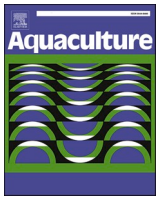
Aquaculture 597 (2025) 741925

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



High plant protein diet impairs growth performance and intestinal integrity in greater amberjack (*Seriola dumerili*): Molecular and physiological insights

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ARTICLE INFO

Keywords:
Greater amberjack
Fishmeal replacement
Intestine
Integrity
Amino acid

ABSTRACT

The Mediterranean aquaculture industry that produces mainly seabream and seabass is exploring alternative plant-based protein sources for fish feeds together with the diversification of fish species. Effective plant-based formulations require diverse sources and additives to maintain fish welfare and growth, which is especially important for carnivorous species. In this scenario, the objective of the present study was to evaluate the impact of a high percentage of fishmeal replacement with protein of plant origin in *Seriola dumerili*, a fast-growing species with a high protein requirement. Two diets were developed: MAP (marine animal protein) with a protein content of 92 % of animal origin and PPB (plant protein blend) with a 50 % replacement of protein of animal origin with plant protein. We combined electrophysiology measurements with expression analysis of claudins and members of the SLC superfamily of solute carriers to unravel and characterise putative markers of intestinal integrity and absorption. The replacement resulted in shorter and lighter fish with a reduction in growth rate (SGR) from 2.6 to 2. We simultaneously observed lower transepithelial tissue resistance (TER), lower permeability, and decreased *cld12* expression in the anterior intestine. In addition, we demonstrated a strong region-dependent electrogenic transport of essential amino acids, with the mid-intestine having the highest transport capacity. The comparative study performed in the mid-intestine with fish fed the MAP or the PPB diets exposed a negative effect of dietary replacement with plant protein. No significant dietary impact on di- and tri-peptide transporters (SLC15) was found at the molecular level. However, a clear region-dependent expression pattern of *slc15a1*, *slc15a2*, and *slc15a4* was observed, which warrants further investigation. The expression pattern of *slc7a5* showed the effect of the diet, but in contrast, the diet and intestinal region affected the expression of its functionally associated slc3a1 and slc3a2 for amino acid antiport, with the strongest effects in the posterior intestine. The integrity and absorption impairments detected in response to protein source replacement will likely underlie the significant growth differences observed between the two dietary regimes.

1. Introduction

A global pressure build-up exists to explore new raw materials to incorporate into fish feeds. Thus, the paradigm of feed formulation has changed to address sustainability issues in aquaculture. Once overly dependent on fishmeal and oil, new feed formulations targeted replacing fisheries' raw materials with plant ingredients (Hardy, 2010). This strategy enables fish production for human consumption, protecting fisheries and reducing the dependence of aquaculture on wild fish catch (one essential SDG in the FAO 2030 agenda). This approach creates an unprecedented complexity in fish feeds, a complex equation that must balance raw material quality, cost, and availability. Most importantly,

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https://doi.org/10.1016/j.aquaculture.2024.741925
Received 7 July 2024; Received in revised form 17 November 2024; Accepted 18 November 2024
Available online 20 November 2024

the ramifications of novel dietary ingredients for fish nutrition and welfare, especially for carnivorous species, require unravelling some important traits to avoid a negative impact and impairment in terms of fish nutrition, growth performance, and welfare.

Additionally, plant ingredients usually have lower protein contents, imbalanced amino acid profiles, and deficiencies in some micronutrients compared to fishmeal (Hardy, 2010). A considerable replacement of dietary fishmeal by plant protein sources with less than 10 % of marine feed ingredients is now possible (Martos-Sitcha et al., 2018; Aragão et al., 2020) on the plus side. On the minus side, the high inclusion of plant protein approaching levels of sustainability often leads to reduced key performance markers, including growth, feed efficiency, or welfare, due to among other factors, the impairment of intestinal health, integrity, and functionality (Oliva-Teles et al., 2015; Estensoro et al., 2016; Simó-Mirabet et al., 2018, 2018; Fonseca et al., 2023; Vizcaíno et al., 2024). Ensuring proper rearing conditions for farmed aquatic animals aligns with ethical standards and significantly enhances the quality of the final product. Moreover, there is an increasing awareness of the 'One Health' concept, which highlights the importance of integrating environmental, animal, and human health by fostering better production practices.

Another side to the sustainability of aquaculture is the need to favour the industry's growth through diversification, especially with species with fast growth and high economic value. Mediterranean aquaculture is primarily linked to seabream and seabass farming and has reached a high degree of maturity at technical and commercial levels. Recent studies have highlighted not only the aquaculture potential but also the increasing interest in the marine fish farming industry of other species, namely the greater amberjack (*Seriola dumerili,* Risso 1810) (Skaramuca et al., 2001; Donohue et al., 2021), because of its good growth performance. However, post-larvae and juvenile amberjacks require high amounts of protein (60–65 %) in their aquafeed formulations (Navarro-Guillén Conceição et al., 2019), and although seabream can be grown with feeds with up to 90 % of plant protein, the limit for the greater amberjack is not yet known. On the other hand, evidence is piling up that to make replacement with plant-based protein a viable alternative for the industry, different plant sources, feed additives, and enhancers need to be sought and tested for effective balanced formulations (Estensoro et al., 2016; Hossain et al., 2018). To this end, devising, assaying and validating tools and proxies for diet effects is paramount.

Previous work documented using the bioelectrical properties measured with the epithelial electrophysiology approach that the inclusion of plant protein in fish feeds, or the inclusion of different amounts of algae or microalgae, can compromise intestinal barrier function, selectivity, or integrity in cultured fish (Estensoro et al., 2016; Molina-Roque et al., 2022; Aragão et al., 2020; Fonseca et al., 2023). Overall, the intestine is the primary target for changes in the diet's configuration, much more so because the gastrointestinal system is the unique entry point for food and the only organ involved in absorption. Intestinal absorption of protein components (amino acids and small peptides) requires integrated nutrient and ion transport action. This process links nutrient sensing to nutrient absorption through the regulation of ion transport (McCauley et al., 2020), an effect mediated by enteroendocrine cells. Most dietary amino acid and peptide transport occurs *via* Na^+- and H^+- linked transporters (Broer, 2008; Broer and Fairweather, 2019). In mammalian models, digested proteins are transported as free amino acids by specific transporters and as di- or tripeptides *via* PEPT transporters using proton gradient as a driving force (Chen et al., 2010), but ion dependence is still unclarified in fish.

Enterocytes express an assortment of amino acids and peptide transporters on their apical membrane side, whose action secures the efficient removal of amino acids from the intestinal lumen during and after digestion. Several studies have focused on the factors affecting nutrient use, growth rates, and disruption of other physiological processes in fish. However, only some encouraging exceptions (Con et al., 2017; Orozco et al., 2018; Vacca et al., 2019; Wu et al., 2016) explore the molecular level. From these studies, the conclusion that comes across clearly is that molecular regulation of intestinal amino acid transporters is species-specific in tissue distribution, ontogenetic development, and regulation by feeding.

The objective of the present study was to evaluate the impact of partial fishmeal/oil replacement by raw materials of plant origin in key markers of intestinal function in a highly carnivorous and fast-growing species such as the greater amberjack.

2. Materials and methods

2.1. Fish

Greater amberjack (*S. dumerili*) juveniles supplied by Futuna Blue España S.L. (Puerto de Santa María, Cadiz, Spain) were acclimated to the indoor experimental facilities at the Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, University of Cadiz, Puerto Real, Cadiz, Spain; Spanish Operational Code REGA ES11028000312) with GEMMA silk (from 1.0 to 1.2 mm, Skretting, Norway; Supplementary Table 1), seawater in controlled conditions of salinity (37 ppt), temperature (19 ± 1 °C), and under natural photoperiod at our latitude (September, 36°31′45″ N, 6°11′31″ W). All experimental procedures followed the guidelines of the Animal Welfare and Ethics Committee of the University of Cadiz for the protection of animals used in scientific experiments, according to the principles published in the European Animal Directive (2010/63/EU) and Spanish laws (Royal Decree RD53/2013). In addition, the Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 23/10/2019/176).

2.2. Fish diets and experimental groups

After an initial acclimation period of two weeks, juveniles with 9.4 ± 0.1 g initial mean body mass were randomly distributed in six 400 L tanks in a flow-through open system with tank volume adjusted to obtain 1.5 kg/m^3 initial stocking density (n = 20 fish per tank). Water temperature was set constant at 19–20 °C with a salinity of 37 ‰. The oxygen content of outlet water remained higher than 85 % saturation, and the photoperiod followed the natural changes. The six tanks were randomly separated into two experimental groups (N = 60 fish per experimental group). In each group, fish were fed with one of two isolipidic (18 % CL, crude lipid) and isoproteic (63 % CP, crude protein) diets (Table 1) produced by the *Servicio de Piensos Experimentales* from the University of Almería (https://www.ual.es/universidad/serviciosgenerales/stecnicos/perifericos-convenio/piensos-experimentales), which were named as i) MAP diet, mainly based on fishmeal, fish oil and other sources of marine animal origin (*i.e.* squid, krill, shrimp, mussel), mimicking formulations of commercial aquafeeds for this species, with 92 % of the protein content of marine animal origin and 8 % of plant origin, and ii) PPB diet, a plant protein-based diet, containing 50 % of the protein content of marine animal origin and 50 % of plant origin. Briefly, all ingredients were mixed in a 20 L mixer and ground with a hammer mill (UPZ 100, Hosokawa-Alpine, Augsburg, Germany) to 0.5 mm. The diets were extruded in a five-section twin-screw extruder (Evolum 25, Clextral, Firminy, France) fitted with 2, 3 or 4 mm die holes. The pellets were dried after extrusion at 27 °C using a drying chamber (AIRS 1070 I PLC, Airfrio, Almería) and cooled at room temperature until use.

2.3. Experimental design

The amberjack juveniles were used in three consecutive experiments. The first and second experiments were done by the end of the acclimation period with fish eating the commercial diet. In the first experiment, we determined plasma ion composition in amberjack to establish the conditions for the electrophysiology assays in Ussing chambers. In

Table 1
Ingredients and proximal composition (% dry matter) of the experimental diets MAP and PPB.

