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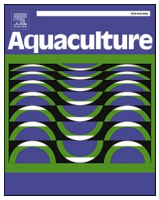

Impact of feeding diets with enhanced vegetable protein content and presence of umami taste-stimulating additive on gastrointestinal amino acid sensing and feed intake regulation in rainbow trout

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ABSTRACT

The regulation of feed intake in fish is dependent upon different neuroendocrine and metabolic mechanisms including amino acid sensing in the gastrointestinal tract (GIT). However, there is little information regarding the impact of diets on such mechanisms. Therefore, in this study, we fed rainbow trout (*Oncorhynchus mykiss*) with 3 diets: a diet with high content of fishmeal and low content of soybean protein concentrate (SPC) (HF), a diet with a reduced content of fishmeal and high content of SPC (LF), and the LF diet supplemented with an umami taste-stimulating additive (LFU). Fish were fed ad libitum once a day for 4 weeks, with no significant differences being registered in feed intake among groups. At the end of the feeding trial, we collected samples of different areas of the GIT (stomach, proximal and distal intestine) and hypothalamus at different times: after 48 h of fasting (time 0), and 1 h, 4 h, and 24 h after feeding. We evaluated the activity of pepsin in the stomach and trypsin and chymotrypsin in the proximal intestine, as well as mRNA abundance of transcripts encoding amino acid transporters and taste receptors, intracellular signalling molecules, and hormones. Moreover, we assessed the hypothalamic mRNA abundance of neuropeptides involved in feed intake regulation. Feeding rainbow trout with LF did not result in marked alterations in parameters related to digestive function and amino acid sensing in the rainbow trout GIT, nor in the expression of gastrointestinal hormones (except *cck*) and hypothalamic neuropeptides. In contrast, supplementation of the LF diet with an umami taste-stimulating additive resulted in a general improvement of digestive or absorptive function (increased protein, dry matter and energy digestibility, and earlier peak in plasma amino acid levels) and activation of gut-brain axis mechanisms involved in feed intake regulation through the transcriptional activation of amino acid transporters, taste receptors, signalling molecules, and hormones. These results demonstrate that the dietary inclusion of umami receptor stimulants has the potential to improve fish physiological responses to the rise in levels of vegetable protein in the diet.

1. Introduction

One of the most important objectives in aquaculture research is to find new formulations that allow reducing fishmeal and fish oil as the main ingredients for aquafeed production. Accordingly, considerable research has been performed during the last years, looking for suitable ingredients for aquafeeds. A good alternative protein source must meet several criteria such as being nutritionally appropriate and digestible, attractive and palatable, and economically sustainable (Gatlin et al., 2007). Some of these ingredient sources are plant proteins, and soybean products, in particular, are one of the most widely used in diet formulations for many fish species (Glencross et al., 2020). However, its incorporation must be done with caution due to limitations of essential amino acids, and the presence of anti-nutritional factors (Gatlin et al., 2007; Hardy, 2010; Krogdahl et al., 2010). Anti-nutritional factors, such as gastrointestinal enzyme inhibitors among others, can decrease digestibility (alterations in enzyme activities) and amino acid absorption, induce intestinal inflammation like enteritis (Bakke-McKellep et al.,

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2000a; Urán et al., 2008), or even affect protein and lipid metabolism (Santigosa et al., 2008; Xu et al., 2016; Lazzarotto et al., 2018). These limitations can be reduced with the use of soybean protein concentrate (SPC) which, given the way it is processed, has reduced levels of anti-nutritional factors (Deng et al., 2006).

Feed intake is usually evaluated in studies dealing with the effect of inclusion of alternative ingredients in aquafeeds. The inclusion of plant proteins has been associated in some cases with a lowered feed intake and feeding efficiency, particularly in the more carnivore fish species (Gómez-Requeni et al., 2004; Hansen et al., 2007; Bonaldo et al., 2011). In other studies, the presence of soybean protein did not result in differences in growth and feed intake (Hernández et al., 2007; Bonaldo et al., 2011; Vélez-Calabria et al., 2021). However, only a few of those studies evaluated the simultaneous impact in feed intake and in parameters related to its regulation (Soengas et al., 2018), such as changes in levels of peripheral appetite-regulating hormones (like Ghrelin, Cck or Leptin) and hypothalamic neuropeptides (Npy, Agrp, Pomc, and Cart). Some examples are studies performed in dourado (*Salminus brasiliensis*, Sabioni et al., 2022), channel catfish (*Ictalurus punctatus*, Schroeter et al., 2018), grass carp (*Ctenopharyngodon idellus*, Liang et al., 2019), pacu (*Piaractus mesopotamicus*, Volkoff et al., 2017), red seabream (*Pagrus major*, Huong et al., 2020), turbot (*Scophthalmus maximus,* Dan et al., 2022), and Atlantic salmon (*Salmo salar*, Sissener et al., 2013). In addition to hormones and neuropeptides, gastrointestinal sensing of nutrients is also involved in feed intake regulation, as demonstrated in rainbow trout (*Oncorhynchus mykiss*) for amino acids (Calo et al., 2021, 2023a) and fatty acids (Calo et al., 2023b, 2023c). Few studies have assessed the impact of partial replacement of fish meal by vegetable ingredients in the function of these systems in fish (Ostaszewska et al., 2010a, 2010b; Brezas et al., 2021). Therefore, using as a model a fish species relevant in aquaculture such as rainbow trout, this study aims to characterize changes in digestive function (activity of proteases and capacity of transporters), and amino acid sensing mechanisms along the gastrointestinal tract (GIT) in response to dietary inclusion of a plant protein source like SPC. We also aim to further understand how these changes can modulate feed intake through appetite-regulating factors like GIT hormones and hypothalamic neuropeptides.

On the other hand, feed intake can be enhanced by additives (Hossain et al., 2023), which can help improve the reduced palatability of diets with increased amounts of vegetable protein (Bai et al., 2022). One promising strategy is to supplement feeds with substances stimulating umami taste receptors. These receptors are present in most animal species, both in oral and extraoral (including the GIT) tissues, being activated by an ample spectrum of amino acids, and potentiated by certain nucleotides (Nelson et al., 2002; Roura et al., 2008). In fish, umami or amino acid taste activation is even more remarkable, as in most fish species for which we have pharmacological evidence so far, the homologue of the sweet taste specific subunit (T1r2) is duplicated several-fold and forms functional receptor dimers with T1r3 that are broadly tuned to L-amino acids (Comesaña et al., 2020; Angotzi et al., 2020, 2022). The activation of these taste receptors by amino acids is key to enable the animal to identify protein sources and make sensible food choices (Morais, 2017). For this reason, in this study, we also assessed if the additional presence of an umami taste-stimulating additive alters the responses induced by partial fishmeal replacement by SPC.

2. Materials and methods

2.1. Fish

Juvenile rainbow trout of 60 ± 2 g body mass were obtained from a local fish farm (A Estrada, Spain; eggs originating from ACUINUGA S.L.) and housed in a flow-through system (flow rate of 1.5 L.min^{-1}) of 100 L tanks with well-aerated, dechlorinated tap water, filtered through a biological and mechanical system consisting of a vertical quartz sand filter and an activated carbon filter. Water quality parameters were monitored daily with values suitable for rainbow trout (pH 6.6–6.8, dissolved oxygen ≥7 mg.l^{-1}, ammonia concentration < 0.5 mg.l^{-1}, nitrite concentration < 0.2 mg.l^{-1}). During a 10-day acclimatisation period, fish were maintained under controlled conditions of photoperiod (12 h light: 12 h darkness) and temperature (15 °C) and were fed daily on a single dose of the commercial feed of the farm of origin (proximate analysis: 44% crude protein, 2.5% carbohydrates, 21% crude fat, and 17% ash; 20.2 MJ/kg of feed; Biomar, Dueñas, Spain) until visual satiety.

The experimental procedures described were carried out in accordance with the Guidelines of the Council of the European Union (2010/63) and the Spanish Government (RD 55/2013, RD 118/2021) for the use of animals in research. They were approved by Xunta de Galicia (ES360570181401/FUN01/FIS02/JLSF01) after receiving a favourable report from the Ethics and Animal Welfare Committee of University of Vigo. The procedures were performed by personnel with required training in a facility authorised by Xunta de Galicia (REGA ES360570181401).

2.2. Experimental design

2.2.1. Diets

Three diets were specifically formulated and prepared by Sparos (Portugal), as shown in Table 1. These diets were formulated to be isoenergetic (22 MJ.kg^{-1}), isoproteic (39% crude protein), and isolipidic (18% crude fat) with a pellet size of 4.5 mm. Diet composition was determined by manufacturer. Moreover, yttrium oxide was included in the diets at 0.2 g.kg^{-1} as an inert marker for the determination of apparent digestibility coefficients (ADCs). We used a diet with high fishmeal content and reduced levels of SPC (HF), a diet with a lower fishmeal content, partially replaced by SPC (LF), and a LF diet supplemented with 0.25% of an umami taste-stimulating additive (LFU). This

Table 1
Ingredients and proximate composition of the three experimental diets used to feed rainbow trout in this experiment.

		HF	LF	LFU
Ingredients (%)	Fishmeal Super Prime	20.00	10.00	10.00
	Soy protein concentrate	10.00	20.00	20.00
	Pea protein concentrate	4.00	4.00	4.00
	Wheat gluten	6.90	6.90	6.90
	Corn gluten meal	7.50	7.50	7.50
	Soybean meal 48	5.00	7.50	7.50
	Wheat meal	19.88	15.83	15.58
	Faba beans (low tannins)	5.00	5.00	5.00
	Vitamin and mineral premix	1.00	1.00	1.00
	Antioxidant	0.20	0.20	0.20
	Monocalcium phosphate	1.20	1.75	1.75
	L-Lysine HCl 99%	0.40	0.40	0.40
	Yttrium oxide	0.02	0.02	0.02
	Fish oil	7.56	7.96	7.96
	Soybean oil	11.34	11.94	11.94
	Umami taste-stimulating additive (1)			0.25
Proximate composition (% feed)	Crude protein	39.0	39.0	39.0
	Crude fat	21.0	21.0	21.0
	Fiber	1.4	2.0	2.0
	Starch	17.9	16.3	16.1
	Ash	6.4	6.2	6.2
	Gross energy (MJ/kg feed)	22.0	22.0	22.0

(1) **Luctamax Umami Fish** from Lucta S.A. (Spain)
HF, diet with high fishmeal content and reduced levels of soy protein concentrate.
LF, diet with lower fishmeal content partially replaced by soy protein concentrate.
LFU, LF diet supplemented with an umami taste-stimulating additive.

additive (Luctamax Umami Fish from Lucta S.A., Spain) contains sources of amino acids and nucleotides, as well as peptides, from synthetic and natural (yeast extracts and vegetable protein hydrolysates) origins.

2.2.2. Feeding trial and tissue collection

Fish were distributed into 3 tanks per dietary treatment ($n = 60$/diet, $n = 20$/tank) and feed intake was measured for 4 weeks, registering also the initial and final weight of each tank for the estimation of weight gain. Fish were fed once per day at the same hour (10:00) until visual satiation. After feeding with a known amount of feed, uneaten pellets were removed, dried, and weighed to calculate the amount of feed consumed as the difference from feed offered. The feeding rate was readjusted daily depending on the amount of uneaten feed pellets recovered from the tank. At the end of the feed intake trial, and after 48 h of fasting, fish were sampled at 4 different times. The first sampling was performed after the 48 h fasting period (0 h), to obtain a basal reference of every parameter to be assessed, collecting all 3 parts of the GIT assessed in this study (stomach, proximal intestine and distal intestine) as previously described (Calo et al., 2023a). Subsequently, the remaining fish were fed again with their corresponding diets. Then, we sampled the following postprandial periods: 1 h, to assess changes in parameters in stomach and proximal intestine; 4 h, as an intermediate point to evaluate all 3 regions; and finally, 24 h, to focus on proximal and distal intestines. The sampling times were established based on Bogevik et al. (2021), according to where feed could be found in the GIT. Moreover, samples of the hypothalamus and blood were collected every time.

