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Postprandial kinetics of digestive function in rainbow trout (*Oncorhynchus mykiss*): genes expression, enzymatic activity and blood biochemistry as a practical tool for nutritional studies

G. Pascon, E. Daniso, G. Cardinaletti, M. Messina, F. Campagnolo, D. Zuccaccia, F. Tulli *

Dept. of Agriculture, Food, Environment and Animal Science, University of Udine, Italy

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ABSTRACT

Postprandial kinetics of genes expression of gastric (chitinase, pepsinogen) and intestinal (alkaline phosphatase, maltase) digestive enzymes and nutrient transporters (peptide transporter 1, sodium-glucose transporter 1), Brush Border Membrane (BBM) enzymes activity (alkaline phosphatase, leucine aminopeptidase, maltase, saccharase) and blood biochemistry (triglycerides, cholesterol, protein, albumin, glucose, amino acids) through NMR spectroscopy, were investigated in rainbow trout (*Oncorhynchus mykiss*) fed a commercial aquafeed. For this purpose, fish were starved 72 h and digestive tract and blood were sampled before the meal and at 1.5, 3, 6, 9, 12, and 24 h after feeding (T0, T1.5, T3, T6, T9, T12 and T24). The postprandial kinetic showed that the expression of the genes involved in digestion and nutrient transport, the activity of BBM enzymes, and the presence of metabolites in blood were stimulated in different ways by the presence of feed in the digestive tract. The expression of most genes peaked 3 h after meal except gastric pepsinogen and maltase in distal intestine that peaked at T9 and T12, respectively. The activity of BBM enzymes were stimulated differently based on the intestine tract. The plasma proteins level increased from T1.5 until T9, while the other blood parameters unvariated during the postprandial period. This study supplied useful information about the physiological effects a single meal as a potential tool for planning nutritional studies involving the digestive functions.

1. Introduction

Over three decades, the aquaculture sector has been one of the fastest-growing sectors of food production systems (Naylor et al., 2009, 2021). Plant-origin ingredients, insects, terrestrial by-products, and single cell ingredients (bacteria, microalgae, yeasts) considering their nutritional profile and possible nutraceutical properties on animal welfare (Cardinaletti et al., 2018; Cerri et al., 2021; Marchi et al., 2023; Maulu et al., 2022; Pascon et al., 2021; Sealey et al., 2011; Van Doan et al., 2023) are used in feed formulations to increase their sustainability. It is well known that diet composition affects fish digestive activity and protein and lipid muscle deposition (Mock et al., 2019) and it is important to understand how the metabolism of fish and their gastrointestinal physiology react to these new ingredients (Aragão et al., 2022). Balanced nutrition is essential to guarantee fish welfare and digestive ability; consequently, the functional changes along the gastrointestinal tract are usually considered when new feed formulations are provided to farmed fish (Cardinaletti et al., 2019, 2022). Comprehensive nutritional studies integrate zootechnical performance and biometric indices and nutrient digestibility as well as physiology and integrity of the digestive tract, blood parameters, immunity status, microbiota composition, and inflammatory response (Cardinaletti et al., 2019, 2022; Gaudioso et al., 2021; Pascon et al., 2021; Weththasinghe et al., 2021; Zarantoniello et al., 2018). Parameters such as zootechnical performance or biometric indices have an endpoint evaluation, and the practice of 24 h fasting before animal sampling is already consolidated (García-Meilán et al., 2023; Renna et al., 2017); parameters related to immune status or inflammatory response are not immediately modulated by feed intake, since these processes take longer time to show some evidence (Henry et al., 2018; Kumar et al., 2021). However, other processes directly linked to feed intake, such as digestive enzymes activity and nutrients absorption exhibit a kinetic pattern related to the stimulus determined by the meal. Some studies investigating the digestive enzymes activity and the circulating metabolites concentration have reported postprandial kinetics to a different extent for the same fish species. For instance, Yamamoto et al. (1998) reported a postprandial

* Corresponding author.
E-mail address: francesca.tulli@uniud.it (F. Tulli).

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pattern of plasma-free amino acid concentrations with a peak concentration at 12 h and Larsen et al. (2012) reported a first peak for all amino acids at 6–8 h and an additional peak after 12 h for some amino acids, in rainbow trout fed fish meal-based diet. For the same species, Borey et al. (2016) reported a postprandial dynamic of the gene expression involved in the digestion and absorption of nutrients, with a peak at 12 h postprandial when fed a fish meal-based diet. Anyway, the limited number of studies on the postprandial dynamics of rainbow trout metabolism under similar conditions does not allow us to make a well-considered sampling time in the postprandial dynamic for the digestive tract and blood. Therefore, since it is not possible to follow the entire kinetics for each parameter every time, it would be useful to understand the postprandial pattern for each investigated parameter to define which sampling time after the meal would be better for results standardization in the different finfish species.

Due to the limited knowledge about gastric and intestinal postprandial physiological response to a single meal in rainbow trout this study was focused on the gene expression and enzymatic activity of the main gastric and intestinal digestive enzymes (chitinase, pepsinogen, alkaline phosphatase, leucine aminopeptidase, maltase, sucrase), the principal intestinal nutrient transporters (peptide transporter 1 and sodium-glucose transporter 1), and the major plasma and serum metabolites at different postprandial sampling time after a meal in reared rainbow trout (*Oncorhynchus mykiss*).

2. Materials & methods

2.1. Ethics

The feeding trial and the final sampling were executed comparatively with European legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU) approved by the Committee of the University of Udine for animal Ethics (prot. n° 8/2021).