	Experimental diets*	
Ingredient composition	MAP	PPB
Fishmeal LT94 [1]	50.5	41.3
Squid meal [2]	6.0	–
CPSP90 [3]	6.0	0.5
Krill meal [4]	5.0	0.5
Shrimp meal [5]	2.0	–
Mussel meal [6]	6.0	–
Wheat gluten [7]	5.0	13.0
Pea protein concentrate [8]	1.8	14.9
Soybean protein concentrate [9]	1.8	14.0
Fish oil [10]	6.7	9.4
Soybean lecithin [11]	1.0	1.0
Wheat meal [12]	4.2	1.4
Choline cloride [13]	0.2	0.2
Betain [14]	0.2	0.2
Vitamin and Mineral premix [15]	1.5	1.5
Vitamin C [16]	0.1	0.1
Guar gum [17]	2.0	2.0
Crude protein	63.9	62.9
Crude lipid	18.7	18.4
Ash	12.9	10.8
Moisture	5.9	6.1

1 69.4 % crude protein, 12.3 % crude lipid (Norsildemel, Bergen, Norway).
2, 3, 4, 5, 6 purchased from Bacarel (UK). CPSP90 is enzymatically pre-digested fishmeal.
7 78 % crude protein (Lorca Nutrición Animal SA, Murcia, Spain).
8 Pea protein concentrate, 85 % crude protein, 1.5 % crude lipid (Emilio Peña SA, Spain).
9 Soybean protein concentrate, 50 % crude protein, 8 % crude lipid (LorcaNutrition, Spain).
10 AF117DHA (Afamsa, Spain).
11 P700IP (Lecico, DE).
12 Local provider (Almería, Spain).
13, 14 Lorca Nutrición Animal SA (Murcia, Spain).
15 Lifebioencapsulation SL (Almería, Spain). Vitamins (mg kg^{-1}): vitamin A (retinyl acetate), 2000,000 UI; vitamin D3 (DL-cholecalciferol), 200,000 UI; vitamin E (Lutavit E50), 10,000 mg; vitamin K3 (menadione sodium bisulfite), 2500 mg; vitamin B1(thiamine hydrochloride), 3000 mg; vitamin B2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine (Betafin S1), 50,000 mg. Minerals (mg kg-1): Co (cobalt carbonate), 65 mg; Cu (cupric sulfate), 900 mg; Fe (iron sulfate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulfate) 750 mg; Ca (calcium carbonate), 18.6 %; (186,000 mg); KCl, 2.41 %; (24,100 mg); NaCl, 4.0 % (40,000 mg).
16 TECNOVIT, Spain.
17 EPSA, Spain.
* Dietary codes: MAP contains 92 % marine animal protein and 8 % plant protein; PPB contains 50 % plant protein and 50 % marine animal protein.

the second experiment, electrogenic amino acid transport (EAAT) was measured in the intestinal epithelium, using Ussing chambers to ascertain putative intestinal region-dependency effects. For the third experiment, set up after the acclimation period, fish fed with MAP or PPB diets were analysed to test the impact of replacing protein of animal origin with plant-based protein on fish growth performance and somatic indexes, intestinal electrophysiology and expression analysis of key genes involved in integrity and amino acid transport. Experimental diets (MAP and PPB) were offered to satiety (*ad libitum*) 5 times per day (9.00 h, 10.30 h, 12.00 h, 13.30 h, and 18.00 h), 6 days per week for 93 days (October 2020–January 2021). The feeding test was carried out blindly. The two aquafeeds were labelled with different colours but without reference to their composition, eliminating any source of subjectivity when feeding the animals. Feed intake was recorded gravimetrically for each experimental replicate to calculate growth performance parameters.

At the end of the experimental feeding period, four fish from each experimental tank (12 fish/diet) were randomly selected, deeply anesthetized with a lethal dose of 2-phenoxyethanol, and then individually weighed and measured. After that, each fish was cervically sectioned, and the entire intestine was removed for length measurement from the pyloric caeca to the anal sphincter. Then, samples of discrete sections of the intestine were obtained and mounted for electrophysiological protocols as described below or taken for gene expression analysis and preserved in an appropriate volume (1/10, *w/v*) of RNAlater (Ambion, Austin, Texas, USA). Finally, the remaining fish of each experimental tank were weighed and measured to obtain the growth performance and biometric parameters for the total of animals assayed.

2.4. Characterization of plasma ions in greater amberjack

Greater amberjack juveniles (12–25 g, $n = 7$) were randomly selected, and deeply anesthetized with an overdose of 2-phenoxyethanol (1 mL/L seawater). Blood was collected from the caudal peduncle into heparinized (50 units Li-heparin/mL blood, Sigma, Madrid, Spain) 1 mL syringes. Plasma obtained by centrifugation of whole blood (10,000 *g* for 3 min) was stored at −20 °C until analysis. The osmolality of 10 μL plasma was measured with a Vapro 5520 osmometer (Wescor, South Logan, Utah, USA). Sodium concentrations were measured by flame photometry (BWB-XP Performance Plus, BWB Technologies, UK). Chloride was determined by Coulomb-metric titration (SAT-500, DKK-TOA, Japan). Calcium and magnesium were measured by colorimetric tests using commercial kits (References 1,001,060 and 1,001,286, Spinreact, Reactivos Spinreact, SA, Girona, Spain), according to the manufacturer detailed protocol, using a Multi-Mode Microplate Reader BioTek SynergyTM 4 (BioTek Instruments, Winooski, VT, USA). The results were expressed as mmol/L.

2.5. Electrophysiology in Ussing chambers

Intestinal tissues used in our experiments were the anterior intestine, the mid intestine, and the posterior intestine (rectum), following the description presented for greater amberjack by Navarro-Guillen et al. (2022). For voltage clamp in Ussing chambers, the intestinal tissue was isolated and mounted as previously described (Fuentes et al., 2018) with apical (luminal) and basolateral (blood side) sides of the tissue identified on a tissue holder of 0.25–0.30cm^2 and positioned between two half-chambers (P2400 or P2300, Physiologic Instruments, Reno, Nevada, USA) containing 2 mL of physiological saline prepared according to the previous characterization of amberjack plasma ion composition (see below, in the Results section).

The tissue was bilaterally gassed with humified air during the experiments, and the experiments ran at room temperature (20 °C). Short-circuit current (Isc, μA/cm^2) was monitored by clamping the epithelia to 0 mV. In a subset of experiments bumetanide (Sigma. Madrid), a specific inhibitor of NKCC co-transporters, was applied at 200 μM to the apical membrane to expose the dietary effects of bumetanide-sensitive currents. Voltage clamping and current injections were performed using VCC MC8 and VCC MC6 voltage-clamp amplifiers (Physiologic Instruments, Reno, Nevada, USA). Bioelectrical parameters for each tissue were recorded continuously during the *in vitro* period onto Labscribe4 running in a MacIntosh computer using IWorx188 and Lab-Trax-4 data acquisition systems (IWorx Inc., Dover, New Hampshire, USA) from the time of mounting for 90 min. Epithelial resistance (Rt, Ω/cm^2) was manually calculated (using Ohm's law) from the current deflections induced by bilateral +2 mV pulses of 3 s every minute. The apical side of the preparation was considered as the ground. Therefore, negative currents are absorptive, while secretory currents are positive.

2.6. Electrogenic amino acid transport (EAAT)

The intestine of greater amberjack juveniles was isolated and mounted as described above. In this type of assay, the apical side of the preparation is stimulated with an amino acid mixture (M 5550 MEM [50×], Sigma, Madrid, Spain). The amino acid pool consists of a complex mixture of essential amino acids: L-Arginine•HCl, L-Cystine • 2HCl, L-Histidine•HCl•H2O, L-Isoleucine, L-Leucine L-Lysine•HCl L-Methionine L-Phenylalanine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine (Supplementary Table 2).

The principle of the test is that the presence of amino acids stimulates the epithelium, thus generating a change in the current due to the cotransport of amino acids with ions (Broer, 2008 and several references therein). EAAT assays were performed in experiments two and three. In experiment two, we performed a set of assays to establish putative region-dependent effects in electrogenic amino acid transport using the anterior, mid, and posterior intestinal regions of fish fed the commercial diet. In experiment three, we performed a comparative response in the intestine of juveniles fed with MAP or PPB diets. In the greater amberjack intestine, the response was concentration-dependent, relatively quick, and plateaus within 30 min of amino acid addition. Therefore, sequential effects of different concentrations can be quantified in each epithelial preparation, and sequential concentrations of 4, 8, and 16 mM were used here. For data presentation, Delta Isc ($\mu A/cm^2$) was calculated as the difference between pre-stimulation values and the steady-state current measured after each stimulation.

2.7. Permeability assay

Permeability assays were performed as previously described (Leal et al., 2022) in the anterior intestine using fish fed with MAP and PPB diets. In brief, after 20–30 min of tissue stabilization after mounting *in vitro*, the saline solution was replaced with a fresh, well-gassed solution to a final volume of 2.2 mL per chamber. Enough Fluorescein isothiocyanate–dextran (FITC, average mol wt ~4000, Sigma, Madrid, Spain) and Rhodamine B isothiocyanate–dextran (RITC, average mol wt ~70,000, Sigma, Madrid, Spain) prepared as concentrated stocks of 100 mg/mL were added to final concentrations of 0.5 mg/mL to the apical chamber. A sample (0.2 mL) was collected from the apical and the basolateral compartments after 15 min of mixing to establish time zero. After exactly 1-h, new samples from the donor and receiver compartments were collected into fresh vials.

Fluorescence measurements were performed using a Multi-Mode Microplate Reader BioTek Synergy 4 (BioTek Instruments, Winooski, Vermont, USA) set for excitation wavelength at 492 nm and emission wavelength at 520 nm for FITC and set for excitation wavelength at 530 nm and emission wavelength at 590 nm for RITC. Concentration standards in the 0.2–2000 ng/mL range were used to establish concentrations in both the apical and basolateral chambers.

The apparent permeability (Papp) was estimated using the equation (Arnold et al., 2019): $Papp = (V*dC)/(A*C_0*dT)$, where Papp is the permeability in centimeters per second (cm/s), V is the volume of the receiver chamber, A is the surface area of the tissue in square centimeters, C_0 is the starting concentration in the donor compartment (apical), and dC/dT is the rate of concentration change (ng/s) of FITC and RITC in the receiving chamber (basolateral).

2.8. Molecular analysis

Intestinal tissue samples from the anterior intestine, mid-intestine, and posterior intestine (rectum) of fish fed the experimental diets in the medium/long-term feeding trial were collected from individual fish and stored for 24 h in RNA later at 4 °C and then at −20 °C until used for RNA extraction (within 3 weeks).