At each sampling time, 8 fish per diet were captured and anaesthetized in buffered tricaine methane sulphonate (MS-222). In each sampling point the fish were captured from one of the replicate tanks per treatment to minimize stress. Collected samples were immediately frozen in dry ice and stored at −80 °C until further analysis.

2.2.3. Chemical analysis, digestibility and zootechnical parameters calculation

After tissue collection, fish remaining in each tank were fed for an additional 1 week to obtain faecal samples. Fish were fed to satiation and, after 5 h, anaesthetized in tanks, and samples removed by manual stripping excluding urinary excretion. The same process was repeated after 7 days to obtain again faeces from each tank. Faeces from each tank obtained at both times were pooled to have 1 sample per tank, three samples per treatment.

Diets and faeces were analysed for dry matter, protein, and fat, in duplicates, following the methods described by AOAC (2006). Dry matter was measured after 24 h at 105 °C (method 930.15); ash was quantified after combustion (550 °C for 6 h, method 942.05); crude protein (N × 6.25, method 990.03) was measured by automatic flash combustion (Leco FP428; Leco Corporation, Michigan, USA); fat was determined using the Soxhlet (dichloromethane) extraction (method 920.39). Gross energy was measured in an adiabatic bomb calorimeter (Werke C2000 basic; IKA, Staufen im Breisgau, Germany). Yttrium oxide concentrations in diets and faeces were determined by SGS Multilab (Évry, France) using inductively coupled plasma atomic emission spectroscopy (ICP-AES Bartlesville, USA).

Apparent digestibility coefficient (ADC) was calculated as follows: ADC (%) = 100 – ((% nutrient in faeces/% nutrient in diet) × (% yttrium in diet/% yttrium in faeces)).

Feed conversion ratio (FCR) was calculated as: Total feed intake (kg) divided by the biomass growth in the tank (kg final biomass – kg initial biomass).

Weight gain rate (WGR) was calculated as: ((final weight) - (start weight))/ (start weight) × 100.

2.3. Assessment of levels of metabolites in plasma

Levels of glucose and lactate in plasma ($n = 8$) were measured by enzymatic determination using commercial kits (Spinreact, Barcelona, Spain). Total α-amino acid levels in plasma (n = 8) were assessed using the colorimetric nynhydrin method (Moore, 1968) with alanine as standard.

2.4. Quantification of mRNA abundance by real-time PCR

Total RNA was extracted by mechanical homogenization from tissues of 8 fish from each group using Trizol reagent (Life Technologies, Grand Island, NY, United States). After checking RNA purity by optical density (OD) absorption ratio (OD 260 nm/280 nm) using a NanoDrop 2000c (Thermo, Vantaa, Finland), cDNA was synthesized from 2 μg of total RNA using Superscript II reverse transcriptase and random hexamers (Promega) in a final volume reaction of 50 μL. Real-time quantitative PCRs were performed in an iCycler iQ (Bio-Rad, Hercules, CA, United States), using MAXIMA SYBR Green qPCR Mastermix (Life Technologies). Transcripts were amplified in duplicate using a 96-well plate loaded with 1 μL of cDNA (or water and RNA for controls) and 500 nM of each forward and reverse primer in a final volume of 10 μL. Thermal cycling was initiated with incubation at 95 °C during 10 min, followed by 40 cycles consisting of heating at 95 °C for 15 s and specific annealing and extension temperatures (transcript-dependent) for 30 s. Following the final PCR cycle, melting curves were systematically performed and monitored (temperature gradient at 0.5 °C/5 s from 70 to 94 °C) to ensure that only one fragment was amplified. Relative expression of target transcripts was calculated using *actb* (β-actin) and *eef1a1* (elongation factor 1α) as references, both stably expressed in this experiment, following the Pfaffl (2001) method. Sequences of primers used for target and reference genes are shown in Table 2, and correspond to previously described primers in the same species (To et al., 2019; Brezas et al., 2021; Calo et al., 2021). For qPCRs of tissue distribution study, representative samples of each tissue were also run on 2% agarose gels. In the case of primers not previously tested (*slc43a2*), a conventional PCR was carried out, and run on 2% agarose gels to confirm the amplification of a single product of the expected size. Then, a real-time PCR was performed to check primers efficiency (90–120%).

2.5. Assessment of enzyme activities

2.5.1. Assessment of pepsin activity in stomach

Pepsin activity in the stomach was assessed following the protocol described in Rungruangsak-Torrissen et al. (2006). Samples were homogenized by mechanical disruption with 3 times their weight in ultrapure water. Then, homogenates were centrifuged at 12,000×*g* for 30 min at 4 °C, and the crude enzyme extract in the supernatant was collected and kept at −80 °C. For assaying enzyme activity, 200 μL of 1% casein substrate (prepared in 60 mM HCl) was added to 200 μL of crude enzyme extract, previously diluted 10 times. Following 15 min incubation at 37 °C, the reaction was stopped by adding 1 mL of 5% trichloroacetic acid. The mixture was left at room temperature for 30 min, and then centrifuged for 20 min at 5000×*g*. Then, 0.5 mL of supernatant was collected and 1 mL of 0.5 M NaOH was added, followed by 0.3 mL of Folin-Ciocalteau reagent (1:3 dilution). After 10 min at room temperature, the absorbance was measured spectrophotometrically at 720 nm in a 96-well plate in an INFINITE 200 Pro microplate reader (TECAN, Germany), and compared with L-tyrosine standard curve.

2.5.2. Assessment of trypsin and chymotrypsin activities in proximal intestine

Crude enzyme extract was obtained from proximal intestine, as described above for stomach. For trypsin activity assaying (Busti et al., 2020), 15 μL of crude enzyme extract (diluted 5 times) was added to 200 μL of BAPNA substrate (diluted to working concentration) with a buffer containing 50 mM Tris-HCl (pH 8.5) and 20 mM $CaCl_2$. For chymotrypsin activity assaying (Rungruangsak-Torrissen et al., 2006), 25 μl of crude enzyme extract (diluted 1/150) was added to 175 μL of substrate

Table 2
Primers used for assessing mRNA abundance of specific transcripts with their GenBank accession numbers (except Tigr for *pomca1*) and annealing temperature (T).

Transcript	Accession number	Forward primer	Reverse primer	T (°C)
actb	NM 001124235.1	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59
agrp1	NM_001146677	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60
cartpt	XM_021596562.2	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60
cck	NM_001124345.1	GGGTCCCAGCCACAAGATAA	TGGATTTAGTGGTGGTGCGT	60
eef1a1	AF498320	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59
gcg	NM_001124698.1	AGGAGTGGTGCTCCATCCAAA	TCCTGATTTGAGCCAGGAAACA	59
ghrl	AB096919.1	GGTCCCCTTCACCAGGAAGAC	GGTGATGCCCATCTCAAAAGG	60
gnai1	CU073912	GCAAGACGTGCTGAGGACCA	ATGGCGGTGACTCCCTCAAA	60
gprc6a	XM_021574849.1	ATGGGGATCAGCAGAATTTGG	CCGGCACCTTGTTTCTCTTTG	60
itpr3	XM_021616029.1	GCAGGGGACCTGGACTATCCT	TCATGGGGCACACTTTGAAGA	59
npy	NM_001124266	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	59
plcb3	XM_021577635.1	ATAGTGGACGGCATCGTAGC	TGTGTCAGCAGGAAGTCCAA	60
pomca1	XM_036963012.1	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60
pyy	XM_021557532.1	GGCTCCCGAAGAGCTGGCCAAATA	CCTCCTGGGTGGACCTCTTTCCA	60
slc1a1	XM_036958100.1	CCATGACAGTGGTAGAGAAAGG	GTACAAGACAACGCGCAAAG	60
slc1a5	XM_021587427.2	AAAGAGTCGGTCATGTAGAG	GAGAGAAGACACAAGGAGAG	60
slc7a7	XM021614955.1	GAGGACTCAACGCTTCTATC	CAACACACAGGTAGACCAA	60
slc7a9	XM_036964115.1	CTTCACCAGGAAGGAACTCAA	CTTGTCTATGATCGGTGCTAGG	60
slc6a19	XM_036971451.1	GGTCCATCCTGTTCTTCAT	TGACACCAGACAGACAATAC	60
slc15a1	XM_036973781.1	GTTTGAAGACCACCAGGAGAA	GACAGTAGACAGGAGACTACCA	60
slc36a1	XM_036969760.1	GGCTGAGAAGGCACTCAATA	GTCACTGAGGAAGACGAAGTAG	60
slc43a2	XM_021582086.2	GGATCGAGCGACCCCAAGAA	GGAAGGTGACAGCGGAGGAT	60
tas1r1	XM_021614415.1	GTTGTGTTCTCCAGCAAAAGC	TCTGTCCCTATCCACACCTTG	60
tas1r2a	MT240253	ATAGTTTTTGCCGGGCAGAGC	CCTGCAATCCACACTTTGCTG	59
tas1r2b	XM_021625831.1	GATGAGTGGGCCAGGAATGG	CCTCCCACCGGCTGACTTTA	59

actb, gene encoding β-actin; *agrp1*, gene encoding agouti-related protein 1; *cartpt*, gene encoding cocaine- and amphetamine-regulated transcript prepropeptide; *cck*, gene encoding cholecystokinin; *eef1a1*, gene encoding elongation factor 1α; *gcg,* gene encoding proglucagon; *ghrl*, gene encoding ghrelin; *gnai1*, gene encoding guanine nucleotide-binding protein G subunit alpha 1; *gprc6a*, gene encoding G-protein-coupled receptor family C group 6 member A; *itpr3*, gene encoding inositol 1,4,5-trisphosphate receptor type 3; *npy*, gene encoding neuropeptide Y; *plcb3*, gene encoding phospholipase C-β3; *pomca1*, gene encoding proopiomelanocortin a1; *pyy*, gene encoding peptide tyrosine-tyrosine; *slc1a1*, gene encoding excitatory amino acid transporter 3 (EAAT3); *slc1a5*, gene encoding neutral amino acid transporter b(0) (AAAT); *slc7a7*, gene encoding Y^+L amino acid transporter 1 (Y^+LAT-1); *slc7a9*, gene encoding b(0,+)-type amino acid transporter 1 (B^{0+}AT1); *slc6a19*, gene encoding sodium-dependent neutral amino acid transporter b(0)at1 (B^0AT1); *slc15a1*, gene encoding solute carrier family 15 member 1 (Pept1); *slc36a1*, gene encoding proton-coupled amino acid transporter 1 (Pat1); *slc43a2,* gene encoding large neutral amino acid transporter small subunit 4 (Lat4); *tas1r1/tas1r2a/tas1r2b*, genes encoding taste receptor 1 family members 1, 2a, and 2b.

Succinyl-Ala-Ala-Ala-Pro-Phe-p-nitroanilide diluted to working concentration with 0.2 M Tris-HCl buffer (pH 8.5). Once 96-well plates were loaded, reactions were allowed to proceed at 405 nm, 37 °C for 15 min. Reaction rates of enzymes were determined by the appearance of p-nitroaniline per min and per mg protein, which was assayed in homogenates according to the bicinchoninic acid method with bovine serum albumin (Sigma-Aldrich) as standard.

2.6. Statistical analysis

We used the statistical software GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California, USA). After verifying that the ANOVA assumptions were met, two-way ANOVA was performed on the different variables, with dietary condition and time as main factors. *P*-values of two-way ANOVA are shown in Table 3. When significant interaction occurred, groups were compared by post-hoc Student-Newman-Keuls test. Statistical differences among diets for feeding efficiency, growth and ADCs were assessed by one-way ANOVA followed by post-hoc Student-Newman-Keuls test.

