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Effect of dietary chitin on growth performance, nutrient utilization, and metabolic response in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The dietary inclusion of ingredients containing chitin has been claimed to be beneficial to fish health. However, controversial results on growth performance and nutrient digestibility have been reported in literature. The present study aimed at assessing the response of rainbow trout (*Oncorhynchus mykiss*) fed with increasing levels of chitin (0, 1.5, 3.0, and 4.5 %) in a semi-purified basal diet. Protein and lipid digestibility was assessed and after 10 weeks of feeding, 6 h after the last meal, fish were euthanized for the evaluation of growth performance, blood biochemistry, brush border membrane and chitinolytic enzyme activity, gene expression of enzymes and nutrient transporters as well as the major pro-inflammatory intestinal cytokines, and mid intestine microbiota. Dietary chitin inclusion of up to 3 % did not hamper growth performances, nutrient apparent digestibility, and levels of most blood enzymes and metabolites. Diet including 4.5 % chitin negatively affected growth performances and nutrient digestibility. The application of a multidisciplinary approach highlighted that the biomarkers related to gut digestion functionality and inflammation response were altered also in fish fed the diets including 1.5 and 3 % of chitin, while gut microbiota did not differ between dietary treatments, even if some taxa suggested to be important in fish physiology were not recovered. Overall, the results demonstrate a good tolerance of rainbow trout to diets including chitin up to 3 %.

1. Introduction

In the last decade, a growing number of feeding trials has demonstrated that insect meals can entirely or substantially substitute fish meal and vegetable ingredients in aquafeeds without threatening production or fish welfare (Cardinaletti et al., 2019, 2022; Homska et al., 2022; Maulu et al., 2022; Motte et al., 2019; Ratti et al., 2023; Rimoldi et al., 2021; Stadtlander et al., 2017; Terova et al., 2019). Among the eight insect species approved by the European authority to be used in aquafeed production (EU Reg 2017/893), promising results were obtained with black soldier fly (*Hermetia illucens*, diptera, BSF) meal. This is due to its high protein value and feed conversion efficiency (Mohan et al., 2022, Sharifinia et al., 2019), low environmental requirements, and benefit of being reared on organic byproducts, which promote sustainability and circular economy (Smetana et al., 2019). Beyond the sustainable properties, BSF larvae meal shows an amino acid profile very close to fish meal which makes it a valuable alternative ingredient (Mohan et al., 2022). BSF larvae meal has been successfully included in diet formulations for Nile tilapia (*Oreochromis niloticus*) (up to 10 % inclusion in the diet) (Tippayadara et al., 2021), rainbow trout (*Oncorhynchus mykiss*) (up to 15 %) (Caimi et al., 2020) and Atlantic salmon (*Salmo salar*) (up to 20 %) (Belghit et al., 2019). In addition to the high nutritional value, insect meals contain bioactive compounds that appear to have positive impacts on fish metabolism (Gasco et al., 2020). Among them, chitin has been reported to have immunostimulant characteristics (Ringø et al., 2012), bacteriostatic effect on several pathogenic gram-negative bacteria (Udayangani et al., 2017; Zhou et al., 2013) and advantageous effects on fish gut microbiota modulation (Bruni et al., 2018; Henry et al., 2018). Despite these positive effects, chitin is generally considered a limiting factor associated to the use of insect meal in aquafeeds, due to the reduction of nutrient digestibility resulting in fish growth impairment (Cardinaletti et al., 2019; Karlsen et al., 2017; Kroeckel et al., 2012; Lindsay et al., 1984; Rust, 2003; Shiau and Yu, 1999). However, the dietary inclusion of BSF larvae meal at 40 % (corresponding to a chitin content of 2 g/100 g DM) reduced protein digestibility without affecting growth in rainbow trout (Renna et al.,

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2017). Furthermore, chitin is cross-linked to a protein matrix in the insect exoskeleton (Hahn et al., 2018), which could interfere with the intestinal proteins and lipids absorption.

It has been shown that fish have the ability to degrade chitin fiber by the action of a specific set of chitinolytic enzymes (chitinase and chitobiase) (Fines and Holt, 2010; Gutowska et al., 2004; Mengkrog Holen et al., 2022; Messina et al., 2022) and insect meal appears to have a dose response effect on nutrient digestibility and growth performance when included in aquafeed. Anyway, it is still unclear if such effect is linked to the chitin level for every fish species.

The current study aimed to find out more concerning the presence of chitin in aquafeed and its impact on growth performance, nutrient digestibility, gut functionality, and metabolic state in rainbow trout (*O. mykiss*). For this purpose, a feeding trial was carried out using four semi-purified diets with different chitin levels selected to simulate different inclusion levels of BSF meal, to study the effect of chitin devoid of all the numerous components present in insect meals, which could also interfere with the abovementioned parameters.

2. Material and methods

2.1. Ethics

Feeding trial and sampling were carried out in agreement with the European legislation frameworks pertaining to the protection of animals used for scientific reasons (Directive, 2010/63/EU), adopted by the Italian law (D.L. 26/2014). The experimental protocol was approved by the Animal Ethical Committee of the University of Udine (Approval No: 8/2021).

2.2. Test diets formulation

Four experimental diets were formulated to be grossly isoproteic and isolipidic. The control diet (CHI0) was formulated as semi-purified diet, containing high digestible ingredients as reported in Table 1. Three experimental diets (CHI1.5, CHI3 and CHI4.5) were added with increasing amounts of pure chitin (1.5 g, 3.0 g and 4.5 g chitin/100 g control diet) (shrimp shell chitin, Merck®, Darmstadt, Germany) to simulate different inclusion levels of BSF larvae meal (15, 30 and 45 g 100 g^{-1} diet). The levels of chitin would simulate the values found in a commercial BSF prepupae meal (9.7 % chitin on 100 g of dry matter) (Protix, Dongen, Netherlands). To estimate the protein and lipid apparent digestibility, was included in the formulation an indigestible indicator (Celite®, Acros Organics, New Jersey, USA). The diets were fortified with vitamins and minerals to satisfy the requirements levels for rainbow trout (Hardy, 2002). The four diets were added with 700 mL/kg water and alginate, and cold extruded with a meat mincer into 3 mm pellets. The pellets were dried for 24 hours at 40 °C and kept at 4 °C until used.

Table 1
Formulation and proximal composition of the experimental diets (g/100 g).

g/100 g	CHI0	CHI1.5	CHI3	CHI4.5
Fish meal (67 %)	34	34	34	34
Casein	28	28	28	28
Fish oil	12	12	12	12
Dextrine	3.4	3.3	3.2	3
Wheat flour	14	14	14	14
Cellulose	4.2	2.8	1.4	0.1
Soy lecithin	1	1	1	1
Chitin	0	1.5	3	4.5
Vitamin mix #	0.7	0.7	0.7	0.7
Mineral mix $	0.7	0.7	0.7	0.7
Agar agar	1	1	1	1
Celite®	1	1	1	1
% DM	**CHI0**	**CHI1.5**	**CHI3**	**CHI4.5**
Dry matter	93.57	92.43	92.36	93.24
Crude protein	50.15	50.14	50.67	50.01
Ether extract	15.8	15.51	15.81	16.3
Crude fiber	1.12	2.36	3.37	4.36
Ashes	7.24	7.42	7.43	7.95
Chitin*	0	1.5	2.88	4.08
NFE#	25.69	23.07	19.84	17.3

*Chitin calculated as ADF – ADL as reported by Hahn et al. (2018); #NFE = 100 – (%EE + %CP + %Ashes + %CF).
Composition of the vitamin mix (% mix): choline chloride, 84; Stay C® DSM, 4.5; myoinositol, 3.2; vit E, 3.2; niacin, 2.1; riboflavin, 0.4; piridoxine HCl, 0.2; cyanocobalamine, 0.2; calcium pantotenate, 0.6; folic acid, 0.1; biotin vit.H 1.1; thiamine HCl, 0.2; menadione vit K3, 0.2; vit A (2500 IU/kg diet), 0.03; vit D3 (2400 IU/kg diet) 0.05.
$ Composition of the mineral mix (% mix): HPO4·2H2O, 78.9; NaCl, 17.65; MgO, 2.725; FeCO3, 0.335; KI, 0.005; ZnSO4·H2O, 0.197; MnSO4·H2O, 0.094; CuSO4·5H2O, 0.027; Na Selenite, 0.067.