Total RNA was extracted with the Total RNA Kit I (E.Z.N.A., Omega Bio-tek, Norcross, Georgia, USA), including a DNase supplementary treatment (DNA-free Kit - RNase-Free DNase I Set, Omega Bio-tek, Norcross, Georgia, USA) following the manufacturer detailed protocol. After assessing the RNA quantity and quality (NanoPhotometer NP80, IMPLEN GmbH, Munich, Germany), reverse transcription of RNA into cDNA was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Barrington, Illinois, USA) with 500 ng of total RNA in a reaction volume of 20 μL.

Real-time qPCR amplifications were performed in duplicate with a final volume of 6 μL with 3 μL PerfeCTaSYBR Green SuperMix, ROX (Quanta BioSciences, Beverly, Massachusetts, USA), approximately 1 ng cDNA (based on RNA input in the cDNA synthesis), and 0.3 μM of each forward and reverse primers (Table 2). Amplifications were performed in 384-well plates using the BIO-RAD CFX connect Real-time system (Bio-Rad Laboratories, Hercules, California, USA) with the following protocol: denaturation and enzyme activation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, and primer-pair specific annealing temperature (60 °C) for 10 s. After the amplification phase, a temperature-determining dissociation step was carried out, gradually increasing 5 °C every 15 s, between the 60–95 °C range. All samples were run in parallel using 18S ribosomal RNA, β-actin, and α-tubulin to normalise expression. Based on the performance of the 3 genes assessed using NormFinder (Andersen et al., 2004), we used the geometric mean of β-actin and α-tubulin to represent relative expression.

A 10-fold serial dilution (from 100 to 0.001 pg) standard curve was generated for each primer pair from a cDNA pool that included all the intestinal samples to estimate qPCR efficiencies. Standard curves represented the cycle threshold value as a logarithmic function of the number of copies generated, defined arbitrarily as one copy for the most diluted standard. All calibration curves exhibited correlation coefficients $R^2 > 0.99$, and the corresponding real-time PCR efficiencies ranged between 95 % and 99 %. Assayed genes, respective primer sequences, and other details are given in Table 2.

2.9. Statistics

All results are shown as mean ± standard error of the mean (mean ± SEM). After assessing the homogeneity of variance and normality, statistical data analysis was carried out as appropriate by *t*-test (growth performance, somatic indexes and permeability assays), one-way ANOVA (Ussing chamber bioelectrical parameters in different regions of the intestine), two-way ANOVA (molecular expression, region-dependent bioelectrical analysis in Ussing chambers, region dependent electrogenic essential amino acid transport), or regression analysis (region-dependent electrogenic essential amino acid transport and dietary effects on electrogenic essential amino acid transport) using Prism 10 (GraphPad Software for McIntosh, Boston, Massachusetts USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Electrophysiology basal measurements

Based on plasma levels measured in juveniles (Table 3), we designed an amberjack-specific saline (Table 4), to be used in Ussing chamber experiments, which mimic the ion composition of the plasma. Basal bioelectrical characterization of discrete regions of the intestine of greater amberjack juveniles fed a commercial diet showed that all intestinal regions are in net anion absorption, as shown by negative short circuit current values (Isc $\mu Amp.cm^{-2}$) (Table 5), without apparent region-specific changes. In contrast, tissue resistance (Rt, $\Omega.\ cm^2$) showed significant ($p < 0.05$, one-way ANOVA) region-dependent changes with the highest values in the posterior intestine and the lowest in the mid-intestine (Table 5).

Table 2
Primer pairs used for expression analysis in the intestinal tissue of *Seriola dumerili* and qPCR parameters for the genes used for the expression analysis. Ta, annealing temperature.

Gene	Primer	Sequence 5′ to 3’	NCBI Access No	Ta (°C)	Size (bp)
18S	sq18SF-1	GACTCAACACGGGAAACCTC	AY850370	60	139
	sq18SR-1	AGACAAATCGCTCCACCAAC			
EF1α	sdef1aF1	CCCAAGTTCGTCAAGTCTGG	KP455300	60	76
	sdef1aR1	GGGCTTCTGTGGAATGAGTT			
β-actin	sdactbF1	AGGTTCCGTTGCCCAGAG	KX570957	60	85
	sdactbR1	TGCTGTTGTAGGTGGTCTCG			
α-tubulin	XF_alpha_tubulin_FW	CCATACAACTCCATCCTGACC	XM_022762947	60	141
	XF_alpha_tubulin_RV	CAGCTTGTTGAGGTTGGTGT			
SLC15a1	sdSLC15-1F2	TGTGCTTATTGTCGCTGAGG	XM_022746601	60	164
	sdSLC15-1R2	GCTCCTTCTGGGCAAACTG			
SLC15a2	sdSLC15-2F2	CTGTTGCATTTGGGAATGTG	XM_022741102	60	111
	sdSLC15-2R2	AGATGATGAAGACGGCCAAC			
SLC15a4	sdSLC15a4F1	TGTTTGAAGCGTTTCCACTG	XM_022757647	60	94
	sdSLC14a4R1	ATGGCACCACTCCTCAAGTC			
SLC3a1	sdSLC3-a1F2	TCAATGCAGGCTTCAACAAC	XM_022763639	60	194
	sdSLC3-a1R2	ACACTGGCATCAGAGTGGA			
SLC3a2	sdSLC3-a2F2	TCCACAGCAGACTTCCCAGT	XM_022758349	60	109
	sdSLC3-a2R2	GGCACCATCTTTAGCCCATA			
SLC7a5	sdSLC7-a5F1	TTCCTCATCGTCGTCTCCTT	XM_022746216	60	176
	sdSLC7-a5R1	ACCACCTCCATCACCTTCTG			
Cld11	sd-cldn11F2	GCGAGGGTCTGATGTCTTTC	XM_022767762	60	114
	sd-cldn11R2	GGGTCCTGATGTCGTTGC			
Cld12	sd-cldn12F2	CTACTCCACCCGCTCACG	XM_022766158	60	99
	sdcldn12R2	TCAATGTGCCGAGGTTTACA			
Cld19	sd-cldn19F2	AAGAAACAACAAACACATACATACGA	XM_022745164	60	91
	sd-cldn19R2	GGCTACTTGGAAACAACCACA			
Cld20	sd-cldn20F2	ATGCCACCATTCCTCTGTTC	XM_022750463	60	113
	sd-cldn20R2	ATCAGCGTTCACTTGTCAGC			

Table 3
Characterization of plasma ion levels in greater amberjack juveniles. Results are shown as mean ± SEM ($n = 7$).

Na^+ (mmol.L^{-1})	173 ± 3
Cl^- (mmol.L^{-1})	172 ± 8
Ca^{2+} (mmol.L^{-1})	2.6 ± 0.1
K^+ (mmol.L^{-1})	6.5 ± 0.3
Osmolality (mOsm.kg^{-1})	423 ± 6

Table 4
Ionic composition of saline used for greater amberjack intestinal experiments *in vitro* in Ussing chambers.

	mM
NaCl	161
KCl	6.5
$NaHCO_3$	7
$MgSO_4$	1
$CaCl_2$	2.5
Na_2HPO_4	2.5
HEPES	5
Glucose	5
pH = 7.80	

3.2. Region-dependent electrogenic amino acid transport

Fig. 1 summarizes the characterization of electrogenic essential amino acid transport in discrete regions of the great amberjack intestine fed a commercial diet. The sequential apical addition of 4, 8, and 16 mM essential amino acid pools evoked changes in short circuit current (Delta Isc, μA.cm^{-2}). Regardless of the essential amino acid pool concentration tested, the Delta Isc observed was always significantly higher in the mid-intestine, as substantiated by a regression analysis of the amino acid concentration tested *versus* Delta Isc for each region. All regions showed a linear response of Delta Isc to increasing levels of essential amino acids with R^2 of 0.83, 0.99 and 0.95, and slopes of 0.21, 0.86 and 0.06 for the anterior intestine, mid intestine and posterior intestine, respectively. The slope in the mid intestine was significantly steeper than the anterior intestine ($F = 29.62$, $p = 0.0321$) and the posterior intestine ($F = 120.6$, $p = 0.0082$).

Table 5
Bioelectrical properties. Short circuit current (Isc μAmp.cm^{-2}) and tissue resistance (Rt, Ω.cm^2) of discrete regions of the intestine of greater amberjack juveniles mounted in Ussing chambers under voltage clamp (Vt = 0 mV). Isc (μAmp/cm^2) negative values for absorption of anions. Results are shown as the mean ± SEM of the number of fish in brackets. Groups with different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

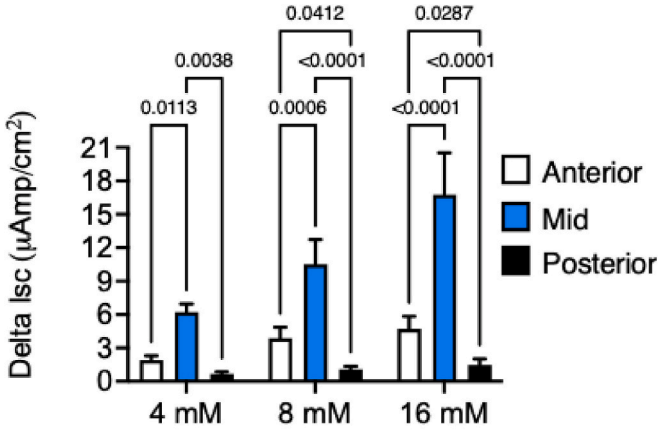
	Anterior Intestine (20)	Mid Intestine (20)	Rectum (7)
Isc (μA.cm^{-2})	−5.1 ± 1.9	−8.6 ± 1.7	−4.1 ± 1.5
Rt (Ω.cm^2)	134.5 ± 10.2 ^{A}	82.7 ± 6.8^{B}	237.6 ± 21.8^{C}

3.3. Experimental diets and growth trial

Both MAP and PPB diets were well accepted by greater amberjack. Nevertheless, the 50 % replacement of marine animal protein with plant protein for 93 days in feeds of greater amberjack (MAP *vs.* PPB) had a significant impact ($p < 0.05$, *t*-test) on final weight, final length, specific growth rate, and feed intake but no significant effect on the intestinal length index (Table 6).

