using sequences for *S. salar* obtained in the GenBank database (NCBI) and the Atlantic salmon Gene Index, and further designed and analyzed using the PrimerQuest and OligoAnalyzer tool available at the web page of Integrated DNATechnologies (https://www.idtdna.com/site), with the exception of *cofilin 2* (primer previously published; Morais et al., 2011) (Table 2). The evaluated genes of interest were fatty acid synthase (*fas*), delta-6 fatty acyl desaturase *(d6fad)*, forkhead box O3 (*Foxo 3*), 78-kDa glucose-regulated protein (*grp78*), C-reactive protein (*crp/sap-1a*). Determination of gene expression was carried out by RT-qPCR on an AriaMX Real-Time PCR System (Agilent, Santa Clara, CA, USA) using LightCycler 480 SYBR Green I Master kit (Roche, Indianapolis, IN, USA). The concentration of cDNA loaded was 2 ng in a final 10 μL PCR. Nuclease-free water was used as negative control. Each reaction was carried out in triplicate. Amplification efficiency of RT-PCR for each gene was determined using a standard curve with 5 different concentration points. The PCR cycle conditions were an initial denaturation step of 95 °C for 10 min, followed by 45 cycles at 95 °C for 5 s, 65 °C for 10 s and 72 °C for 10 s. For each gene, specific amplifications were sequenced to confirm their identity. In each assay, the melting curve was analyzed to determine unspecific amplifications. Genes of interest expression output were normalized against the geometric mean of 3 reference genes; elongation factor 1-alpha 1 (*elf-1α*), β-actin and cofilin 2 (*cofilin-2*). Gene expression data was analyzed following the model ($2^{-\Delta\Delta Ct}$) reported by Livak and Schmittgen (2001).

**Table 2**
Primer sequences for Atlantic salmon (*Salmo salar)* used in real-time polymerase chain reaction.

| Gene | Primer sequence (5′- 3′) | Accession number |
|---|---|---|
| Fatty acid synthase (*fas*) | F: GTGCCCACTGAATACCATCC<br>R: ATGAACCATTAGGCGGACAG | CK876943[1] |
| Delta-6 fatty acyl desaturase *(d6fad)* | F: CCCCAGACGTTTGTGTCAG<br>R: CCTGGATTGTTGCTTTGGA | AY458652[1] |
| Forkhead box O3 (*Foxo 3*) | F: TTTACCAGGTTCTCCGTGGC<br>R: CTCCTCGCCTGCATATTCGT | XM_014,145,259[1] |
| 78-kDa glucose-regulated protein (*grp78*) | F: TTCCGTAAAGCTTGCTGATGATG<br>R: TGCCGATGAGGACAAGAAGT | AM042306[1] |
| C-Reactive protein (*crp/sap-1a*) | F: GTTATGGTGAACATCAAGATCTC<br>R: CATGAGACTGGGTTGCCAGA | NM_001123671.1[1] |
| Elongation factor 1-alpha 1 (*elf-1α*) | F: ATGGGCTGGTTCAAGGGATG<br>R: GGGTGGTTCAGGATGATGAC | AF321836[1] |
| *β-actin* | F: AGTGAGCAGGACTGGGTGCT<br>R: CAACTGGGACGACATGGAGA | AF012125[1] |
| *Cofilin-2*[2] | F: AGCCTATGACCAACCCACTG<br>R: TGTTCACAGCTCGTTTACCG | - |

[1] GenBank (http://www.ncbi.nlm.nih.gov/).
[2] Primer previously published by Morais et al. (2011).

### 2.9. Histology

From the initial population, liver samples were collected (one fish per tank) for histology evaluation; however, these samples were used to corroborate the health status of the liver, and thus were not considerate for further statistical analysis at the end of the trial. Liver samples were dehydrated through graded alcohol series (70% to 100%), cleared in xylene and embedded in paraffin. Paraffin sections of 5 μm of thicknesses were stained with hematoxylin–eosin and red Sirius stain for cell morphology evaluation, the presence of cytoplasmic vacuoles (fat droplets-derived vacuoles) and collagen deposition (liver fibrosis), respectively. We used no specific stain technique for lipid, since effort was addressed to detect the formation of cytoplasmatic vacuoles derived from fat droplets to grade steatosis based on a semi-quantitative scoring method similar to Karanjia et al. (2016). Briefly, the grade of steatosis is based on the proportion of hepatocytes containing visible macrovacuoles (fat droplets) and is expressed semi-quantitatively on a scale from 0 to 4 (0, <5%; 1, 5% to 25%; 2, 25% to 50%; 3, 50% to 75%; 4, >75%). The assessment is only semi-quantitative, being a two-dimensional estimation of the proportion of hepatocytes including lipid, and not considering the volume of droplets. A total of 22 sites per sample were analyzed, with a counting frame of 70 × 70 μm per site. All histological

samples were analyzed under an Olympus CX21 light microscope (Olympus, Center Valley, PA, USA). The analyses were performed at Laboratorio de Morfología, Programa de Anatomía y Biología del Desarrollo, Facultad de Medicina, Universidad de Chile.

### 2.10. Statistical analyses

Statistical analyses were carried out using R version 3.4.3 (http://www.R-project.org). Fish growth data were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). One-way analysis of variance (ANOVA) was performed in order to detect significant effect of feeding different carbohydrate-to-protein ratio to fish on growth parameters and hepatic gene expression between experimental groups. Post-hoc tests (Tukey's HSD Test) were performed to identify experimental groups that differed significantly. Kruskal–Wallis rank sum test was run to determine significant differences in liver steatosis score between experimental groups. Then, a post-hoc pairwise comparisons using Wilcoxon rank sum test, with Bonferroni-based adjustment of *P*-values, was conducted to identified which groups differed. Spearman correlation matrix was computed to determine association between multiple variables (continues and ordinal) of interest. We built a correlation matrix between variables of interest using the R packages Hmisc (Harrell and Dupont (2020)) to calculate correlation matrices containing both correlation coefficients and *P*-values, and the R packages corrplot (Wei et al., 2017) for plotting correlograms. Further, we conducted a principal component analysis (PCA) via the R package factoextra (Kassambara and Mundt (2019)) using the functions prcomp to reduce the highly dimensional multivariate data set to a lower dimensional set, and further plotting the outcome of the analysis. Microbiota analyses were performed in R with accompanying packages Phyloseq and Vegan (Oksanen et al., 2011). Alpha diversity measured by the Shannon and Simpson diversity index and species richness measured by Chao 1 was calculated using Phyloseq (McMurdie (2013)). The normality was tested with the Shapiro–Wilk test. Unweighted and Weighted UniFrac distances were calculated as beta diversity measures using the phyloseq package in R (McMurdie (2013)). To statistically test diet effects on the homogeneity of microbial community composition, we performed permutational multivariate analysis of variance (PERMANOVA) using package Vegan analyses on distance metrics. Differential taxa abundance was performed using LefSe (Segata et al., 2013). This method involves the Kruskal–Wallis (KW) sum-rank test between classes of data followed by an (unpaired) Wilcoxon rank-sum test to conduct pairwise tests among subclasses. Linear discrimination analysis (LDA) is then used to estimate the effect size for each of the identified taxa. We used LEfSe (Galaxy Version 1.0) with default parameters (KW = 0.05; Wilcoxon $n = 0.05$; LDA score threshold = 2.0) as well as using the all-against-all strategy for multi-class analysis. All other comparisons were made using either Welch's *t*-test or Kruskal–Wallis (KW). Finally, Spearman's rank correlation coefficient between bacteria genera and steatosis index as well as Pearson correlation coefficient between bacteria genera and *d6fad*, *fas*, HSI and VSI, were determined.

## 3. Results

### 3.1. Growth performance, productive parameters and chemical composition of whole body of atlantic salmon fed the experimental diets for 12 weeks

The growth trajectories of Atlantic salmon fed either experimental diet were similar during the first 4 weeks of the study; however, the growth dynamic subsided for fish fed the HC/LP diet beyond 4 weeks (Fig. 1). Fish growth parameters of experimental groups are summarized in Table 3. After 12 weeks of feeding, the group fed the HC/LP diet showed significantly ($P = 0.005$) lower final weight compared both with the group fed the LC/HP diet and the group fed the MC/MP diet. Similarly, the group fed the HC/LP diet showed significantly ($P = 0.003$) lower weight gain compared both with the group fed the LC/HP diet and the group fed the MC/MP diet. Feed intake was significantly ($P = 0.047$) lower in the group fed the HC/LP diet compared with the group fed the MC/MP diet. Feed conversion ratio (FCR) was significantly ($P = 0.033$) poorer in fish fed the HC/LP diet compared with fish fed the LC/HP diet. On the other hand, the daily growth coefficient (DGC) was significantly ($P = 0.003$) better in fish fed the LC/HP diet and the MC/MP diet compared with fish fed the HC/LP diet. No differences in protein retention and protein efficiency ratio between experimental groups were observed at the end of the trial. HSI and VSI were significantly ($P = 0.026$ and $P = 0.018$) greater in fish fed the HC/LP diet compared with either fish fed the LC/HP diet or fish fed MC/MP diet (Table 3). Finally, we observed no differences in any variable associated with whole-body proximate composition of fish fed either experimental diet at the end of the trial (Table S1).

### 3.2. High-throughput sequence data

After quality filtering, database alignment and removal of chimeras, a total of 1,702,157 good-quality sequences were retained for analysis. Details of the number of reads retained after each step of the pipeline are presented in Table S2. Briefly, samples were analysed considering 37,339 sequences as an average per sample; LC/HP group were in average 36,354 sequences, MC/MP group were in average 38,133 sequences and HC/LP group were in average 37,530 sequences. Dataset was representative of bacterial communities due to Good's coverage estimators for all samples were greater than 99%. The sequencing depth was evaluated by observing the saturation phase of the rarefaction curves based on species richness.

### 3.3. Diversity analysis of distal intestine digesta microbiota between experimental groups

The taxonomic diversity across a diet in different time periods using metrics of alpha diversity that incorporate species richness and diversity, as the Shannon and Simpson indexes, were compared (Fig. 2). We observed no significant ($P > 0.05$) difference for Chao 1 richness estimator, Shannon and Simpson index between groups fed experimental diets in 3 sampling times (Table S3). Beta diversity was estimated using both unweighted (i.e., qualitative) and weighted (i.e., quantitative) UniFrac phylogenetic distance matrices, and visualized in principal coordinates analysis (PCoA) plots (Fig. 3). The composition of distal intestine digesta microbiota was significantly different between groups fed the experimental diets in weeks 4 and 8. In week 4, group fed HC/LP diet showed significant ($P = 0.007$ and $P = 0.008$) difference compared with either group fed MC/MP diet or group fed LC/MP, respectively, when considering unweighted distances. In week 8, significant ($P = 0.04$) difference between fish fed HC/LP diet and fish fed MC/MP diet when considering unweighted distances was observed (Table 4).