3. Results

Survival was 95–98% across treatments during the experimental period (data not shown). No significant differences occurred in feed intake, either expressed as cumulative feed intake (Fig. 1A) or as a percentage of average body weight per day (Fig. 1D). The mRNA abundance of hypothalamic appetite-regulating neuropeptides *npy* (Fig. 1B), *cartpt* (Fig. 1C), *agrp1* (Fig. 1E), and *pomca1* (Fig. 1F) was also not significantly different among diets. However, values of *npy* in fish fed the LFU diet were significantly increased at 4 h compared to its basal level at 0 h (Fig. 1B).

No differences were observed in feed conversion ratio and specific growth rate among dietary treatments (Table 4). Digestibility of dry matter, protein and energy was significantly higher in fish fed the LFU diet than in fish fed the HF and LF diets (Table 4). Conversely, fat digestibility was lower in fish fed the LFU diet than in the other two treatments. Moreover, digestibility of dry matter was lower in fish fed the LF diet compared with the HF and LFU treatments.

The activity of GIT enzymes is shown in Fig. 2. Pepsin activity in stomach (Fig. 2A) increased in fish fed the LF and LFU diets compared with those fed HF 1 h after feeding, which was also significantly higher than that at 0 h in the LF group. No significant changes occurred in the activity of trypsin (Fig. 2B) and chymotrypsin (Fig. 2C) in proximal intestine.

Fig. 3 shows changes in metabolite levels in plasma. Glucose levels (Fig. 3A) increased 24 h after feeding the LFU diet compared with fish fed the other diets, and also compared with fish fed the same diet at 0 h, 1 h, and 4 h. Lactate levels did not show significant changes (Fig. 3B). Levels of amino acids (Fig. 3C) after 4 h were significantly higher in fish fed the LFU diet compared with fish fed the other diets. Moreover, fish fed the LF diet displayed higher values than fish fed the HF diet at 4 h and 24 h after feeding. Changes in plasma amino acid levels over time showed differences depending on the treatment. While fish fed the HF diet displayed a significant reduction at 4 h compared with 0 h, 1 h and 24 h, in fish fed the LF diet values at 4 h were higher than those at 1 h, and in fish fed the LFU diet amino acid levels were significantly raised at 4 h compared to 0 h and 1 h (Fig. 3C).

The dietary treatments also produced significant changes in mRNA abundance of a peptide transporter and of transporters of amino acid of cationic and anionic nature in intestinal regions (Fig. 4). In proximal intestine, *slc15a1* (also known as *pept1*) was up-regulated at 4 h after feeding in fish fed the LF diet compared with fish fed the other diets, and this value was also higher than in fish fed the same diet at the other sampling times (Fig. 4A). An up-regulation of *slc7a9* was also observed

Table 3
P-values obtained after two-way analysis of variance. Dietary condition (D) and time (T) are the main factors, and dietary condition x time (D x T) is the first order interaction.

			Diet	Time	D x T
	Tissue	Parameter			
Metabolites	Plasma	Glucose	0.0088	<0.0001	0.0011
		Lactate	0.0154	0.7507	0.9885
		Amino acids	0.0582	<0.0001	<0.0001
Enzyme activities	Stomach	Pepsin	0.0391	0.0023	0.1401
	Proximal intestine	Trypsin	0.1801	0.2549	0.6783
		Chymotrypsin	0.0270	0.0388	0.2740
Neuropeptides	Hypothalamus	*npy*	0.5151	0.0098	0.1998
		agrp1	0.0608	0.5179	0.9737
		cartpt	0.9109	0.2614	0.2636
		pomca1	0.3218	0.3010	0.1621
		tas1r1	0.3593	0.6526	0.9693
	Stomach	*tas1r2a*	0.0080	0.1336	0.3893
		tas1r2b	0.2392	0.6845	0.4700
		tas1r1	0.1305	<0.0001	0.0044
Receptors	Proximal intestine	*tas1r2a*	0.0231	0.0477	0.2773
		tas1r2b	0.1801	0.0111	0.0528
		tas1r1	0.4504	0.0747	0.4469
	Distal intestine	*tas1r2a*	0.0028	0.6733	0.8796
		tas1r2b	0.0575	0.3487	0.7211
		gprc6a	0.4477	0.0619	0.5897
		gnai1	0.0046	0.0265	0.3735
	Stomach	*plcb3*	<0.0001	0.0017	0.0126
		itpr3	<0.0001	0.0044	0.0176
		gnai1	0.6293	<0.0001	0.0013
Cell signalling	Proximal intestine	*plcb3*	0.7567	0.0003	0.0092
		itpr3	0.3377	0.0500	0.0403
		gnai1	0.1680	0.0195	0.1641
	Distal intestine	*plcb3*	0.0267	0.0087	0.0335
		itpr3	0.3159	0.0006	0.0460
	Stomach	*ghrl*	0.0098	0.0065	0.1187
		cck	0.0004	<0.0001	<0.0001
Hormones	Proximal intestine	*pyy*	0.0039	0.4802	0.2438
		gcg	0.1483	0.6680	0.1211
	Distal intestine	*cck*	0.0115	0.0027	0.0002
		gcg	0.8879	0.3103	0.7024
		slc1a5	0.0002	0.0133	0.0052
	Stomach	*slc36a1*	0.5233	0.8801	0.5203
		slc43a2	0.0041	0.0003	0.0020
		slc15a1	0.0147	0.0138	0.0123
		slc7a9	0.0140	0.0027	0.1082
		slc7a7	0.0412	0.0322	0.0215
	Proximal intestine	*slc1a5*	<0.0001	0.0062	0.0063
Transporters		*slc36a1*	0.0839	0.0868	0.1612
		slc43a2	0.3994	0.0271	0.0138
		slc6a19	0.0347	<0.0001	0.0002
		slc1a1	0.2778	0.5477	0.0038
		slc7a9	0.0053	0.0729	0.0005
	Distal intestine	*slc36a1*	<0.0001	0.0002	<0.0001
		slc43a2	0.1029	0.1525	0.0098
		slc6a19	0.3782	0.1245	0.2075

after 4 h, in this case in fish fed the LFU diet compared with the HF group, and this value was also higher than in fish fed the same diet at 1 and 24 h (Fig. 4B). Also in proximal intestine, the expression of *slc7a7* was higher in fish fed the LFU diet than in fish fed HF diet at 1 h after feeding, and higher than the other two diets at 24 h; within the LFU group, values were significantly higher at 1 h and 24 h than at 0 h and 4 h (Fig. 4C). In distal intestine, the expression of *slc1a1* (Fig. 4D) was significantly increased at 4 h in fish fed the LF diet compared with fish fed the other diets, whereas after 24 h fish fed the LFU diet displayed elevated mRNA levels compared to the other diets; in both cases values were also higher than those of fish fed the same diets at the other sampling times. Finally, also in distal intestine, the mRNA abundance of *slc7a9* was higher after 4 h in fish fed the LF diet than both other groups, and higher than in fish fed the same diet at the other times assessed (Fig. 4E).

Changes in mRNA abundance of transporters for neutral amino acids are shown in Fig. 5. In the stomach, the values of *slc1a5* (Fig. 5A) were higher at 0 h in the LFU group than in the other groups at the same time, or at the other times assessed. In the same tissue, no changes occurred in *slc36a1* (Fig. 5B). The expression of *slc43a2* (Fig. 5C) was elevated at 0 h in the LF group compared to the other groups, or with the same treatment at the other sampling times, whereas in fish fed the HF diet *slc43a2* expression was higher than in the other groups at 4 h after feeding. In proximal intestine, the expression of *slc1a5* (Fig. 5D) was higher in fish fed the LFU diet than in fish fed the HF (at 1 h, 4 h, and 24 h) and those fed the LF (at 1 h and 24 h) diets; furthermore, within the LFU group, values were higher at 1 h and 24 h than at 0 h and 4 h. In the same tissue, no changes occurred in *slc36a1* (Fig. 5E). The levels of *slc43a2* (Fig. 5F) in proximal intestine were elevated at 24 h after feeding in fish fed the LFU diet compared with fish fed the other diets at the same time, and its expression was also higher than in fish fed the same diet at 0 and 4 h. Also in proximal intestine, the expression of *slc6a19* was higher in the LFU group than in fish those feeding on the other diets at 24 h, or in fish fed the same diet at the other times assessed (Fig. 5G). At 0 h there were

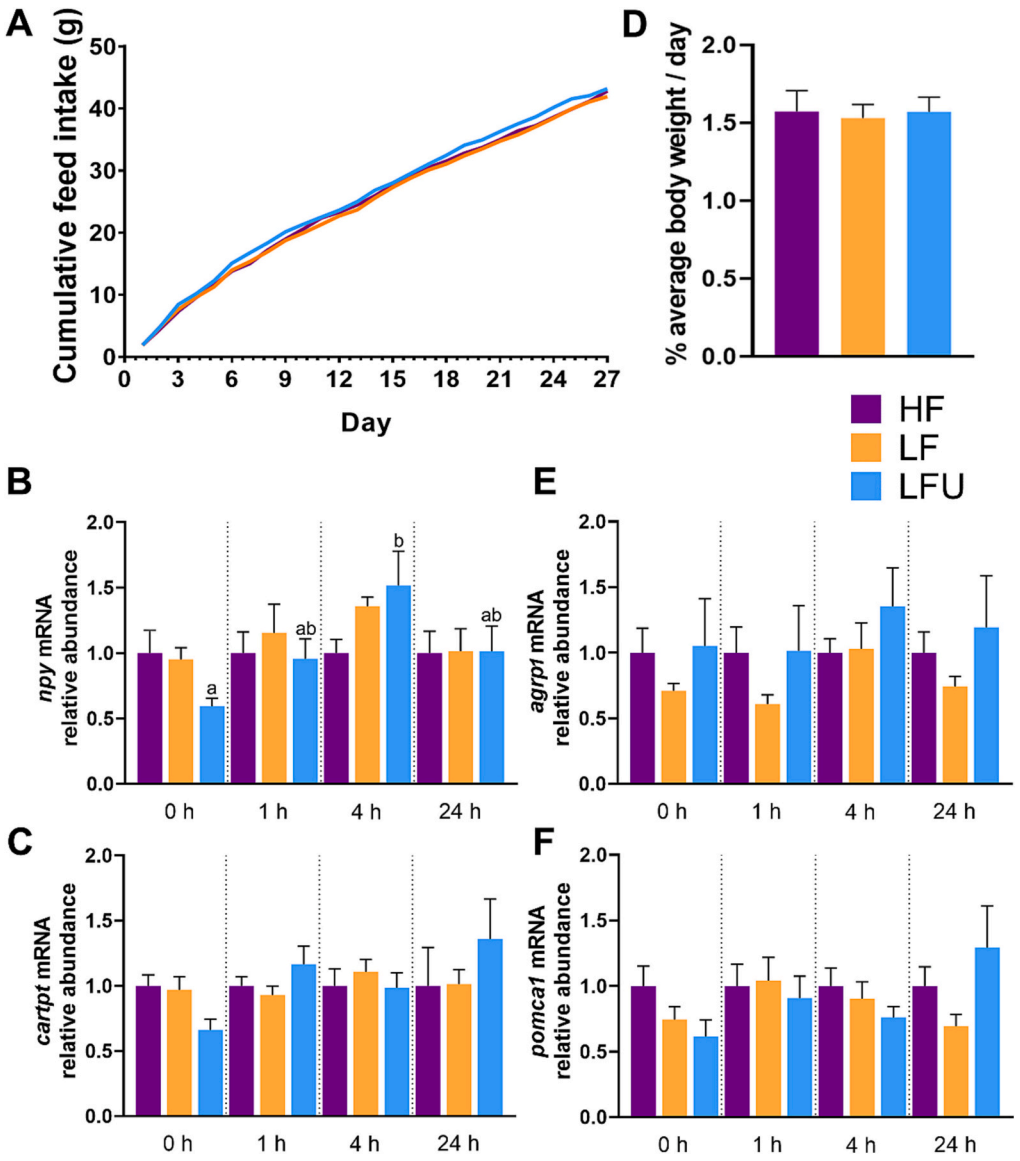

Fig. 1. Feed intake in rainbow trout fed either a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Feed intake during 27 days is shown as cumulative values (A), and % average body weight per day (D). In the later data are shown as mean ± SEM of $n = 3$ tanks per treatment. Effects of feeding diets on mRNA abundance of key appetite-regulating neuropeptides is indicated for npy (B), cartpt (C), agrp1 (E), and pomca1 (F) in the hypothalamus after 48 h of fasting (0 h), and 1 h, 4 h and 24 h after feeding. Quantification of mRNA abundance was performed by RT-qPCR, normalizing data to mRNA abundance of actb and eef1a1 as reference genes. Data are shown as mean ± SEM of $n = 8$ fish per treatment relative to the HF group. Statistical differences among groups were assessed by two-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment.

also significant differences in the expression of *slc6a19*, which was significantly lower in fish fed the LF and LFU diets compared to the HF group, subsequently increasing in the following sampling times, significantly at 4 h and 24 h in both LF and LFU groups (Fig. 5G). In distal intestine, values of *slc36a1* (Fig. 5H) and *slc43a2* (Fig. 5I) increased at 4 h after feeding in fish fed the LF diet compared to fish fed the other diets, and their expression was also higher than in fish from the same group at 0 h and 24 h for *slc36a1*, and at 24 h for *slc43a2*. Finally, no changes occurred for *slc6a19* (Fig. 5J).