3.4. Electrophysiology

The PPB diet significantly decreased Rt measured in Ussing chambers ($p < 0.05$, Two-way ANOVA), and the Bonferroni pos-hoc comparisons showed that this effect was significant in the anterior intestine ($p = 0.0057$), but not in the mid intestine ($p = 0.4232$). In addition, PPB also



Source of Variation	% of total variation	P value
Interaction	8.942	0.0110
aa concentration	11.91	0.0003
Intestinal region	54.60	<0.0001

Fig. 1. Region-dependent electrogenic essential amino acid transport in anterior intestine, mid intestine, and rectum in greater amberjack juveniles recorded *in vitro* in Ussing chambers. Changes in short circuit current (Delta Isc, μA. cm^{-2}) are evoked by sequential apical addition of an essential amino acid pool of 4, 8, and 16 mM. Each column represents the average + SEM (n = 7). In the two-way ANOVA analysis, intestinal region and aa concentration are the main factors. A Bonferroni post-hoc test was performed, indicating significant differences between intestinal regions.

Table 6
Growth performance and somatic indexes of *Seriola dumerili* juveniles fed with MAP and PPB diets* for 92 days.

Biometric	MAP	PPB	p
Initial weight (g)	9.40 ± 0.01	9.41 ± 0.02	0.728
Final weight (g)	106.9 ± 4.3	60.5 ± 3.5*	0.002
Final length (cm)	18.3 ± 0.5	15.4 ± 0.7*	0.008
SGR (%/day)	2.64 ± 0.16	2.02 ± 0.11*	0.002
FE	1.11 ± 0.08	0.81 ± 0.09*	<0.001
FI (g/fish/day)	1.04 ± 0.09	0.74 ± 0.04*	0.012
ILI (%)	78.2 ± 2.7	75.9 ± 4.0	0.636

* MAP contains 92 % marine animal protein and 8 % plant protein; PPB contains 50 % plant protein and 50 % marine animal protein. Specific growth rate (SGR, %/day) = 100× (ln final body weight − ln initial body weight)/days. Feed efficiency (FE) = weight gain/total feed intake. Feed Intake (FI) = (grams of aquafeed consumed/fish)/day. Intestine length index (ILI) = (100 × intestine length)/fork length. Data on body weight, feed intake, and growth indexes are the mean ± SEM of triplicate tanks. Data on somatic indexes are the mean ± SEM of 12 fish. Asterisks represent significant differences between dietary treatments based on the Student's *t*-test, *p* values are provided for each comparison.

had a significant impact on short circuit current, which was significantly stimulated in the anterior intestine ($p = 0.0419$, Two-way ANOVA, Bonferroni pos-hoc comparison) but not in the mid intestine ($p = 0.1449$). In light of the significant effect of the diet on the anterior intestine, we aimed to confirm if the absorptive current was related to NKCC stimulation and applied its specific inhibitor, bumetanide, at 200 μM. This experimental manipulation showed that the absorptive current was almost completely bumetanide-sensitive in MAP-fed fish but not in the PP-fed group (Fig. 2).

3.5. Permeability

Replacement of MAP with PPB significantly increased Permeability (Papp, cm/s) to FITC dextran (4kD) and RITC dextran (70kD) in the anterior intestine ($p < 0.05$, t-test) of juvenile greater amberjack (Fig. 3).

3.6. Electrogenic amino acid transport

Replacement of 50 % marine animal protein with plant protein significantly modified electrogenic amino acid transport in the mid-intestine of the greater amberjack (Fig. 4). We substantiate this observation by regression analysing the amino acid concentration tested *versus* Delta Isc for each diet. In the MAP-fed group, there was a linear response of Delta Isc to increasing levels of essential amino acids with R^2 of 0.98 and a slope of 0.65. However, in the PPB-fed group, the response plateaus at aa levels >8 mM and provides a lower R^2 of 0.76 and a calculated slope of 0.29.

3.7. Molecular analysis

Fig. 5 shows the relative expression of claudin 11 (*cld11*), claudin12 (*cld12*), claudin19 (*cld19*), and claudin 20 (*cld20*) in different intestinal regions of greater amberjack in response to MAP and PPB diets. The general expression pattern of all four claudins showed higher levels in the anterior intestine and a trend to decrease towards the posterior intestine ($p < 0.05$, Two-way ANOVA, Bonferroni pos-hoc comparison). Dietary replacement of fishmeal had only minor effects on the expression of the claudins analysed. Among those, it would be essential to highlight the significantly decreased expression of *cld12* in the anterior intestine and the increased expression of *cld20* in the mid intestine in response to dietary replacement (50 %) of animal protein with plant protein.

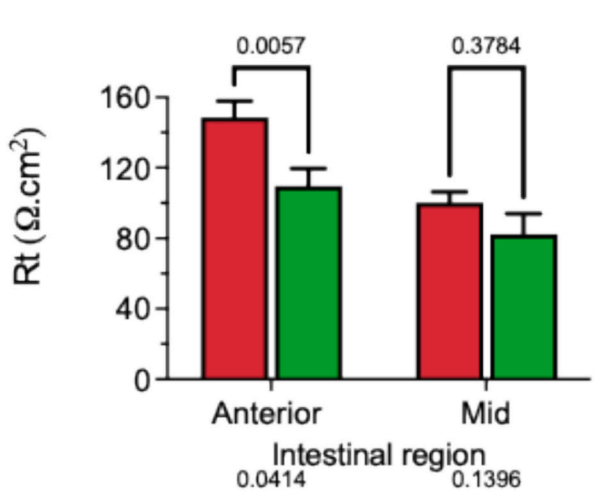
Fig. 6 shows the relative expression of the members of the slc15 family, *slc15a1*, *slc15a2*, and *slc15a4*, in different intestinal regions of greater amberjack in response to MAP and PPB diets. Diet manipulation did not affect the expression of either of the members of the slc15 family analysed. However, the analyses uncovered significant and contrasting region-specific expression patterns for each gene in the greater amberjack intestine ($p < 0.05$, Two-way ANOVA).

Fig. 7 shows the relative expression of *slc3a1*, *slc3a2*, and *slc7a5* in different intestinal regions of greater amberjack in response to MAP and PPB diets. The expression of *slc7a5* was observed in all intestinal regions, but it showed no regional expression pattern and was unaffected by dietary manipulation ($p < 0.05$, Two-way ANOVA). In contrast, the expression of *slc3a1* and *slc3a2* in the intestine of greater amberjack showed clear region-dependent expression patterns and a significant response to dietary manipulation, especially evident in an increased expression in the posterior intestine of fish fed the PPB diet in both cases ($p < 0.05$, Two-way ANOVA).

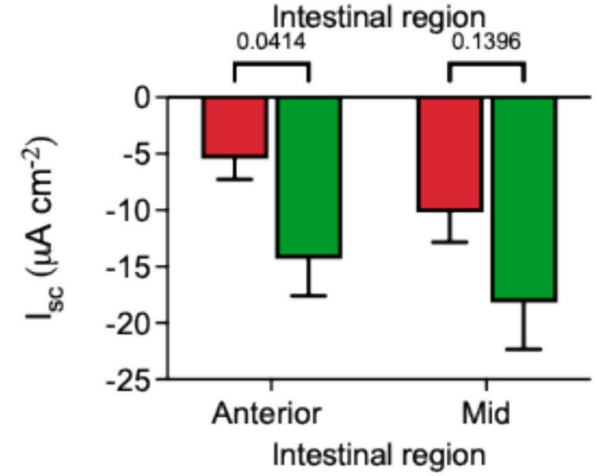
4. Discussion

We embarked on this study to comprehend how growing greater amberjack (*Seriola dumerili*) would respond to the dietary replacement (50 %) of marine animal origin ingredients with plant protein.

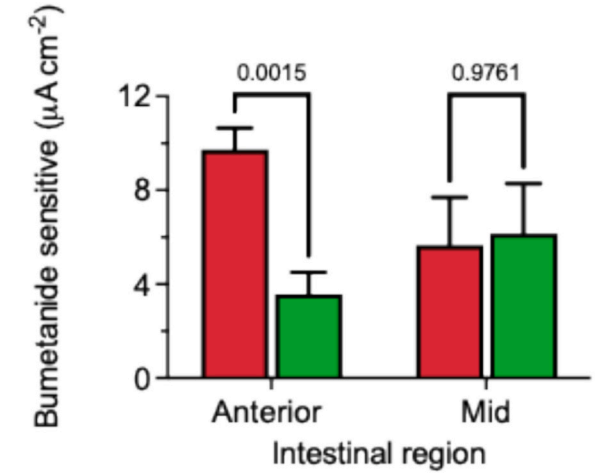
One observation we made in the current study was the impairment of growth-related parameters. Fish fed without replacement showed a specific growth rate (SGR) of 2.62 %/day. However, dietary replacement (50 %) of marine animal origin ingredients with PPB resulted in lighter and shorter fish, with a specific growth rate of 2.02 %/day. Nonetheless, although it is clear that the nutritional requirements of this species vary when compared with other commonly farmed fish in the Mediterranean aquaculture, the Specific Growth Rate could be considered far superior, even at temperatures deemed sub-optimal for the species (19–20 °C), as those reported for gilthead seabream (1.22–1.30 %/day in aquafeeds with 46 % crude protein and 17–18 % crude lipids, in Parma et al., 2020; Perera et al., 2020) or European seabass (0.97–1.1 %/day in aquafeeds with 44–49 % crude protein and 15–23 % crude lipids, in Bonvini et al., 2018), thus pointing out the importance of



Source of Variation	% of total variation	P value
Interaction	1.517	0.2707
Intestinal region	19.66	0.0002
Diet	11.13	0.0040



Source of Variation	% of total variation	P value
Interaction	0.04262	0.8761
Intestinal region	3.960	0.1376
Diet	15.11	0.0050



Source of Variation	% of total variation	P value
Interaction	14.35	0.0240
Intestinal region	1.078	0.5122
Diet	10.60	0.0489

Fig. 2. Effect of plant protein inclusion on tissue resistance (Rt, Ω cm^2; upper panel), short-circuit current (Isc, μA.cm^{-2}; mid panel), and apical (200 μM) bumetanide-sensitive current (Bum-Delta Isc, μAmp.cm^{-2}; lower panel) in the anterior and mid intestine of greater amberjack juveniles recorded *in vitro* in Ussing chambers. Each column represents the average + SEM ($n = 12$). In the two-way ANOVA analysis, intestinal region and diet are the main factors. A Bonferroni post-hoc test was performed, indicating significant differences between experimental groups.

providing an adequate aquafeed to reach the maximum potential in terms of growth performance for each species."