### 3.4. Compositional analysis of microbial communities and core microbiota in distal intestine digesta of atlantic salmon fed experimental diets for 12 weeks

Three major phyla, including Firmicutes, Actinobacteria and Proteobacteria, were observed in distal intestine digesta microbiota in Atlantic salmon fed either experimental diet at weeks 4 and 8. However, there was a shift in the dominant hierarchy at the end of

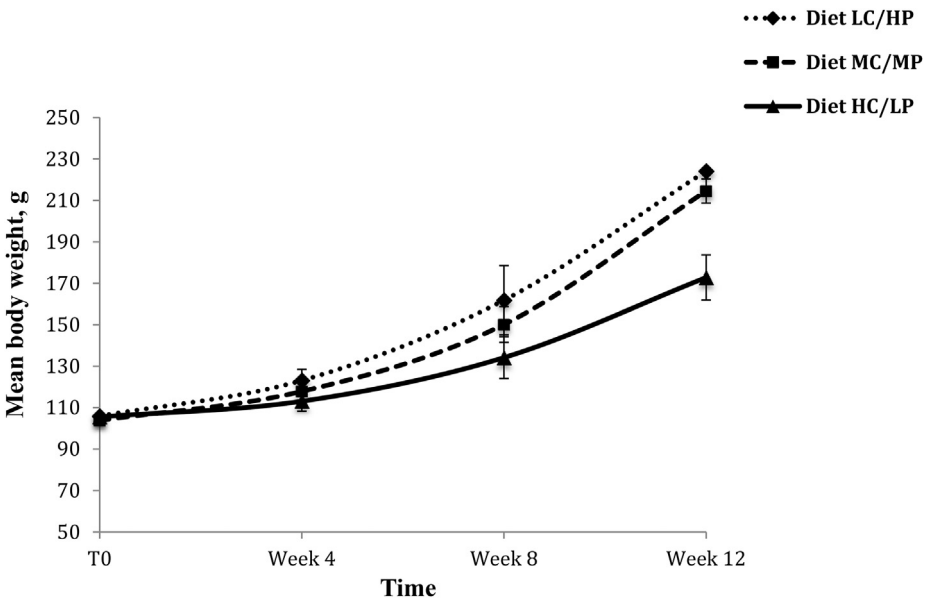


**Fig. 1.** Growth measurement. Growth trajectory of *Salmo salar* fed 3 experimental diets during a 12-week feeding trial. LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

**Table 3**
Fish growth parameters of experimental groups fed either experimental diet for 12 weeks [1].

| Item | Diets | | | | | | ANOVA |
|---|---|---|---|---|---|---|---|
| | LC/HP | | MC/MP | | HC/LP | | |
| | Mean | SEM | Mean | SEM | Mean | SEM | *P*-value |
| Initial weight, g/fish | 105.9 | 1.5 | 103.8 | 0.5 | 105.5 | 1.5 | 0.493 |
| Final weight, g/fish | 224.2[a] | 1.2 | 214.6[a] | 5.9 | 172.8[b] | 10.9 | 0.005 |
| Weight gain, g[2] | 118.3[a] | 2.5 | 110.8[a] | 6.0 | 67.3[b] | 9.9 | 0.003 |
| Feed intake as fed, g/fish[3] | 155.4[ab] | 16.4 | 164.5[a] | 5.6 | 118.0[b] | 6.8 | 0.047 |
| FCR[4] | 1.0[a] | 0.1 | 1.2[ab] | 0.0 | 1.5[b] | 0.1 | 0.033 |
| DGC[5] | 1.6[a] | 0.0 | 1.5[a] | 0.1 | 1.0[b] | 0.1 | 0.003 |
| Protein retention, %[6] | 31.1 | 3.3 | 30.4 | 0.5 | 33.7 | 2.3 | 0.609 |
| Protein efficiency ratio[7] | 1.4 | 0.1 | 1.4 | 0.0 | 1.5 | 0.1 | 0.893 |
| Hepatosomatic index, %[8] | 0.7[a] | 0.1 | 0.9[ab] | 0.1 | 1.2[b] | 0.1 | 0.026 |
| Viscerosomatic index, %[9] | 6.1[a] | 0.4 | 6.7[ab] | 0.3 | 7.4[b] | 0.2 | 0.018 |

LC/HP = low carbohydrate-to-high protein, MC/MP = medium carbohydrate-to-medium protein; HC/LP = high carbohydrate-to-low protein.
[a,b]Within a row, different letters indicate significant difference between treatments ($P < 0.05$).
[1] Mean values with their SEM for 3 tanks per group ($n = 3$).
[2] Weight gain (g/fish) = Mean final weight (g) - Mean initial weight (g).
[3] Feed intake = Total amount of ingested food (g as fed)/The number of fish.
[4] Feed conversion ratio (FCR) = Feed intake/Wet weight gain.
[5] Daily growth coefficient (DGC) = (Mean final weight$^{1/3}$ - Mean initial weight$^{1/3}$)/Number of days × 100.
[6] Protein retention = 100 × (Final body weight × Final body nutrient content − Initial body weight × Initial body nutrient content)/Nutrient intake.
[7] Protein efficiency ratio = Live weight gain (g)/Protein intake (g).
[8] Hepatosomatic index = 100 × (Liver weight/Body weight).
[9] Viscerosomatic index = 100 × (Viscera weight/Body weight).

the trial with Actinobacteria highlighting as the most abundant phylum followed by Firmicutes and Proteobacteria (Fig. 4). Briefly, in week 4, dominant phylum was Firmicutes (29% to 96%), followed by Actinobacteria (2% to 35%) and Proteobacteria (2% to 11%) (Fig. 4A). In week 8, similar composition was observed at the phylum level; however, greater relative abundance of Firmicutes (55% to 96%) was detected. Actinobacteria (3% to 34%) and Proteobacteria (2% to 13%) were the second and third major phyla observed, respectively (Fig. 4B). Alternatively, at the phylum level, the composition of the distal intestine digesta microbiota of fish fed either experimental diet showed differences in the hierarchal structure at week 12 in comparison with either week 4 or 8 sampling time. We observed Actinobacteria to be the most abundant phylum (9% to 84%), followed by the phylum Firmicutes (10% to 79%) and Proteobacteria (1% to 13%) (Fig. 4C). At the genus level, in week 4, distal intestine digesta microbiota revealed a core microbiota of 12 genera present at least in 70% of samples from each dietary treatment (Fig. 5A). This core microbiota included *Clostridium sensu stricto*, *Clostridium XI*, *Geobacillus*, *Lactobacillus*, *Legionella*, *Lentibacillus*, *Leuconostoc*, *Macrococcus*, *Paenibacillus*, *Pseudoclavibacter*, *Streptococcus* and *Weisella*. In week 4, bacteria genera, including *Anaerosalibacter*, *Bacillus*, *Oceanobacillus*, *Sporosarcina* and *Tepidimicrobium* were exclusive for fish fed the LC/HP diet. Alternatively, in week 4, bacteria genera including *Atopostipes*, *Blastopirellula*, *Haematobacter*, *Neochlamydia*, *Nocardioides*, *Photobacterium* and *Singulisphaera* were only observed in distal intestine digesta microbiota of fish fed the HC/LP diet. In fish fed the MC/MP diet, unique genera were *Fusobacterium*, *Marinococcus* and *Ralstonia* (Fig. 5A). Overall, the dominant genus

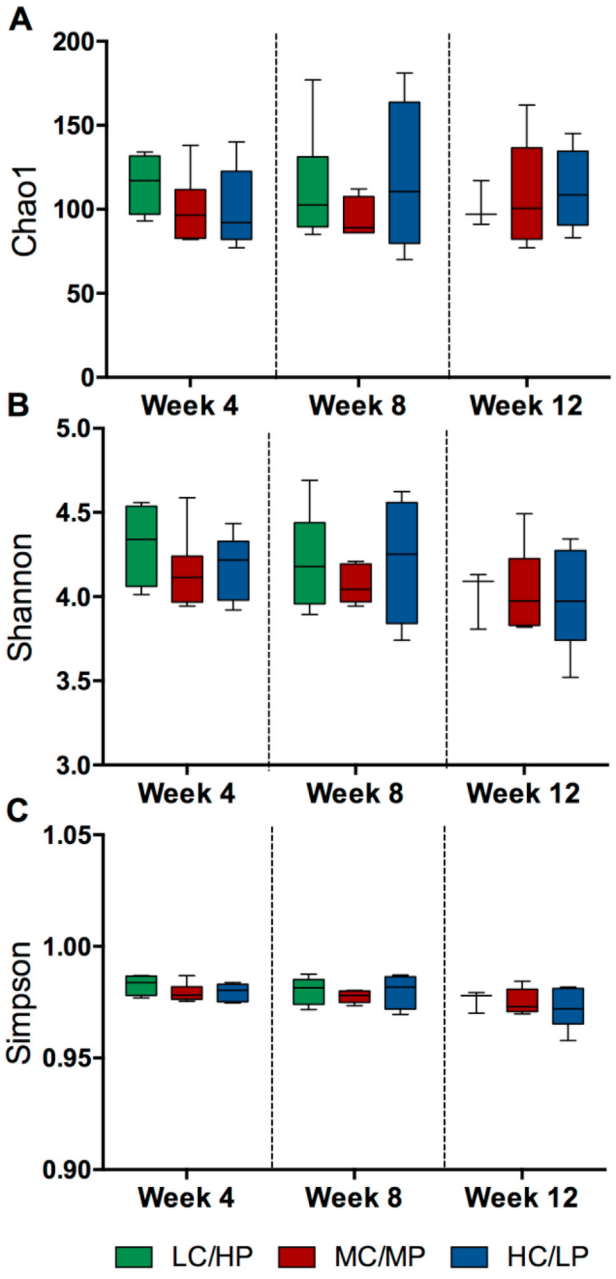


**Fig. 2.** Alpha diversity. Comparison of alpha diversity indexes (A) Chao 1 (B) Shannon and (C) Simpson, across experimental diets at 3 sampling times (weeks 4, 8 and 12). LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

(greatest relative abundance) observed in either experimental group corresponded to *Pseudoclavibacter* (Fig. S2), and bacteria genera showing greater relative abundance were found among the components of the core microbiota identified in the 3 experimental groups (Fig. 5B). In week 8, the core microbiota of fish distal intestine digesta from the 3 experimental groups, consisted of 9 bacteria genera, including *C. sensu stricto*, *Lentibacillus*, *Streptococcus*, *Staphylococcus, Geobacillus*, *Lactobacillus, Leuconostoc*, *Pseudoclavibacter*, and *Weisella* of which the last 5 genera showed greater relative abundance (Fig. 5C and D; Fig. S2). The unshared components of distal intestine microbiota of fish fed LC/HP diet were *Acinetobacter*, *Anaerosalibacter*, *Brevibacterium*, *Anoxibacillus*, *Legionella*, *Naxibacter*, *Oceanobacillus* and *Tissierella*. *Blastopirellula*, *Macrococcus*, *Peptococcus* and *Pseudochrobactrum* were exclusive for fish fed HC/LP diet. However, *Photobacterium*, *Pseudomonas*, *Ralstonia*, Sphingomonas, *Tepidimicrobium*, and *Ureibacillus* were unique for distal intestine digesta microbiota of fish fed MC/MP diet in week 8. At the end of the trial (week 12), the core microbiota of distal intestine digesta encompassed 6 bacteria genera, including *Lactobacillus*, *Legionella*, *Leuconostoc*, *Pediococcus*, *Pseudoclavibacter* and *Streptococcus* (Fig. 5E). Highlighting *Leuconostoc, Lactobacillus* and *Pseudoclavibacter* as bacteria genera with higher values of relative abundance (Fig. 5F; Fig. S2). The unshared bacteria genera of the distal intestine digesta microbiota of fish fed LC/HP diet were *Anaerosalibacter*, *Blastopirellula, Clostridium* XI, *Geobacillus, Lactococcus, Marinococcus, Mycobacterium, Oceanobacillus, Peptococcus and Sphingobacterium*, while Enterococcus, *Macrococcus* and *Ureibacillus* were only detected in fish fed HC/LP diet. Finally, the genus *Bacillus* was unique for fish fed MC/MP diet.