Changes occurring in mRNA abundance of taste receptors along the GIT are shown in Fig.6. In stomach, no changes were noted for *tas1r1* (Fig. 6A) and *tas1r2b* (Fig. 6C) whereas *tas1r2a* (Fig. 6B) was up-regulated in fish fed the LFU diet at 4 h after feeding, relative to the other treatments and sampling times. In proximal intestine, *tas1r1* (Fig. 6D) increased after 1 h in the LFU group compared with fish fed the HF and LF diets, and values were higher than in fish fed the same diet at the other times assessed. Similarly, *tas1r2a* mRNA abundance (Fig. 6E) increased after 1 h in fish fed LFU diet, but differences were only significant compared to the LF group, and with 0 h and 4 h within the same treatment. Also in proximal intestine, *tas1r2b* mRNA levels (Fig. 6F) increased after 4 h in fish fed the LF diet compared with fish fed the other two diets, which was higher than in fish from the same group at the other times assessed. In distal intestine, no changes occurred in *tas1r1* (Fig. 6G), *tas1r2b* (Fig. 6I), and *gprc6a* (Fig. 6J) expression. However, the mRNA abundance of *tas1r2a* was lower in the distal intestine of fish fed the LFU diet compared with those fed the HF diet at 0 h, whereas fish fed the LF diet displayed lower values than those fed the HF diet at 4 h (Fig. 6H).

The mRNA abundance of intracellular signalling molecules responsible for transducing taste signals downstream from the activation of

Table 4
Feed conversion ratio (FCR), weight gain rate (WGR), and apparent digestibility coefficients (ADC) of dry matter, protein, fat, and energy in rainbow trout fed a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content partially replaced by soy protein concentrate (LF), and LF diet supplemented with an umami taste-stimulating additive (LFU) for 27 days.

		HF	LF	LFU
Initial weight (g)		59.1 ± 2.29	61.24 ± 2.09	62 ± 1.93
Final weight (g)		99 ± 5.79	107.02 ± 5.28	105.36 ± 4.69
FCR		1.017 ± 0.001	0.857 ± 0.055	0.946 ± 0.054
WGR		52.9 ± 5.79	64.52 ± 10.29	58.55 ± 8.72
ADC (%)	Dry matter	57.99 ± 0.53 a	53.94 ± 0.56 b	66.37 ± 0.65 c
	Protein	84.68 ± 0.47 a	83.99 ± 0.04 a	90.45 ± 0.13 b
	Fat	90.25 ± 0.05 a	91.72 ± 0.94 a	86.57 ± 0.27 b
	Energy	69.61 ± 0.67 a	68.46 ± 0.22 a	74.97 ± 0.47 b

FCR calculated as total feed intake divided by the biomass growth in the tank. WGR calculated as ((final weight) - (start weight)) / (start weight) × 100. Data are shown as mean ± SEM of n = 3 tanks per treatment for FCR, WGR, and ADC. For ADC, faeces samples from each tank result from pooling faeces obtained at two different times. Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among dietary treatments.

taste G-coupled protein receptors, including TAS1R's, in the GIT of vertebrates is shown in Fig. 7. In stomach, the expression of *gnai1* was higher in fish fed the LFU diet compared with those fed the HF diet at 0 h and 4 h, and within the LFU group its expression was also significantly higher at 4 h in relation to 1 h after feeding (Fig. 7A). In the same tissue, the mRNA abundance of both *plcb3* (Fig. 7B) and *itpr3* (Fig. 7C) was elevated in fish fed the LFU diet compared with fish fed the other two diets at 0 h and 4 h, and levels were also higher than in fish fed the same diet at 1 h. In proximal intestine, *gnai1* mRNA abundance increased in fish fed the LFU diet 1 h after feeding compared with fish fed the other diets at the same time, wich was higher in this group at 1 h than at the other times assessed (Fig. 7D). In the same tissue, the expression of *plcb3* (Fig. 7E) increased in fish fed the LFU diet compared with those fed the HF diet at 1 h, and this value was also higher than in fish fed the same diet at 0 h and 24 h. Additionally, at 4 h the mRNA abundance of *plcb3* was higher in both the LF and LFU groups, compared to HF, being also significantly higher than the expression at 0 h in fish fed the LF diet. Also in proximal intestine, the mRNA abundance of *itpr3* decreased in fish fed the LFU diet compared with those fed the other diets at 24 h, and this value was also lower than that of fish fed the same diet at 0 h and 1 h (Fig. 7F). In distal intestine, *gnai1* mRNA abundance was higher at 4 h than at 24 h in fish fed the LF diet, and higher at 0 h than at 24 h for fish fed the LFU diet (Fig. 7G). In the same tissue, the expression of *plcb3* 4 h after feeding the LF diet was higher than in the HF and LFU groups at the same time, and higher than in fish fed the same diet after 24 h (Fig. 7H). In the case of fish fed the LFU diet, *plcb3* expression was higher at 0 h than at 24 h. Finally, the abundance of *itpr3* (Fig. 7I) at time 0 h was higher in fish fed the LFU diet compared to fish fed the HF diet, which was also higher at 0 h than at 4 h (in fish feed LF) and at 0 h compared to

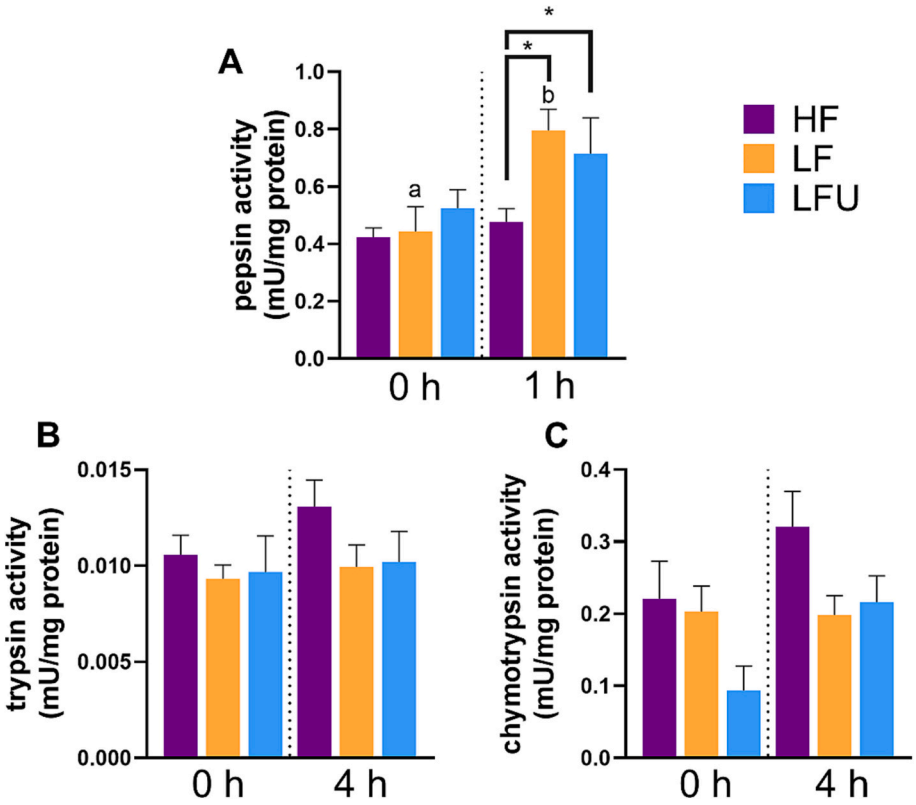

Fig. 2. Enzyme activities of pepsin in the stomach (A) and trypsin (B) and chymotrypsin (C) in proximal intestine of rainbow trout after 48 h fasting (0 h), and after 1 h (pepsin) or 4 h (trypsin and chymotrypsin) of feeding a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Data are shown as mean ± SEM of $n = 8$ fish per treatment. Statistical differences among groups were assessed by two-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment. Significant differences ($p < 0.05$) among dietary treatments within sampling times are denoted by bars with asterisks.

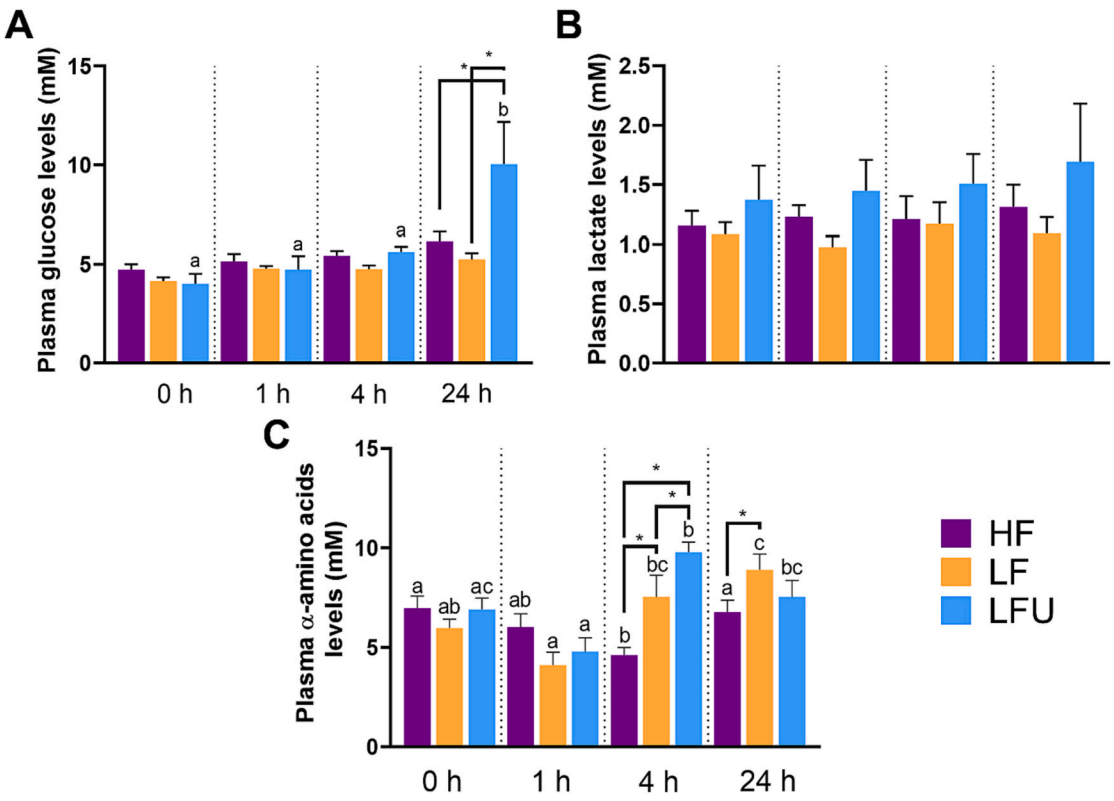

Fig. 3. Plasma levels of glucose (A), lactate (B) and α-amino acid (C) in rainbow trout after 48 h fasting (0 h) and 1 h, 4 h and 24 h after feeding a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Data are shown as mean ± SEM of $n = 8$ fish per treatment. Statistical differences among groups were assessed by *two*-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment. Significant differences ($p < 0.05$) among dietary treatments within sampling times are denoted by bars with asterisks.