2.3. Chemical composition analysis

Experimental diets (Table 1) and feces were analyzed for chemical composition (AOAC, 2000). Dry matter (DM) was determined by drying the samples in an oven at 105 °C for 4 hours before weighting (930.15). Ashes were determined by incineration of the samples in a muffle at 550 °C for 4 hours while acid insoluble ashes were determined after further ashes dilution with hydrochloric acid (10 %), and muffle incineration at 550 °C. Crude protein (CP, N×6.25) was measured according to Kjeldahl method (976.05). Ether extract (EE) was determined following Soxhlet method (2003.05). Crude fiber (CF) was measured according to Weende's method (962.09). The dietary nitrogen-free extract (NFE) of the experimental diets was calculated as follows: NFE = 100 – (% EE + % CP + % Ashes + % CF). The chitin content of the experimental diets was determined by difference between acid detergent fiber (ADF) and acid detergent lignin (ADL) (Van Soest et al., 1991), according to Hahn et al. (2018).

2.4. Experimental protocols and fish rearing conditions

The trial was carried out at the indoor aquaculture facilities of the Department of Agrifood, Environmental and Animal sciences, University of Udine, Italy. The *in vivo* digestibility measurements were performed using 4 units designed according to the Guelph system (Cho, 1992). Each unit of 3 tanks (75-L) was assigned randomly to the experimental diets. Each tank within a unit was randomly stocked with 15 rainbow trout (*O. mykiss*) juveniles selected from a resident batch with an individual weight of 27.4 ± 2.38 g/fish. The four units received a continuous supply of well water with a water renewal rate of 16 L min^{-1}. Water quality parameters were daily monitored for temperature (13.6 ± 0.7 °C), pH (8.2 ± 0.1) and dissolved oxygen (9.4 ± 0.3 mg/L). Before beginning the feces collection, the fish had been acclimated to the breeding conditions for 21 days. Fish were hand-fed one meal each day at 1 % live weight (9:00 a.m.) by adjusting the ratio according to the fish biomass. Three fecal samples were collected over 10 days to obtain three independent samples per diet. To avoid contaminating the feces collection, uneaten pellets were removed from the bottom of the tank as soon as the fish finished eating. With the aim of measuring the nutrients apparent digestibility, feces were collected, before the morning meal, from the settling columns and subsequently centrifuged at 12000 × *g* at 5 °C for 20 min.

2.5. Sampling

The feces were pooled and stored at −20 °C until the end of each collection period (10 days) and freeze-dried. The protein and lipids apparent digestibility coefficients (ADC) of the experimental diets were computed as follows:

$$ADC_{Diet} = 1 - \frac{\%\ \text{indicator in the diet}}{\%\ \text{indicator in feces}} \times \frac{\%\ \text{nutrient in feces}}{\%\ \text{nutrient in the diet}}$$

At the end of the experiment, after a 6 h fasting period, 18 fish per dietary treatment (6 fish per tank) were sacrificed with an overdose of anesthetic (tricaine methansulphonate, 300 mg L^{-1}). Immediately after, all fish were subjected to blood sampling from the caudal vessels according to Pascon et al. (2024). After blood sampling, the digestive tract was collected and separated into stomach (S), pyloric caeca (PC), proximal intestine (PI), and distal intestine (DI). Tissue samples of S and PI from 2 fish per tank (6 fish per dietary treatment) were collected for the analysis of the activity of chitinase, chitobiase, and brush border membrane (BBM) enzymes. PC, PI, and DI from 2 fish per tank (6 fish per dietary treatment) were utilized for the gene expression analysis. Two fish per tank (6 fish per dietary treatment) of CHI0 and CHI3 treatments were frozen at – 80 °C for downward microbiota analysis. All sacrificed fish were used to evaluate the growth performances and feed utilization, under the same breeding conditions (13.6 ± 0.7 °C; pH 8.2 ± 0.1; D.O. 9.4 ± 0.3 mg/L) over 70 days. At the beginning and at the end of the trial, the initial and final fish body weight was recorded per tank. The real amount of feed intake was recorded every day for each tank. The growth performance parameters were calculated as follows:

RFI (relative feed intake): feed intake / (final body weight + initial body weight) / 2 / days

FCR (feed conversion rate): feed intake / weight gain

SGR (specific growth rate): 100 × [(*ln* final body weight − *ln* initial body weight) / 70 days].

2.6. Plasma parameters and serum metabolites

Plasma cholesterol (Chol, mg dL^{-1}), triglycerides (Trig, mg dL^{-1}), total proteins (TP, g dL^{-1}), albumin (Alb, g dL^{-1}), glutamic-pyruvic transaminase (GPT, U L^{-1}), glutamic-oxalacetic transaminase (GOT, U L^{-1}), and lactate dehydrogenase (LDH, U L^{-1}) were analyzed with commercial kits (Paramedical s.r.l., Salerno, Italy).

The serum glucose, lactic acid, and free amino acids (mg dL^{-1}) were determined using NMR spectra acquisition, as detailed in Pascon et al. (2024) protocol. An in-house library was created for the identification and quantification of 35 molecules (betaine, isoleucine, leucine, lysine, methionine, tryptophan, valine, alanine, taurine, arginine, histidine, asparagine, aspartic acid, ornithine, proline, glutamine, glutamic acid, fumaric acid, cystine, tyrosine, alanine, carnitine, citrulline, glycine, creatine, glucosamine, choline, serine, phenylalanine, threonine, creatinine, benzocaine, trehalose, glucose and lactic acid) of interest.

2.7. BBM enzymes and Chitinase and chitobiase activity

The brush border membrane (BBM) enzymes activity was determined according to Messina et al. (2019). One unit of enzyme activity (U) corresponded to the amount of enzyme that transforms or hydrolyses 1 μmole of substrate mL^{-1} min^{-1}. The specific enzymatic activity was calculated as U/g wet tissue.cAfter thawing, the S and PI samples were weighed and diluted 1:10 w/v with Tris-HCl buffer 50 mM at 5 and 7.4 pH, respectively. The diluted samples were homogenized by TissueLyser II (Qiagen, Germany) and centrifuged at 13500*xg* for 10 min at 4 °C. The chitinase and chitobiase enzyme activities, measured in the supernatant (U = μm of p-nitrophenol mL^{-1} min^{-1}) was performed by a Chitinase Assay Kit (CS0980, Sigma-Aldrich) following the manufacturer instructions and adapting the volume of the samples to the fish matrix. The specific enzymatic activity of chitinase and chitobiase was expressed as U/g tissue.

2.8. Expression analysis of digestive enzymes, nutrient transporters and pro-inflammatory cytokines

Total RNA was extracted from S, PI, and DI using PureLink™ RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, Rodano, Italy). To evaluate the RNA concentration a NanoDrop™ One Microvolume UV–Vis Spectrophotometer was used (Thermo Scientific™ Inc., Italy) and 28 S and 18 S ribosomal RNA bands integrity were checked with standard agarose gel electrophoresis (1.2 %). Afterwards, the complementary DNA (cDNA) synthesis was carried out using the PrimeScript™RT reagent Kit with gDNA Eraser (Takara Bio Europe SAS, France) following the manufacturer's protocol. The RT-qPCR was performed using SYBR SsoFast™ EvaGreen (Biorad, Germany). The thermal profile for all reactions was 3 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C according to Cardinaletti et al. (2019). The target genes selected and evaluated were gastric chitinase (*chia*) and pepsinogen (*pga*) in the stomach, while maltase (*mal1*), oligopeptide transporter 1 (*slc15a1*), neutral amino acid-transporter solute carrier (*slc1a5*), intestinal alkaline phosphatase (*alpi*), intestinal phospholipase (*pla2*), fatty acid binding protein 2 (*fabp2*), interleukin 1β (*il-1β*), interleukin 8 (*il-8*), tumor necrosis factor α (*tnf-α*) in the PI and DI. Glucose transporter 2 (*slc2a2*) was evaluated only in the PI. Ribosomal protein 60 S (*60 S*) and beta-actin (*β-Act*) were selected as housekeeping genes (HKGs). The target and housekeeping genes primers are listed in Table 2. They were designed based on the cDNA sequences accessible in the GenBank database for *Oncorhynchus mykiss* or recovered from published sequences. Relative expression levels were calculated using the Δ-Δ Ct method (Pfaffl, 2001) by applying the CFX Maestro™ Software selection tool (user guide version 1.1, Biorad). The same software allowed the selection of the appropriate reference genes based on the average M value and analyzed gene stability by means of the reference genes selection tools. The *60 S* and *β-Act* were then used to normalized gene expression.