### 3.5. Differential abundance at bacteria taxa levels in distal intestine digesta microbiota of atlantic salmon fed experimental diets at 3 sampling times

The LEfSe analysis revealed significant differences in the relative abundance (LDA effect side score greater than or equal to 3.5; Fig. 6A and B and C) at different taxon levels (using phylum to genus-level data) between fish fed experimental diets at 3 sampling times (weeks 4, 8 and 12, respectively). A total of 39 bacteria taxa showed differential abundance when considering all pairwise comparison between experimental dietary groups at week 4 (Fig. 6A). At this sampling time, the LC/HP vs HC/LP pairwise comparison revealed 8 taxa, including 1 order (Vibrionales), 3 bacteria families (Nocardioidaceae, Vibrionaceae and Clostridiaceae 1) and 4 bacteria genera (*Leuconostoc, Neochlamydia, Photobacterium* and *Nocardioides*), were significantly more abundant in the HC/LP diet group. On the other hand, 8 taxa, including 1 class (Clostridia), 1 order (Clostridiales) and 6 genera (*Sporosarcina, Anaerosalibacter, Anoxibacillus Bacillus Staphylococcus* and *C. sensu stricto*), were significantly more abundant in the LC/HP diet group. Further, the HC/LP vs MC/MP pairwise comparison showed 15 taxa, including 1 phylum (Planctomycetes), 1 class (Planctomycetia), 3 orders (Bifidobacteriales, Rhodobacterales and Planctomycetales), 7 families (Bifidobacteriaceae, Carnobacteriaceae, Nocardioidaceae, Rhodobacteraceae Planctomycetaceae, Streptococcaceae and Pseudonocardiaceae) and 4 genera (*Nocardioides, Haematobacter Singulisphaera* and *Lactococcus*), were significantly more abundant in the HC/LP diet group (Fig. 6A). The LC/HP vs MC/MP pairwise comparison revealed 8 bacteria taxa, including 1 class (Clostridia), 1 order (Clostridiales), 1 family (Clostridiaceae 1) and 5 genera (*Oceanobacillus Anaerosalibacter, Sporosarcina, Lentibacillus* and *Clostridium* sensu stricto), were significantly more abundant in the LC/HP group (Fig. 6A). In week 8, a total of 18 bacteria taxa exhibited differential abundance when considering all pairwise comparison between experimental dietary groups (Fig. 6B). In the LC/HP vs HC/LP pairwise comparison, the family Clostridiales incertae sedis XI was the only taxa showing significant differences in relative abundance between both groups, being more abundant in the LC/HP group. In the case of HC/LP vs MC/MP pairwise comparison, we observed 2 taxa, including 1 genus (*Enterococcus*) and 1 family (Enterococcaceae) were significantly more abundant in the HC/LP group. However, 6 taxa, including 1 order (Vibrionales), 2 families (Clostridiales incertae sedis XI and Vibrionaceae) and 3 genera (*Photobacterium, Tepidimicrobium* and *Streptococcus*) were significantly more abundant in the MC/MP group. The LC/HP vs MC/MP pairwise comparison showed 4 taxa, including 1 order (Vibrionales), 2 families (Vibrionaceae and Streptococcaceae) and 1 genus (*Photobacterium*), significantly more abundant in the MC/MP group, whilst 2 families (Moraxellaceae and Enterococcaceae) and 3

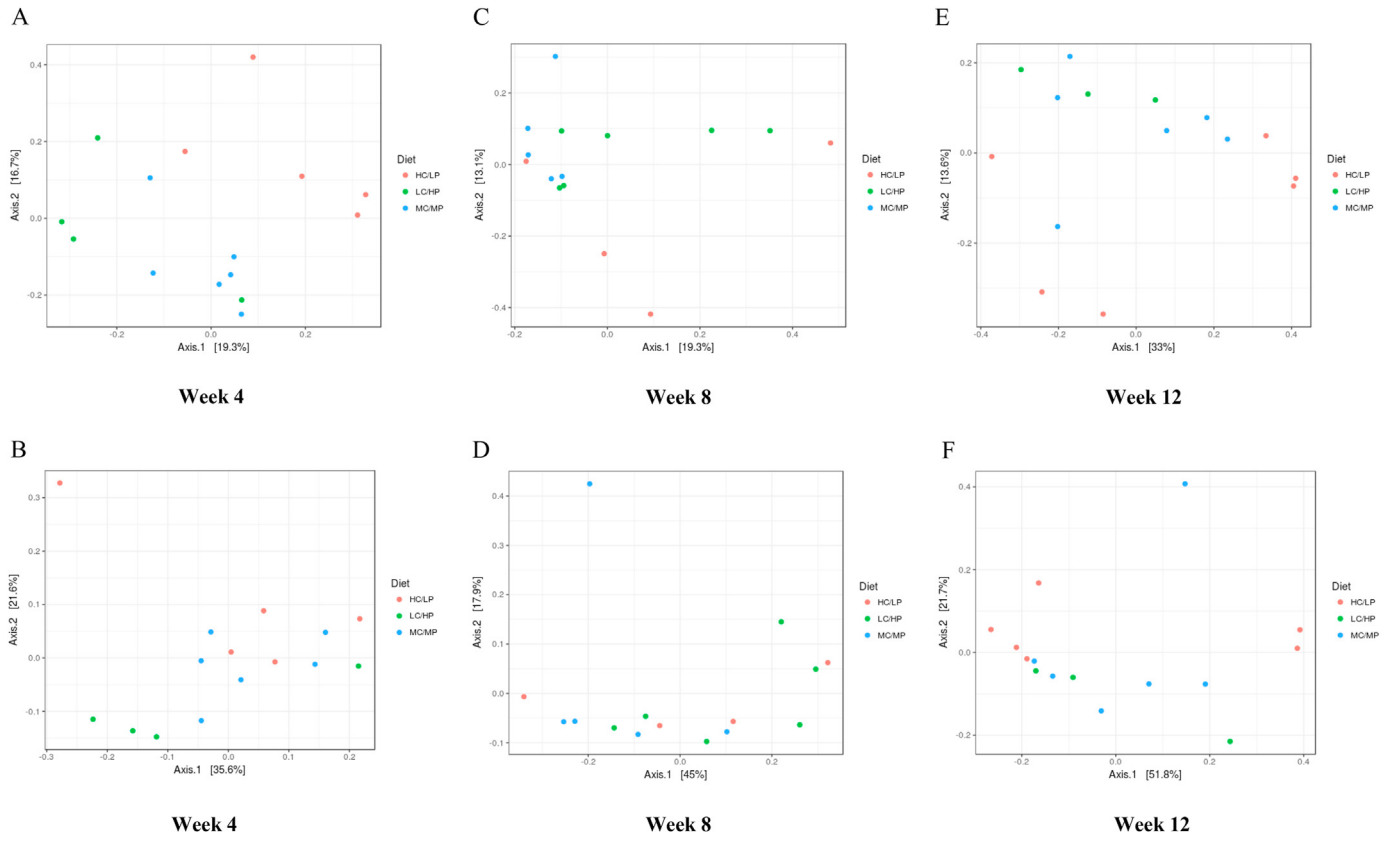


**Fig. 3.** Beta diversity. Principal coordinates analysis (PCoA) of the bacterial communities derived from the unweighted (A, C and E) and weighted (B, D and F) UniFrac distance matrix. Dots represent individual samples from *Salmo salar* distal intestine digesta microbiota. Red dots correspond to samples from fish ($n$ = 5, 4, 6 for weeks 4, 8 and 12, respectively) fed the HC/LP diet, light blue dots correspond to samples derived from fish ($n$ = 6, 5, 6 for weeks 4, 8 and 12, respectively) fed MC/MP diet, and green dots correspond to samples derived from fish ($n$ = 4, 6, 3 for weeks 4, 8 and 12, respectively) fed the LC/HP diet. LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

**Table 4**
PERMANOVA analysis results for the comparison of similarities in distal intestine digesta microbiota composition between fish fed grading levels of carbohydrates in 3 different sampling times, during a 12-week trial.

| | | Unweighted UniFrac | | | Weighted UniFrac | | |
|---|---|---|---|---|---|---|---|
| Time | Comparison | Pseudo F | $R^2$ | $P$-value | Pseudo F | $R^2$ | $P$-value |
| Week 4 | LC/MP vs MC/MP | 1.0026 | 0.02949 | 0.445 | 0.49996 | 0.01492 | 0.835 |
| | HC/LP vs MC/MP* | 1.9656 | 0.17925 | 0.007 | 1.238 | 0.12092 | 0.219 |
| | HC/LP vs LC/HP* | 2.0106 | 0.07178 | 0.008 | 0.97987 | 0.03632 | 0.392 |
| Week 8 | LC/MP vs MC/MP | 1.2871 | 0.12511 | 0.136 | 1.9811 | 0.18041 | 0.092 |
| | HC/LP vs MC/MP* | 1.4677 | 0.17333 | 0.044 | 0.82278 | 0.10518 | 0.594 |
| | HC/LP vs LC/HP | 1.0154 | 0.11263 | 0.394 | 0.62062 | 0.07199 | 0.68 |
| Week 12 | LC/MP vs MC/MP | 0.8326 | 0.10630 | 0.641 | 0.51337 | 0.06833 | 0.851 |
| | HC/LP vs MC/MP | 1.0792 | 0.09741 | 0.328 | 1.0168 | 0.09229 | 0.379 |
| | HC/LP vs LC/HP | 1.3750 | 0.16418 | 0.245 | 1.0404 | 0.12939 | 0.404 |

PERMANOVA = permutational multivariate analysis of variance.
* Comparison with superscript asterisk showed significant differences between experimental groups ($P$ < 0.01).

genera (*Acinetobacter*, *Enterococcus* and *Anoxibacillus*) were significantly more abundant in the LC/HP group (Fig. 6B). Finally, in week 12, a total of 21 bacteria taxa showed differential abundance when considering all pairwise comparison between experimental dietary groups (Fig. 6C). The LC/HP vs HC/LP pairwise comparison, identified 13 taxa, including 1 phylum (Bacteroidetes), 1 class Sphingobacteria, 2 orders (Sphingobacteriales and Lactobacillales), 3 families (Peptostreptococcaceae, Sphingobacteriaceae and Leuconostocaceae) and 6 genera (*Marinococcus, Clostridium XI, Staphylococcus, Lactobacillus, Weisella* and *Sphingobacterium*) were significantly more abundant in the LC/HP group. The HC/LP vs MC/MP pairwise comparison revealed 2 taxa, including 1 genus (*Enterococcus*) and 1 family (Enterococcaceae), significantly more abundant in the HC/LP group, whilst 5 taxa, including 1 order (Lactobacillales), 1 family (Leuconostocaceae) and 3 genera (*Streptococcus, Lactobacillus* and *Leuconostoc*) were significantly more abundant in the MC/MP group. Finally, the genus *Clostridium XI* was the only taxa showing significantly differential abundance from the LC/HP vs MC/MP pairwise comparison (Fig. 6C).