The reduction in feed intake could partially justify the impaired growth in greater amberjack in the PPB group in our trial. However, the intestine serves as the only entry point for feed components. Therefore, our main focus was on the intestinal response of partially replacing dietary animal protein with plant protein in greater amberjack intestinal physiology. We initially investigated intestinal integrity in the anterior and mid-intestine using electrophysiological measurements of trans-epithelial tissue resistance (TER) in Ussing chambers as a marker. Accordingly, we observed decreased tissue integrity in the anterior intestine in response to increased dietary plant protein. Interestingly, this decrease in integrity was not observed in the mid-intestine, where tissue resistance in both feeding regimes remained at similar levels. To validate the electrophysiological findings in the anterior intestine, we conducted permeability measurements using fluorescent markers of different sizes (4 and 70 kD). Nutritional studies have shown that dietary replacement with protein of plant origin often challenges intestinal integrity and barrier function, sometimes resulting in persistent diarrhea. Considering the reported effects in salmon (Krogdahl et al., 2020; Kumar et al.,

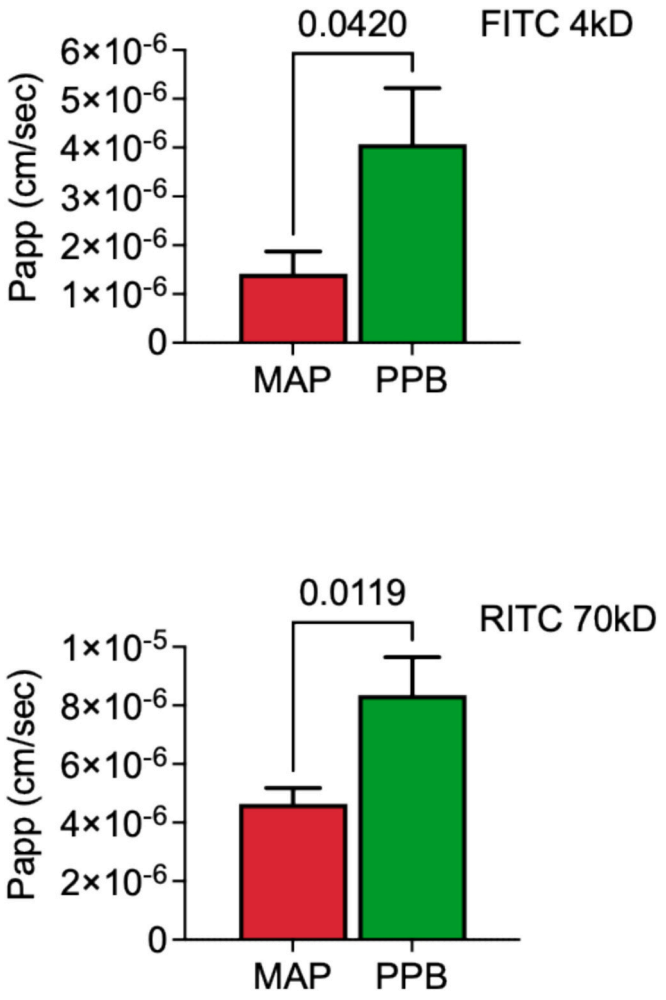
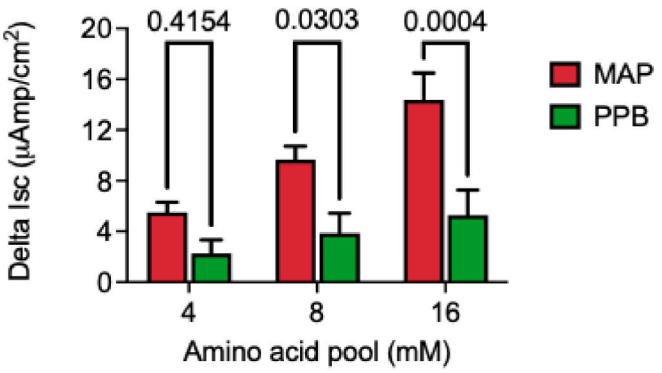


Fig. 3. Effect of plant protein inclusion on Permeability (Papp, cm/s) to FITC dextran (4kD, upper panel) and RITC dextran (70kD, lower panel) in the anterior intestine of juvenile greater amberjack. Each column represents the average + SEM (n = 12). Values of p (Student t-test) are shown.



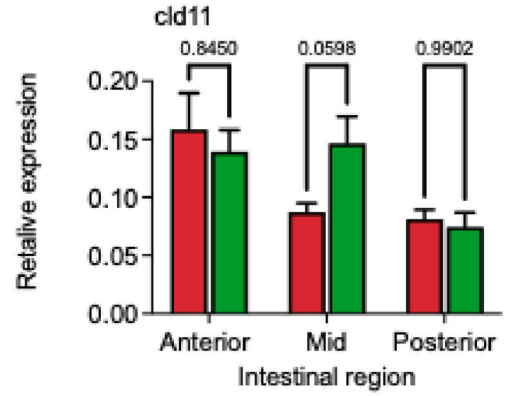
Source of Variation	% of total variation	P value
Interaction	4.403	0.1674
aa concetration	18.02	0.0015
Diet	28.01	<0.0001

Fig. 4. Effect of plant protein inclusion on electrogenic essential amino acid transport in the mid intestine of greater amberjack juveniles recorded *in vitro* in Ussing chambers). Changes in short circuit current (Delta Isc, $\mu A.cm^{-2}$) were evoked by sequential apical addition of an essential amino acid pool of 4, 8, and 16 mM. Each column represents the average + SEM (n = 12). Concentration and diet are the main factors in the two-way ANOVA analysis. A Bonferroni post-hoc test was performed, indicating significant differences between experimental groups.

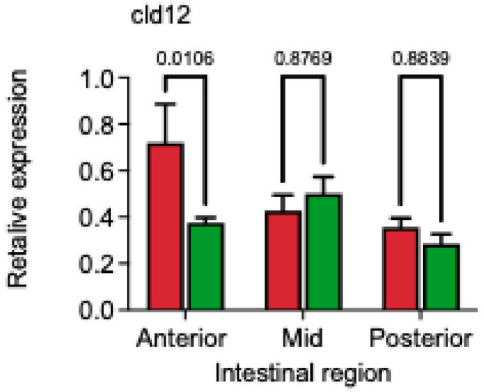
2021), meagre (de Rodrigáñez et al., 2013), *Seriola dorsalis* (Viana et al., 2019), and many other species, our results confirmed the anticipated challenge to tissue integrity (predicted by TER measurements) caused by including PPB in fish feeds in greater amberjack, as indicated by the increased permeability observed with both size markers.

The intestinal epithelial surface has a selective barrier function and is permeable to ions, water, and macromolecules. This barrier is created by the enterocyte layer connected apically by tight junctions, which are cell-to-cell adhesion complexes and the essential components of the epithelial barrier. Claudins, a family of proteins with at least 24 members (Lal-Nag and Morin, 2009), are integral membrane proteins in tight junctions and express in all known epithelial tissues. Some claudins form channels that drive paracellular ions and water movements, while others insert into the cell membrane and interact with claudins from neighboring cells to contribute to form the paracellular barrier (Günzel and Yu, 2013). Here, we analysed the expression pattern and responses of expression of claudin 11 (*cld11*), claudin12 (*cld12*), claudin19 (*cld19*), and claudin 20 (*cld20*) to understand if the observed barrier dysfunction in the anterior intestine could be exposed from a molecular angle. In line with this approach, of the 4 targeted claudins, only *cld12* was down-regulated in the anterior intestine in response to dietary replacement (50 %) of marine animal origin ingredients with PPB. It was surprising to find upregulation of *cld20* in the mid-intestine in response to the inclusion of plant protein, which was not paralleled by a TER decrease. We do not intend to overstate the dietary effect on *cld12* as a marker of epithelial dysfunction, especially in light of the newly recognised mechanism of interclaudin interference, where other claudins can negatively regulate a claudin (Shashikanth et al., 2022). However, it is noteworthy that lower TER, permeability, and *cld12* expression occur simultaneously in the anterior intestine. Following another line of evidence, a recent study in knockout mice (Beggs et al., 2021) demonstrates that claudin-2 and claudin-12 play important complementary roles in paracellular calcium transport, affecting calcium homeostasis and subsequent bone mineralisation. Although we focused on *cld12* in the context of barrier dysfunction, it is important to note that the anterior intestine is the most significant intestinal fragment for calcium uptake in marine fish intestine (Fuentes et al., 2006). Considering the larger implications, *cld12* is certainly interesting for future nutritional tests.

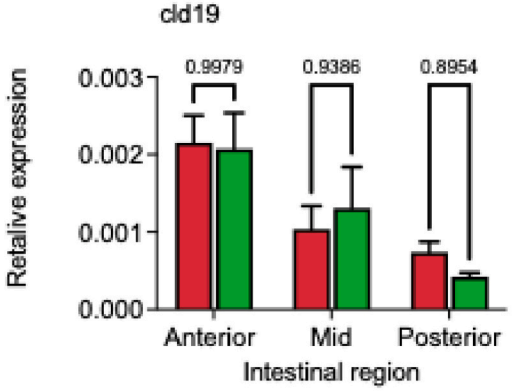
In mammals, the small intestine is the most critical contributor to the plasma profile of amino acids, while the colon has a less vital role but contributes to transport (Broer and Fairweather, 2019). Such regional distribution of relative importance in amino acid transport still needs to be established in fish, although some gene expression studies point to species-specificity in intestinal distribution. Our preliminary results from transport studies show a region-dependent electrogenic transport of essential amino acids in the intestine of greater amberjack. The mid-intestine appears to have the highest electrogenic essential amino acid transport capacity. In light of this observation, we performed a comparative study of amino acid transport capacity in the mid-intestine with fish fed the MAP or the PPB diets and exposed a negative effect of dietary replacement (50 %) of marine animal origin ingredients with plant protein. This transport impairment likely underlies the significant growth differences observed between the two dietary regimes. Considering this observation, one might wonder about the mechanisms behind this impaired electrogenic response, leading to several questions. The first is related to the possibility of molecular changes in the expression profiles of amino acids and small peptide transporters. Amino acid transport across the plasma membrane is facilitated by diverse amino acid transporter systems within the solute carrier (SLC) superfamily (Pizzagalli et al., 2021). Consequently, we targeted first the slc15 family