2.9. Intestinal microbiota sampling and DNA extraction and sequencing

During thawing, fish abdomen was cleaned with 70 % ethanol and cut open with disinfected tools. The intestinal tract was clamped right after the last pyloric caecum, and at the end of the mid intestine (MI), *i.e.*, at the increase in the intestinal diameter. The clamped segments were transferred to a disinfected Petri dish and opened longitudinally. When present, the whole MI content (MIC) was gently collected with a spatula and placed into a 1.5 mL sterile tube. MI tissue was rinsed in autoclaved phosphate-buffered saline 3 times to remove traces of content. The DNA from MI and MIC samples was extracted using the FastDNA™ SPIN kit for soil (MP Biomedicals, Solon, OH, USA) with some modifications (Mannelli et al., 2018) in comparison to the manufacturer's protocol and further adapted to fish samples as follows: each sample was transferred to a lysis tube and thawed on ice, then 90 μL of MT buffer and 724 μL of phosphate buffer were added, and the mixture was homogenized with a Retsch MM300 disrupter (90 s at 30 cycles) twice, with a break of 1 minute in ice bath for cooling down the samples, when necessary. The samples were incubated for 20 min at 70 °C and inverted 5 times every 5 minutes, then centrifuged at 14 000 × *g* at 4 °C for 5 minutes. The supernatant was transferred to a sterile 1.5 mL tube, 185 μL of PPS were added, the tubes were inverted for 8 minutes, and then centrifuged at 14 000 × *g* at 4 °C for 5 minutes. The supernatant was recovered and processed according to the manufacturer's specification, with a double washing step with SEWS-M solution. The samples were eluted in 100 μL of distilled water provided in the kit. Two extraction blanks (EB1, EB2) were extracted along the samples to assess the presence of contaminant sequences in the kit and in the environment. The extracted DNA was quantified using Qubit® (Life Technologies, Carlsbad, CA, USA). The extracted DNA was diluted to 1 ng μL^{-1} and then sent to BMR Genomics (Padova, Italy) and, when meeting the quality thresholds for sequencing set by the sequencing company, processed following the Illumina protocol (Illumina, 2013). The V3-V4 region of the 16 S rRNA gene was amplified with primer pair Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014) with 35 PCR cycles, added with Illumina adapters (Nextera XT index

Table 2
Sequence of primers used for RT-qPCR (S: stomach; PI: proximal intestine; DI: distal intestine).

Analyzed organ	Gene	Primer Forward (5' – 3')	Primer Reverse (5' – 3')	Efficiency (%)	Accession number	Reference
S	*chia*	AACAGCAACCTGAAGACTCTG	CGACTCTTCGAGGACCAACC		GenBank, EU877960	(Cardinaletti et al., 2022)
S	*pga*	GTTTCGGTGGGAGGCATCT	TGTTTCCGAAAGACACCACA		Sigeneae, CX136077.s.om.10	(Borey et al., 2016)
PI	*Mal1*	GCAGCAGGAATACCCTACGA	GGCAGGGTCCAGTATGAAGA		TIGR, TC3451	(Borey et al., 2016)
PI+DI	*Slc15a1*	CCTGTCAATCAACGCTGGT	CACTGCCCATAATGAACACG		GenBank, EU853718	(Borey et al., 2016)
PI+DI	*Slc1a5*	ACCTCAAAACCTGCGACTTG	CCACCGTTCCTTCTATGCTG		KY775397	(Cardinaletti et al., 2022)
PI+DI	*Slc5a1*	GGGCTGAACATCTACCTTGCT	CTCATAACCTCCCACCTCATTG	98.9	GenBank, AY210436	Present study
PI+DI	*Alpi*	GAAACACCGGACCCAGAGTA	GCAGTCACTTGGCTCAAACA	98.9	XM021610191.2	Present study
PI	*Slc2a2*	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	100.8	GenBank, AF321816	Present study
PI+DI	*Pla2*	TGTGTCATGTAGTAAGGAACCTGAA	TTTGTAACCATCGCTGCATT	100	Genoscope, GSONMT00024336001	Present study
PI+DI	*Fabp2*	GTAGAGCCAGTCCAACCCTT	AATTCCATATCCAGCGTGCG	99.0	GenBank, XM020497032.2	Present study
PI+DI	*Il-1β*	TTGGGCCTCTACGATCAGGA	CAGGGGCGCTTACCACAATA	99.2	NM001124347	Present study
PI+DI	*Il-8*	ATTGAGACGGAAAGCAGACG	CGCTGACATCCAGACAAATCT	104.9	NM001124362.1	Present study
PI+DI	*Tnf-α*	AGGTTGGCTATGGAGGCTGT	TCTGCTTCAATGTATGGTGGG	97.8	NM001124357	Present study
HKG	*60 S*	TTCCTGTCACGACATACAAAG	GTAAGCAGAAATTGCACCATC		XM021601278	(Cardinaletti et al., 2022)
HKG	*β-actin*	AGACCACCTTCAACTCCATCA	AGAGGTGATCTCCTTCTGCAT		AJ537421	(Cardinaletti et al., 2022)

kit). An Illumina MiSeq platform with paired-end 2 × 300 bp sequencing chemistry was used.

2.10. Statistical analyses and bioinformatics

Growth performance, digestibility, plasma and serum parameters, and enzyme activity data are presented as mean value ± standard deviation (SD). Data on gene expression are shown as mean value ± standard error of the mean (SEM). All data were checked for normality and variance homogeneity using the Shapiro-Wilk and Levene tests. One-way ANOVA was used to analyze differences between the four treatments. Duncan's post hoc test ($p < 0.05$) was employed when applicable. Data analysis was performed using the SPSS program (Release 17.12, SPSS Inc., Chicago, IL, USA) was used.

The raw sequence data of the intestinal microbiota are deposited at the NCBI SRA database under the BioProject number PRJNA703401. The raw sequences were processed with the QIIME2 pipeline (Bolyen et al., 2019) and R (R Core Team, 2018) following Li et al. (2021) and Bruni et al. (2022) with adaptations; the summary of the pipeline and the codes used are contained in the Figure S1. Alpha-diversity indices (observed ASVs, Pielou's evenness and Faith's phylogenetic diversity) were tested with two one-way Kruskal-Wallis tests, while beta-diversity (built on Bray-Curtis metrics) data were tested with two one-way permutational multivariate analysis of variance (PERMANOVA).

3. Results

3.1. In vivo digestibility of the experimental diets

The protein and lipid apparent digestibility coefficients (ADC) diets were affected by the inclusion level of dietary chitin. In fact, diet CHI4.5, characterized by the highest chitin content, resulted in significant lower values of both protein and lipid ADC than the other experimental diets (P=0.007 and P=0.004, respectively) (Fig. 1).

3.2. Growth performance

Growth performance parameters are detailed in Table 3. Fish fed with the highest inclusion level of chitin (CHI4.5) showed the significantly highest relative feed intake (RFI) and feed conversion ratio (FCR), and the significantly lowest final body weight (FBW) and, specific growth rate (SGR) respect to the other groups. Fish fed the CHI3 diet showed a significant improvement in SGR respect to the fish fed CHI0 diet while a significant decrease was observed in fish fed the highest chitin inclusion level. Because of the worst growth and the increased RFI, the FCR significantly got worse with the CHI4.5 diet compared to the other treatments.

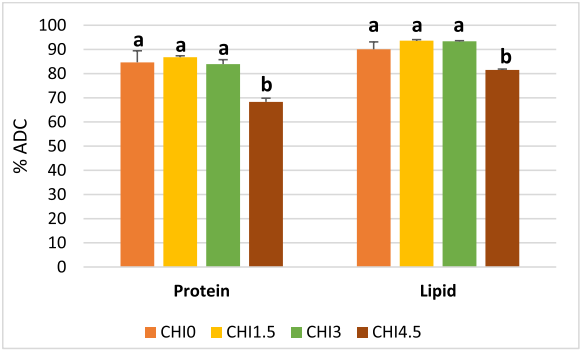

Fig. 1. Effect of dietary inclusion of chitin on protein and lipid apparent digestibility coefficients. For each nutrient, different superscript letters indicate significant differences among diets (mean ± SD, n = 3). a, b: p < 0.05. Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet).