### 3.6. Hepatic gene expression

The effect of feeding grading levels of dietary digestible carbohydrates to Atlantic salmon on the expression of genes

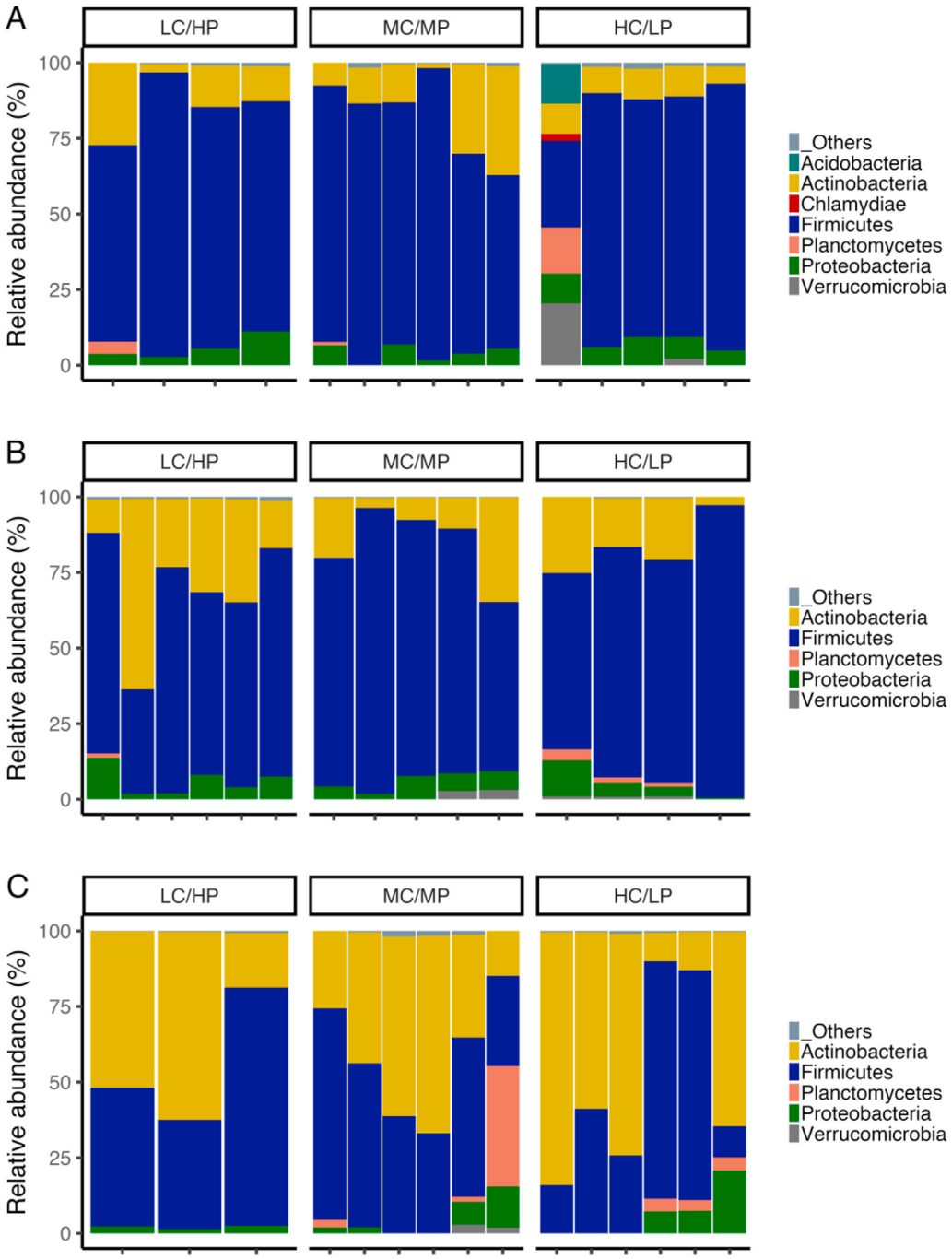


**Fig. 4.** Microbiota composition. Relative abundance of the major phyla expressed as the % of total sequences obtained from each sample in distal intestine digesta microbiota from fish fed either experimental diet: LC/HP diet, MC/MP diet or HC/LP diet at 3 different sampling times (**A**) week 4 ($n = 4$, 6 and 5, LC/HP, MC/MP and HC/LP, respectively) (**B**) week 8 ($n = 6$, 5 and 4, LC/HP, MC/MP and HC/LP, respectively) and (**C**) week 12 ($n = 3$, 6 and 6, LC/HP, MC/MP and HC/LP, respectively). "Others" represent phyla with <1% of relative abundance. LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

associated with lipid synthesis (*fas* and *d6fad*), metabolic regulation (*foxo 3*), endoplasmic reticulum stress response (*grp78*) and inflammation marker (*crp/sap-1a*) in liver was assessed at the end of the 12-week trial (Fig. 7). The expression level of *d6fad* was significantly greater ($P = 0.001$ and $P = 0.001$) in fish fed the HC/LP diet compared with either fish fed the LC/HP diet or fish fed the MC/MP diet, respectively. Similarly, the expression level of *fas* was significantly greater ($P < 0.0001$ and $P = 0.043$) in fish fed the HC/LP diet compared with either fish fed the LC/HP diet or fish fed the MC/MP diet, respectively. No significant differences in the expression level of other evaluated genes of interest between experimental groups was detected.

### 3.7. Hepatic histology evaluation

Based on the semi-quantitative scoring system to quantify fat on the basis of visible hepatic lipid droplets-derived vacuoles within fish hepatocytes, the observed median of each

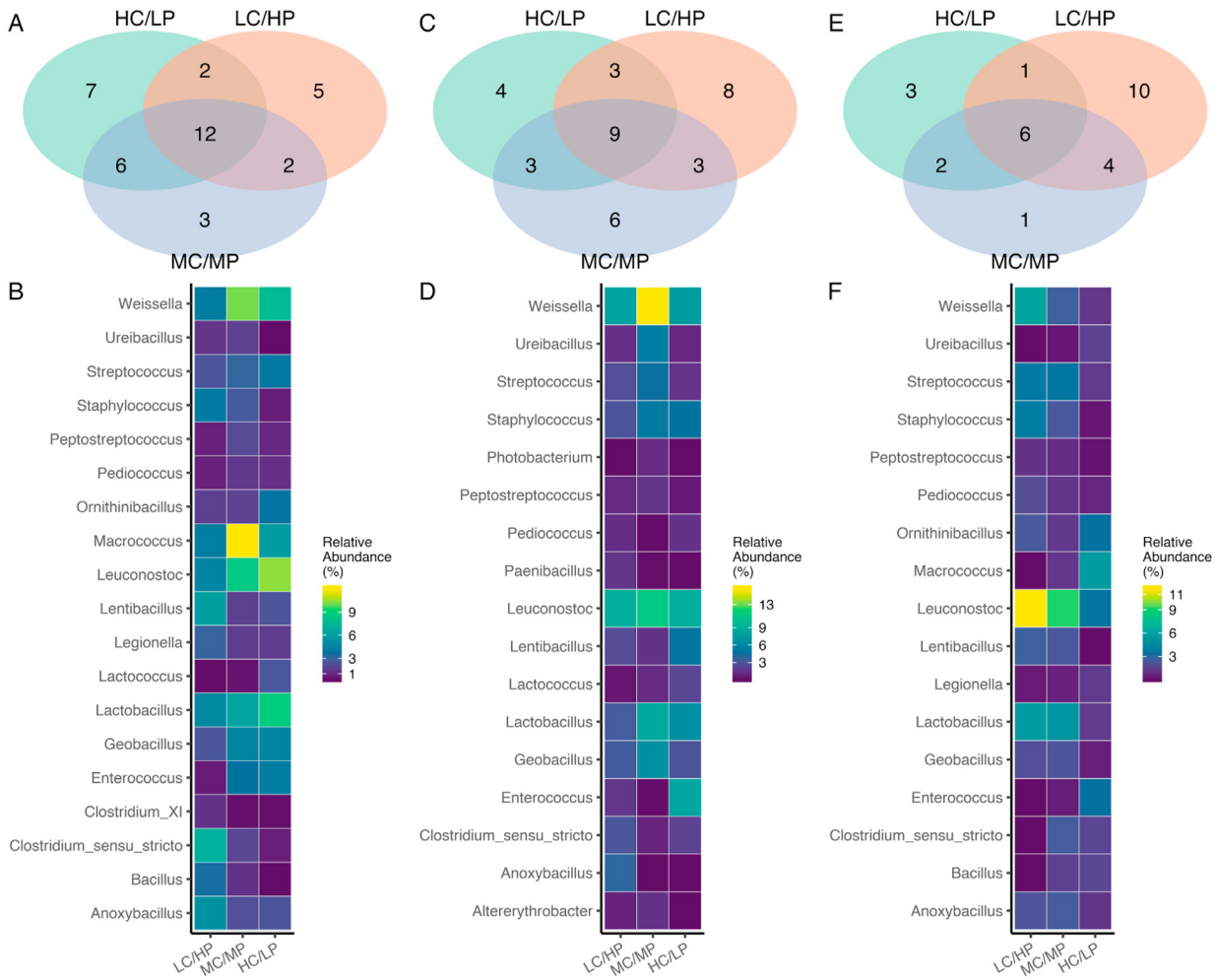


**Fig. 5.** Core microbiota (A, C and E) illustrate Venn diagrams representing common genera detected in distal intestine digesta of fish fed either experimental diet at 3 sampling times (weeks 4, 8 and 12, respectively). Diagram shows numbers of genera present in at least 70% of all samples from each experimental group (B, D and F) depict heatmap displaying relative abundance of most representative bacteria genera in distal intestine digesta of fish fed either experimental diet: LC/HP diet, MC/MP diet or HC/LP diet at 3 sampling times (weeks 4, 8 and 12, respectively). *Pseudoclavibacter* genus was excluded for illustration purpose only (Fig. S1). LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

experimental group—the LC/HP group, the MC/MP group and the HC/LP group—was 0.5, 3.0 and 4.0, respectively. The Kruskal–Wallis rank sum test revealed significant ($P < 0.0001$) differences in liver steatosis score between experimental groups. Further, the post-hoc pairwise comparisons series using Wilcoxon rank sum test with Bonferroni's correction showed the median of the liver steatosis score of the HC/LP group was significantly ($P = 0.007$) different compared with the LC/HP group. However, no difference between the HC/LP group and the MC/MP group was observed. The median of the liver steatosis score of the MC/MP group was significantly ($P = 0.024$) different compared with the LC/HP group (Fig. 8;Fig. S3).