4 h and 24 h (in fish fed LFU).

Changes in mRNA abundance of gastrointestinal hormones are shown in Fig. 8. In stomach, levels of *ghrl* increased 4 h after feeding in fish fed the LFU diet compared with fish fed the HF diet at the same time, and levels were also higher than in fish fed the same diet at 0 and 1 h (Fig. 8A). In proximal intestine, the abundance of *cck* mRNA increased after 1 h in fish fed the LF diet compared with fish fed the other diets, which was higher when compared with fish fed the same diet at the other times (Fig. 8B). The mRNA abundance of *pyy* decreased at 4 h after feeding in fish fed the LFU diet compared with fish fed the HF diet (Fig. 8C). In the same tissue, no changes occurred in mRNA abundance of *gcg* (Fig. 8D). In distal intestine, the expression of *cck* (Fig. 8E) was higher 24 h after feeding in fish fed the LFU diet compared with those fed the other diets, which was also higher than in fish fed the same diet at 0 h and 4 h. Finally, no changes occurred in mRNA abundance of *gcg* in distal intestine (Fig. 8F).

4. Discussion

Rainbow trout successfully tolerates diets with soybean protein levels above 20–30% (Burrells et al., 1999) as long as they are adequately supplemented. The low availability of essential amino acids in most plant proteins makes it crucial to supplement plant-based diets with some key amino acids such as methionine and lysine (Sveier et al., 2001; Rolland et al., 2015; Deborde et al., 2021). However, these supplementations do not improve feed intake, which can be seriously affected by the poor palatability of plant proteins (Mambrini et al., 1999). The addition of natural feeding attractants such as squid and krill meal, which play an important role as olfactory and gustatory stimulants, has been seen to improve feed intake, as demonstrated for instance in red sea bream fed with diets where fishmeal was partially replaced with soybean meal (Kader et al., 2012). In our study, we tested supplementing the LF diet with a sensory additive (LFU diet) that was specifically formulated to contain amino acids, nucleotides and peptides, from synthetic and natural (yeast extracts and vegetable protein hydrolysates) origins. These substances are known to activate umami taste receptors in vertebrates (Carr et al., 1996; Servant and Frerot, 2022), and hence are good candidates to enhance the palatability and attractiveness of aquafeeds and, accordingly, improve feed intake. However, no significant differences were observed between any of the treatments, including the HF and LF diets, in terms of daily feed intake or final average body weight and specific growth rate. Feed conversion ratio was also not significantly affected, although FCR was slightly lower in fish fed the LF than in those fed the HF diet. Although the duration of the feeding trial and fish weight gain achieved in that period is not sufficient to enable concluding on the effects of diets in terms of growth and feeding performance, the observed results are in line with available studies in which comparable replacement levels of fishmeal by SPC in rainbow trout (Mambrini et al., 1999) or soybean meal in rainbow trout (Dalsgaard et al., 2012) and Atlantic salmon (Øverland et al., 2009) did not affect feed intake and growth performance. However, the feed intake results were not fully consistent with the mRNA abundance of hypothalamic neuropeptides involved in the homeostatic regulation of feed intake, particularly with that of *npy*, which is one of the most potent orexigenic peptides found in vertebrate brains including fish (Soengas et al., 2018). The mRNA abundance of *npy* in the hypothalamus of fish fed the LFU diet was significantly raised above its basal (0 h) level 4 h after feeding, which suggests a potential feed intake stimulatory effect of this diet. Rises in the level of *npy* in the brain have been described to decrease the latency and increase the motivation to eat, or to delay

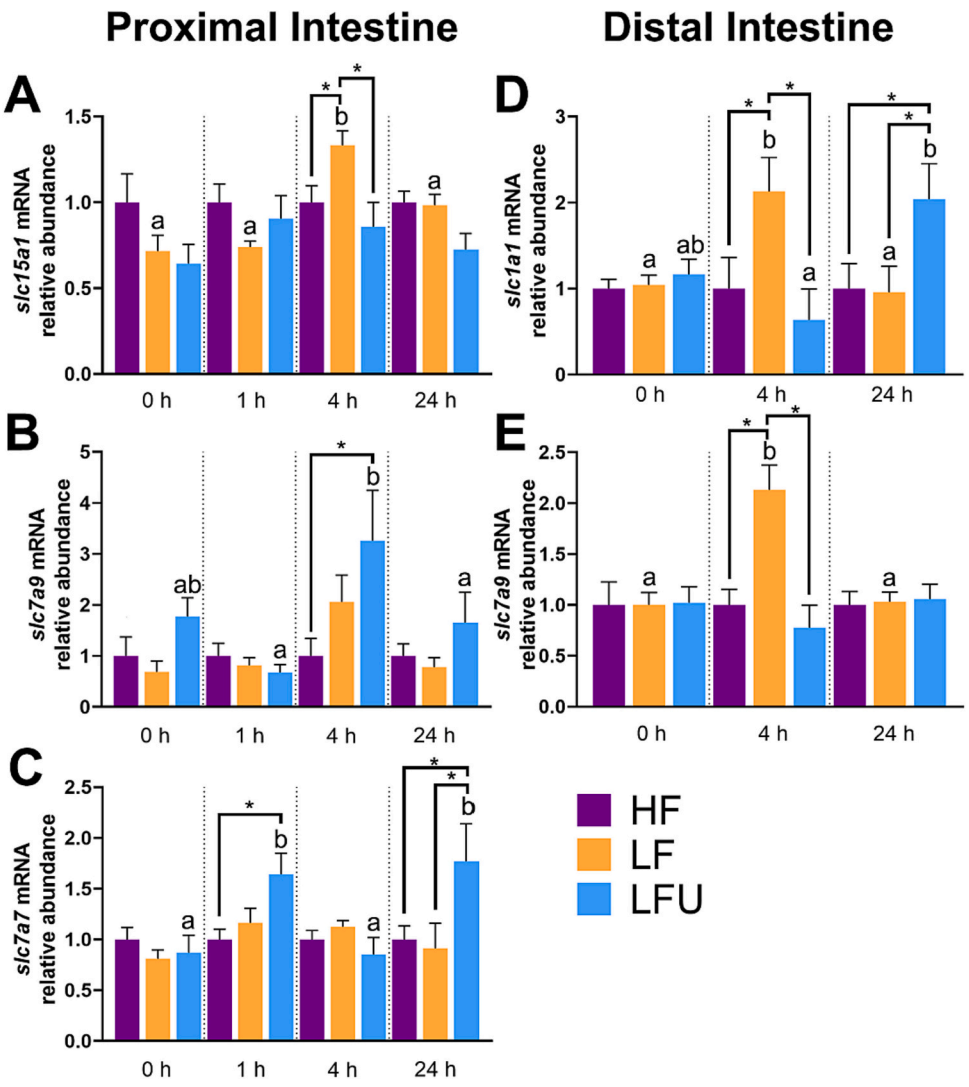

Fig. 4. mRNA abundance of *slc15a1* (gene encoding peptide transporter Pept1) in proximal intestine (A), *slc7a9* (gene encoding cationic amino acid transporter $B^{0+}AT1$) in proximal (B) and distal (E) intestine, *slc7a7* (gene encoding cationic amino acid transporter $Y^{+}LAT$-1) in proximal intestine (C), and *slc1a1* (gene encoding anionic amino acid transporter EAAT3) in distal intestine (D) of rainbow trout after 48 h fasting (0 h) and 1 h, 4 h and 24 h after feeding a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Quantification of mRNA abundance was performed by RT-qPCR, normalizing data to *actb* and eef1a1 reference genes. Data are shown as mean ± SEM of $n = 8$ fish per treatment relative to the HF group. Statistical differences among groups were assessed by two-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment. Significant differences ($p < 0.05$) among dietary treatments within sampling times are denoted by bars with asterisks.

satiety by augmenting meal size in vertebrates (Beck, 2006). However, the fact that this was not reflected in changes in feed intake in the present study could relate to the feeding protocol, in which a single meal was offered per day, even though feeding was done to satiation. Thus, changes in satiety will have not affected the time to initiate the subsequent meal. By 24 h, appetite was likely high in all treatments, minimizing differences in feed intake among treatments. Seeing how the peak in *npy* expression in the LFU treatment was observed at 4 h after feeding (with a similar numerical trend in *agrp1*, also orexigenic), one might speculate that this could have affected the subsequent meal if feed had been available ad libitum, or at least multiple meals had been offered per day. A previous study performed with rainbow trout looking at changes in *npy* mRNA abundance in brain of fish fed at different feeding frequencies revealed an upward trend at 4 h after feeding, which was suggested to indicate that the fish were entering again a state of fasting, and a second feeding would be required at this time (Nahayat et al., 2022). Furthermore, it was observed that the best feeding regime for rainbow trout in this study was 3 times per day (morning, noontime, and before sunset), leading to better growth and FCR, which coincided with the timings of elevated *npy* mRNA abundance in brain indicating a rise in appetite. Therefore, it is plausible that if the feeding experiment had been performed with multiple feedings per day, it could have evidenced higher differences between the diets. Moreover, as a corollary to this hypothesis, fish fed the LFU diet seem to have reached a rise in appetite earlier than the other two treatments (which at 4 h still did not have significantly elevated mRNA levels of *npy* relative to its basal level at 0 h), and hence might have potentially resumed eating earlier. In addition, since fish were attuned to a 24-h feeding schedule, and thus should have been sampled after 24 h of fasting to capture the physiological state in anticipation of feeding and corresponding response postprandially, we cannot discard the possibility that comparisons among groups might have been affected by the 48 h of fasting.