Table 3
Effect of dietary inclusion of chitin on growth performance of rainbow trout after 70 days of feeding trial. Different superscript letters in the same row indicate significant differences among diets (mean ± SD, n = 6). a, b, c: p < 0.05.

Item[a]	Experimental groups[b]				
	CHI0	CHI1.5	CHI3	CHI4.5	*P value*
RFI	9.67 ± 0.5[b]	10.30 ± 0.9[ab]	10.16 ± 0.3[ab]	11.4 ± 0.8[a]	*0.031*
FBW	56.65 ± 4.7[a]	54.94 ± 4.9[a]	59.03 ± 2.9[a]	42.48 ± 3.4[b]	*0.002*
SGR	0.97 ± 0.2[b]	1.08 ± 0.1[ab]	1.18 ± 0.1[a]	0.73 ± 0.1[c]	*0.005*
FCR	1.07 ± 0.2[a]	1.02 ± 0.0[a]	0.93 ± 0.1[a]	1.65 ± 0.2[b]	*0.001*

[a] RFI = relative feed intake (g/kg/day); FBW = final fish body weight (g); SGR = specific growth rate (%) =100*[(ln final body weight - ln initial body weight) / days]; FCR= feed conversion ratio (feed given (g)/ body weight gain (g).
[b] Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet).

3.3. Plasma metabolic parameters

Dietary chitin affected several plasma metabolic parameters, depending on its dietary content (Table 4). Cholesterol, triglycerides, total protein, albumin and glutamic-oxalacetic transaminase levels were significantly lower in fish fed the CHI4.5 diet compared to the other treatments ($p<0.001$). Intermediate values were observed in glutamic-pyruvic transaminase level where the lowest value was detected in fish fed CHI4.5 diet, while the highest value was observed in fish fed CHI1.5 diet. Lactate dehydrogenase resulted unaffected in response by the dietary chitin.

3.4. Serum metabolites

H NMR spectroscopy allowed the detection of 11 of the 34-targeted metabolites in each sample of fish serum. The metabolites were identified by comparing them to an in-house database of standard substances (Pascon et al., 2024). Serum concentrations of the metabolites are shown in Table 5. Dietary chitin affected 6 out of 11 serum metabolites. Phenylalanine and histidine decreased in fish fed CHI4.5 diet and increased in fish fed the CHI1.5 and CHI3 diet, although the differences were not significantly different with respect to the fish fed CHI0 diet ($p>0.05$). Valine significantly decreased in fish fed diet with the highest amount of chitin while increased in fish fed the CHI1.5 diet respect to the fish fed the CHI0 and CH3 diets ($p<0.001$). Isoleucine and leucine showed a significant decrease in fish fed CHI4.5 diet in comparison with the other treatments ($p<0.05$). Choline showed a significant increase in fish fed the CHI3 diet with respect to the fish fed the CHI0 diet, while tryptophan, glutamine, alanine, glucose, and lactic acid were unaffected by the chitin dietary inclusion ($p > 0.05$).

3.5. Brush border membrane enzymes activity

The activity of alkaline phosphatase, leucine aminopeptidase, sucrase-isomaltase and maltase resulted affected by the dietary chitin content (Table 6).

In PC, Alp activity significantly decreased when fish were fed the CHI3 diet, while L-anp activity significantly decreased when fish were fed the CHI4.5 diet, SI significantly decreased when fish were fed with more than 3 % of chitin. No significant differences were detected in Malt

Table 4
Effect of dietary inclusion of chitin on metabolites in plasma biochemical parameters measured in rainbow trout after 70 days of feeding trial. Different superscript letters in the same row indicate significant differences among diets (mean ± SD, n = 9) (a, b, c: $p < 0.05$; A, B: $p < 0.001$).

	CHI0	CHI1.5	CHI3	CHI4.5	*p value*
mg/dl					
Chol	197.0 ± 43.6^{A}	216.9 ± 40.7^{A}	226.5 ± 36.5^{A}	122.1 ± 36.3^{B}	*0.000*
TG	168.3 ± 55.0^{A}	179.1 ± 42.1^{A}	170.5 ± 41.4^{A}	84.9 ± 28.5^{B}	*0.000*
g/dl					
TP	2.2 ± 0.2^{A}	2.3 ± 0.2^{A}	2.1 ± 0.2^{A}	1.7 ± 0.2^{B}	*0.000*
Alb	1.8 ± 0.2^{A}	1.6 ± 0.2^{A}	1.6 ± 0.2^{A}	1.3 ± 0.2^{B}	*0.000*
U/L					
LDH	593.1 ± 108.6	713.2 ± 175.6	777.8 ± 206.9	599.9 ± 122.8	*0.108*
GPT	8.3 ± 1.4^{ab}	10.6 ± 4.1^{a}	8.3 ± 1.2^{ab}	6.7 ± 1.2^{b}	*0.029*
GOT	31.4 ± 4.9^{A}	38.6 ± 13.9^{A}	38.1 ± 5.7^{A}	14.8 ± 5.1^{B}	*0.000*

[a] Cholesterol (Chol), Triglycerides (TG), total protein (TP), albumin (Alb), lactate dehydrogenase (LDH), glutamic-pyruvic transaminase (GPT), glutamic-oxalacetic transaminase (GOT)

[b] Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet.

Table 5
Effect of dietary inclusion of chitin on metabolites (mg dL^{-1}) in serum of rainbow trout after 70 days of feeding trial. Different superscript letters in the same row indicate significant differences among diets (means ± SD, n = 6) (a, b, c: $p < 0.05$; A, B, C: $p < 0.001$).

mg dL^{-1}	CHI0	CHI1.5	CHI3	CHI4.5	*P value*
Tryptophan	0.42 ± 0.19	0.70 ± 0.06	0.59 ± 0.37	0.31 ± 0.09	*0.101*
Phenylalanine	3.28 ± 0.48^{ab}	4.21 ± 0.89^{a}	4.11 ± 0.79^{a}	2.35 ± 0.46^{b}	*0.008*
Histidine	2.01 ± 0.35^{ab}	2.49 ± 0.56^{a}	2.25 ± 0.71^{a}	1.13 ± 0.15^{b}	*0.012*
Valine	12.60 ± 0.80^{B}	16.60 ± 2.71^{A}	10.50 ± 0.81^{BC}	8.21 ± 2.40^{C}	*0.000*
Isoleucine	6.16 ± 0.47^{ab}	8.27 ± 1.45^{a}	5.37 ± 2.62^{bc}	2.81 ± 0.72^{c}	*0.003*
Leucine	14.00 ± 0.90^{a}	18.20 ± 3.30^{a}	14.61 ± 5.40^{a}	8.70 ± 2.70^{b}	*0.016*
Glutamine	6.38 ± 0.89	7.04 ± 0.92	7.90 ± 3.65	4.75 ± 1.02	*0.212*
Alanine	6.30 ± 1.65	6.89 ± 1.04	7.54 ± 2.18	5.90 ± 2.23	*0.626*
Choline	0.37 ± 0.08^{bc}	0.50 ± 0.12^{ab}	0.57 ± 0.10^{a}	0.30 ± 0.07^{c}	*0.007*
Glucose	134.10 ± 30.61	116.10 ± 25.51	123.41 ± 28.01	100.31 ± 16.90	*0.350*
Lactic acid	65.40 ± 12.51	73.20 ± 4.01	76.51 ± 23.10	52.01 ± 14.70	*0.159*

[b] Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet).

Table 6
Effect of dietary inclusion of chitin on BBM specific enzyme activities (U/g wet tissue) in pyloric caeca (PC), proximal (PI) and distal (DI) intestine of rainbow trout after 70 days of feeding trial. Different superscript letters indicate significant differences among diets (means ± SD, n = 6) (a, b, c: $p < 0.05$; A, B, C: $p < 0.001$).