### 3.8. Principal component analysis and correlation matrix of productive variables analyzed at the end of the growth trial

The PCA analysis shows the dimensionality of the growth-related multivariate data was reduced to 2 principal components that can be visualized graphically in Fig. 9. The 2 main components or dimensions explain 91.9% of the observed variance (first component or dimension “Dim 1”, 78.7% and second component or dimension “Dim 2”, 13.2%). Further, the PCA analysis revealed the formation of 3 clusters (each cluster formed by variables found in the same quadrant) based on the contribution of each variable to the overall variance (Fig. 9). Variables that are correlated with Dim1 and Dim2 explain the major fraction of the variability in the data set. Steatosis index, HSI and VSI constitute the first cluster, DGC, weight gain and final weight constitute the second cluster and FCR constitutes the third cluster. In Dim1, the first, the second and the third cluster account for 39.4%, 49.2% and 11.4% of the variability provided by this component, respectively. For its part, in Dim 2, the first, the second and the third cluster account for 59.1%, 20.5% and 20.4% of the variability explained by this component, respectively. Further, a PCA on individual samples revealed 3 individuals-based clusters using carbohydrate level in diet as grouping variable (Fig. 9B). Finally, among the major findings revealed by the Spearman's correlation matrix, carbohydrate level in diet showed a statistically significant positive association with HSI, VSI and hepatic steatosis score; however, carbohydrate level in diet showed a significant negative association with weight gain, final weight and DGC (Fig. 10).

### 3.9. Correlating distal intestine digesta microbiota with hepatic steatosis index, HSI, VSI and lipogenic gene expression

Spearman's rank correlation coefficient between bacteria genera and steatosis index was calculated (Fig. 11A). We observed a

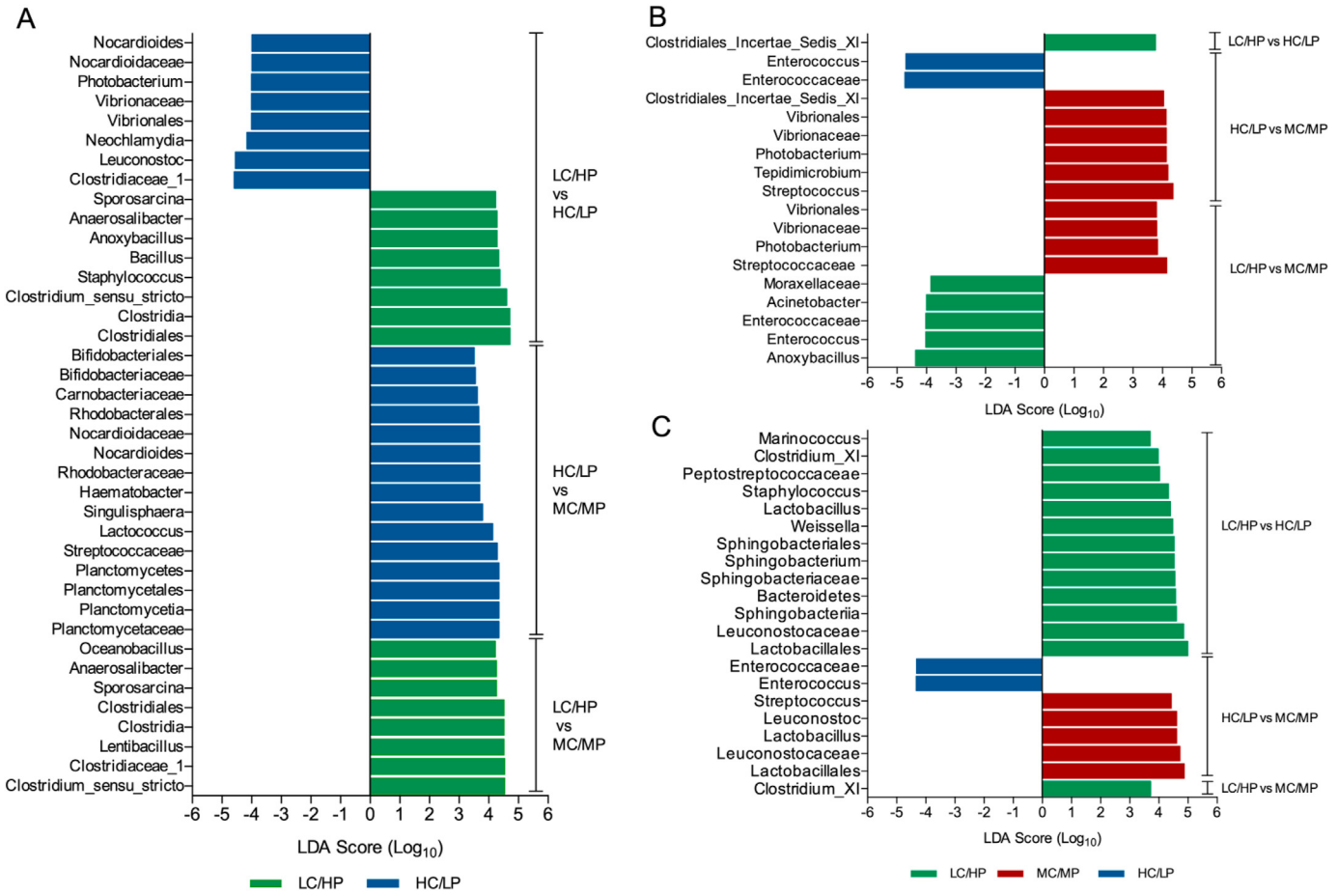


**Fig. 6.** Linear discriminant analysis effect size (LEfSe). LEfSe analysis identifying taxa showing significant differences in relative abundance in pairwise comparison of distal intestine digesta microbiota of *Salmo salar* fed either experimental diet: LC/HP diet, MC/MP diet or HC/LP die at 3 sampling times (A) week 4, (B) week 8 and (C) week 12. The bar length represents a $\log_{10}$-transformed linear discriminant score. The colors represent different experimental diet; LC/HP (green), MC/MP (red) and HC/LP (blue). LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

significant negative correlation between *Leuconostoc, Lactobacillus* and *Weissella* with hepatic steatosis index ($P = 0.026$, $P = 0.017$ and $P = 0.015$, respectively). On the other hand, *Blastopirellula* was the only bacteria genera showing a significant positive correlation with hepatic steatosis index ($P = 0.033$). Further, Pearson correlation coefficient between bacteria genera and *d6fad*, *fas*, HSI and VSI was measured (Fig. 11B). We observed significant negative correlation between *Leuconostoc* and *Lactobacillus* with *d6fad*, *fas*, HSI and VSI (for *Leuconostoc*, $P = 0.012$, $P = 0.013$, $P = 0.011$, $P = 0.001$, respectively; for *Lactobacillus*, $P = 0.005$, $P = 0.004$, $P = 0.031$, $P = 0.019$, respectively). *Weissella* showed significant negative correlation with HSI ($P = 0.011$) and *Staphylococcus* revealed significant negative correlation with *d6fad*, *fas* and VSI ($P = 0.026$, $P = 0.028$ and $P = 0.03$, respectively). On the other hand, *Blastopirellula* showed significant positive correlation with *d6fad*, *fas* and VSI ($P = 0.004$, $P = 0.005$ and $P = 0.014$, respectively), *Lentibacillus* revealed significant positive correlation with *d6fad* and *fas* ($P < 0.001$ and $P < 0.001$, respectively) and *Bacillus* showed significant positive correlation with VSI ($P = 0.005$).

## 4. Discussion

Macronutrient proportions-based differences of diet energy composition distinctly affect fish growth (Saravanan et al., 2012; Overturf et al., 2016). In experimental studies, carnivorous fish, such as Atlantic salmon, fed high digestible polysaccharides diets tend to show reduced growth performance when compared with their control groups. It is well documented gut microbiota plays a major role mastering metabolism and physiology in host, and it can be modulated by diet composition as well as feed intake patterns (Sommer and Bäckhed (2013); Klingbeil and de La Serre (2018)). The effect of feeding digestible carbohydrate to Atlantic salmon on gut microbiota has been poorly explored in the past.

We conducted a study to determine the effect of feeding different carbohydrate-to- protein ratio diets to Atlantic salmon in gut microbiota composition at 3 sampling times during a 12-week trial. Our results showed that increasing carbohydrate-to-protein ratio in diets did not affect species richness (Chao 1 index) and species diversity (Shannon index) of the microbiota of distal intestine digesta in experimental groups, regardless of the sampling time. The effect of modulating dietary components on alpha diversity indexes in fish is not consistent across the fish literature. Previous works have either reported no effect or marginal effect of modulating protein ingredients in alpha diversity indexes in fish (Apper et al., 2016; Rimoldi et al., 2018; Michl et al., 2019). Conversely, other studies have described significant effect of modulating protein ingredients in both species richness and species diversity in fish (Michl et al., 2017; Huyben et al., 2019; Rimoldi et al., 2019). Further, Limbu et al. (2020) observed no differences in species richness abundance-based coverage estimator (ACE) and Chao 1 index and the diversity indices (Shannon and Simpson) of gut microbiota in Nile tilapia (*Oreochromis*