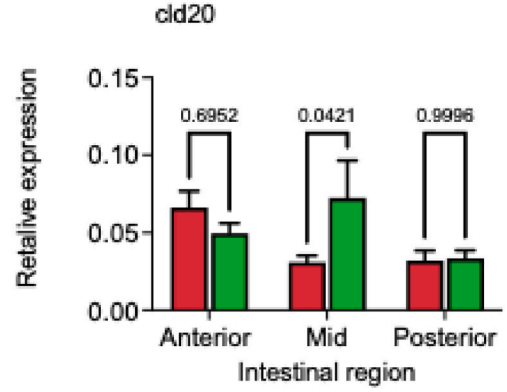
Source of Variation	% of total variation	P value
Interaction	6.949	0.0678
Int. Region	19.78	0.0008
Diet	0.7195	0.4480



Source of Variation	% of total variation	P value
Interaction	9.152	0.0337
Int. Region	11.13	0.0170
Diet	4.025	0.0807

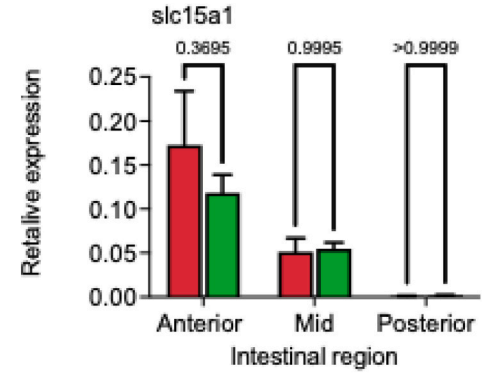


Source of Variation	% of total variation	P value
Interaction	1.011	0.7174
Int. Region	29.15	0.0003
Diet	0.03152	0.8858

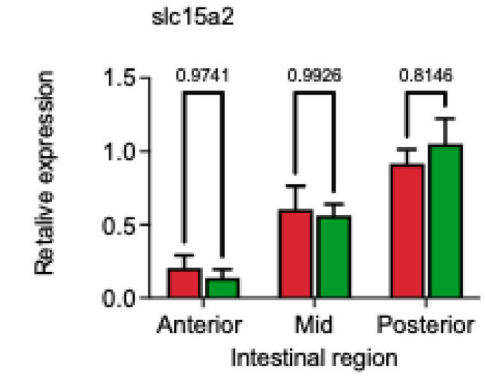


Source of Variation	% of total variation	P value
Interaction	8.895	0.0469
Int. Region	7.154	0.0834
Diet	1.184	0.3582

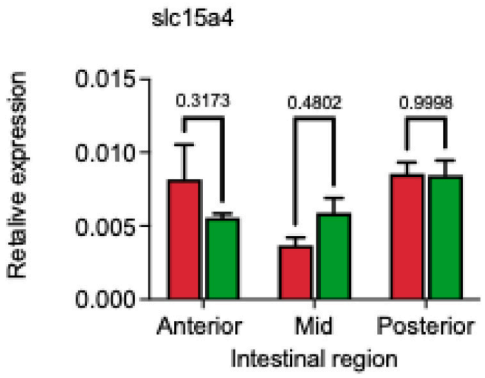
Fig. 5. Relative expression of claudin 11 (*cld11*), claudin12 (*cld12*), claudin19 (*cld19*), and claudin 20 (*cld20*) in different intestinal regions of greater amberjack in response to MAP and PPB diets. Normalization of expression used the geometric mean of beta-actin and alpha-tubulin. Results are mean + SEM of 10–11 individual regions per group. Statistical analysis was performed by a two-way ANOVA considering intestinal region and diet as main factors (please refer to the tables). A Bonferroni post-hoc test was carried out, indicating significant differences between experimental groups.



Source of Variation	% of total variation	P value
Interaction	1.739	0.4513
Int. Region	34.51	<0.0001
Diet	0.6990	0.4239



Source of Variation	% of total variation	P value
Interaction	0.7959	0.6641
Int. Region	42.28	<0.0001
Diet	0.004609	0.9451

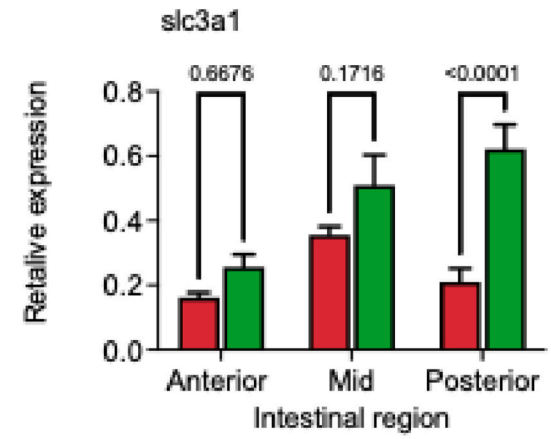


Source of Variation	% of total variation	P value
Interaction	5.745	0.1337
Int. Region	14.26	0.0086
Diet	0.04396	0.8589

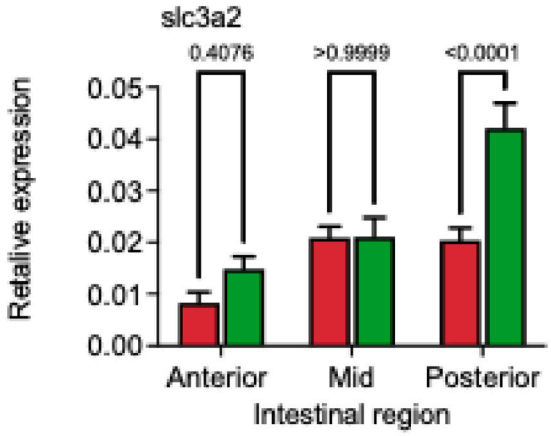
Fig. 6. Relative expression of *slc15a1*, *slc15a2*, and *slc15a4* in different intestinal regions of greater amberjack in response to MAP and PPB diets. Normalization of expression used the geometric mean of beta-actin and alpha-tubulin. Results are mean + SEM of 10–11 individual regions per group. Statistical analysis was performed by a two-way ANOVA considering intestinal region and diet as main factors (please refer to the tables).

formerly known as PepT1 (*slc15a1*), PepT2 (*slc15a2*), and PhT1 (*slc15a4*) (PhT2, *i.e.*, slc15a3 is absent in fish; Huang et al., 2015) that play key roles in the cellular uptake of di/tripeptides (Smith et al., 2013). Our objective when targeting the intestinal expression of *slc15a1*, *slc15a2*, and *slc15a4* was to understand if the dietary replacement of marine animal origin ingredients with PPB had an impact on di- and/or tri-peptide transport on the intestine of greater amberjack, based on expression analysis. The short answer is no, at least at this level of protein source manipulation. Our results agree with previous reports on fish in different nutritional scenarios, such as *Misgurnus anguillicaudatus* (Gonçalves et al., 2007) or cod larvae (Amberg et al., 2008). But in the case of PEPT1 (slc15a1), at least, contrast with the results for grouper hybrids in response to hydrolyzed porcine mucosa (Yang et al., 2021), or rainbow trout fry (Ostaszewsk et al., 2010) in response to protein source (dipeptides, free amino acids, and intact protein). Regardless of the lack of dietary effects of protein sources, one observation emerged from our study: a clear region-dependent expression pattern of *slc15a1*, *slc15a2,* and *slc15a4* was observed. In further studies, it will be interesting to understand if this region-dependency is functional and entails a manifestation of the substrate preference of each of the slc15 members along the intestinal tract based on the affinity/capacity of different isoforms (Kottra et al., 2013).

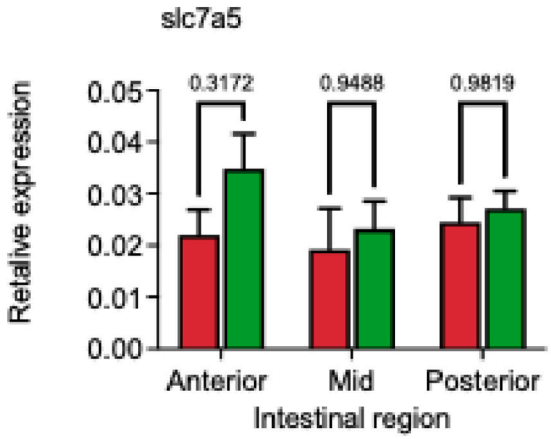
Our study also focused on *slc7a5* (LAT1), one of seven members of the *slc7* family known for its functional association (forming heterodimeric transmembrane protein complexes) with members of the *slc3* family, namely *slc3a1* and *slc3a2* (Napolitano et al., 2015). While the mechanisms underlying heterodimer function remain elusive, it is evident that *slc7a5* plays a crucial role in distributing essential amino acids within cells through amino acid antiport, serving as the sole transport-competent subunit of the heterodimer (Napolitano et al., 2015). In our investigation on the greater amberjack intestine, we observed that *slc7a5* is expressed throughout the different intestinal regions. Interestingly, we noted a significant influence of diet on expression patterns, albeit without discernible regional variations.



Source of Variation	% of total variation	P value
Interaction	8.103	0.0215
Intestinal Region	15.77	0.0009
Diet	20.14	<0.0001



Source of Variation	% of total variation	P value
Interaction	9.808	0.0037
Int. Region	30.22	<0.0001
Diet	10.52	0.0006



Source of Variation	% of total variation	P value
Interaction	2.236	0.4872
Int. Region	6.098	0.1467
Diet	6.337	0.0469

Fig. 7. Relative expression of *slc3a1*, *slc3a2*, and *slc7a5* in different intestinal regions of greater amberjack in response to MAP and PPB diets. Normalization of expression used the geometric mean of beta-actin and alpha-tubulin. Results are mean + SEM of 10–11 individual regions per group. Statistical analysis was performed by a two-way ANOVA considering intestinal region and diet as main factors (please refer to the tables). A Bonferroni post-hoc test was carried out, indicating significant differences between experimental groups.

Considering the pivotal role of slc7a5 in essential amino acid transport and our findings from electrogenic amino acid transport assays, it is plausible that the tested dietary regime, featuring a 50 % replacement of marine animal origin ingredients with PPB, compromises the bioavailability of essential amino acids crucial for absorption and somatic growth, thus promoting a molecular response at the level of *slc7a5*.

