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Uncovering the physiological impacts of soybean meal replacement by Narbonne vetch (*Vicia narbonensis*) meal in rainbow trout *(Oncorhynchus mykiss*) diets: Towards the future and sustainable European aquaculture.

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ABSTRACT

The identification and implementation of raw materials to replace fish meal (FM) or its major alternative, soybean meal (SBM), among other factors, is crucial for the sustainable growth of aquaculture. Narbonne vetch (*Vicia narbonensis*) meal (NVM) has previously been identified as a promising alternative raw material locally produced in Europe to replace SBM. Previous work has showed that 33% replacement of SBM by NVM treated with a commercially available phytase (Rovabio® PHY) does not compromise fish growth performance in a 63-day trial. Here, a deeper characterization of the potential use of NVM to replace SBM was performed to unveil any potential undesired physiological impact in rainbow trout (*Oncorhynchus mykiss*) juveniles with an initial weight of 38.04 ± 0.07 g. After a 63-day feeding trial, high (66%) SBM replacement by NVM (even when treated with Rovabio® PHY) had a negative impact on rainbow trout: decreasing the activity of alkaline proteases, trypsin, chymotrypsin, and α-amylase; inducing cell shrinkage in hepatocytes; and reducing plasma triglycerides and hepatic vitamin E (VE) levels. No

Abbreviations: ActB, actin β; Amy2b, α-amylase 2 b; ANF, anti-nutritional factors; Attpb, α-tocopherol transfer protein b; BAPNA, N-α-benzoyl-DL-arginine p-nitroanilide; Cd36, cluster determinant 36; FM, fish meal; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GLC, gas-liquid chromatography; GPx, glutathione peroxidase; GST, glutathione S-transferase; Npc1l1, Niemann-pick c1-like protein 1; NSP, non-starch polysaccharides; NVM, Narbonne vetch meal; RAS, recirculating aquaculture system; SAAPNA, N-succinyl-ala-ala-pro-phe p-nitroanilide; SBM, soybean meal; Slc2a2 or Glut2, solute carrier family 2, facilitated glucose transporter member 2; Slc2a4b or Glut4, solute carrier family 2, facilitated glucose transporter member 4 b; SOD, superoxide dismutase; Srb1, scavenger receptor class B member 1; VA, vitamin A; VD, vitamin D; VE, vitamin E; VK1, phylloquinone; VK2, menaquinone; VK3, menadione; Ubq, ubiquitin.

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biochemical alterations were observed in blood plasma glucose, cholesterol or redox enzymes, regardless of the % of SBM replacement and/or Rovabio® PHY treatment. In fish fed diets with 33% replacement of SBM by NVM (regardless of being treated with Rovabio® PHY or not), only a reduced content of plasma triglycerides, and a slight modification in the position of the nucleus in enterocytes (more intermediate) from the proximal intestine, were reported. Altered metabolism of both carbohydrates and VE was also confirmed at transcriptional level, possibly being related to the presence of non-starch polysaccharides (NSPs) in NVM. The present study identifies future strategies to improve the use of NVM as an alternative raw material to replace SBM, allowing greater sustainability, and a lower carbon footprint in European aquafeeds to be achieved.

1. Introduction

The use of some alternative raw materials such as single cell proteins or insect meals have shown promising results in replacing fish meal (FM) in aquafeeds. Nevertheless, plant-derived products, mainly soybean meal (SBM), are still the most widely implemented ingredients (Nie and Hallerman, 2021). Traditional legume seeds locally produced in Southern European countries might reduce the dependency of European aquaculture on SBM imports from third countries (e.g. USA), as well as the carbon footprint of European aquafeeds, thus improving local economies and population maintenance within low settlement density areas.

The successful implementation of any alternative ingredient in animal feeds requires a complete evaluation of different factors such as nutritional composition, palatability, digestibility and metabolic use, as well as the modification of its physical and chemical properties after processing (Glencross, 2020). In this sense, the more frequently evaluated parameters are total content in protein, lipids and carbohydrates, amino acid and fatty acids profiles, content in minerals, and the presence of anti-nutritional factors (ANFs) (Prabhu et al., 2016; Fernández et al., 2018; Lall and Kaushik, 2021). Another important issue is not only the total amount, but also the presence of different types of carbohydrates (Maas et al., 2020), particularly in "glucose-intolerant" fish such as rainbow trout (*Oncorhynchus mykiss*; Kostyniuk et al., 2019). Furthermore, the assessment of digestive enzymes, intestinal and liver functionality (Agboola et al., 2022), redox balance in blood plasma (e.g. Liu et al., 2022) and/or hepatic content of fat-soluble vitamins (essential nutrients to perform almost all biological processes (Fernández et al., 2018)) are also regularly monitored to infer the potential physiological impact of alternative raw materials in feeds for farmed fish.