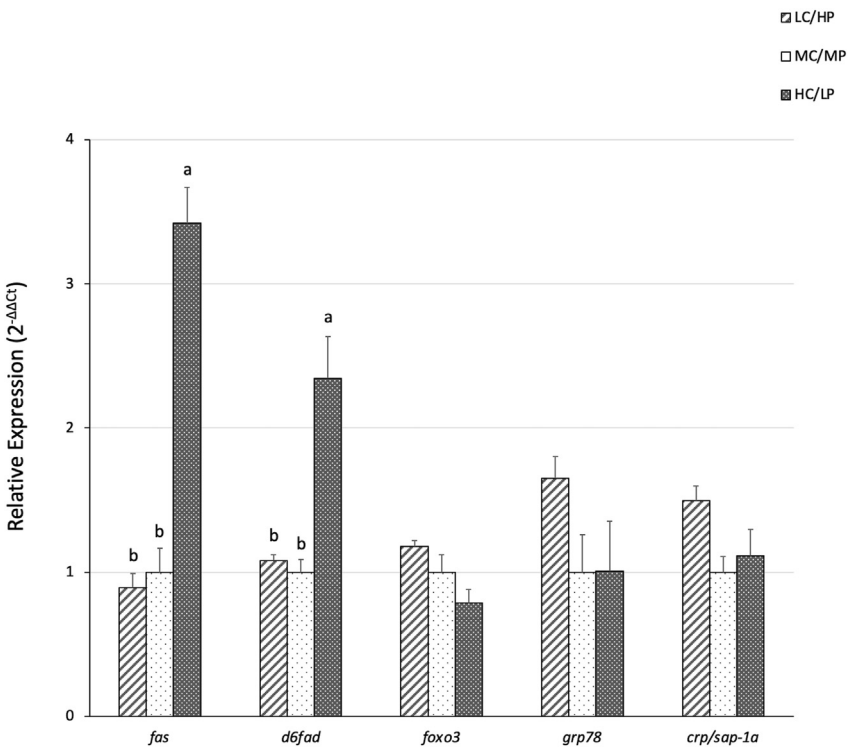


**Fig. 7.** Gene expression analysis. Genes of interest analyzed at the end of the 12-week trial. Relative mRNA expression of fatty acid synthase (*fas*) and delta-6 fatty acyl desaturase (*d6fad*), metabolic regulation (*foxo 3*), endoplasmic reticulum stress response (*grp78*) and inflammation marker (*crp/sap-1a*) analyzed in the liver of Atlantic salmon fed either experimental diet: LC/HP diet, MC/MP diet or HC/LP diet. For each gene, the expression of the MC/MP diet was set to a baseline of 1, with the expression of all other treatments being presented relative to it at the end of the trial. Results were normalized to the geometric mean of elongation factor 1-alpha 1 (*elf-1α*), β-actin (*β-actin*) and cofilin 2 (*cofilin-2*) using the delta–delta Ct method ($2^{-\Delta\Delta Ct}$). Bars represent the mean ± SEM of the relative mRNA expression ($n = 6$). For each gene, bars without a common letter are significantly different ($P < 0.05$). LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

*niloticus*) fed either on medium carbohydrate diet or high carbohydrate diet with or without oxytetracycline supplementation. Several drivers of microbial diversity, including fish trophic level, dietary component under evaluation and range of variation in test diets, energy density of experimental diets, feeding strategy, husbandry practices and life stage of fish, can be accounted for the apparent inconsistency between reports. In terms of how different the intestinal microbial composition between groups fed experimental diets during the 12-week trial, our study showed differences at weeks 4 and 8, when comparing the group fed the HC/LP diet with both the group fed the MC/MP diet and the group fed the LC/HP diet, and when comparing the group fed the HC/LP diet with the group fed the MC/MP diet, respectively. Significant differences were observed when considering the presence or absence (unweighted UniFrac) of bacteria in distal intestine digesta but not for the case of considering abundance of observed bacteria (weighted UniFrac). However, we detected no such effect at the end of the trial. In agreement with this small effect of digestible polysaccharides on intestinal microbiota composition in carnivorous fish, Geurden et al. (2014) reported feeding a high carbohydrate-to-protein ratio diet (40% starch +20% glucose) to alevines and juvenile rainbow trout (*O. mykiss*) either during a short-term (5 days) or long-term period (65 days) did not affect dissimilarities in bacterial profile on intestinal microbiota composition. Similarly, Castro et al. (2019) observed digestible carbohydrate level (0% starch vs 20% starch) did not affect intestinal microbiota profile in gilthead sea bream juveniles. Interestingly, Limbu et al. (2020) observed a small change on the number of operational taxonomic units which abundance was affected by the level of dietary carbohydrates in Nile tilapia fed either a medium carbohydrate diet or a high carbohydrate diet (35.5% and 45.5% starch, respectively). Therefore, to some extent increasing digestible polysaccharide, such as starch, exerts a marginal effect on intestinal microbiota composition in fish. The digestive system in fish, including intestinal microbiota, has not been target of selection pressure driven by digestible polysaccharides, since this macronutrient is not abundant in the base of the marine trophic pyramid. Consequently, we expect carnivorous fish intestinal microbiota will be less responsive towards digestible polysaccharides compared with changes in dietary inclusion levels of protein and lipids. Carnivorous fish belong to a high trophic level which natural feed is rich in protein (>40%) and very poor in carbohydrates (<1%) (Panserat et al., 2013b), and thus natural selection pressure has shaped their capacity to better deal with changes in dietary protein source rather than changes in digestible polysaccharides. On the other hand, omnivorous species and non-filter herbivorous species belong to a lower trophic level whose natural diet include macroalgae intake, which major organic constituent are non-digestible polysaccharides, such as alginates, xylans, agars, carrageenans, among others (Øverland et al., 2019). Natural selection pressure has shaped their capacity, including microbial digestion and fermentation capacity, to utilize these types of energy-yielding substrates (Mountfort et al., 2002; Maas et al., 2020). However, we cannot rule out the existence of a threshold, influenced by fish trophic level and water-environmental temperature of the species, for dietary intake of digestible polysaccharide, above which intestinal microbiota composition could display major changes due to a fibre-like effect of undigested starch. Hemre et al. (1995) proposed that undigested starch can evoke a fibre-like effect in salmon fed a high

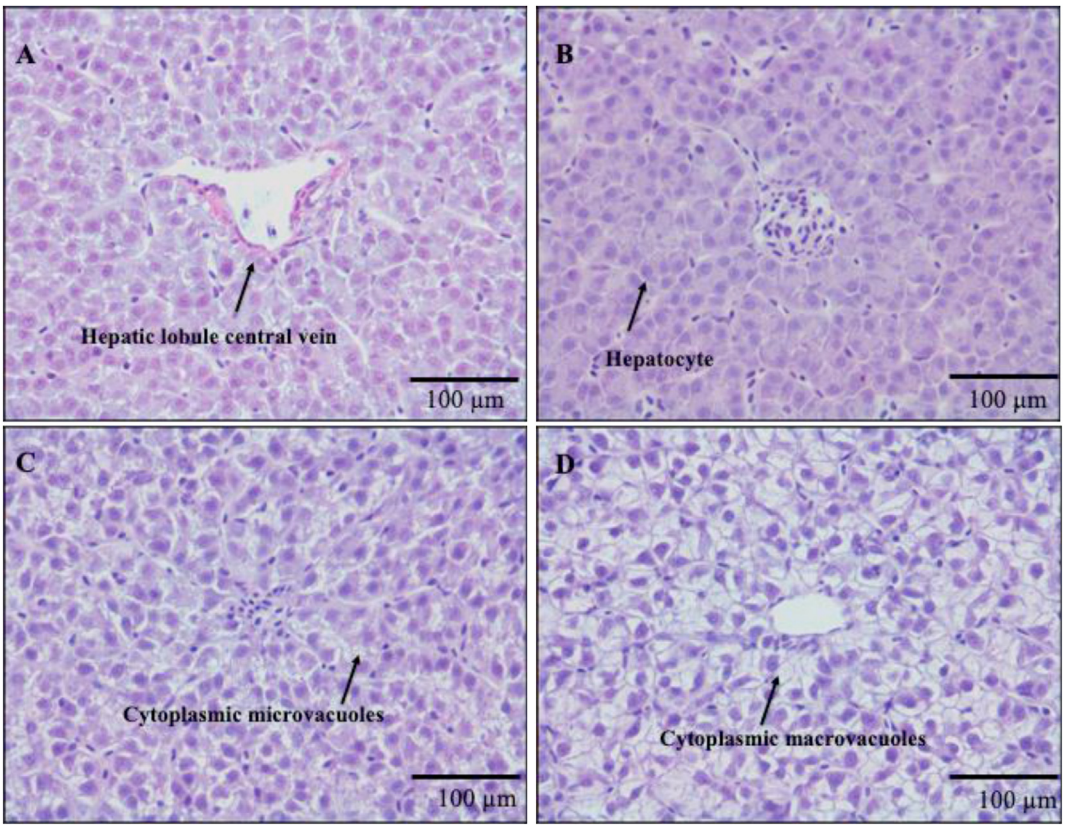


**Fig. 8.** Histological analysis. Red Sirius stain liver sections of *Salmo salar* ($n = 6$) fed experimental diets with different carbohydrate-to-protein ratio (A) liver sections taking at the beginning of the trial from *Salmo salar* either fed the acclimation diet (MC/MP) for 2 weeks (B) on a LCHP diet (C) on a MC/MP diet or (D) on a HCLP diet for 12 weeks. Image (A) shows hepatocytes with an incipient level of cytoplasmic microvacuoles. Image (B) reveals hepatic tissue showing healthy histological structure, quite homogenous cytoplasm and sinusoids. Image (C) reveals hepatic tissue with cytoplasmic microvacuoles and hepatocytes with a central nucleus. Image (D) reveals hepatic tissue with cytoplasmic macrovacuoles showing a certain level of displacement of the nucleus towards the cell membrane. LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

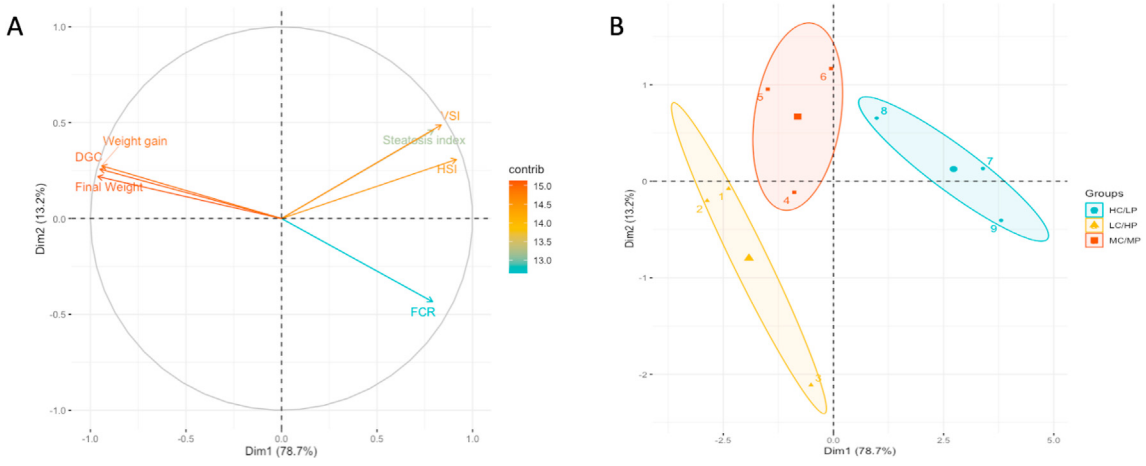


**Fig. 9.** Principal component analysis (A) Contributions of the seven variables to first component (Dim 1) and second component (Dim 2) obtained from the principal component analysis (PCA) represented inside the circle of correlations. The angles of the variables to the 2 axes (x or y) and the percentage of total contribution given by the colour coding are indicative of the percentage of contribution to either component. Positively correlated variables are grouped together in the same quadrant, whilst negatively correlated variables are positioned on opposite quadrant. Variables values represent pooled data obtained from 6 fish per tank (3 tank per treatment, $n = 3$) (B) PCA results on individual samples (experimental tanks; numbering from 1 to 9) using diet as grouping variable; LC/HP diet, MC/MP diet or HC/LP diet. The coordinates for a given group, is calculated as the mean (central point in the group) coordinates of the individuals in the group. For illustration purpose, individual samples from a same group were confined inside concentration ellipses. LC/HP = low carbohydrate-to-high protein diet; MC/MP = medium carbohydrate-to-medium protein diet; HC/LP = high carbohydrate-to-low protein diet.