2.2. Experimental protocol and fish rearing conditions

The experiment was carried out indoor, at the aquaculture facilities of the University of Udine (Pagnacco (UD), Italy). One hundred forty rainbow trout (*O. mykiss*) with an individual body weight of 121.4 ± 25.2 g were selected from a resident batch. Fish were randomly allocated among 14 tanks (2 tanks per sampling time), each consisting of 10 fish and reared in 300 l cylindrical fiberglass tanks. Each tank was connected to a flow–through system supplied with well water, ensuring a complete volume renewal/h with 18–20 l·min^{-1} and optimal water quality (temperature 13.65 ± 0.7 °C; dissolved oxygen 9.38 ± 0.3 mg·l^{-1}; pH 8.23 ± 0.1; total ammonia-N, <0.01 mg·l^{-1}; Nitrite-N, <0.02 mg·l^{-1}). Fish were adapted to the culture conditions for 3 weeks and were hand-fed once daily (9:00 a.m.) to 1% live weight with a commercial diet for rainbow trout (ARCTIC SF, ALLER AQUA A/S; Denmark). The photoperiod was the natural one for May at the latitude of 46°06′52″ N, 15 h light and 9 h darkness.

2.3. Sampling

Based on a previous study (Borey et al., 2016), at the end the feeding trial, fish were starved for 72 h and then received a single meal after the first sampling time. Eight fish (four fish from 2 tanks) were randomly sampled at the end of the fasting period (T0) and after 1.5, 3, 6, 9, 12, 24 h the single meal (T1.5, T3, T6, T9, T12 and T24). Fish were sacrificed with an overdose (300 mg·l^{-1}) of the tricaine methan sulphonate, MS-222 (PHARMAQ, Fordingbridge Hampshire, UK), and immediately subjected to blood sampling. Blood was withdrawn from the caudal vessel in heparinized (17 U/ml) and non-heparinized tubes to obtain plasma and serum, respectively. Plasma was obtained after centrifugation at 1500 x*g* for 15 min at 4 °C, while serum samples were obtained after clotting for 2 h at 4 °C and centrifugation at 1500 x*g* for 20 min at 4 °C. Plasma and serum samples were immediately stored at −20 °C for the subsequent analysis of metabolic profile performed by enzymatic assay and through NMR spectroscopy, respectively. Immediately after blood sampling the abdomen was opened and the digestive tract was excised and divided into stomach (S), pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI). Samples of PC, PI and DI were rinsed with iced saline water, put in individual plastic tubes and stored at −80 °C until brush border membrane enzymes activity analysis. Small samples of S, PI, and DI were retrived in individual plastic tubes containing RNAlater™ solution (Invitrogen, Thermo Fisher Scientific Inc., Italy) following the manufacturer's instructions until the subsequent gene expression analysis.

2.4. Gene expression

Total RNA was extracted from S, PI, and DI (approximately 90 mg) using PureLink™ RNA Mini Kit (Invitrogen by Thermo Fisher Scientific, Italy) following the producer's instructions. To evaluate the RNA concentration NanoDrop™ One Microvolume UV–Vis Spectrophotometer was used (Thermo Scientific™ Inc., Italy) and 28S and 18S ribosomal RNA bands integrity were checked with standard agarose gel electrophoresis (1.2%). Subsequently, the synthesis of complementary DNA (cDNA) was performed using the PrimeScript™RT reagent Kit with gDNA Eraser (Takara Bio Europe SAS, France) following the manufacturer's protocol. The genes selected and evaluated in the stomach were pepsinogen (*pga*) for protein luminal digestion and gastric chitinase (*chia*) for chitin digestion. Our interest for this latter gene is to elucidate, for the first time its postprandial expression pattern since it has been previously demonstrated that: (i) fish, including Salmonids (Holen et al., 2022a), exhibit chitinase activity in the stomach (Ikeda et al., 2017); (ii) *chia* expression is species-specific (Ohno et al., 2013); (iii) *chia* expression is related to feeding habit (Tabata et al., 2018). The target genes selected and evaluated in the PI and DI were maltase (*mal1*) for membrane maltose digestion, intestinal alkaline phosphatase (*alpi*) as marker of intestine homeostasis, glucose transporter sodium-glucose transporter 1 (*slc5a1*) and peptide transporter 1 (*slc15a1*). Ribosomal protein 60S (*60S*) and beta-actin (*β-act*) were selected as reference genes (housekeeping genes HKGs). The target and housekeeping genes primers are listed in Table 1, they were designed based on the cDNA sequences accessible in the GenBank database for *Oncorhynchus mykiss* or found from published sequences. The RT-qPCR was performed following the protocol by Cardinaletti et al. (2022).

2.5. Brush border membrane enzyme activity

The brush border membrane enzymes were analyzed according to Messina et al. (2019). Briefly, the intestine was thawed and the remaining content was squeezed out. The gut tissue was added with iced saline buffer (1:10 *w/v*) and mashed with a tissue-lyser (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 1 min. Samples were centrifuged at 13,500 x*g* for 15 min at 4 °C and the BBM enzymes activity was measured in the supernatant. The hydrolysis of maltose and sucrose, by the maltase and the sucrase-isomaltase (SI), was determined according to Harpaz and Uni (1999). Alkaline phosphatase (ALP), as a marker of intestinal homeostasis, was determined using Paramedical kits (Pontecagnano Faiano, SA, Italy) following the manufacturers' protocol. The proteolytic enzyme leucine aminopeptidase (L-ANP) was determined according to Vizcaíno et al. (2014). The specific enzymatic activity was calculated as U of enzyme activity per g of tissue for all the BBM enzymes. One unit (U) of enzyme activity corresponded to the amount of enzyme that transforms or hydrolyses 1 μmole of substrate ml^{-1} min^{-1}.

2.5.1. Serum filtration/NMR sample preparation

The serum glucose, lactic acid and free amino acids (phenylalanine (Phe), histidine (His), valine (Val), isoleucine (Ile), tryptophan (Trp),

Table 1
Sequence of primers used for RT-qPCR.