U/g tissue[a]	CHI0	CHI1.5	CHI3	CHI4.5	*P value*
PC					
Alp	90.6±10.8^{a}	98.6±23.2^{a}	48.7±16.3^{b}	74.6±21.8^{a}	*0.003*
L-anp	1224.3 ±187.7^{a}	996.8 ±244.4^{a}	996.9 ±283.0^{a}	569.1 ±141.3^{b}	*0.002*
SI	742.3 ±150.5^{a}	667.5 ±167.0^{ab}	494.1 ±135.0^{bc}	380.7 ±51.6^{c}	*0.010*
Malt	4931.3 ±1255.1	4502.2 ±1642.7	4025.5 ±1396.4	2471.9 ±755.1	*0.092*
PI					
Alp	128.8 ±36.5^{C}	238.0 ±66.7^{B}	115.0 ±31.4^{C}	352.4 ±99.3^{A}	*0.000*
L-anp	695.4 ±151.4^{b}	1144.2 ±310.0^{a}	637.7 ±178.1^{b}	1142.4 ±412.9^{a}	*0.015*
SI	586.6 ±137.4	964.2 ±295.3	816.9 ±332.2	1137.1 ±376.8	*0.116*
Malt	2831.2 ±716.9	4276.3 ±1521.8	3657.8 ±1222.7	3810.6 ±968.5	*0.386*
DI					
Alp	38.3±11.6^{b}	101.4±24.4^{a}	56.9±18.8^{b}	63.0±29.6^{b}	*0.003*
L-anp	560.7 ±165.1^{c}	1346.4 ±347.9^{a}	1107.8 ±211.9^{ab}	904.7 ±193.0^{b}	*0.001*
SI	650.5 ±187.2^{b}	1164.6 ±408.9^{a}	946.7 ±308.9^{ab}	550.4 ±99.8^{b}	*0.033*
Malt	1603.6 ±337.4^{b}	3389.3 ±1609.7^{a}	2792.7 ±886.7^{ab}	1594.0 ±278.6^{b}	*0.046*

[a] Pyloric Caeca (PC); Proximal intestine (PI); Distal intestine (DI); Alkaline phosphatase (Alp); Leucine aminopeptidase (L-anp); Sucrase-isomaltase (SI); Maltase (Malt).

[b] Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet.

activity.

In PI, Alp and L-anp activity significantly increased in fish fed diet CHI1.5 and CHI4.5. No significant differences were detected in SI and Malt activity.

In DI, Alp activity significantly increased in fish fed the CHI1.5 diet while L-anp activity significantly increased in fish fed all the chitin-containing diets with the highest activity in fish fed the CHI1.5 diet. Both disaccharidases activities significantly increased in fish fed the CHI1.5 diet.

3.6. Chitinase and chitobiase activity

The activity of chitinase enzymes was not the same along the digestive system. In fact, chitinase activity was higher in the intestine than in the stomach while chitobiase activity was higher in the stomach with respect to the intestine (Table 7). Chitobiase and chitinase activity in the stomach were not affected by the inclusion of chitin in the diets, while the intestinal chitinase activity was significantly reduced in fish fed the CHI4.5 diet.

3.7. Gene expression

Expression of genes involved in chitin (*chia*) and protein (*pga*) digestion measured in rainbow trout stomach is presented in Fig. 2. Both genes were significantly upregulated in fish fed diet containing 3 % and 4.5 % chitin (p=0.000 for both genes).

The expression of the genes involved in the absorption of peptides (*slc15a1*), amino acid transport (*slc1a5*), digestion and absorption of carbohydrates (*mal1, slc5a1 and slc2a2*), lipids (*pla2* and *fabp2*), and intestinal alkaline phosphatase (*alpi*) was investigated in PI and DI and the results are reported in Figs. 3a and 3b, respectively.

Overall, the expression of the studied genes in the proximal intestine was almost double than in the distal intestine. In the proximal intestine, gene expression of *slc1a5, mal1 and pla2* was significantly down-regulated by dietary chitin (p=0.000, p=0.001, p=0.001, respectively) while the expression of *slc2a2* and *alpi* presented contradictory values (p=0.041, p=0.035, respectively). Genes as *slc15a1, slc5a1* and *fabp2* seemed not to be affected by the presence of chitin (p=0.231, p=0.123, p=0.051, respectively) (Fig. 3a).

Distal intestine was less responsive to different levels of chitin in the diet than the proximal one. The expression of gene *alpi* resulted the only one affected by the inclusion of 1.5 % of chitin in the diet (p=0.000) (Fig. 3b).

The inflammatory response was studied in the proximal and distal intestine through the gene expression of interleukin 1β (*il-1β*), interleukin 8 (*il-8*), and tumor necrosis factor α (*tnf-α*) (Fig. 4a,b). In PI, both interleukins were upregulated in fish fed the CHI3 diet, while no significant differences were detected for *tnf-α* (*il-1β*, p=0.016; *il-8*, p=0.006; *tnf-α*, p=0.059) (Fig. 4a). Chitin presence in the diets did not affect the gene expression of *il-1β*, *il-8*, and *tnf-α* in DI (Fig. 4b). For these parameters too, the values were lower in the distal tract.

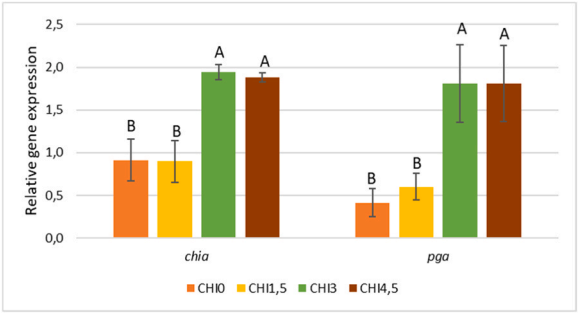

Fig. 2. Effect of dietary inclusion of chitin on expression of genes involved in the chitin and protein digestion (*chia* and *pga*) in the stomach of rainbow trout after 70 days of feeding trial. For each gene, different superscript letters indicate significant differences among diets (mean ± SEM, n = 6) (A, B: $p < 0.001$). Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet.

3.8. Intestinal microbial communities

The microbial communities revealed by sequencing the V3-V4 region of the 16 S rRNA gene did not show major differences between dietary groups (CHI0 and CHI3) nor between sample type (MI, MIC). The taxa bar plots (Fig. 5) display the relative abundances of the phyla and of the genera found in the biological samples. The sample numbers vary across the different sample type and diets depending on the number of samples meeting the quality thresholds for sequencing set by the sequencing company and on the samples passing the rarefaction step (figure S2). Except for CHI3-MI fish number 18 and the CHI3-MIC fish numbers 17 and 18, the relative abundances followed the same patterns in all samples. Altogether, more than 95 % of the sequences were assigned to Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, and Spirochaetes, and Actinobacteria and Tenericutes mainly composed the rest. The most abundant genera were *Fusobacterium*, *Comamonas*, *Leptospira*, *Streptococcus*, a bacterium belonging to the Bacteroidales order, *Bacteroides*, *Pseudobutirivibrio*, a bacterium belonging to the Lachnospiraceae family, *Succinivibrio*, *Staphylococcus*, *Prevotella*, *Butyrivibrio*, *Lachnospira*, *Shuttleworthia*, *Bibersteinia*, *Escherichia*, *Lysinibacillus*, and *Oribacterium*. Other genera did not appear consistently among samples, among others: *Lactococcus* (in CHI3-MIC 16 and 18, but in very low relative abundance in other few MIC samples), a bacterium belonging to the Caulobacterales order (only present in CHI3-MI 18 and CHI0-MIC 2). The observed ASVs and the Faith's phylogenetic diversity did not show significant differences taking diet or sample type into consideration, but the values of the two indices tended to be lower in MI than in MIC. A small but significantly higher Pielou's evenness was noticed in MI in comparison to MIC (p=0.033). The PCoA built using the Bray-Curtis metrics (figure S3) did not show clusters according to either factor. As a confirmation of the visual inspection, the PERMANOVA analyses tested on diet and on the sample type showed no statistical significance (results not shown).

Table 7
Effect of dietary inclusion of chitin on chitinase and chitobiase activity (U/g wet tissue) in the stomach and proximal intestine of rainbow trout after 70 days of feeding trial. Different superscript letters in the same row indicate significant differences among diets (means ± SD, n = 6) (a, b: $p < 0.05$).