How the expression patterns of both *slc3a1* and *slc3a2* fit with the effects observed for *slc7a5* remains unknown. In higher vertebrates, the slc3a1 is involved in sodium-independent transport of cystine, neutral, and dibasic amino acids across the cell membrane. slc3a2 is in the basolateral membrane and is a sodium-independent transporter of large neutral amino acids such as leucine, arginine, tyrosine, and phenylalanine (de La Ballina et al., 2018). The information on the expression of the members of the slc3 family in fish is scarce. In tilapia (Nitzan et al., 2017), *slc3a1* is more expressed in the anterior and mid intestine than in the posterior intestine. This observation contrasts with our results for the

greater amberjack in that the diet significantly affects the expression of both *slc3a1* and *slc3a2*, an effect particularly strong in the posterior intestine, *i.e.* the rectum. Recent reports indicate that in addition to its role in amino acid transport, slc3a2 is a novel endoplasmic reticulum stress-related signaling protein that could be used as an early marker of apoptosis (Liu et al., 2018). Using an approach of gain and loss-of-function in mouse models with genetically manipulated CD98 (slc3a2) expression, specifically in intestinal epithelial cells Nguyen et al. (2011) showed that overexpression of slc3a2 was linked to its capacity to induce barrier dysfunction and to stimulate cell proliferation and production of proinflammatory mediators. If this chain of events holds true for the greater amberjack intestine (especially in the posterior intestine or rectum), *slc3a2* expression could be used as a reporter of a nutritionally challenged intestine.

In summary, this study used a combination of *in vivo*, *ex-vivo*, and molecular approaches to expose and integrate the effects of dietary replacement (50 %) of marine animal origin ingredients (MAP diet) with plant protein (PPB). At *in vivo* levels, we showed a clear impairment of growth indexes in greater amberjack fed with partial protein replacement with material of plant origin. *Ex vivo*, we characterized the intestinal regionalization as a proxy to be used as a diagnostic tool involved in the gut integrity and functionality, revealing an impact on barrier function in the anterior intestine and providing evidence of impaired electrogenic amino acid transport in the mid-intestine in response to plant proteins. Finally, the expression patterns of the genes targeted in our molecular approach have disclosed important clues for putative markers to be pursued in future nutritional studies.

Funding

This study was partially supported by the Research Projects SeriBlue (Knowledge Transfer and Business Innovation Project in the Field of the Blue Economy, CEI·MAR 2020), IRISer (PID2023-146411OB-C21 and PID2023-146411OB-C22, funded by Spanish Ministry of Sciences, Innovation and Universities), and the Spin-off LifeBioencapsulation S.L. CIMA receives Portuguese national funds from FCT - Foundation for Science and Technology through project UIDP/00350/2020 (doi: 10.54499/UIDP/00350/2020 and doi: 10.54499/UIDB/00350/2020). ARNET, Associate Laboratory, receives Portuguese national funds from FCT - Foundation for Science and Technology through project LA/P/0069/2020. CCMar receives Portuguese national funds from FCT - Foundation for Science and Technology through projects UIDB/04326/2020, UIDP/04326/2020 and LA/P/0101/2020.

Filomena Fonseca was supported by a grant from Fundación Carolina during her sabbatical stay at UCA.

CRediT authorship contribution statement

Juan Fuentes: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Filomena Fonseca:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Silvia F. Gregorio:** Methodology, Formal analysis. **Larissa Kussaba:** Methodology, Formal analysis. **Erick Perera:** Methodology, Investigation. **Francisco J. Alarcón-López:** Resources, Funding acquisition. **Juan A. Martos-Sitcha:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data availability

Data will be made available on request.

Acknowledgements

The authors wish to thank the University of Almería (Experimental feeds Service, https://www.ual.es/universidad/serviciosgenerales/stecnicos/perifericos-convenio/piensos-experimentales) for its support in elaborating aquafeeds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741925.

References

Amberg, J.J., Myr, C., Kamisaka, Y., Jordal, A.E., Rust, M.B., Hardy, R.W., Koedijk, R., Rønnestad, I., 2008. Expression of the oligopeptide transporter, PepT1, in larval Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 150, 177–182. https://doi.org/10.1016/j.cbpb.2008.02.011.

Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496.

Aragão, C., Cabano, M., Colen, R., Fuentes, J., Dias, J., 2020. Alternative formulations for gilthead seabream diets: towards a more sustainable production. Aquacult. Nutr. 26, 444–455. https://doi.org/10.1111/anu.13007.

Arnold, Y.E., Thorens, J., Bernard, S., Kalia, Y.N., 2019. Drug transport across porcine intestine using an Ussing chamber system: regional differences and the effect of Pglycoprotein and CYP3A4 activity on drug absorption. Pharmaceutics 11, 139. https://doi.org/10.3390/pharmaceutics11030139.

Beggs, M.R., Young, K., Pan, W., O'Neill, D.D., Saurette, M., Plain, A., Rievaj, J., Doschak, M.R., Cordat, E., Dimke, H., Alexander, R.T., 2021. Claudin-2 and claudin-12 form independent, complementary pores required to maintain calcium homeostasis. Proc. Natl. Acad. Sci. U. S. A. 118, e2111247118. https://doi.org/10.1073/pnas.2111247118.

Bonvini, E., Bonaldo, A., Parma, L., Mandrioli, L., Sirri, R., Grandi, M., Fontanillas, R., Viroli, C., Gatta, P.P., 2018. Feeding European sea bass with increasing dietary fibre levels: impact on growth, blood biochemistry, gut histology, gut evacuation. Aquaculture 494, 1–9. https://doi.org/10.1016/j.aquaculture.2018.05.017.

Broer, S., 2008. Amino acid transport across mammalian intestinal and renal epithelia. Physiol. Rev. 88, 249–286. https://doi.org/10.1152/physrev.00018.2006.

Broer, S., Fairweather, S.J., 2019. Amino acid transport across the mammalian intestine. Comp. Physiol. 9, 343–373. https://doi.org/10.1002/cphy.c170041.

Chen, M., Singh, A., Xiao, F., Dringenberg, U., Wang, J., Engelhardt, R., Yeruva, S., Rubio-Aliaga, I., Nassl, A.M., Kottra, G., Daniel, H., Seidler, U., 2010. Gene ablation for PEPT1 in mice abolishes the effects of dipeptides on small intestinal fluid absorption, short-circuit current, and intracellular pH. Am. J. Physiol. Gastrointest. Liver Physiol. 299, G265–G274. https://doi.org/10.1152/ajpgi.00055.2010.

Con, P., Nitzan, T., Cnaani, A., 2017. Salinity-dependent shift in the localization of three peptide transporters along the intestine of the Mozambique Tilapia (*Oreochromis mossambicus*). Front. Physiol. 8, 8. https://doi.org/10.3389/fphys.2017.00008.

de La Ballina, L.R., de Garay, T., Féral, C.C., Palacín, M., 2018. SLC3A2. In: Choi, S. (Ed.), Encyclopedia of Signaling Molecules. Springer, Cham. https://doi.org/10.1007/978-3-319-67199-4_101895.

de Rodrigáñez, M.A.S., Fuentes, J., Moyano, F.J., Ribeiro, L., 2013. In vitro evaluation of the effect of a high plant protein diet and nucleotide supplementation on intestinal integrity in meagre (*Argyrosomus regius*). Fish Physiol. Biochem. 39, 1365–1370. https://doi.org/10.1007/s10695-013-9790-x.

Donohue, C.G., Partridge, G.J., Sequeira, A.M.M., 2021. Bioenergetic growth model for the yellowtail kingfish (*Seriola lalandi*). Aquaculture 531. https://doi.org/10.1016/j.aquaculture.2020.735884.

Estensoro, I., Ballester-Lozano, G., Benedito-Palos, L., Grammes, F., Martos-Sitcha, J.A., Mydland, L.-T., Calduch-Giner, J.A., Fuentes, J., Karalazos, V., Ortiz, Á., Øverland, M., SitjàBobadilla, A., Pérez-Sánchez, J., 2016. Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. PlosONE 11, e0166564. https://doi.org/10.1371/journal.pone.0166564.

Fonseca, F., Fuentes, J., Vizcaíno, A.J., Alarcón, F.J., Mancera, J.M., Martos-Sitcha, J.M., 2023. From invasion to fish fodder: inclusion of the brown algae *Rugulopteryx okamurae* in aquafeeds for European sea bass *Dicentrarchus labrax* (L., 1758). Aquaculture 568, 739318. https://doi.org/10.1016/j.aquaculture.2023.739318.

Fuentes, J., Figueiredo, J., Power, D.M., Canário, A.V., 2006. Parathyroid hormone-related protein regulates intestinal calcium transport in sea bream (Sparus auratus). Am. J. Physiol. Regul. Integr. Comp. Physiol. 291, R1499–R1506. https://doi.org/10.1152/ajpregu.00892.2005.

Fuentes, J., Ribeiro, L., Aragão, C., 2018. Bile salts regulate ion transport in the intestine of Senegalese sole. Aquaculture 495, 842–848. https://doi.org/10.1016/j.aquaculture.2018.06.050.

Gonçalves, A.F., Castro, L.F., Pereira-Wilson, C., Coimbra, J., Wilson, J.M., 2007. Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish? Comp. Biochem. Physiol. Part D Genomics Proteomics. 2, 345–355. https://doi.org/10.1016/j.cbd.2007.08.002.