On the other hand, the LFU treatment showed the highest ADC of dry matter, protein and energy, although it also led to a lower fat digestibility. Many studies relate changes in diet composition to effects on digestive functions, such as GIT motility and enzymatic activities. Many

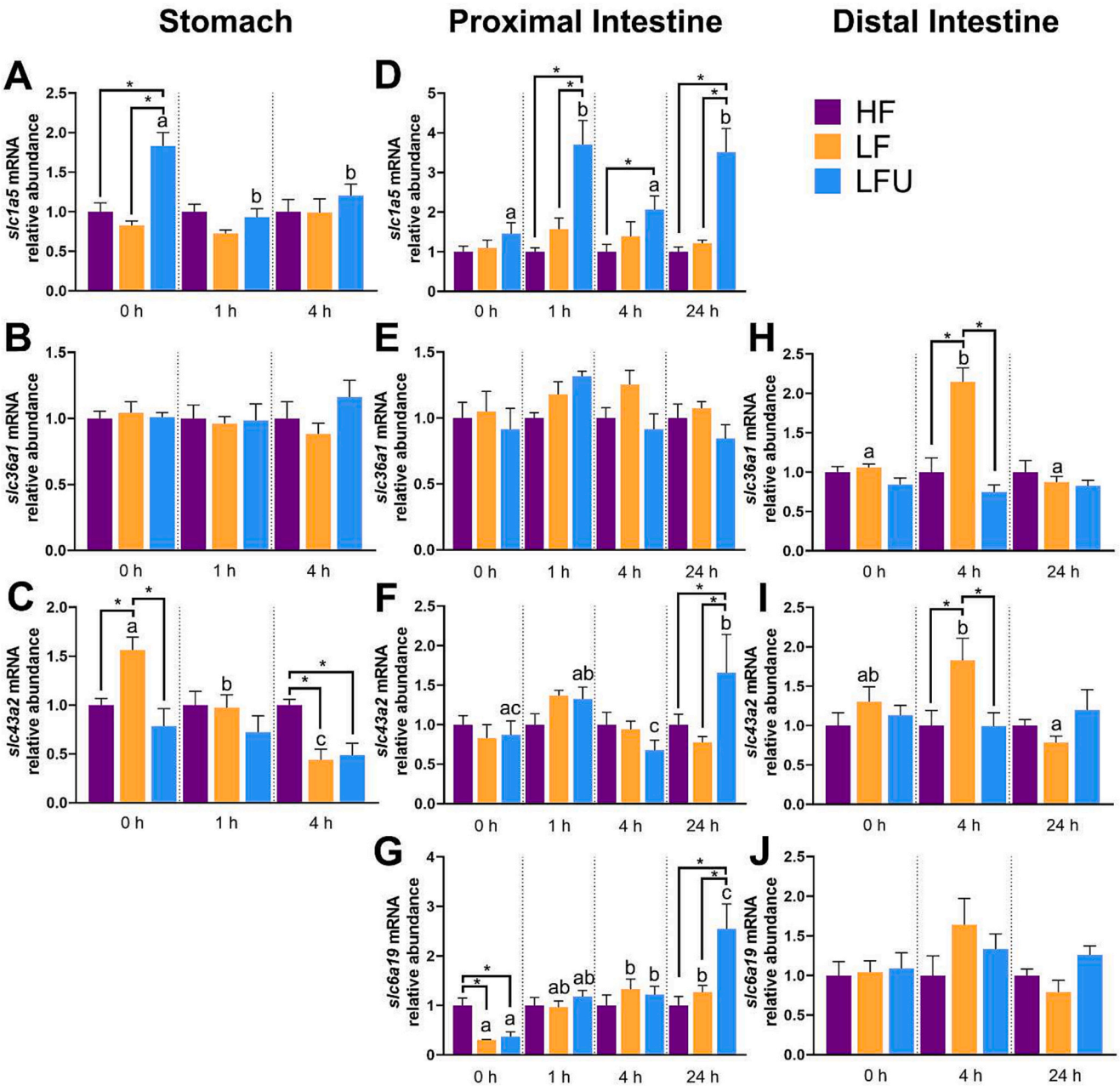

Fig. 5. mRNA abundance of *slc1a5* (gene encoding neutral amino acid transporter AAAT) in stomach (A) and proximal intestine (D), *slc36a1* (gene encoding neutral amino acid transporter Pat1) in stomach (B), proximal intestine (E) and distal intestine (H), *slc43a2* (gene encoding neutral amino acid transporter Lat4) in the stomach (C), proximal intestine (F) and distal intestine (I), and *slc6a19* (gene encoding neutral amino acid transporter B^0AT1) in the proximal intestine (G) and distal intestine (J) of rainbow trout after 48 h fasting (0 h) and 1 h, 4 h and 24 h after feeding a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Quantification of mRNA abundance was performed by RT-qPCR, normalizing data to *actb* and *eef1a1* reference genes. Data are shown as mean ± SEM of $n = 8$ fish per treatment relative to the HF group. Statistical differences among groups were assessed by *two*-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment. Significant differences ($p < 0.05$) among dietary treatments within sampling times are denoted by bars with asterisks.

plant feedstuffs including soybean meal, possess anti-nutritional factors like lectins, with the ability to hinder digestion and decrease intestinal enzyme activity (Krogdahl et al., 2003), and protease inhibitors able to inhibit digestive proteases in fish GIT, which are particularly sensitive to them (Gatlin et al., 2007). Studies in salmonids have found that the reduction of trypsin activity in intestine produced by protease inhibitors can be accompanied by an increase in pancreatic trypsin synthesis and secretion (Krogdahl et al., 1994; Olli et al., 1994). Moreover, a decrease in trypsin activity affects protein and lipid digestibility (Krogdahl et al., 1994, 2010; Lilleeng et al., 2007). However, in the present study we used SPC instead of soybean meal, thus having reduced levels of anti-nutritional factors. This could explain the absence of significant changes in trypsin and chymotrypsin activities in proximal intestine 4 h after feeding the LF diet. However, a significant increase was observed in pepsin activity in stomach 1 h after feeding both the LF and LFU diets, compared with fish fed the HF diet. Digestion starts in the stomach, and pepsin production takes place quickly after pH decrease. An acidic pH between 1.5 and 3.5 triggers pepsinogen conversion to pepsin in fish and

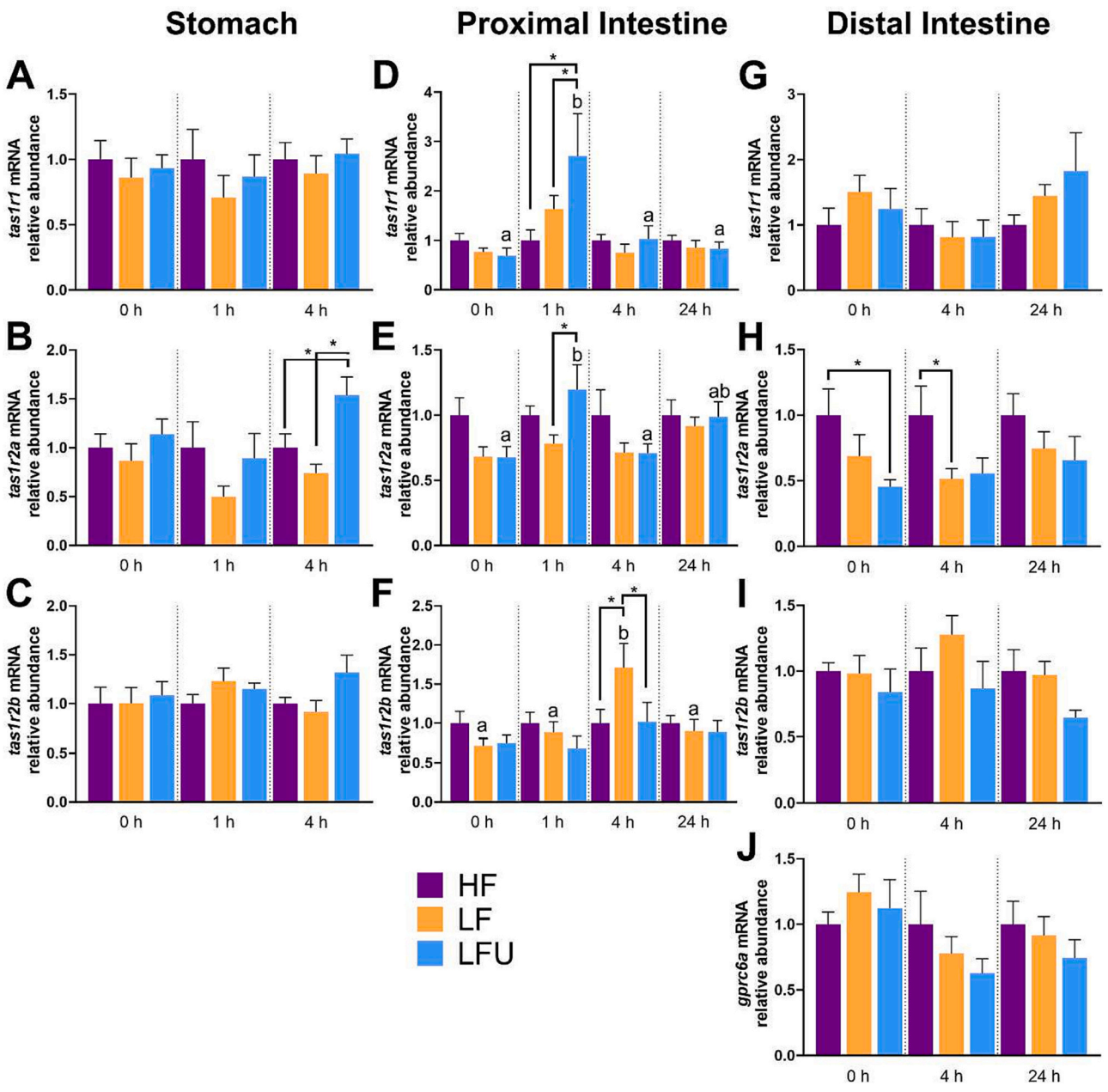

Fig. 6. mRNA abundance of taste receptors *tas1r1* in the stomach (A), proximal intestine (D) and distal intestine (G), *tas1r2a* in the stomach (B), proximal intestine (E) and distal intestine (H), *tas1r2b* in the stomach (C), proximal intestine (F) and distal intestine (I), and *gprc6a* in distal intestine (J) of rainbow trout after 48 h fasting (0 h) and 1 h, 4 h and 24 h after feeding a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Quantification of mRNA abundance was performed by RT-qPCR, normalizing data to *actb* and *eef1a1* reference genes. Data are shown as mean ± SEM of $n = 8$ fish per treatment relative to the HF group. Statistical differences among groups were assessed by *two*-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment. Significant differences ($p < 0.05$) among dietary treatments within sampling times are denoted by bars with asterisks.

diets have a big influence on pH alterations (Bucking and Wood, 2009; Martínez-Llorens et al., 2021). Each diet has a unique buffering capacity able to affect pepsin activity (Bucking and Wood, 2009). Furthermore, plant ingredients usually have a lower buffering capacity than fishmeal (Giger-Reverdin et al., 2002; Parma et al., 2019; Fabay et al., 2022; Ciavoni et al., 2023). Therefore, it is possible that our LF and LFU diets (with the same basal formulation) presented a lower buffering capacity enabling to acidify gastric pH more quickly, which resulted in higher pepsin activity. Still, this result is puzzling, considering how LF presented the lowest (numerically) and LFU the highest (significantly) digestibility of protein, dry matter, and energy, which could indicate that a different mechanism, unrelated to digestive enzyme activity, was responsible for the observed differences in protein digestibility. Similarly, no explanation can be offered at the moment for the inverse results of lipid digestibility, although we did not measure lipase activity in this study.