U/g tissue		Experimental groups[a]												
		CHI0			CHI1.5			CHI3			CHI4.5			*P value*
Chitinase	**stomach**	15.7	±	6.3	27.5	±	11.1	26.2	±	9.1	24.8	±	4.9	*0.135*
	intestine	173.3	±	35.8[a]	201.8	±	41.5[a]	204.3	±	43.4[a]	97.4	±	21.0[b]	*0.001*
Chitobiase	**stomach**	12.9	±	1.0	20.6	±	8.0	19.9	±	6.6	21.2	±	10.6	*0.295*
	intestine	3.0	±	1.6	2.6	±	0.7	2.0	±	0.3	2.6	±	0.8	*0.440*

[a] Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet.

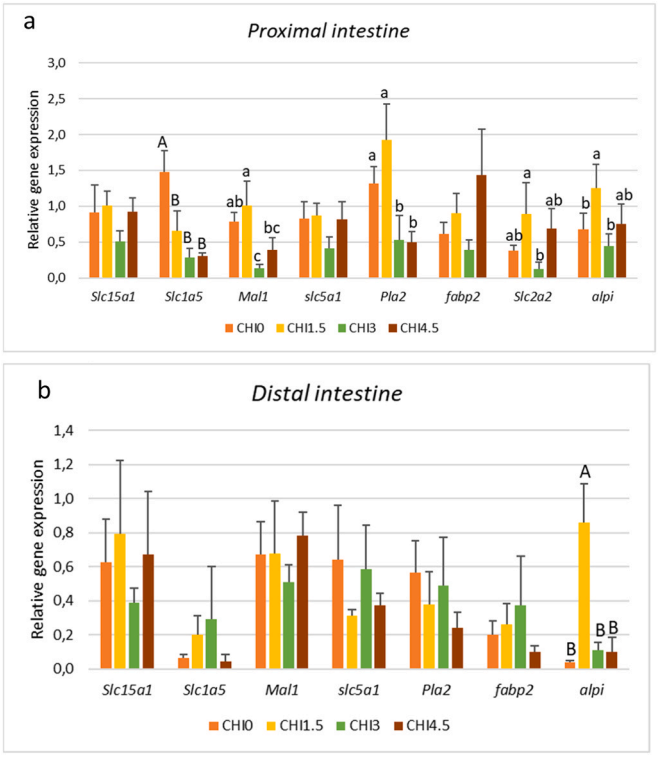

Fig. 3. Effect of dietary inclusion of chitin expression of genes involved in the absorption of di-tripeptides (*slc15a1*), amino-acid transport (*slc1a5*), digestion and absorption of carbohydrates (*mal1* and *slc5a1*), digestion and absorption of lipids (*pla2* and *fabp2*) and intestinal alkaline phosphatase (*alpi*) in proximal (a) and distal (b) intestine of rainbow trout after 70 days of feeding trial. Glucose transporter 2 (*slc2a2*) was evaluated only in PI. For each gene, different superscript letters indicate significant differences among diets (mean ± SEM, n = 6) (a, b, c: $p < 0.05$; A, B: $p < 0.001$). Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet.

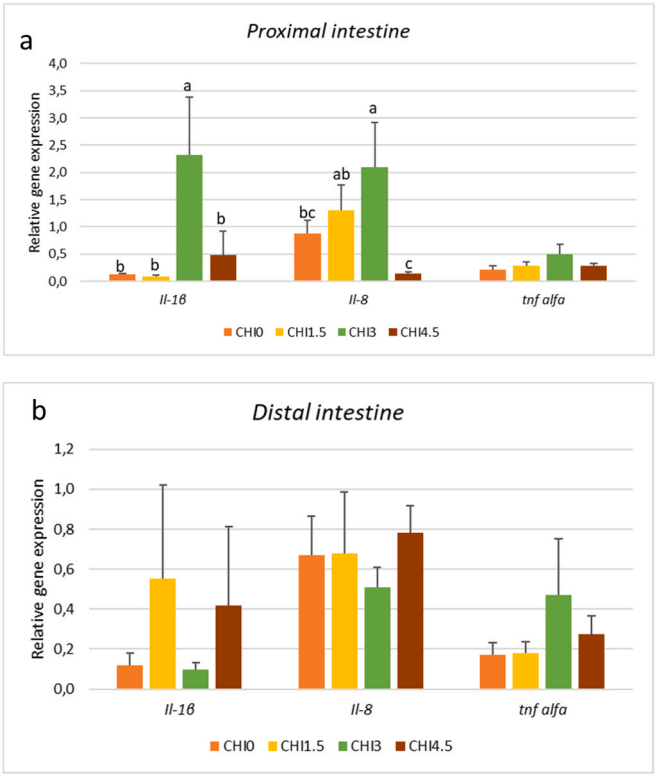

Fig. 4. Effect of dietary inclusion of chitin expression of genes involved in the inflammatory response (interleukin 1β (*il-1β*), interleukin 8 (*il-8*), and tumor necrosis factor α (*tnf-α*)) in proximal (a) and distal (b) intestine of rainbow trout after 70 days of feeding trial. For each gene, different superscript letters indicate significant differences among diets (mean ± SEM, n = 6) (a, b, c: $p < 0.05$). Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet.

4. Discussion

To the authors knowledge, the effects of graded levels of chitin in aquafeeds on growth performances, nutrient digestibility, gut functionality, and blood metabolic parameters have not comprehensively studied, yet. For this reason, results are discussed referring to previous scientific works where chitin was added by the inclusion of shrimp or insect meal in the diet.

Furthermore, at the time of the experiment planning no insect breeding or insect meal manufacturing company was able to provide purified chitin, so commercial chitin from Merck (shrimp shell chitin) was used. The crystalline form (α-chitin) of chitin from insects is similar to the one of commercial chitin from crustaceans and other aquatic invertebrates (Abidin et al., 2020). The main difference lies on the surface morphology which, in BSF, presents a honeycomb-like structure with the presence of pores according to the larvae development stages. However, if we take into consideration the prepupae stage, which is the stage preferentially used as feed ingredient, the surface morphology is comparable to the one of commercial shrimp chitin (Soetemans et al., 2020). We believe, therefore, that the use of commercial chitin, in addition to being the only possibility at the time of the experiment, has not affected the approach of our study.

4.1. *In vivo digestibility of the experimental diets*

Digestibility is usually referred to the apparent digestibility (ADC) because the contribution of endogenous material such as enzymes, secretions from the digestive tract, body residues, mucosal cells and microflora residues are not considered (Bai et al., 2022). The protein and lipid ADCs registered in the present study were similar to the ones observed in turbot fed diets including 300 g/kg (3.2 % chitin) BSF meal (protein ADC: 81.1 %; lipid ADC: 92.8 %; Kroeckel et al., 2012). Despite no significant differences were detected with diets containing up to 3 % chitin, a significant decrease in the protein and lipid ADCs was recorded with higher dietary chitin level (diet CHI4.5). Similarly, a significant decrease in protein ADC was observed in Atlantic salmon (*Salmo salar*) fed diet containing 5 % chitin (Karlsen et al., 2017). On the contrary, rainbow trout fed diets including 25 % BSF meal (chitin level 4.4 %) showed protein and lipid ADCs higher than the ones observed in the present study (protein ADC: 85.1 % *vs* 68.3 %; lipid ADC: 92.8 % *vs* 81.54 %) (Eggink et al., 2022). Similarly to our study, Piccolo et al. (2017) observed a significant decrease in protein and lipid digestibility in gilthead seabream (*Sparus aurata*) fed diet including 50 % *Tenebrio molitor* larvae meal that contained 4.6 % chitin respect to control diets. In the present study, the decrease in nutrients ADC at the highest dietary chitin level justifies and supports the declining growth performances and feed utilization observed in rainbow trout fed CHI4.5 diet.

The low nutrient availability of chitin-containing ingredients in aquafeed could be due to the molecule structure and properties. In fact, being an insoluble fiber with a high water-binding capacity, chitin forms ionic bonds with several compounds (protein and lipid), stimulating fecal bulking and shortening intestinal transit time (Mudgil, 2017; Tharanathan and Kittur, 2010). To overcome such limits, research efforts on the possibility of increasing the digestibility of chitin-containing ingredients through additives (mainly enzymes) are under study (Bolton et al., 2021; Liceaga, 2021).