Günzel, D., Yu, A.S., 2013. Claudins and the modulation of tight junction permeability. Physiol. Rev. 93, 525–569. https://doi.org/10.1152/physrev.00019.2012.
Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aqua. Res. 41, 770–776. https://doi.org/10.1111/j.1365-2109.2009.02349.x.
Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., Islam, M.J., Maekawa, M., Fujieda, T., 2018. Substitution of dietary fishmeal by soybean meal with inosine administration influences growth, digestibility, immunity, stress resistance and gut morphology of juvenile amberjack *Seriola dumerili*. Aquaculture 488, 174–188. https://doi.org/10.1016/j.aquaculture.2018.01.037.
Huang, Q., Vera Delgado, J.M., Seni Pinoargote, O.D., Llaguno, R.A., 2015. Molecular evolution of the Slc15 family and its response to waterborne copper and mercury exposure in tilapia. Aquat. Toxicol. 163, 140–147. https://doi.org/10.1016/j.aquatox.2015.04.011.
Kottra, G., Spanier, B., Verri, T., Daniel, H., 2013. Peptide transporter isoforms are discriminated by the fluorophore-conjugated dipeptides β-ala- and d-ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid. Physiol. Rep. 1 (7), e00165. https://doi.org/10.1002/phy2.165.
Krogdahl, Å., Kortner, T.M., Jaramillo-Torres, A., Gamil, A.A.A., Chikwati, E., Li, Y., Schmidt, M., Herman, E., Hymowitz, T., Teimouri, S., Storebakken, T., 2020. Removal of three proteinaceous antinutrients from soybean does not mitigate soybean-induced enteritis in Atlantic salmon (*Salmo salar*, L.). Aquaculture 514, 734495. https://doi.org/10.1016/j.aquaculture.2019.734495.
Kumar, V., Fawole, F.J., Romano, N., Hossain, M.S., Labh, S.N., Overturf, K., Small, B.C., 2021. Insect (black soldier fly, *Hermetia illucens*) meal supplementation prevents the soybean meal-induced intestinal enteritis in rainbow trout and health benefits of using insect oil. Fish Shellfish Immunol. 109, 116–124. https://doi.org/10.1016/j.fsi.2020.12.008.
Lal-Nag, M., Morin, P.J., 2009. The claudins. Genome Biol. 10, 235. https://doi.org/10.1186/gb-2009-10-8-235.
Leal, E., Angotzi, A.R., Godino-Gimeno, A., Gregório, S.F., Rotllant, J., Saera-Vila, A., Fuentes, J., Cerdá-Reverter, J.M., 2022. Agouti overexpression in a transgenic model regulates integrity, permeability and electrogenic amino acid transport in zebrafish intestine. Frontiers in marine. Science 9. https://www.frontiersin.org/articles/10.3389/fmars.2022.1011702.
Liu, C., Li, X., Li, C., Zhang, Z., Gao, X., Jia, Z., Chen, H., Jia, Q., Zhao, X., Liu, J., Liu, B., Xu, Z., Tian, Y., He, K., 2018. SLC3A2 is a novel endoplasmic reticulum stress-related signaling protein that regulates the unfolded protein response and apoptosis. PloS One 13 (12), e0208993. https://doi.org/10.1371/journal.pone.0208993.
Martos-Sitcha, J.A., Simó-Mirabet, P., Piazzon, M.C., De Las Heras, V., Calduch-Giner, J. A., Puyalto, M., Tinsley, J., Makol, A., Sitjà-Bobadilla Pérez-Sánchez, J., 2018. Dietary sodium heptanoate helps to improve feed efficiency, growth hormone status and swimming performance in gilthead sea bream (*Sparus aurata*). Aquacult. Nutr. 24, 1638–1651. https://doi.org/10.1111/anu.12799.
McCauley, H.A., Matthis, A.L., Enriquez, J.R., Nichol, J.T., Sanchez, J.G., Stone, W.J., Sundaram, N., Helmrath, M.A., Montrose, M.H., Aihara, E., Wells, J.M., 2020. Enteroendocrine cells couple nutrient sensing to nutrient absorption by regulating ion transport. Nat. Commun. 11, 4791. https://doi.org/10.1038/s41467-020-18536-z.
Molina-Roque, L., Bárany, A., Sáez, M.I., Alarcón, F.J., Tapia, S.T., Fuentes, J., Mancera, J.M., Perera, E., Martos-Sitcha, J.M., 2022. Biotechnological treatment of microalgae enhances growth performance, hepatic carbohydrate metabolism and intestinal physiology in gilthead seabream (*Sparus aurata*) juveniles close to commercial size. Aquac. Rep. 25, 101248. https://doi.org/10.1016/j.aqrep.2022.101248.
Napolitano, L., Scalise, M., Galluccio, M., Pochini, L., Albanese, L.M., Indiveri, C., 2015. LAT1 is the transport competent unit of the LAT1/CD98 heterodimeric amino acid transporter. Int. J. Biochem. Cell Biol. 67, 25–33. https://doi.org/10.1016/j.biocel.2015.08.004.
Navarro-Guillén Conceição, L.E.C., Pinto, W., Siguero, I., Urrutia, P., Moyano, F.J., Yúfera, M., 2019. Fast growing greater amberjack post-larvae require a high energy-high protein weaning diet. Aquaculture 499, 195–202. https://doi.org/10.1016/j.aquaculture.2018.09.037.
Navarro-Guillen, C., Yúfera, M., Perera, E., 2022. Biochemical features and modulation of digestive enzymes by environmental temperature in the greater amberjack, *Seriola dumerili*. Front. Mar. Sci. 9, 960746. https://doi.org/10.3389/fmars.2022.960746.
Nguyen, H.T., Dalmasso, G., Torkvist, L., Halfvarson, J., Yan, Y., Laroui, H., Shmerling, D., Tallone, T., D'Amato, M., Sitaraman, S.V., Merlin, D., 2011. CD98 expression modulates intestinal homeostasis, inflammation, and colitis-associated cancer in mice. J. Clin. Invest. 121, 1733–1747. https://doi.org/10.1172/JCI44631.
Nitzan, T., Rozenberg, P., Cnaani, A., 2017. Differential expression of amino-acid transporters along the intestine of Mozambique tilapia (*Oreochromis mossambicus*) and the effect of water salinity and time after feeding. Aquaculture 472, 71–75. https://doi.org/10.1016/j.aquaculture.2016.01.020.
Oliva-Teles, A., Enes, P., Peres, H., 2015. In: Davis, D. (Ed.), Feed and Feeding Practices in Aquaculture. Woodhead Publishing Ltd, Cambridge, pp. 203–223. https://doi.org/10.1016/B978-0-08-100506-4.00008-8.
Orozco, Z.G.A., Soma, S., Kaneko, T., Watanabe, S., 2018. Spatial mRNA expression and response to fasting and refeeding of neutral amino acid transporters *slc6a18* and *slc6a19a* in the intestinal epithelium of *Mozambique tilapia*. Front. Physiol. 9, 212. https://doi.org/10.3389/fphys.2018.00212.
Parma, L., Pelusio, N.F., Gisbert, E., Esteban, M.A., D'Amico, F., Soverini, M., Candela, M., Dondi, F., Gatta, P.P., Bonaldo, A., 2020. Effects of rearing density on growth, digestive conditions, welfare indicators and gut bacterial community of gilthead sea bream (*Sparus aurata*, L. 1758) fed different fishmeal and fish oil dietary levels. Aquaculture 518, 734854. https://doi.org/10.1016/j.aquaculture.2019.734854.
Perera, E., Sánchez-Ruiz, D., Sáez, M.I., Galafat, A., Bárany, A., Fernández-Castro, M., Vizcaíno, A.J., Fuentes, J., Martínez, T.F., Mancera, J.M., Alarcón, F.J., Martos-Sitcha, J.A., 2020. Low dietary inclusion of nutraceuticals from microalgae improves feed efficiency and modifies intermediary metabolisms in gilthead sea bream (Sparus aurata). Sci. Rep. 10, 18676. https://doi.org/10.1038/s41598-020-75693-3.
Pizzagalli, M.D., Bensimon, A., Superti-Furga, G., 2021. A guide to plasma membrane solute carrier proteins. FEBS J. 288, 2784–2835. https://doi.org/10.1111/febs.15531.
Shashikanth, N., France, M.M., Xiao, R., Haest, X., Rizzo, H.E., Yeste, J., Reiner, J., Turner, J.R., 2022. Tight junction channel regulation by interclaudin interference. Nat. Commun. 13, 3780. https://doi.org/10.1038/s41467-022-31587-8.
Simó-Mirabet, P., Felip, A., Estensoro, I., Martos-Sitcha, J.A., De Las Heras, V., Calduch-Giner, J., Puyalto, M., Karalazos, V., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2018a. Impact of low fish meal and fish oil diets on the performance, sex steroid profile and male-female sex reversal of gilthead sea bream (*Sparus aurata*) over a three-year production cycle. Aquaculture 490, 64–74. https://doi.org/10.1016/j.aquaculture.2018.02.025.
Skaramuca, B., Kožul, V., Teskeredzic, Z., Bolotin, J., Onofri, V., 2001. Growth rate of tank-reared Mediterranean amberjack, Seriola dumerili (Risso 1810) fed on three different diets. J. Appl. Ichthyol. 17, 130–133. https://doi.org/10.1111/j.1439-0426.2001.00038.x.
Smith, D.E., Clémençon, B., Hediger, M.A., 2013. Proton-coupled oligopeptide transporter family SLC15: physiological, pharmacological and pathological implications. Mol. Aspects Med. 34, 323–336. https://doi.org/10.1016/j.mam.2012.11.003.
Vacca, F., Barca, A., Gomes, A.S., Mazzei, A., Piccinni, B., Cinquetti, R., Del Vecchio, G., Romano, A., Rønnestad, I., Bossi, E., Verri, T., 2019. The peptide transporter 1a of the zebrafish *Danio rerio*, an emerging model in nutrigenomics and nutrition research: molecular characterization, functional properties, and expression analysis. Genes Nutr. 14, 33. https://doi.org/10.1186/s12263-019-0657-3.
Viana, M.T., Rombenso, A.N., Del Rio-Zaragoza, O.B., Nomura, M., Díaz-Argüello, R., Mata-Sotres, J.A., 2019. Intestinal impairment of the California yellowtail, *Seriola dorsalis*, using soybean meal in the diet. Aquaculture 513, 734443. https://doi.org/10.1016/j.aquaculture.2019.734443.
Vizcaíno, A.J., Sáez, M.I., Galafat, A., Galindo-Melero, R., Perera, E., Casa-Porras, I., Zubía, E., Veja, J., Figueroa, F.L., Martínez, T.F., Martos-Sitcha, J.A., Alarcón, F.J., 2024. Effects of feeding European seabass (*Dicentrarchus labrax*) juveniles with crude, hydrolysed and fermented biomass of the invasive macroalga *Rugulopteryx okamurae* (Ochrophyta). Aquac. Rep. 34, 101877. https://doi.org/10.1016/j.aqrep.2023.101877.
Wu, P., Li, Y., Cheng, J., Chen, L., Zheng, M., Wu, Y., Wang, J., Zhang, J., Chu, W., 2016. Transcriptome analysis and postprandial expression of amino acid transporter genes in the fast muscles and gut of Chinese perch (*Siniperca chuatsi*). PloS One 11 (7), e0159533. https://doi.org/10.1371/journal.pone.0159533.
Yang, X., Wang, G., Zhao, X., Dong, X., Chi, S., Tan, B., 2021. Addition of hydrolysed porcine mucosa to low-fishmeal feed improves intestinal morphology and the expressions of intestinal amino acids and small peptide transporters in hybrid groupers (*Epinephelus fuscoguttatus* ♀ × *E. Lanceolatus* ♂). Aquaculture 535, 736389. https://doi.org/10.1016/j.aquaculture.2021.736389.