It is also known that the composition of the diet can additionally affect nutrient absorption (Debnath and Saikia, 2021). The source of protein, the presence of anti-nutritional compounds or additives can affect the absorption capacity through intestinal transporters (Santigosa et al., 2011). In the present study, levels of amino acids in plasma peaked 4 h after feeding in the case of the LFU diet and later (4 h to 24 h) in fish

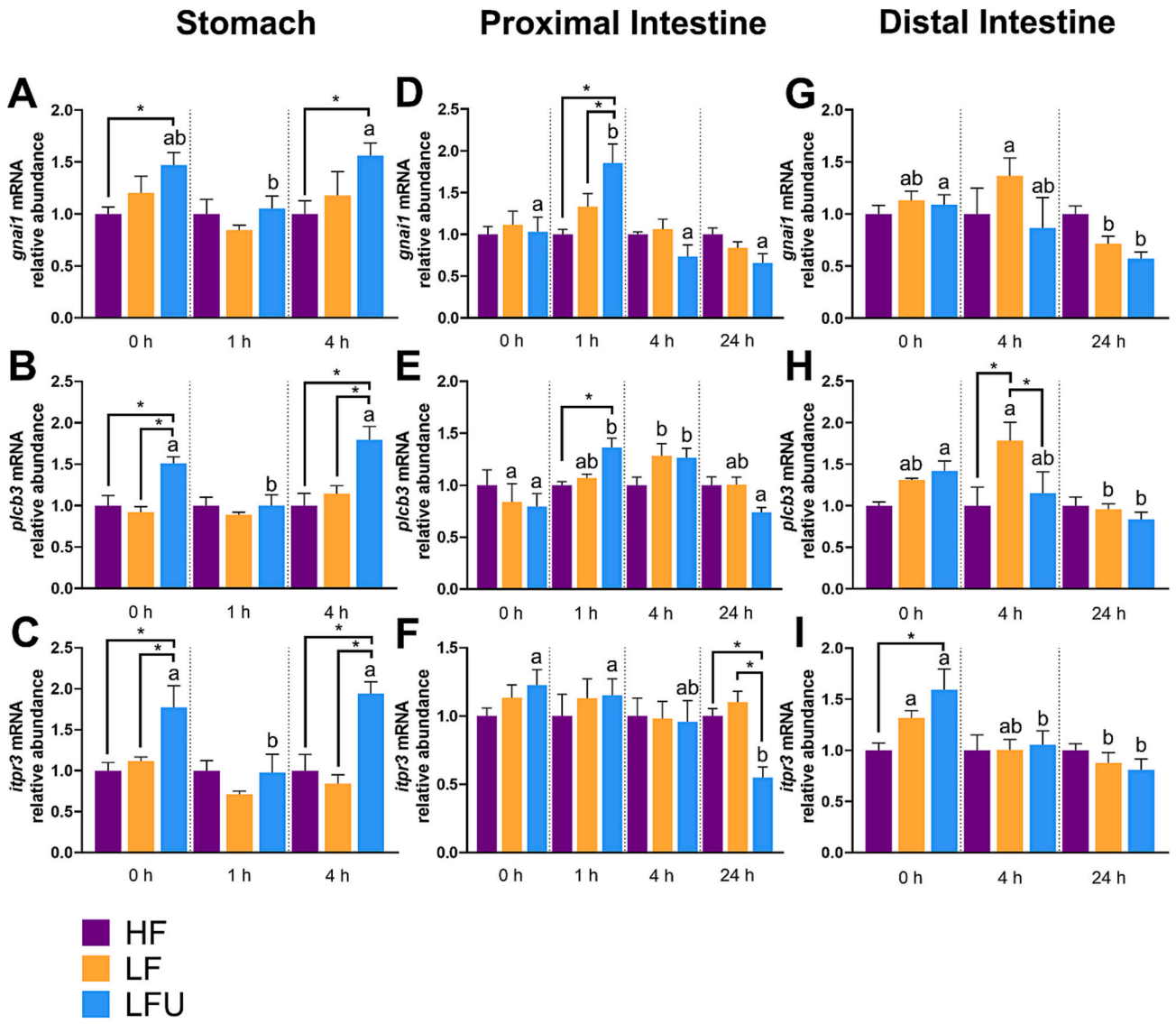

Fig. 7. mRNA abundance of intracellular signalling elements *gnai1* in the stomach (A), proximal intestine (D) and distal intestine (G), *plcb3* in the stomach (B), proximal intestine (E) and distal intestine (H), and *itpr3* in the stomach (C), proximal intestine (F) and distal intestine (I) of rainbow trout after 48 h fasting (0 h) and 1 h, 4 h and 24 h after feeding a a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Quantification of mRNA abundance was performed by RT-qPCR, normalizing data to *actb* and *eef1a1* reference genes. Data are shown as mean ± SEM of $n = 8$ fish per treatment relative to the HF group. Statistical differences among groups were assessed by *two*-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment. Significant differences ($p < 0.05$) among dietary treatments within sampling times are denoted by bars with asterisks.

fed the LF diet. A previous study showed that levels of amino acids in plasma peaked within 6–8 h after feeding when rainbow trout is fed with fishmeal, while feeding a vegetable diet produced a 4 h delay, which the authors attributed to a slower digestive processing of plant based proteins resulting in a slower uptake of amino acids (Larsen et al., 2012). Based on this previous study, it is possible that we missed the peak of amino acid absorption in the HF treatment. On the other hand, the apparently faster absorption of amino acids in fish fed the LFU diet correlated with this treatment having the highest protein digestibility. Moreover, several amino acid transporters were up-regulated in this treatment at different times, mostly in proximal intestine, which is an active area for amino acid absorption (Wang et al., 2017; Orozco et al., 2018). In mammals, a relationship between the stimulation of umami taste receptors and up-regulation of amino acid absorption through increased mRNA abundance of amino acid transporters in the GIT has been demonstrated (Mace et al., 2009). In the present study we found a higher mRNA abundance of cationic amino acid transporters B^{0+}AT1 (*slc7a9*) at 4 h and Y^{+}LAT1 (*slc7a7*) at 1 h and 24 h in proximal intestine of fish fed the LFU diet compared with those fed the HF diet (and LF diet in the case of *slc7a7* at 24 h). A similar response was observed for the neutral amino acid transporters AAAT (*slc1a5*) at 1 h and 24 h (compared to HF and LF), and 4 h (relative to HF), LAT4 (*slc43a2*) and B^{O}AT1 (*slc6a19*) at 24 h, the latter being significantly up-regulated in the proximal intestine of fish fed the LFU diet compared to both the HF and LF treatments. In distal intestine, only the expression of *slc1a1* was significantly elevated in the LFU treatment, relatively to HF and LF, at 24 h after feeding. Based on these results, it is reasonable to hypothesize that the presence in the LFU diet of free amino acids and small peptides able to stimulate T1r taste receptors (umami and/or "sweet" homologues), or directly up-regulate amino acid receptors, might be responsible for the significant increase in the expression of most amino acid transporters in this treatment. The only transporter that was

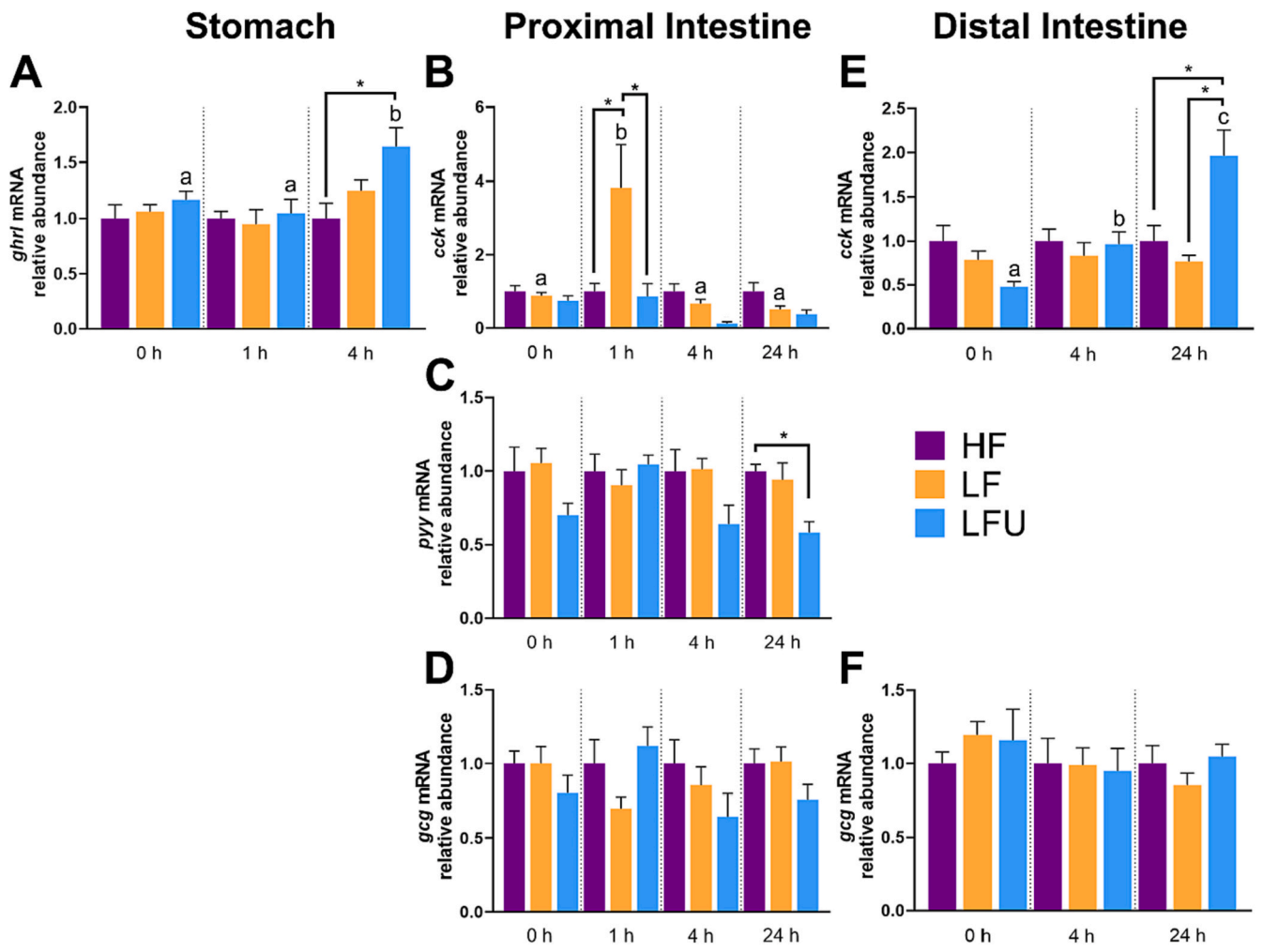

Fig. 8. mRNA abundance of gastrointestinal hormones *ghrl* in the stomach (A), cck in proximal intestine (B) and distal intestine (E), *pyy* in proximal intestine (C), and *gcg* in proximal intestine (D) and distal intestine (F) of rainbow trout after 48 h fasting (0 h) and 1 h, 4 h and 24 h after feeding a diet with high fishmeal content and reduced levels of soy protein concentrate (HF), a diet with lower fishmeal content, partially replaced by soy protein concentrate (LF), or the LF diet supplemented with an umami taste-stimulating additive (LFU). Quantification of mRNA abundance was performed by RT-qPCR, normalizing data to *actb* and *eef1a1* reference genes. Data are shown as mean ± SEM of $n = 8$ fish per treatment relative to the HF group. Statistical differences among groups were assessed by *two*-way ANOVA and post-hoc Student-Newman-Keuls test using GraphPad software. Different letters denote significant differences ($p < 0.05$) among sampling times within each dietary treatment. Significant differences ($p < 0.05$) among dietary treatments within sampling times are denoted by bars with asterisks.

significantly up-regulated in the proximal intestine of fish fed the LF diet was Pept1 (*slc15a1*). This is the main transporter of tri- and dipeptides into the enterocytes, with low affinity but high capacity in fish, and is primarily expressed in foregut (specially in pyloric caeca), with lower levels in stomach and distal intestine (Wang et al., 2017). This transporter has been shown to respond to fasting and re-feeding conditions, with Pept1 levels decreasing after a fasting period and increasing during refeeding (Ostaszewska et al., 2010a; Xu et al., 2016; Wang et al., 2017). In the present study, mRNA abundance of Pept1 was upregulated 4 h after feeding LF diet. This is consistent with results obtained in turbot where Pept1 peaked 2 h after feeding and with studies in carp and rainbow trout where feeding plant-based diets increased Pept1 levels in proximal intestine (Ostaszewska et al., 2010a, 2010b) while no changes occurred in mRNA abundance in GIT of gilthead seabream fed different plant protein sources (Terova et al., 2013). Interestingly, no changes were observed in the expression of Pept1 over time in the LFU or HF treatments.

In contrast, in distal intestine, amino acid transporters were more responsive to the LF diet, with most of them displaying an increased expression at 4 h compared with fish fed the HF and LFU diets, as seen in the anionic transporter EAAT3 (*slc1a1*), cationic transporter B^{0+}AT1 (*slc7a9*), and neutral transporters Pat1 (*slc36a1*) and LAT4 (*slc43a2*). In fish, middle and distal intestines are critical for the digestion and absorption of nutrients (Bakke-McKellep et al., 2000b). Furthermore, the distal intestine has a greater capacity to absorb dipeptides and intact proteins by endocytosis (Sundell and Rønnestad, 2011). There is currently no clear explanation for the upregulation of amino acid transporters in this tissue in fish fed the LF diet. However, it is possible that this could be linked to the lower protein digestibility of the diet, compared to the LFU treatment, which may require an enhancement of protein absorption in the distal intestine. Interestingly, fish fed the HF diet showed a striking constancy in the expression of all amino acid and peptide transporters in all areas of the GIT over time.