Narbonne vetch (*Vicia narbonensis*) is a leguminous seed that can be cultivated in dry and/or semiarid habitats and grows rapidly in winter, rendering nearly 1.50 t of grains per ha (El Moneim, 1992; Berger et al., 2003). In a previous study (Toledo-Solís et al., 2022), Narbonne vetch meal (NVM) was selected among different locally produced crops using a multiparametric approach, including parameters such as buffering capacity, contents in soluble protein, soluble phosphorus and phenolic compounds, inhibition of alkaline protease activity, presence of anti-nutritional factors after exogenous enzyme pretreatment, and nutrients bioavailability. A posterior 9-week growth trial demonstrated that NVM could be considered as an interesting ingredient in diets for juvenile rainbow trout since 33% of SBM replacement by enzymatically pretreated NVM did not affect growth performance or the amino acid profile in fish fillet. Proximal composition, apparent digestibility and nutritional content of experimental diets as well as the corresponding growth data can be found in Toledo-Solís et al. (2022). The present work goes one step further, exploring the potential physiological impact of using NVM to partially replace SBM and offering insights into the possibilities and limitations of using NVM in the diet of rainbow trout. To this end, not only the specific content of NVM in non-starch polysaccharides (NSPs), but also different metabolic (activities of digestive enzymes, blood plasma contents in glucose, triglycerides and cholesterol, activity of redox enzymes, and hepatic content of fat-soluble vitamins) and histopathological parameters (tissue integrity in liver and proximal intestine) were evaluated in this study. Also, the expression of some genes involved in the metabolism of glucose and fat-soluble vitamins were evaluated to obtain an insight into the specific molecular pathways involved in some of the physiological alterations induced by replacing SBM with NVM. The objective was to unveil key limiting factors associated with SBM replacement by NVM, as well as to suggest new feed processing strategies and/or formulations to overcome such negative physiological impacts. This will certainly raise the standards of sustainability in European aquaculture.

2. Materials and methods

2.1. Ethical statement

All experiments were conducted following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Percie du Sert et al., 2020), according to 2010/63/EU of the European Parliament and Council, guideline 86/609/EU of the European Union Council and Spanish legislation (RD53/2013), and were previously approved by the Bioethical Committee of ITACyL (approval number: 2018/31/CEEA).

2.2. Experimental diets

Five experimental isonitrogenous and isoenergetic diets were formulated and produced at the Experimental Feeds Unit of the University of Almeria (Almeria, Spain). SBM was replaced in diets at 0, 33 or 66% by NVM previously treated or not with exogenous

enzyme mixture Rovabio® PHY. Experimental groups were named accordingly as Control (0% SMB replacement), A33 (33% SMB replacement with non-treated NVM), A33+E (33% SMB replacement with NVM treated with Rovabio® PHY), A66 (66% SMB replacement with non-treated NVM), and A66+E (66% SMB replacement with NVM treated with Rovabio® PHY). Diets were supplemented with methionine to fulfill the nutritional requirements of rainbow trout (Table S1). Proximate composition and contents in soluble protein, pentoses and reducing sugars were quantified, these data being detailed in Toledo-Solís et al. (2022).

2.3. Fish and sampling

A total number of 195 all-female rainbow trout juveniles (38.04 ± 0.07 g of mean wet weight and 15.10 ± 0.07 cm of furcal length) were randomly distributed in 21 experimental 500 L tanks connected to a recirculating aquaculture system (RAS). Fish were hand-fed to apparent satiation once per day (until a maximum of 3% daily feed intake) for 63 days. The concentration of ammonia and nitrite were checked, and water was renewed (up to 15%) when necessary to maintain its quality. Standard rearing conditions, and results in fish growth performance, apparent digestibility and fish fillet proximate composition and amino acid profile can be found in Toledo-Solís et al. (2022).