Organ	Gene	Primer Forward (5′ – 3′)	Primer Reverse (5′ – 3′)	Accession number	Reference
Stomach	*pga*	GTTTCGGTGGGAGGCATCT	TGTTTCCGAAAGACACCACA	Sigeneae, CX136077.s.om.10	(Borey et al., 2016)
Stomach	*chia*	AACAGCAACCTGAAGACTCTG	CGACTCTTCGAGGACCAACC	GenBank,**EU877960**	Present study
Proximal & Distal intestine	*Mal1*	GCAGCAGGAATACCCTACGA	GGCAGGGTCCAGTATGAAGA	**TIGR, TC3451**	(Borey et al., 2016)
Proximal & Distal intestine	*slc15a1*	CCTGTCAATCAACGCTGGT	CACTGCCCATAATGAACACG	GenBank, **EU853718**	(Borey et al., 2016)
Proximal & Distal intestine	*alpi*	GAAACACCGGACCCAGAGTA	GCAGTCACTTGGCTCAAACA	**XM 021610191.2**	Present study
Proximal & Distal intestine	*slc5a1*	GGTGACCATGCCAGAGTACT	GATATTCAGCCCCAGAGCCT	**AY210435.1**	Present study
HKG	*60S*	TTCCTGTCACGACATACAAAGG	GTAAGCAGAAATTGCACCATCA	**DT044641.1**	(Cardinaletti et al., 2019)
HKG	*β-act*	AGACCACCTTCAACTCCATCAT	AGAGGTGATCTCCTTCTGCATC	**AJ537421**	(Cardinaletti et al., 2019)

and leucine (Leu)) concentrations (mmol l^{-1}) were determined using NMR spectra acquisition, according to Schock et al. (2012). To remove high molecular weight molecules, serum were passed through a cartridge size-exclusion filter. Briefly, 500 µl of serum was transferred to a pre-conditioned centrifuge filter tube (Vivacon® 500, 30,000 MCWO HY, Sartorius, Germany). The filter tubes were washed by centrifugation (8200 ×*g* for 20 min at 4 °C) ten times with Milli-Q water. Consequently, serum were centrifuged at 7600 ×*g* for 20 min at 4 °C and filtrates (approximately 160 µl) were diluted with 500 µl potassium phosphate buffer (0.1 M, pH 7.3) prepared in D_2O (Deuterium Oxide, containing 0.05% weight of D4-TMSP acid, sodium salt, 99.9% of D atoms (AB571341 | CAS 7789-20-0, abcr, Germany). The samples (660 µl) were transferred to 5 mm NMR tube (Bruker® SampleJet NMR tubes, Merck, Germany).

2.5.2. NMR: spectra acquisition and processing

The H-NMR spectra of the samples were acquired using a Bruker Avance III HD 400 MHz spectra (Bruker, Czech Republic) equipped with a broadband 5 mm probe (^{1}H/BBF iProbe) with a z-axis gradient (50G/cm). The spectra were obtained using a one-dimensional pulse sequence for water suppression based on the noesygppr1d pulse sequence. All the spectra were recorded at 298 K with a spectral width of 13 ppm, 4 s of relaxation delay, 1024 scans (transients), and 4 dummy scans (steady state scans), generating a free induction decay (FID) with a time domain of 65,536 data points. The FIDs were processed performing apodization with an exponential function (line broadening set to 1.00 Hz), and zero-filling was carried out, adding 64 k points before Fourier transform. Then the spectra were manually phased, auto-baseline corrected, and referenced to the internal standard (TMSP).

2.5.3. NMR: data analysis

The assignment of the metabolites in the NMR spectrum of the filtered rainbow trout samples was carried out by comparing chemical shift and spin coupling to an in-house produced database. Bruker's Assure NMR and literature reference work (Schock et al., 2012) were used to verify the assignments. The metabolites concentration was determined by integration relative to the internal standard (TMSP).

2.5.4. NMR: generation of the metabolite database

An in-house library of molecules of interest was created for the identification and quantification of the studied molecules. The selected metabolites were histidine, arginine, isoleucine, lysine, methionine, phenylalanine, leucine, threonine, tryptophan, valine, glucose, and lactic acid. All the metabolites (1 mmol) were dissolved in a deuterium oxide phosphate buffer solution containing 0.05% TMSP, which was obtained by dissolving 30 mg (2.4 mmol, 5 eq) of potassium phosphate monobasic and 7 mg (0.5 mmol, 1 eq) of potassium phosphate dibasic in 2 ml of D_2O. Following the procedure by Schock et al. (2012), the solution was analyzed via ^{1}H NMR spectroscopy, with the number of transients set to 128. The resulting spectrum was included in the metabolite database created in Bruker's Assure NMR 2.2 and Amix 4.0.2.

2.6. Plasma metabolites

Plasma triglycerides (mg dl^{-1}), total cholesterol (mg dl^{-1}), albumin (g dl^{-1}) and total proteins (g dl^{-1}) contents were determined using commercially available kits (Paramedical s.r.l., Salerno, Italy) following the manufacturer's protocols

2.7. Statistical analyses

Data of plasma and serum parameters and enzyme activity are presented as mean value ± SD. Gene expression data are presented as mean value ± SEM. Normality and homogeneity of data were tested using Shapiro–Wilk's and Levene's tests, respectively. All data related to plasma and serum metabolites, enzyme activity and gene expression were subjected to one-way ANOVA to analyze differences among the seven sampling times. When appropriate, the Duncan's post hoc test ($p < 0.05$) was used. Different letters in the graphs indicate significant differences of the same parameter at different times (lowercase letter: $p < 0.05$; uppercase letter: $p < 0.001$). SPSS package (SPSS Inc., Chicago, IL, USA) was utilized to analyze all data.

3. Results

3.1. Gene expression

The expression of the genes involved in the digestive process in the stomach, proximal intestine and distal intestine were significantly affected over the sampling times. The expression of gastric *pga* (Fig. 1) started to increase immediately after the meal (T1.5), and a bimodal pattern of *pga* expression can be detected with the highest values at T9 and T12 (0.59 ± 0.02, 1.00 ± 0.10, 0.81 ± 0.06, respectively). The expression of *chia* showed a basal level throughout the postprandial period with an upregulation at T1.5, although not significant, reaching the highest value (1.00 ± 0.13) after the meal.