To our knowledge, this is the first study comparing the response of amino acid transporters in different sections of the GIT in fish fed diets with different content in vegetable protein, although at least one study evaluated changes in the whole intestine (Brezas et al., 2021). Other studies (Santigosa et al., 2008, 2011; Brezas and Hardy, 2020) have shown that fish modify their pattern of intestinal absorption when fed vegetable-based diets. Moreover, an up-regulation of amino acid transport capacity has been suggested to occur as a compensatory mechanism to a nutritional deficit (Santigosa et al., 2011). These changes in

absorption efficiency can relate to intestine histopathological modifications but also to changes in mRNA abundance of peptide and amino acid transporters in the intestinal brush border membrane in response to different protein sources in the diet (Xu et al., 2016; Song et al., 2017; Debnath and Saikia, 2021). However, whatever the mechanism explaining the differential regulation of amino acid transport by the LF and HF diets, likely associated to the composition of the dietary protein, it is noteworthy that the supplementation of the LF basal diet with just 0.25% of the umami taste-stimulating additive (LFU treatment) radically changed the pattern of expression of amino acid transporters in the fish GIT. In particular, there was a clear regional difference in the effect of these two diets, with LFU mainly stimulating transporters in proximal intestine and LF in distal intestine. With the existing knowledge, it is challenging to mechanistically explain these results. However, it is plausible that the elevated expression of amino acid transporters induced by the LFU diet in the proximal intestine could be linked to the significantly higher protein ADC value in this treatment.

In a previous study carried out in our laboratory, we demonstrated the presence of different receptors involved in amino acid sensing and putative taste-signalling elements along the GIT of rainbow trout, such as T1r family (T1r1 and T1r2), Gprc6a, and Casr receptors, and Gnai1, Plcβ3 and 4, and Itpr3 signalling molecules (Calo et al., 2021). Furthermore, it was shown that these parameters can respond with changes in their mRNA abundance to the presence of the amino acids valine, proline or glutamate in the GIT, but the response varied according to the specific amino acid, with L-glutamate (the predominant amino acid in proteins) exerting the most significant transcriptional changes (Calo et al., 2021). This previous study, as well as others (Oike et al., 2007; Wang et al., 2012; Cai et al., 2018; Angotzi et al., 2020), supported a growing body of evidence that taste receptors, and particularly the umami (T1r1-T1r3) and sweet (T1r2-T1r3) taste receptor homologues function as broadly tuned amino acid sensors in the fish GIT and are likely key elements of the gut-brain axis regulating appetite and gut function (e.g., modulation of gut transit and digestive, absorptive or metabolic processes) in fish, similarly to mammalian vertebrates (Depoortere, 2014; Angotzi et al., 2022). In the present study, besides evaluating the response to predominantly fishmeal-based or plant-based diet formulations, we aimed to assess whether the supplementation with a sensory additive containing natural chemostimulants of T1r taste receptors (mostly free amino acids, short peptides, and nucleotides) further modulates the response to a plant-based diet. The activation of T1r family taste receptors in the GIT has been implicated in the regulation of gastric emptying rate, intestinal motility, and digestive enzyme secretion in mammals through GLP-1 and CCK release (Treesukosol et al., 2011; Raka et al., 2019; Sarnelli et al., 2019), and a similar involvement has been suggested for teleost fish (Angotzi et al., 2022). The basal diet formulation appeared to have little effect in the transcriptional regulation of the studied taste receptor subunits. The HF treatment showed a higher expression of *tas1r2a* (significantly higher than LFU at 0 h and LF at 4 h), but this was only observed in the distal intestine. This result was somewhat surprising, considering that fishmeal contains high levels of soluble protein and free amino acids (Cho and Kim, 2011; Calo et al., 2023a) that could easily leach out from feeds. However, a previous study had already suggested fairly minor effects of the soluble fraction of fishmeal (including free amino acids and peptides) in transcriptionally regulating taste receptors and taste signalling pathways in the GIT of trout (evaluated at 20 min and 2 h after administration), except for an up-regulation of *tas1r1* and *gnai1* in proximal intestine and of *tas1r1* in distal intestine at 2 h, and of *itpr3* and *plcb3* in middle intestine at 2 h (Calo et al., 2023a). The effect of the LF diet was also minor (only *tas1r2b* was up-regulated at 4 h in proximal intestine), which could have been expected in this case, as vegetable meals typically contain low levels of free amino acids (Li et al., 2009; Cho and Kim, 2011). A study in grass carp found that *t1r2a* mRNA levels increased in fish fed a totally plant-based diet compared with fish fed a diet containing fishmeal (Liang et al., 2019). In our study, *t1r2a* mRNA abundance was lower in LF than in HF-fed fish in the distal intestine, but neither the diet formulations nor the species (carnivore versus herbivore) are comparable. The main effects observed in the present study were those elicited by the LFU diet, which up-regulated *tas1r1* in proximal intestine 1 h after feeding, and *tas1r2a* in stomach and proximal intestine at 1 h and 4 h, respectively. The enhanced mRNA abundance of these genes in anterior intestine at 1 h after feeding is consistent with the hypothesis that diet supplementation with a sensory additive designed to quickly deliver taste-active substances into the GIT (ahead of significant nutrient release through food processing and digestion) might be an effective strategy to stimulate gut sensing mechanisms signalling the arrival of nutrients. However, the up-regulation of *tas1r2a* in stomach must be reflecting a different mechanism, since at 4 h gastric digestion should already be well underway.

Confirming a previous study (Calo et al., 2023a), the cellular signalling molecules putatively responsible for transducing taste signals appeared to be more responsive, in terms of transcriptional regulation, than taste receptors. These markers corroborated that the LFU diet had a clear effect in up-regulating taste or GPCR-coupled signalling pathways in different areas of the GIT, at different times. In stomach, *gnai1*, *plcb3*, and *itpr3* were all up-regulated in fish fed the LFU diet, in both basal (0 h) conditions and 4 h after feeding. In proximal intestine, changes were similarly only observed in response to the LFU treatment, at 1 h after feeding (up-regulation of *gnai1* and *plcb3*) or at 24 h after feeding (down-regulation of *itpr3*), while in distal intestine *itpr3* was up-regulated in basal conditions. Hence, it would appear that these genes are not just responding to the presence of tastants or nutrients in the GIT, and that a more long-lasting modulatory effect might occur. However, we are not able to mechanistically explain these results at present and further studies should be performed to corroborate them.

The activation of GIT taste receptors triggers an intracellular signalling cascade within enteroendocrine cells that culminate in the release of GIT hormones including Ghrl, Cck, Pyy, and Glp-1 (Depoortere, 2014; Steensels and Depoortere, 2018). These peptides are responsible for sending enteric nerve and endocrine signals to the central nervous system, where the information is integrated and potentially results in changes in mRNA abundance of appetite modulatory neuropeptides. Such gut sensing mechanisms, part of the gut-brain axis, are well known in mammalian vertebrates, and evidence so far suggests that they might also be operational in fish (Blanco et al., 2021; Calo et al., 2021; Angotzi et al., 2022). In the present study, the HF diet did not affect the mRNA abundance of any of the GIT hormones assessed. However, feeding the LF diet resulted in a remarkable increase in *cck* mRNA abundance in proximal intestine 1 h after feeding. As suggested in Calo et al. (2023a), where cck was also upregulated after intragastric administration of fishmeal aqueous extract, its rapid increase could be related to the inhibition of gastric emptying and/or the stimulation of pancreatic enzymes and bile secretion to improve digestion and nutrient utilization (Debas et al., 1975; Olsson et al., 1999), which might be enhanced in the case of diets containing less free amino acids. On the other hand, consistent with the fact the LFU treatment induced the main effects in the other gut sensing elements analysed, this was also the diet inducing more changes in the expression of gut peptides. Ghrelin has different roles, but the most well established in vertebrates (including fish) are the stimulation of food intake and body weight gain, and modulation of energy homeostasis (Jönsson, 2013; Müller et al., 2015). The highest abundance of ghrelin-producing cells is found in stomach (Sakata and Sakai, 2010; Calo et al., 2021), that secretes and maintains elevated plasma Ghrl levels in fasting conditions, then quickly returning to basal condition after refeeding (Kaiya et al., 2013). The up-regulation of *ghrl* in the stomach of LFU-fed fish at 4 h could potentially indicate a quicker return to a hunger state, meaning that the animals would be ready to feed again earlier, in line with our earlier discussed hypothesis. However, it would have also been expectable to observe a higher expression in not just this treatment, but also in the HF and LF treatments in 48 h fasting (0 h) conditions, compared to the other time

points. On the other hand, both PPY and CCK have been attributed a role in suppressing hunger and inhibiting gastric emptying (Blanco et al., 2021; Volkoff, 2016), while CCK additionally stimulates the secretion of pancreatic enzymes and bile, promoting digestion, and regulates intestinal motility (peristalsis) (Cawthon and de La Serre, 2021). Other roles have also been attributed to PYY in more distal areas of the intestine (Holzer et al., 2012), which are likely unrelated to the results obtained in this study, as the effects were observed in the anterior intestine. However, considering these multiple possible effects of PYY and CCK, it is difficult to pinpoint the potential physiological significance of the changes observed at 24 h in proximal intestine (down-regulation of *pyy*) and in distal intestine (up-regulation of *cck*) of fish the the LFU diet.

5. Conclusions

Feeding rainbow trout with a diet containing an enhanced level of SPC and reduced fishmeal content (LF) did not result in marked alterations in parameters related to digestive function and amino acid sensing in GIT, which were further reflected in little effects in the expression of GIT hormones (except for *cck*) and hypothalamic neuropeptides, compared to the HF diet. However, the 48 h fasting protocol could have affected the results, as it did not capture the physiological state in anticipation of feeding, which would have occurred at 24 h of fasting. Furthermore, there were no significant differences in feed intake, although it is likely that the feeding protocol did not help evidencing these, by not enabling post-ingestive or post-prandial effects to modulate the time to initiate the subsequent meal. In contrast, the supplementation of the LF diet with a sensory additive containing umami chemostimulants (i.e., with the potential to activate T1r taste receptors in fish) resulted in a significant improvement in protein, dry matter and energy digestibility, as well as in the up-regulation of several amino acid transporters in the proximal intestine (mostly) and earlier peak in plasma α-amino acid levels, suggesting an enhanced digestive and absorptive function. Moreover, feeding trout with the LFU diet modified several mechanisms involved in feed intake regulation through the transcriptional increase of taste receptors and signalling effectors, as well as hormone expression in the GIT. These results demonstrate that small amounts of umami taste-active substances added into aquafeeds can be sensed by the fish GIT. The activation of these mechanisms, signalling the arrival of nutrients, are known to stimulate gut sensing and gut-brain axis mechanisms that enable a coordinated GIT response to ensure optimal digestion and nutrient assimilation. Therefore, this study suggests that supplementation of feeds with umami taste-active substances could have an impact in improving feeding efficiency in aquaculture production. However, a longer study following a practical feeding schedule is needed to confirm the results and further assess whether they translate into an enhanced feed intake and performance.

CRediT authorship contribution statement

Jessica Calo: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Sara Comesaña**: Methodology, Validation, Formal analysis, Investigation. **Cristina Fernández-Maestú**: Methodology, Validation, Formal analysis, Investigation. **Ayelén M. Blanco**: Conceptualization, Methodology, Validation, Formal analysis, Writing – Review & Editing, Supervision. **Sofia Morais**: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition. **José** L. **Soengas**: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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