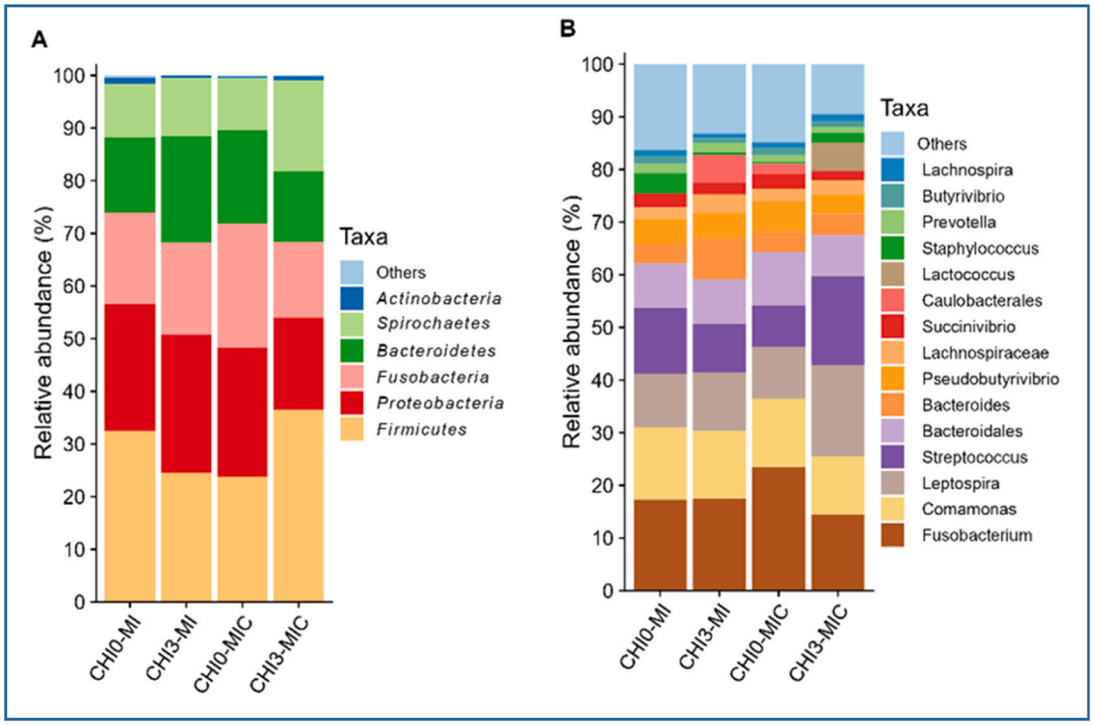

Fig. 5. Effect of dietary inclusion of chitin on mean relative abundance of each *taxon* within the same sample type and diet of the samples at phylum (A) and genus (B) level in rainbow trout after 70 days of feeding trial. [a]mid intestine (MI); mid intestine content (MIC). [b]Fish were fed diets added with increasing amounts of pure chitin: 0 (CHI0); 1,5 (CHI1.5); 3,0 (CHI3) and 4,5 (CHI4.5) g /100 g diet.

4.2. Growth performance

At the end of the 70-day feeding trial, rainbow trout fed diets including up to 3 % of chitin did not register significant differences in growth performances, while the highest chitin level (4.5 %) resulted in a significant impairment of the overall fish growth performances. These results are consistent with previous researches that considered several inclusion levels of insect meal (with less than 3 % chitin) in rainbow trout (Cardinaletti et al., 2019; Chemello et al., 2020; Ratti et al., 2023; Renna et al., 2017; Stadtlander et al., 2017) and other fish species, such as Atlantic salmon (*Salmo salar*) (Belghit et al., 2019), European sea bass (*Dicentrarchus labrax*) (Magalhães et al., 2017), African catfish (*Clarias gariepinus*) (Fawole et al., 2020), Japanese sea bass (*Lateolabrax japonicas*) (Wang et al., 2019) and barramundi (*Lates calcarifer*) (Hender et al., 2021). Nevertheless, results on the inclusion of chitin-containing ingredients in aquafeeds are still contradictory. For instance, dietary inclusion levels of 21 % or 25 % of defatted and full-fat *Tenebrio molitor* meal impaired the growth performance of largemouth bass (*Micropterus salmoides*) (Chen et al., 2023; Gu et al., 2022). Similarly, Nile tilapia (*Oreochromis niloticus*) fed diets including 20 % of full-fat yellow mealworm meal resulted in growth performance reduction (Tubin et al., 2020); similar evidence was observed in meagre (*Argyrosomus regius*) fed diets including 30 % defatted BSF meal (1.6 % chitin) (Guerreiro et al., 2020). On the contrary, no significant differences were reported by Rimoldi et al. (2023) when feeding rainbow trout with shrimp meal inclusion up to 20.4 % (7 % chitin) and by Piccolo et al. (2017) feeding gilthead seabream (*Sparus aurata*) with a diet including 50 % full-fat *T. molitor* meal (4.6 % chitin). The defatting process, the feeding regimes, the method used for feed manufacturing (pelleting or extrusion), the fish species and their developmental stage could all be contributing factors to these contradictory results.

4.3. Plasma and serum metabolic parameters

Hematological parameters are recognized as valid indicators of fish physiological and health status under different nutritional conditions (Fazio, 2019). Dietary chitin inclusion at the highest level negatively affected most of the parameters measured both in serum and plasma. Similar results were reported by Gu et al. (2022) in largemouth bass fed a diet containing over 20 % *T. molitor* meal. Regarding cholesterol, it has been shown that chitin can bind cholesterol, reducing its absorption and leading to excessive excretion (Kipkoech, 2023). Several studies reported a reduction in hematological parameter values in different fish species fed diets including chitin-containing ingredients (Fawole et al., 2020; Gu et al., 2022; Khosravi et al., 2018; Li et al., 2017; Magalhães et al., 2017; Wang et al., 2019). All these authors did not consider other dietary component and attributed this effect to the presence of chitin in the diet.

Plasma aminotransferases, as biomarkers of liver damage, have several physiological functions in the amino acid metabolism and energy homeostasis (McGill, 2016). GPT plays a major role in the alanine-glucose cycle while GOT contributes to maintain the cellular NAD^+/NADH ratio (McGill, 2016). In the present study, plasma GPT and GOT resulted significantly depressed in fish fed the highest chitin-containing diet. Data on the aminotransferase activities are also consistent with the low growth performances and protein digestibility and support the indication that a decreased GPT and GOT activity may result from a limited amino acid utilization (Lin and Luo, 2011).

Serum LDH as well as glucose and lactic acid levels were not affected by the dietary treatments, demonstrating that chitin-containing diets did not induce stress condition in rainbow trout (Begg and Pankhurst, 2004). Similarly, Cardinaletti et al. (2019) and Mastoraki et al. (2022) reported no significant differences in glucose levels fed diets including up 20 % of insect meal (chitin content in diet not reported) in rainbow trout and gilthead seabream. Serum amino acids values resulted consistent with the total protein concentration in plasma. The lowest amino acids levels, especially the essentials, were observed in fish fed diet CHI4.5, where this chitin level consistently affected also both protein digestibility and fish growth.

4.4. Brush border membrane enzymes, digestive enzymes and nutrients transporters

A complementary tool for assessing the digestive system response to dietary modifications is the measurement of BBM enzymes specific activity and the digestive enzymes and nutrient transporters gene expression (Cardinaletti et al., 2022; Pascon et al., 2021; Pleić et al., 2022). It has been demonstrated that fish's natural eating habits support their capacity to hydrolyze chitin and that this capacity is dependent on chitinolytic enzymes (chitinases and chitobiase) (Gutowska et al., 2004). It is noteworthy that rainbow trout expressed the chitinase-related gene (*chia*) and pepsinogen-related gene (*pga*) in the stomach, and that their expression was upregulated in an apparent dose-dependent manner. Moreover, the expression patterns of both genes are corroborated by a previous study (Cardinaletti et al., 2022), where the proteolytic enzymes and chitinase resulted upregulated in rainbow trout fed diet containing 23 and 45 % BSF meal.