Concerning the digestion and absorption of proteins and

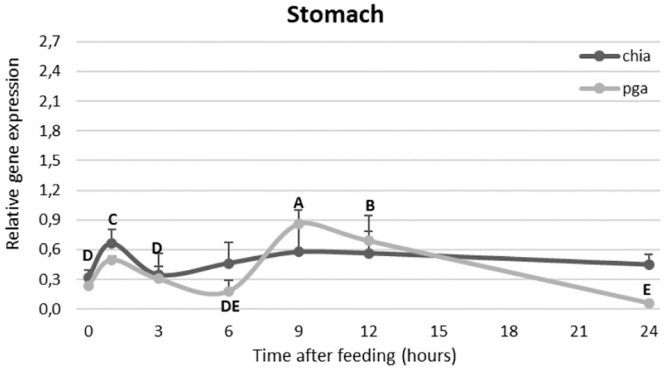

Fig. 1. Postprandial relative normalized expression of gastric pepsinogen and chitinase in rainbow trout after a single meal. Data are reported as mean + SEM ($n = 8$). Different letters indicate significant differences in gene expression of the same gene among different times (A, B = $p < 0.001$).

carbohydrates in the PI, significant differences can be observed in the expression pattern of ALP (Fig. 2A) and *slc5a1* gene (Fig. 2B), with a peak recorded at T3 (1.33 ± 0.04 and 1.43 ± 0.15, respectively). On the contrary, no significant differences were detected for *mal1* (Fig. 2A), and Slc15a1 (Fig. 2B). Nevertheless, a peak value can be observed in the expression of *mal1* T1.5 h after the meal (1.36 ± 0.36).

Regarding the digestion and absorption of proteins and carbohydrates in the DI, both the enzymes and the tranporters showed different expression among the sampling times. The maximum expression of *alpi* (Fig. 2C), *slc5a1* and *slc5a1* (Fig. 2D) were reported at T3 (1.44 ± 0.28, 1.51 ± 0.22, 1.20 ± 0.23, respectively), while the *mal1* expression increased 9 h after the meal reaching a peak value of 1.9 ± 0.45 relative normalized expression at T12.

3.2. BBM enzymes activity

The activity of BBM enzymes in PC, PI and DI of rainbow trout throughout the 24 h postprandial period is reported in Fig. 3 A, B, C, and D.

The specific activity of ALP (Fig. 2 A) showed different patterns along the intestinal tracts (PC, PI and DI). The highest value is observed 3 h after the meal in PC (52.5 ± 15.7 U/g tissue) and PI (181.3 ± 68.7 U/g tissue) ($p < 0.05$) while no significant differences were detected in DI over the 24 h sampling period.

The specific activity of L-ANP (Fig. 3 B) resulted in a peak value 3 and 24 h after the meal in PC (463.8 ± 144 and 529.62 ± 220.4 U/g tissue, respectively) and 3 h after the meal in the PI (1014.6 ± 280.9 U/g tissue). The activity of this enzyme resulted always lower in the PC than in the other two tracts. In the PC and PI, the value at some sampling time resulted significantly different from the basal value at T0, i.e. at T9 in the PI and T3 and T24 in the PC. Differences in the values of the pattern in the DI were not significant ($p > 0.05$).

The maltase activity (Fig. 3 C) showed no significant differences both in PC and DI, while in PI this enzyme activity significantly decreased after 9 h (T9, T12, and T24) postprandial.

The specific activity of sucrase (Fig. 3 D) showed different patterns in PC, PI and DI. This enzyme presented the lowest specific activity value in PC compared with the other two tracts, without any difference among the sampling time. Regarding the PI, although the values at the different sampling times were not statistically different from the basal value at T0, it is possible to individuate a peak value at T3 (1709.7 ± 306.8 U/g tissue) and the lowest value 12 h after the meal. In DI the specific activity presented similar values except at T9 where a peak value (1523.9 ± 171 U/g tissue) was observed.

3.3. Plasma metabolites

Data on the plasma metabolic parameters obtained from samples collected at each sampling time are reported in Figs. 4 A and B. The pattern of plasma Chol and Trig over the postprandial period did not show significant differences among the sampling times ($p > 0.05$). Nevertheless, a minimum value was observed at T12 (144.9 ± 56.5 mg dl^{-1}, 54.7 ± 31.8 mg dl^{-1}, for Trig and Chol respectively). Circulating proteins level significantly increased after meal (T1.5); the total protein level (3.03 ± 0.25 g dl^{-1}) remained at the highest level until 9 h postprandial when albumins as well reached the highest value (2.4 ± 0.24 g dl^{-1}).

3.4. Serum metabolic parameters

^{1}H NMR spectroscopy permitted the detection of 7 of the 12-targeted serum metabolites in the rainbow trout samples. The metabolites were