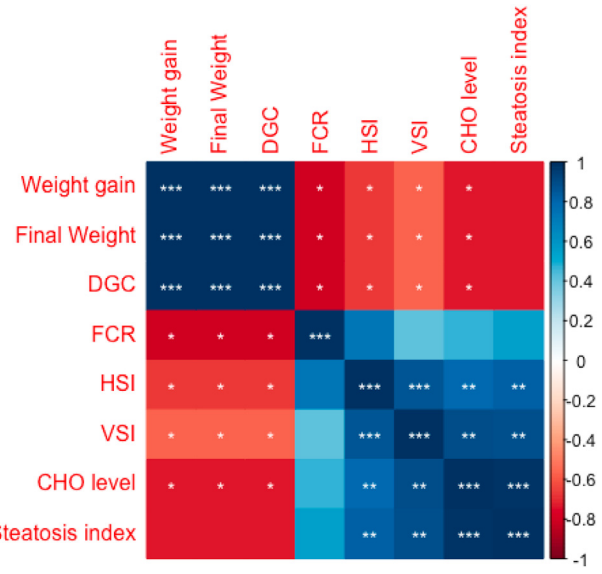


**Fig. 10.** Correlation matrix. Spearman's correlation matrix between productive variables measured in *Salmo salar* fed grading dietary levels of digestible carbohydrate for 12 weeks. Correlations are agglomerate using hierarchical clustering order. Positive correlations are shown in blue and negative correlations in red. Color intensity of boxes are proportional to the correlation values. Significant correlations are highlighted with * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

carbohydrate diet. Similarly, Storebakken (1985) suggested feed carbohydrates can cause a fibre effect when included in high concentration in diets. The modulatory effect of dietary fibre on intestinal microbiota composition in several fish species is well summarized by Ringø et al. (2016), and the relation microbiota/fibre/metabolism reviewed by Cronin et al. (2021).

Overall, in our study, the taxonomic composition of distal intestine digesta microbiota of freshwater-reared Atlantic salmon revealed Firmicutes, Actinobacteria and Proteobacteria as dominant phyla. Despite differences in dominance hierarchy structure, Dehler et al. (2017) and Rudi et al. (2018) found the same phyla to be dominant in gut microbiota in Atlantic salmon reared in freshwater. Notably, changes in the dominance hierarchy structure occurred after fish were transferred into seawater environment in both studies, particularly with Actinobacteria relative abundance decreasing during freshwater to seawater transfers, which indicates water salinity is a crucial factor modulating fish gut microbiota communities in anadromous fish. Further, in the present study, it seems age was a factor modulating distal digesta in fish microbiota composition as trial progresses (Fig. 4). The phyla dominance hierarchy characterized by Firmicutes, Actinobacteria and Proteobacteria was true in all experimental groups at weeks 4 and 8; however, a shift in the hierarchy was observed at the end of the trial (week 12), highlighting Actinobacteria as the dominant phylum in all experimental groups. Previous works have reported changes in gut microbiota composition within a same experimental group during time, suggesting that these differences were associated with age-related physiological changes during the experiment (Desai et al., 2012; Catalán et al., 2018). Supporting this idea, Zhao et al. (2020) observed age as a factor affecting beta diversity of gut microbiota in Chinook salmon (*Oncorhynchus tshawytscha*) reared either in freshwater or saltwater environment. Although Proteobacteria was the most abundant phylum in the adult stages, the authors observed the relative abundance of *Corynebacterium* spp., an Actinobacteria phylum member, to increase in parallel to fish age. Interestingly, Chapagain et al. (2019) reported that the fecal microbiota of slow-growing rainbow trout strain was dominated by Actinobacteria phyla, highlighting the abundance of genus *Corynebacterium_1*. Therefore, it may be worthy to explore the role of Actinobacteria phyla genera members in fish growth, particularly

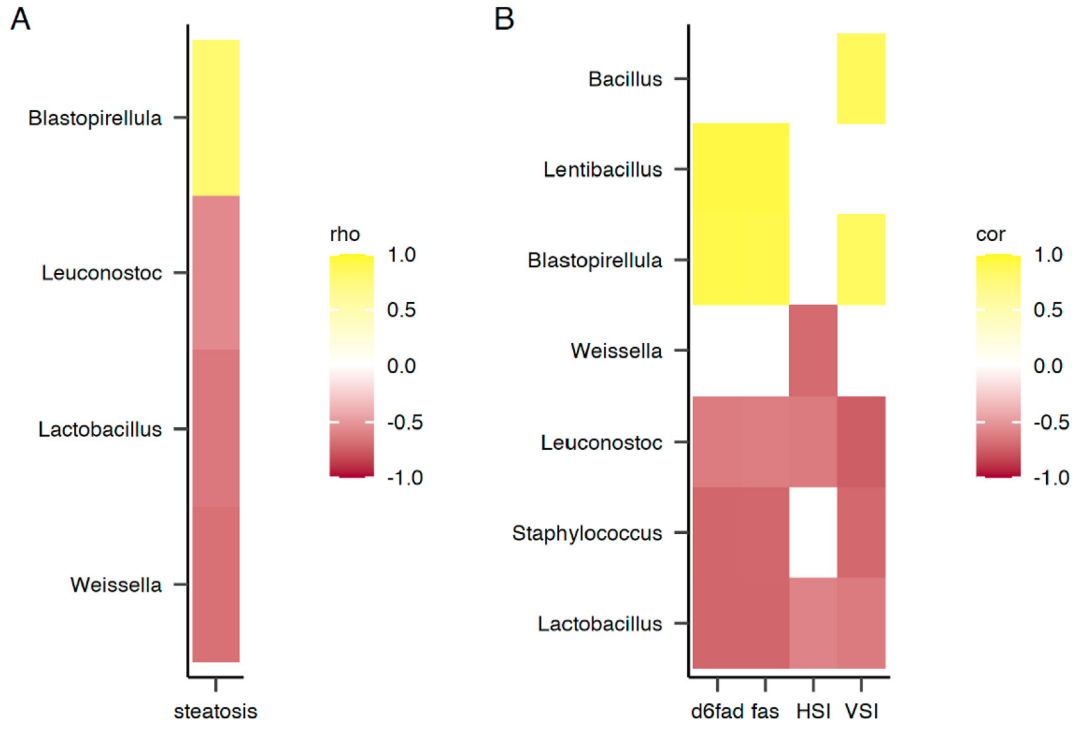


**Fig. 11.** Correlation analysis (A) Spearman's rank correlation coefficient between bacteria genera and steatosis index (B) Pearson correlation coefficient between bacteria genera and fatty acid synthase (*fas*), delta-6 fatty acyl desaturase (*d6fad*), hepatosomatic index (HSI) and viscerosomatic index (VSI). Magenta color represents significantly negative correlations and yellow significantly positive correlations. White spaces show non-significant correlations.

in adult stages, where growth rate is reduced compared with younger life stages for better understating host-gut microbiota interaction in the context of fish growth physiology. Regardless, Actinobacteria is considered a resident of the gut in a variety of fish species, and its relative abundance varies (0% to 50%) between fish species and studies (Mansfield et al., 2010; Desai et al., 2012; Gajardo et al., 2016; Liu et al., 2016; Dehler et al., 2017; Ramírez and Romero 2017; Betiku et al., 2018; Bruce et al., 2018; Ricaud et al., 2018; Rimoldi et al., 2018; Yildirimer and Brown 2018; Huyben et al., 2019; Serra et al., 2021). It has been described that members of the phylum Actinobacteria (i.e., *Streptomyces*) are capable of synthesizing diverse bioactive products which can result in benefits to fish health, including antimicrobial activity against pathogens, inducing immune response, tolerance to gut conditions and growth-enhancing effects (Jami et al., 2015; Balagurunathan et al., 2020). Although we observed a substantial increase in the relative abundance of this phyla across all the experimental groups during trial progress, we observed no significant effect of feeding grading levels digestible carbohydrate in the abundance of this phyla. Thus, the observed significant differences in several of the variables of interest evaluated in our study cannot be associated with this phylum. In terms of defining the most abundant phylum in salmonids gut microbiota, most studies report either Firmicutes or Proteobacteria as dominant phylum with differences based on whether considering fish rearing conditions (i.e., seawater vs freshwater or aquaculture vs wild), the source of dietary protein or dietary lipid (i.e., marine-derived ingredients vs plant-derived ingredients) feeding strategy, husbandry practices, life stage of fish and the type of analyzed sample (i.e., digesta microbiota vs mucosa microbiota or comparing different intestinal segments) (Mansfield et al., 2010; Desai et al., 2012; Gajardo et al., 2016; Liu et al., 2016; Dehler et al., 2017; Ramírez and Romero 2017; Betiku et al., 2018; Bruce et al., 2018; Ricaud et al., 2018; Rimoldi et al., 2018; Rudi et al., 2018; Yildirimer and Brown 2018; Huyben et al., 2019; Serra et al., 2021). Hence all these are factors are required to be taken in consideration when interpreting data across fish gut microbiota literature.