In the present study the chitinolytic enzyme activity was considered at gastric and intestinal level where tissue-specific chitinase hydrolyzes chitin chains into trimers and dimers, and chitobiase further hydrolyzes into monomers (Gutowska et al., 2004). In this case, only the intestinal chitinase was affected by the graded dietary chitin levels, supporting a different/specific tissue response. Similarly, a low ability of rainbow trout to utilize dietary chitin has been reported by Ringø et al. (2012) which reported that rainbow trout has a low ability to utilize chitin. Contrarily, Eggink et al. (2022) reported an increased chitinase activity in the proximal intestine at higher dietary chitin level (4.4 % DM basis), in rainbow trout (*Oncorhynchus mykiss*) and in Nile tilapia (*Oreochromis niloticus*). Furthermore, the enzymatic activity of chitinase in the stomach is not consistent with its expression and it would be useful to deepen the control mechanism that regulates the activity of this enzyme.

The insect meal included in aquafeed formulations has shown that low chitin level did not result in major differences on the expression of genes involved in protein digestion but could have positively affected the protein digestion (Pleić et al., 2022). In general, the expression of transporters and digestive enzymes decreased along the intestine, as reported also by Wang et al. (2017) in a review concerning the membrane transporter PepT1 (*slc15a1*). Furthermore, it can be noted that, in the proximal intestine, the expression of different genes is dose dependent; on the contrary this was not the case in distal intestine, where only limited amounts of chitin stimulate *alpi* gene. Results also highlighted that the distal intestine was less involved in the digestive activity than the proximal intestine, as the expressions in the distal tract were approximately of half of those registered in the proximal intestine. The downregulation of *slc1a5* in fish fed the chitin-containing diets is consistent with lower protein digestibility and lower serum amino acids level in rainbow trout fed the highest chitin-containing diet.

Leucine aminopeptidase activity is used as an indicator of the fish ability to breakdown peptides into aminoacids. The highest *L-anp* activities were observed in proximal and distal intestine irrespectively of the dietary chitin level. This contrasts with the results of Belghit et al. (2018) where a diet including 60 % BSF meal resulted in a marked decrease in intestinal *L-anp* activity in Atlantic salmon. For such effect authors claimed the chitin content of the BSF-containing diet, which may have impaired intestinal homeostasis and caused changes in intestinal turnover. These results were also confirmed by the lowest apparent protein digestibility and circulating amino acid level in fish fed diets with the highest chitin content. The gene expression of both the BBM intestinal enzymatic activities related to the digestion of carbohydrates in response to increasing dietary chitin levels gives a contradictory picture and does not allow to understand if and how the presence of chitin can interfere with the digestion of these complex molecules.

Pla2 catalyzes the hydrolysis of membrane phospholipids releasing fatty acids and its activity appears positively correlated to the dietary phospholipid level (Cahu et al., 2003) in sea bass larvae fed diets containing different phospholipid levels. In the present study, the increase in dietary chitin resulted in a decrease in *pla2* expression together with a decrease in lipid digestibility and in circulating triglycerides.

Intestinal alkaline phosphatase was considered in the present study due to its important role in maintaining intestinal microbial homeostasis and intestinal barrier function, as well as other properties extensively reviewed by Lallès (2020). Specifically alkaline phosphatase was used to verify if chitin levels compromised the intestinal homeostasis and thus induced possible gastrointestinal inflammation. In our study, *alpi* gene expression and BBM *alp* activity were stimulated in all the analysed intestinal tracts of the fish fed chitin-containing diets, similarly to European sea bass fed diets with 8 % of *H. illucens* meal (Pleić et al., 2022). Alkaline phosphatase, as a marker of intestinal homeostasis, suggests a possible inflammatory status of the intestinal mucosa in trout fed chitin-containing diets.

4.5. Pro-inflammatory cytokines

The intestine is a physical barrier against infections and has a significant immunological function (Takiishi et al., 2017). Several immune cells, including lymphocytes, plasma cells, eosinophilic granulocytes, and macrophages, are abundant in the gut mucosa and have the ability to elicit local reactions (Salvo-Romero et al., 2022). These cells communicate by adhesion molecules and production of protein messengers, called cytokines, that are activated by phagocytes during an inflammation process (Calder, 2007). *Tnf-α* is the first cytokine produced systemically in an inflammatory response immediately followed by *il-1β* and *il-6*. These pro-inflammatory cytokines promote the production of chemotactic cytokine such *il-8*, which migrates to the infection site (Suzuki et al., 2020). Consequently, the presence of these cytokines suggests the development of the early events leading to inflammation (Waterhouse and Stadnyk, 1999). In our study, significant differences were recorded for *il-1β* and *il-8* gene expression only in proximal intestine of fish fed the CHI3 diet while no significant differences were detected for *tnf-α* gene expression. This could be due to the time which elapses between the production of *tnf*-α and of the other cytokines as previously reported by Khalil et al. (2023). Similarly, Cardinaletti et al. (2019) showed an upregulation of cytokines (*il-10*, *tnf-α*) in the mid intestine of rainbow trout fed a BSF meal-including diet. The cytokines upregulation in fish fed the CHI3 diet suggests an inflammatory status at this intestinal level.

4.6. Intestinal microbial communities

As previous literature highlighted that dietary insect affected the intestinal microbiota of fish, the present study also investigated whether dietary pure chitin is the actor leading to such effects. The statistical analyses carried out on the rainbow trout microbiota at the end of the experimental period did not show major modification among dietary groups. The phylum pattern in the present study roughly aligns with previous literature on salmonids fed fish meal-based or insect-containing diets (Bruni et al., 2022; Eide et al., 2024; Foysal and Gupta, 2022; Holen et al., 2022; Rimoldi et al., 2023; Sampathkumar et al., 2023), but differed from the insect-fed fish in that the administration of chitin did not increase Actinobacteria nor Firmicutes in our study. In addition, the insect-fed groups of several studies reported higher alpha-diversity indices in comparison to the control, along with a tendency of mucosa samples to be more resilient to dietary changes than the content samples (Bruni et al., 2022; Huyben et al., 2019; Li et al., 2021; Rimoldi et al., 2019, 2021; Terova et al., 2019). Rimoldi et al. (2023) highlighted a modulatory effect of dietary BSF pupal exuviae on rainbow trout gut microbiota, while no significant differences were found in rainbow trout gut microbiota fed shrimp head meal (SHM). Similarly, Holen et al. (2022) found that the distal intestine microbiota of Atlantic salmon fed control or shrimp shell was almost identical, and different from the microbiota of Atlantic salmon fed defatted BSF larvae meal. These observations suggest that chitin alone is not responsible of

the fish gut microbiota modulation but instead the insect-associated microbiota may play this role.

The present study retrieved only one member of the Tenericutes phylum, and no members of the *Mycoplasma* genus. The lack of this genus is of particular interest. *Mycoplasma* is a mutualist symbiont of Atlantic salmon (Cheaib et al., 2020) and one of the prominent subset of gut microflora adapted to the farmed and wild Atlantic salmon (Heys et al., 2020), and retrieved in the majority of the studies on rainbow trout gut microbiota (Brown et al., 2019; Huyben et al., 2018; Kim et al., 2007; Lowrey et al., 2015; Lyons et al., 2016; 2017; Messina et al., 2023; Rasmussen et al., 2021) and indeed was also found in insect-fed salmonids (Bruni et al., 2022; Li et al., 2021; Rimoldi et al., 2019; Terova et al., 2021). This "non-result" of the present study incites further investigations on the role played by these small cell wall-deficient bacteria considering its implication in fatty acids or lipid metabolism (Bruni et al., 2022).

5. Conclusion

The present study highlighted that dietary inclusion of chitin up to 3 % in a semi-purified diet for rainbow trout did not hamper growth performance, nutrients digestibility, blood biochemistry, liver health and gut functionality. This percentage can be used as a reference to formulate feed containing insect meal for rainbow trout. These no differences presence notwithstanding, the application of a multidisciplinary approach revealed that even lower dietary chitin levels were able to alter some fish biomarkers related to gut digestion functionality and inflammation response. Besides, pure chitin did not induce gut microbiota modulations, therefore the attention should be drawn on the differences found in comparison to previous studies, investigating the modulating role of chitin type or other molecules on gut microbiota, as well as the role of core bacteria that were absent in the present study. However, it should be taken into consideration that these results were recorded using a commercial product of shrimp origin which could present differences in surface morphology compared to the one originating from BSF. Further investigations, such as intestinal histomorphology, are necessary to confirm the possibility of an inflammatory status induced at intestinal level.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102244.

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