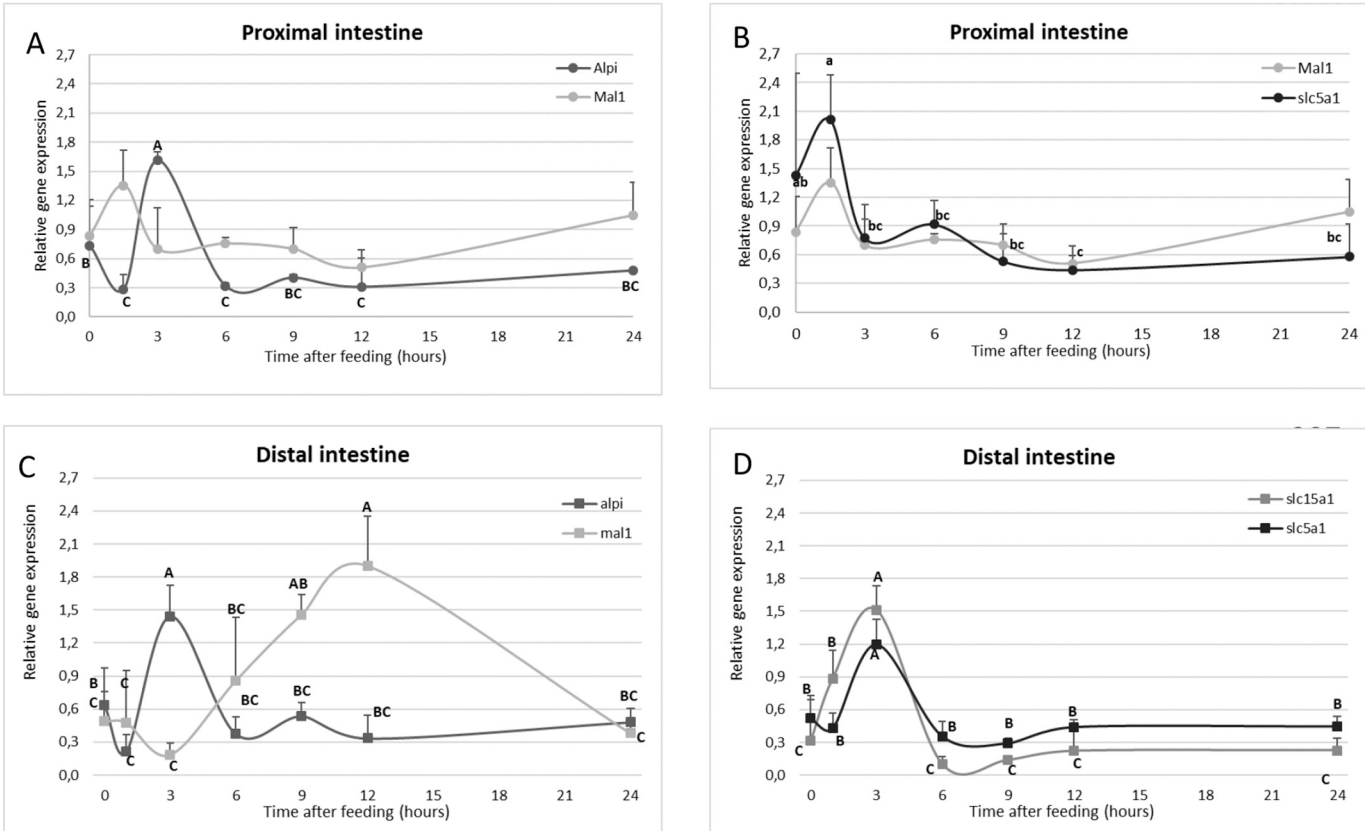

Fig. 2. Postprandial relative normalized expression of intestinal genes involved in the digestion/absorption of nutrients in rainbow trout after a single meal. A and B = proximal intestine, C and D = distal intestine. Data are reported as mean + SEM (n = 8). Different letters indicate significant differences in gene expression of the same gene among different times (a, b = $p < 0.05$; A, B = $p < 0.001$).