In our study, the major impact of feeding different carbohydrate-to-protein ratios to Atlantic salmon took place at week 4, with greater number of taxa (a total of 39) reflecting significant differences in relative abundance between experimental groups pairwise contrasts. However, these numbers decrease to 18 and 21 taxa at weeks 8 and 12, respectively, which suggest the effect of feeding different dietary carbohydrate-to-protein ratio to salmon in intestinal microbiota decreased as trail progresses. In week 4, a total of 23 taxa were detected to be significantly more abundant in the group fed the high carbohydrate diet when considering both the contrast with the group fed the low carbohydrate diet, and the contrast with the group fed the medium carbohydrate diet. Most part of these taxa, share the potential to ferment carbohydrates under anaerobic conditions. It is well known *Planctomycetes* species grow on sugar monomers, as well as disaccharides and polysaccharides. *Planctomycetes* species can use N-acetylglucosamine (NAG) as source of carbon and nitrogen to support growth (Youssef and Elshahed 2014; Kaboré et al., 2020). N-acetylglucosamine is a naturally occurring amino sugar precursor for epithelial glycosaminoglycan synthesis, normally attached to mucin, which is part of the intestinal mucous layer acting as physical barrier that limits the contact between bacteria and host epithelial cells (Sicard et al., 2018). Overall, *Planctomycetes* species can grow on sugar monomers, disaccharides and polysaccharides (i.e., starch) (Youssef and Elshahed 2014). Moreover, species from *Singulisphaera genera* (i.e., *Singulisphaera rosea*), which belong to Planctomycetes phylum, has been reported to use lactate as carbon source (Kulichevskaya et al., 2011). Here, we found both *Singulisphaera* and members of lactic acid bacteria (LAB), including *Leuconostoc* and *Lactococcus* to be significantly more abundant in fish fed the high-carbohydrate diet compared with fish fed the medium-carbohydrate diet at week 4. However, we could not determine whether these findings were pure serendipity or implied an interaction between theses bacteria. For example, by supplying lactic acid to *Singulisphaera* as a source of carbon. Notably, the opposite effect was observed at the end of the study with *Streptococcus*, *Leuconostoc* and *Lactobacillus* showing greater abundance in fish fed a medium-carbohydrate diet compared with the high-carbohydrate group. Further supporting the idea that the effect of feeding grading levels of carbohydrate to salmon in distal digesta microbiota varies as trial progresses, we observed the family Enterococcaceae and the genus *Enterococcus* to be significantly greater in both the group fed the low-carbohydrate diet and the group fed the high-carbohydrate diet when compared with the group fed the medium-carbohydrate diet at week 8, and to be significantly greater in fish fed the high-carbohydrate diet when compared with fish fed the medium-carbohydrate diet at week 12. However, no differences in the abundance of these 2 taxa between either experimental group was detected at week 4. Regarding the core microbiota, we observed a reduction of the percentage of bacteria genera shared by the 3 experimental groups (32.4% in week 4, 25% in week 8 and 22% in week 12) in time. Although, this might be indicative of an acclimation process to dietary treatment in fish, the absence of significant differences in the beta diversity between experimental groups at the end of the trial suggests that Atlantic salmon possess a large intestinal core microbiota (constituted by the more abundant bacteria genera), resilient toward high levels of digestible carbohydrate in diet. This is similar to what was observed by Wong et al. (2013). Authors reported the presence of a large core intestinal microbiota (37.4%) resistant to variation in dietary protein source and rearing density in rainbow trout. However, the core microbiota analyzed in our study correspond to the distal intestine digesta microbiota (transient microbiota), and thus might be not extrapolated to resident core microbiota. Further, we believe differences observed in the intestinal microbial composition (unweighted UniFrac) between experimental groups at weeks 4 and 8 are more likely to be consequences of changes in the carbohydrate-to-protein ratio per se rather than dietary protein source, since all experimental groups were fed on a similar ratio of dietary protein blend of fishmeal/plant-meal (0.8, 0.7 and 0.8 for LC/HP, MC/MP and HC/LP, respectively).

In terms of fish growth performance, feeding the HC/LP diet to salmon significantly reduced DGC, final weight, weight gain and feed efficiency compared with fish fed the LC/HP diet, and significantly reduced growth rate, final weight and weight gain compared with fish fed the MC/MP diet. Further, the PCA showed steatosis index, HSI and VSI were positively correlated and grouped in one cluster, whilst DGC, final weight and weight gain were positively correlated and grouped in a second cluster. FCR was the third cluster, and negatively correlated (opposite quadrant) with DGC, final weight and weight gain (Fig. 9A). In addition, when using dietary carbohydrate level as grouping variable in the PCA, we observed experimental tanks were clustered in 3 different groups with their respective coordinates located in a different quadrant (Fig. 9B). In agreement with our study, previous trials have reported feeding high carbohydrate diets to salmonids species, including Atlantic salmon and rainbow trout, provoked similar effect in same variables (Hemre et al., 1995; Kirchner et al., 2005; Saravanan et al., 2012; Skiba-Cassy et al., 2013). The reduced feed intake observed in these studies may be the result of the fibre-like effect of undigested starch, as previously mentioned and proposed by Storebakken (1985). Interestingly, contrary to our findings, Skiba-Cassy et al. (2013) observed higher feed intake in

trout fed a high carbohydrate-to-low protein diet; however, this difference might obey to differences in crude protein content of the respective high carbohydrate diet between both studies (37.1% vs 41.5% of crude protein). The authors suggest that the increased feed intake in the group fed the high carbohydrate-to-low protein diet was to compensate for the low dietary protein level. On the contrary, in our study, the dietary protein level of the high carbohydrate diet might have been high enough to do not trigger this compensatory response in fish. Interestingly, feed intake has been suggested to be driven either by lipid or energy content in diet where different levels of both dietary proteins and lipids were tested in carnivorous fish, such as yellowtail (*Seriola dumerilii*) (Takeuchi et al., 1992; Jover et al. (1999)), gilthead seabream (*Sparus aurata*) (Lupatsch et al., 2001) and pompano (*Trachinotus carolinus*) (Riche (2009)). However, Overturf et al. (2016) reported feed intake appears to be influenced according to dietary protein level rather than dietary lipid or energy content in rainbow trout. The authors observed feed intake was significantly higher in juvenile rainbow trout fed a low protein/high lipid diet when compared with the group fed the high protein/low lipid diet. Notably, in the present study, the 3 experimental diets resulted to be isocaloric and close in lipid content, and although the crude protein content of the high carbohydrate-to-low protein diet was similar to the low protein/high lipid tested by Overturf and colleagues, we observed different tendency in feed intake response in fish fed the high carbohydrate-to-low protein diet. We believe this inconsistency might be explained by the fact that Overturf and colleagues used juvenile fish (11.2 ± 0.1 g of initial body weight) with higher protein requirement, and thus, increasing feed intake to compensate for the low dietary protein level.

We have demonstrated feeding salmon a high carbohydrate-to-low protein diet induced hepatic steatosis, as observed from higher HSI with greater proportion of hepatocytes exhibiting the formation of cytoplasmic macrovacuoles (macrovacuoles steatosis) at the end of the trial (Fig. 8). Fish fed a medium carbohydrate-to-medium protein diet showed moderate levels of fat deposition in hepatocytes evidenced by the presence of cytoplasmic microvacuoles (microvacuoles steatosis) and hepatocytes with a central nucleus. On the contrary, fish fed a low carbohydrate-to-high protein diet exhibited no vacuolization in the hepatocyte cytoplasm, showing a normal architecture of liver tissue, and highlighting the presence of homogenous hepatic sinusoids. The red Sirius stain did not show deposition of collagen fiber in the hepatic parenchyma in the HC/LP, supporting no histological damage in liver tissue in fish fed the carbohydrate-rich diet. In line with our findings, previous studies have reported feeding digestible carbohydrates-rich diets to carnivorous fish species lead to an increase in lipid deposition in liver and HSI (Hemre et al., 2002; Panserat (2009); Enes et al., 2011; Gatesoupe et al., 2014; Limbu et al., 2020). In the present study, dietary lipids are unlikely to be the factor causing the increment in hepatic lipid deposition of fish fed the carbohydrates-rich diet, since this experimental group exhibit significant lower feed intake and experimental diets had close levels in lipid content. Thus, a higher de novo synthesis of lipids is likely to be the cause of this finding. Supporting this idea, we found significant greater expression of *d6fad* and *fas, 2* genes encoding for enzymes which play a crucial role in hepatic lipogenesis in the group fed the HC/LP diet compared with the group fed either the LC/HP or the MC/MP diet. In agreement with these findings, Betancor et al. (2018) reported farmed Atlantic salmon fed a high carbohydrate-to-low protein diet exhibited upregulation of *fas* and *d6fad* in liver.

Finally, we correlated bacteria genera with some of the variables related with lipid metabolism and hepatic health analyzed at the end of the trial. We found LAB members, including *Leuconostoc* and *Lactobacillus* to be negatively correlated with hepatic steatosis and HSI as well as with the expression level of *d6fad* and *fas*, and *Weissella* negatively correlated with hepatic indexes. In line with this finding, at the end of the trial, genera *Lactobacillus* and *Weissellla,* family Leuconostocaceae and the order Lactobacillales were significantly less abundant in fish fed the high-carbohydrate diet when compared with fish fed the low-carbohydrate diet as well as liver steatosis score was significantly greater in the high-carbohydrate group compared with the group fed the low-carbohydrate diet. Together, these results suggest that a decrease in the abundance of LAB members in gut microbiota might be associated with hepatic steatosis in fish fed a high-carbohydrate diet. Numerous LAB strains have been used as probiotics in finfish aquaculture due to their health beneficial effect, including improved growth performance, feed utilization, digestive enzymes activity, immune response, and disease resistance (Merrifield et al., 2014; Ringø et al., 2018). However, supplementing LAB members to protect liver in carnivorous fish fed diets that promote fatty liver has not yet been conducted. A plethora of works reported supplementing LAB members exerts modulatory effect of lipid metabolism as well as anti-obese and anti-fatty liver condition effect in humans and mammalian models (Abenavoli et al., 2013). Choi et al. (2020) found *Lactobacillus plantarum* LMT1-48 evokes an anti-obesity effect in obese mice fed a high fat diet, particularly by downregulating lipogenic gene expression in liver and adipocytes. Supplementing L. *plantarum* NCU116 to rats with a high fat diet induced non-alcoholic fatty liver disease showed improved liver function and decreased hepatic fat accumulation (Li et al., 2014). The use of *Lactobacillus casei* strain *Shirota* as probiotic was shown both to suppress nonalcoholic steatohepatitis in mice fed a methionine/choline deficient diet (Okubo et al., 2013), and to protect against the onset of fructose-induced nonalcoholic fatty liver diseases in diet-induced obese mice (Wagnerberger et al., 2013). Interestingly, a novel protective mechanism of probiotic *Lactobacillus rhamnosus GG* against hepatic steatosis via reducing host intestinal fatty acid absorption, as result of a bacteria and host competition for fatty acid intestinal absorption, has been recently proposed in mice (Jang et al., 2019). Finally, *Leuconostoc mesenteroides* was reported to prevent metabolic dysfunction, including the reduction of liver steatosis, associated with a high-fat diet-induced obesity in male mice (Castro-Rodríguez et al., 2020). Similarly, *Leuconostoc pseudomesenteroides* was shown to improve liver metabolism and reduce hepatic steatosis in high-fat diet-fed mice (Sun et al., 2020).

## 5. Conclusion

To our knowledge, this study provides the first evidence regarding the effect of feeding Atlantic salmon increasing levels of digestible carbohydrates on dynamics of intestinal microbiota and correlating distal intestinal digesta-associated LAB with diet-induced hepatosteatosis in fish. Our findings highlight 3 relevant ideas: (1) feeding a carbohydrate-rich diet to salmon causes low impact in gut microbial communities of distal intestine digesta, mostly affecting low-abundance bacteria, (2) the above-mentioned effect seems to be true only in the short term since it was less evident at the end of the trial, denoting some level of resilience of the intestinal microbiota in salmonids exposed to this type of nutritional challenge at the end of the trial, and (3) the negative correlation of LAB members with hepatic steatosis, HSI and the expression of *d6fad* and *fas* suggest that a decrease in the abundance of these group of bacteria in gut microbial ecology might be associated with fatty liver in fish fed a high-carbohydrate diet. Therefore, further research to explore the potential use of LAB as probiotics to protect liver in carnivorous fish fed a fatty liver-induced diet is warranted.

## Author contributions

**Alejandro Villasante** and **Jaime Romero** designed research. **Carolina Ramírez, Elías Figueroa, Héctor Rodríguez, Patricio Dangtanan** and **Adrián Hernández** conducted research; **Carolina Ramírez**, **Alejandro Villasante** and **Jaime Romero** analyzed data; and **Alejandro Villasante** and **Jaime Romero** wrote the paper. All authors read and approved the final manuscript.

## Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

## Declaration of competing interest

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

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## Appendix. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.04.003.

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