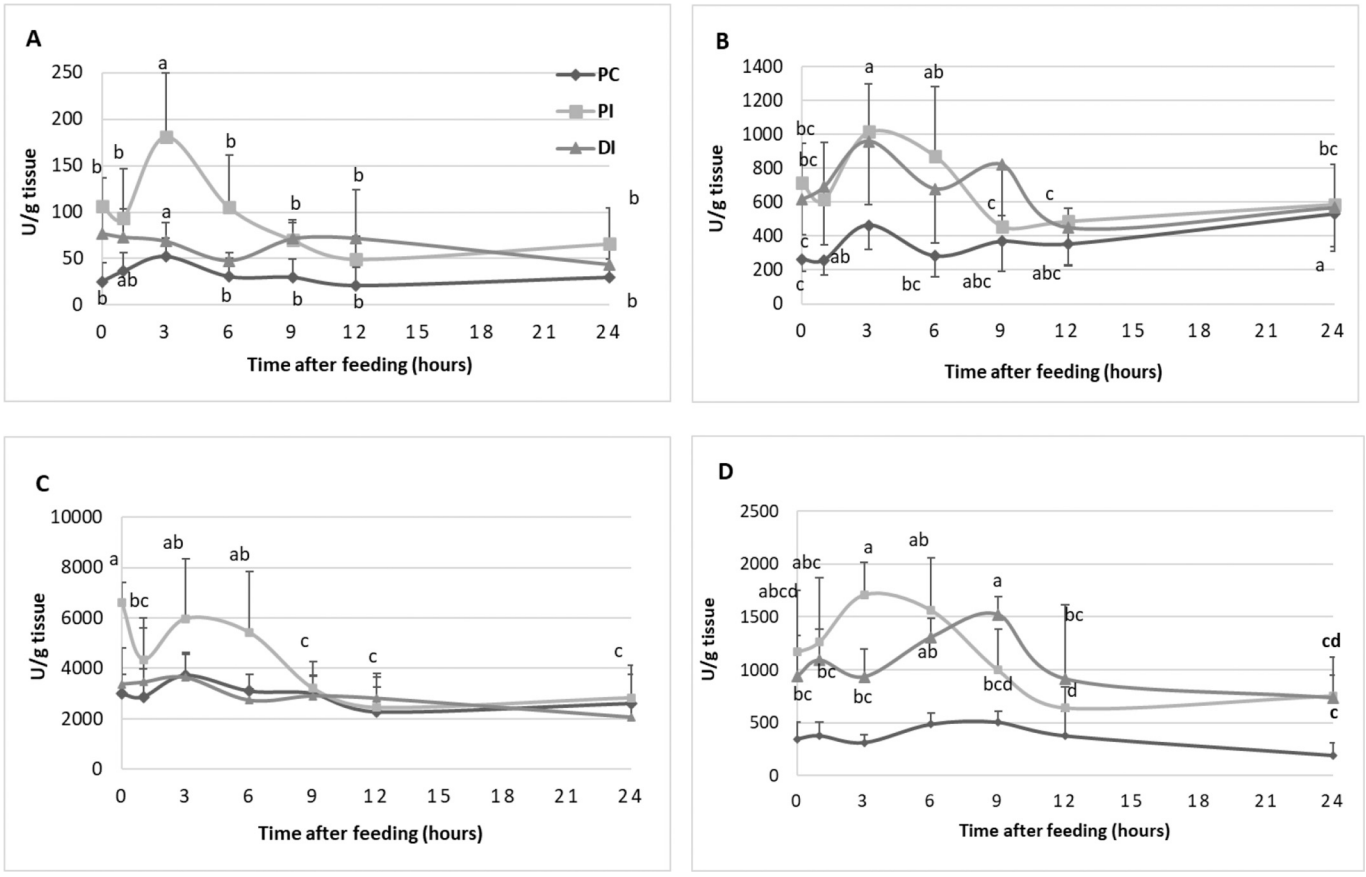

Fig. 3. Postprandial pattern of intestinal enzymes activity (U/g tissue) in rainbow trout after a single meal. A = Alkaline phosphatase, B = Leucine aminopeptidase, C = Maltase D=Sucrase. Data are reported as mean ± SD ($n = 8$). Different letters indicate significant differences in enzyme activity of the same enzyme among different times (a, b, c = $p < 0.05$).

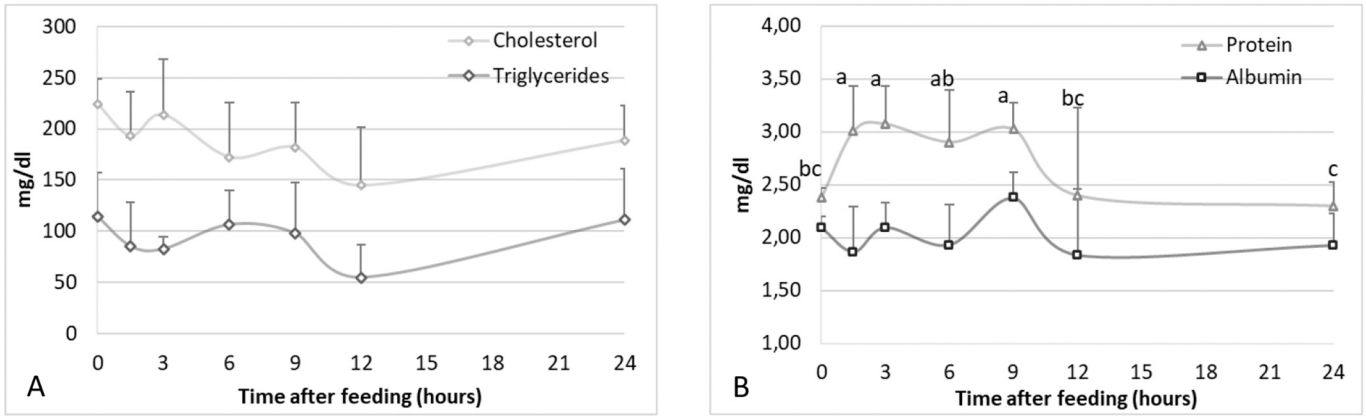

Fig. 4. Postprandial kinetics of plasma metabolic parameters in rainbow trout after a single meal.
A = Cholesterol and Triglycerides (mg dl^{-1}); B = Abumin and Proteins (g dl^{-1}) Data are reported as mean + SD (n = 8). Different letters indicate significant differences of the same plasma metabolite among different times (a, b = $p < 0.05$).

identified in correlation to the in-house database created for this type of fish samples. The following metabolites were accounted for glucose, lactic acid, His, Phe, Trp, Val, Ile, Leu.

The circulating levels of serum glucose, lactic acid, and free amino acids of rainbow trout over time after feeding is reported in Fig. 5. Despite not being statistically significant, an increase in circulating glucose level occurred 6 h after meal while serum lactic acid and amino acid levels remained unchanged throughout the postprandial period.

4. Discussion

Fish digestion is a process associated with the activation of enzymatic and hormonal systems and aims to transform food into simple molecules that can be absorbed by membrane transporters present on enterocytes. The main trigger for the digestion activation is the presence of food in the digestive system that determines a series of events that follow each other in the *post prandium*.

This research can provide useful information about the physiological

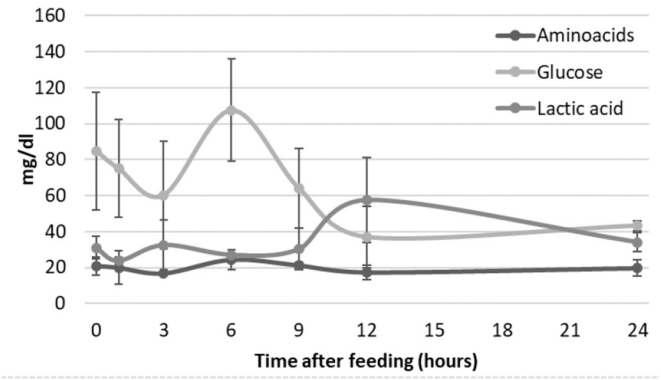

Fig. 5. Postprandial patterns of serum glucose, amino acids and lactic acid in rainbow trout after a single meal. Data are reported as mean + SD (n = 8).

effects after a single meal and could support researchers' decisions when drafting a study involving digestion and nutrients absorption in rainbow trout. Results indicate that 3 h postprandial are needed in rainbow trout to observe an increase in gene expression at intestinal level coupled by a subsequent increase in gut enzyme activity (3–6 h) and finally followed by an increase of circulating metabolites (6 h).

The **postprandial expression of genes** involved in the digestion and absorption of macronutrients showed that the genes were expressed differently over the 24 h period due to the presence of feed along the gut. Pepsinogen is a digestive enzyme secreted by the gastric glandular cells and it is the inactive precursor of pepsin. The *pga* gene expression is normally stimulated in fish by gastrin, a hormone released immediately after the meal (Rust, 2003). In the present study, a substantial increase of the *pga* expression occurred rapidly (1.5 h) after the meal and could be considered the result of the direct feed stimulation. The second peak of *pga* (9–12 h after the meal) seems not related to the feed intake, but rather to the circadian cycle. The presence of a circadian rhythm in mRNA expression of *pga* gene has already been reported by Yúfera et al. (2012) in white seabream (*Diplodus sargus*) and in gilthead seabream (*Sparus aurata*) (Yúfera et al., 2014), where an increase in *pga* expression is reached immediately before the morning meal, likely to supply the subsequent digestion. Even in our case, the results may highlight how the mRNA transcripts, which showed lower levels during the diurnal period, are higher during the nightime (corresponding to 6 and 9 p.m., when the sunset begins in the third decade of May, in Northeast Italy). This second peak at T9 (6 pm) seems to be related to the time of the day when wild trout looks for feed (Landless, 1976). A similar result was observed for the gastric *chia*, although the different expression at the sampling times was not statistically significant. Chitinase is an enzyme that hydrolyzes chitin and recently its presence has been demonstrated in the Salmonids gastric mucosa such as Atlantic salmon (Holen et al., 2022b) and in many fish species (Ikeda et al., 2017) as well as Mammals (Tabata et al., 2018). The highest concentration of this enzyme is detected in the stomach and the pyloric caeca, indicating that either these intestinal parts or the insects containing diets are the main sources of chitinase in fish (Moraes and De Almeida, 2019). In a previous study with European seabass (*D. labrax*) fed diets containing graded levels of black soldier fly (*H. illucens*) pupae meal, the chitinase activity increased with the dietary level of insect meal (0, 8, 16, 32%), mainly in the proximal intestine (Messina et al., 2022). However, a high chitinase activity was measured even in fish fed diet without insect meal demonstrating a basal chitinolytic activity both in the stomach and in the proximal intestine, thus supporting the hypothesis of the role of this enzyme as part of the host defense against chitin-containing pathogens in the gastric contents (Ohno et al., 2013). When the gastric chitinolytic activity was compared in European seabass and rainbow trout, a lower activity was observed in rainbow trout compared to seabass (Messina et al., 2022). This low activity in salmonids was also reported in a review regarding the chitin utilization in aquaculture by Ringø et al. (2012).

In the **proximal intestine**, gene expression of *mal1*, and *slc15a1* did not show any significant difference among the sampling times, even if it reached the highest value 1.5 h after the meal, showing a postprandial pattern of the expression of these genes. On the contrary, *alpi* expression is strongly stimulated by the meal likely because of its specific role in digestion (Lallès, 2020). Many studies reported a positive correlation between feed intake and alkaline phosphatase activity (Lemieux et al., 1999; Xu et al., 2016) in agreement with the highest *alpi* activity at T3 in the present study and the depressive effect of feed deprivation (Abolfathi et al., 2012; Hayes and Volkoff, 2014).

The increase of the ALP enzymatic activity 3 h after the meal is consistent with the ALP expression in the PI at the same time. In the case of MALT in the proximal intestine, there is an increase (even if not significant) in gene expression, followed by an increase in enzymatic activity. These results are compatible with the increase in the expression of *mal1* and *Slc15a1* 2 h after the last meal (Borey et al., 2016) and the peak of *alpi* observed 3 h after the last meal (Santigosa et al., 2008) in rainbow trout while a peak in intestinal *alpi* expression has been reported much later (4–6 h postprandial) in mice fed a high fat diet (Nakano et al., 2009).

In the **distal intestine**, a postprandial pattern could be noticed for all the genes considered. Although overexpression of the *alpi* gene occurred after 3 h, this was not translated into enzyme activity, demonstrating a basal activity throughout the sampling period in DI. Similar pattern was observed for *mal1*, that is overexpressed at 9–12 h, without a related enzymatic activity in DI. The enzymatic activity of L-ANP for protein digestion reaches the highest values in PI and DI 3 h after the feed intake in conjunction with the highest expression value of the *slc15a1* gene for the amino acids transport. This also applies to the glucose transporter, *slc5a1*, whose expression is followed by the enzymatic activity of maltase and sucrase, in particular in PI.

The timing of gene expression seems to be aligned to the enzymes activity for digestion and absorption of proteins and carbohydrates and to the presence of the specific circulating metabolites. Concerning protein, a peak of *pga* in stomach at T1.5 and a constitutive expression of *Slc15a1* in PI followed by a peak of *Slc15a1* in DI at 3 h and a peak of the L-ANP activity between 3 and 6 h was observed; this was followed by a subsequent increase of serum amino acids after 6–9 h. Concerning carbohydrates, the increase of *mal1* and *slc5a1* expression in PI at T1.5 is followed by an increase of *mal1* and *slc5a1* expression at T6 and T3 in DI, respectively. The peak of maltase and sucrase activity occurs in the first 6 h postprandial in PI and only after 9 h for sucrase in DI. Consequently, a peak of serum glucose is detected at T6.

The postprandial expression of genes and the profiles of the BBM enzymes activity highlighted that they were stimulated by the nutrients presence in the rainbow trout digestive tract and by the anticipatory feeding behaviour in case of the disaccharides and leucine aminopeptidase enzymatic activity. The feed anticipatory activity is a behaviour observed in almost all fish (López-Olmeda, 2017). This activity exhibits an increase in fish locomotion and in feeding-related hormones and digestive enzyme activity (Vera et al., 2007; Liew et al., 2020). Although the experiment included a 3-day fasting period, the feed anticipatory activity was observed, as also reviewed by Patton and Mistlberger (2013).

Limited comparison can be performed with previous literature as only a few studies have evaluated the digestive enzyme activities over time and they were focused on the effect of different dietary treatments in sea bass (Castro et al., 2016), Senegalese sole (Rodiles et al., 2012) and rainbow trout and seabass (Santigosa et al., 2008). In general, the contribution of the distal intestine in nutrient digestion and absorption is considered lower than the one of the proximal intestine, as demonstrated by some research on the digestive enzymatic activity and nutrient digestibility studies along the digestive tract of fish (Chikwati et al., 2013; Hartviksen et al., 2014; Nordrum et al., 2000; Olsen et al., 1999; Weinrauch et al., 2019). Although DI shows lower activity than PI,

the activity of sucrase in the DI has peaked at T9, supporting the final digestion of simple carbohydrates in this specific tract.

Plasma cholesterol and triglycerides concentrations did not show a clear postprandial pattern with levels lower than the values reported by Manera and Britti (2006) (247.38 ± 10.32 mg dl^{-1} and 347.5 ± 23.6 mg dl^{-1}, respectively) for rainbow trout. Cholesterol constitutes the cell membranes and it is the principal precursor of steroid hormones. Its blood levels can be affected by sex, sexual development, hepatic activity and nutrition (Banaee et al., 2016; Hamed et al., 2021). Triglycerides form the adipose tissue and their circulating level reflect dietary fat intake (Ahmadian et al., 2017). Despite the low value (which may be due to the 3 days of fasting pre-sampling versus 1 day of fasting in the study performed by Manera and Britti (2006), both cholesterol and triglycerides level did not differ significantly among the different sampling times, as reported also by Borey et al. (2016) for triglycerides in rainbow trout. These results suggest that plasma cholesterol and triglycerides concentrations are maintained by the activity of the liver which metabolizes both cholesterol and triglyceride pouring the most part of them into the blood bound to transport proteins

Proteins represent the principal components of blood plasma and are crucial in evaluating the animal physiological state. Plasma albumin level is of diagnostic importance in animals, since the levels change according to general nutritional status, vascular system integrity and liver function (Pastorino et al., 2022). In fact, albumins are produced in the liver and act as binding proteins for many different kindsof molecules such as hormones, vitamins, ions, fatty acids, cholesterol and have also a role in the acid-base homeostasis and in the colloid-osmotic pressure control. The total protein and albumin levels herein reported are similar to those obtained after fasting and refeeding in rainbow trout by Messina et al. (2023), but resulted lower for protein (3.59 ± 0.13 g dl^{-1}) and higher for albumin (1.38 ± 0.05 g dl^{-1}) than the values reported by Manera and Britti (2006).

Our data on circulating total proteins are consistent with Mente et al. (2017) who reported that postprandial protein synthesis in liver and muscle increases from 4 to 6 h after meal and levelled until it returns to pre-feeding level after 24 h. The remaing part of the total proteins are globulins, also produced by the liver except for the γ-globulins that are synthetized by the white blood cells. The concentrations of these liver proteins seem to be mainly affected by the single meal since the levels of albumins along 24 h does not justify the increase in the total protein levels.

The data registered for the plasma metabolites suggest that the proteins supplied from aquafeed digestion start to occur quite immediately (1.5 h) after the feed intake, while the other plasma metabolites appear not to be affected by the time elapsed after the meal.

Serum amino acids, glucose, and lactic acid concentrations remained unchanged during the postprandial period, demonstrating a sort of balance in blood metabolites concentration. Our results are not in line with a previous study on rainbow trout, which showed a peak of free amino acid concentration around 6–12 h postprandial (Larsen et al., 2012) while the glucose peak 6 h after meal is in agreement with the results on rainbow trout observed by Borey et al. (2016), even if not significant. These results demonstrate that the dynamics of serum glucose and aminoacids are not the only consequence of postprandial absorption from the intestine, but they are also related to other factors such as the distribution and uptake by peripheral tissues and organs and the dietary composition. Blood lactate is the end product of anaerobic metabolism in fish (Olsenl, 1992). The study of lactic acid kinetics provides an improved description of the production and utilization of lactic acid in fish that results particularly relevant also for fillet quality evaluation (Yu et al., 2022). In fact, the increase in blood lactate can affect muscle pH, which in turn can contribute to a faster start of *rigor mortis* and the decrease of the fillet quality (Svalheim et al., 2017). In our study, the fish maintained a basal serum lactate level without changes during the postprandial period.

5. Conclusion

In conclusion, at present there is limited data available (Borey et al., 2016) that considers the physiological responses over time of the postprandial process at different levels in a single study. The results of this study contribute to provide useful information about the postprandial physiological effects as a potential tool for planning future nutritional studies involving the digestive-assorbitive functions. Some specific indications can be suggested as rainbow trout can be fed 3 h before the intestine sampling for the gene expression analysis while the gut enzyme activity peak after 3–6 h, and the blood metabolite concentration peaks 6 h after feed intake. These peaks appear to observe a sequential path, being nutrient absorption and digestion gene expression the first process to be activated around 3 h after feed ingestion. Once expressed, enzymes are active to digest nutrients into monomers, i.e., from 3 to 6 h following a meal. At the end, metabolites appear in the bloodstream approximately 6 h after the meal.

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CRediT authorship contribution statement

G. Pascon: Data curation, Writing – original draft. **E. Daniso:** Formal analysis, Writing – review & editing. **G. Cardinaletti:** Conceptualization, Validation, Writing – review & editing. **M. Messina:** Conceptualization, Methodology, Writing – review & editing. **F. Campagnolo:** Formal analysis, Methodology, Writing – review & editing. **D. Zuccaccia:** Methodology, Writing – review & editing. **F. Tulli:** Conceptualization, Funding acquisition, Writing – review & editing.

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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