A two-step strategy was applied to identify the factors potentially affecting the growth and physiological condition of the fish. In a first step, digestive enzyme activities, histopathological condition and blood biochemistry analyses were conducted in all experimental groups. In a second step, redox enzyme activities, content of fat-soluble vitamins and the expression of genes involved in glucose and fat-soluble metabolism were only analyzed in fish fed with the Control, A33 and A66 diets.

After 30 and/or 63 days post-feeding, samples for different biochemical and molecular analysis were taken from specimens previously fasted for 24 h and sacrificed with an overdose of MS222. Blood samples (up to 2 ml) were taken from the caudal vein with a single-use needle (20 G). Tubes and needles were previously treated with cold buffered sodium citrate (3.8% in 0.01 M PBS). Blood plasma was isolated by differential centrifugation (15 min at 3500 rcf) and collected. No visible signs of hemolysis were noted in collected plasma and samples were immediately stored at − 80 °C. The complete digestive tract was collected from the same specimens and stored at − 80 °C until used to evaluate the activity of different digestive enzymes. In addition, small sections of proximal intestine and liver from another 3 specimens per tank were collected and fixed by immersion in 4% buffered paraformaldehyde (pH 7.4) for 24 h at room temperature. Dehydration of fixed samples was performed by transferring samples in a sequential series of graded alcohol solutions (25%, 50%, 75% and 100%) and stored. A second portion of proximal intestine was washed in DEPC-treated water and stored in 500 μL of TRIzol Reagent (Ambion) at − 80 °C prior to RNA extraction. Small sections of liver were also dissected, washed and stored for gene expression analysis, while most of the liver was stored at − 80 °C to quantify the content of fat-soluble vitamins.

2.4. Profile of non-starch polysaccharide

Dietary fiber was determined according to the AOAC enzymatic–gravimetric method 991.43. The gravimetric residues were treated with H_2SO_4 12 M at 35 °C during 30 min, followed by H_2SO_4 2 M at 100 °C for 1 h. The released neutral sugars from the NSPs were transformed into alditol acetates with acetic anhydride in the presence of 1-methylimidazol and quantified by gas-liquid chromatography (GLC) using β-D-allose (Fluka) as internal standard in a Perkin-Elmer Autosystem Chromatograph (Waltham, USA) equipped with a hydrogen flame ionization detector. The column used was a SP-2330 (30 m long, 0.25 mm i.d., and 0.25 μm film thickness) and nitrogen served as carrier gas. Injector and detector temperatures were 275 °C and oven temperature was 235 °C. Uronic acid content was determined according to the colorimetric method of 3,5-dimethylphenol previously miniaturized and adapted to a microplate reader (Synergy™ HTX Multi-Mode, BioTek, Winooski, VT, USA), using galacturonic acid monohydrate (Merck) as standard. Total NSP was calculated as the sum of both neutral and acid sugars, and results were expressed as g/100 g of raw dry material.

2.5. Digestive enzyme activity analysis

Activities of total acid and alkaline proteases, trypsin, chymotrypsin, leucine-aminopeptidase, alkaline phosphatase, and α-amylase were evaluated from extracts obtained from either the stomach or intestine of 3 fishes from each tank. Acid protease activity was measured using 0.5% hemoglobin as a substrate in 100 mmol L^{-1} glycine–HCl, pH 2, as described by Anson (1938), recording absorbance at 280 nm. Alkaline protease activity was determined using 0.5% casein as a substrate in 100 mmol L^{-1} Tris–HCl, 20 mmol L^{-1} $CaCl_2$, pH 9.0 following the modified methodology by Walter (1984), also at 280 nm. Trypsin activity was measured following Erlanger et al. (1961), using BAPNA (N-α-benzoyl-DL-arginine *p*-nitroanilide) as substrate at 0.5 mmol L^{-1}, in a Tris–HCl buffer (50 mmol L^{-1} + $CaCl_2$ 10 mmol L^{-1} pH 8), recording absorbance at 405 nm. Chymotrypsin activity was assayed according to DelMar et al. (1979), using SAAPNA (N-succinyl-ala-ala-pro-phe *p*-nitroanilide) at 0.5 mmol L^{-1} in a Tris–HCl buffer (50 mmol L^{-1} + $CaCl_2$ 10 mmol L^{-1} pH 8) as substrate, and absorbance at 410 nm. Leucine aminopeptidase activity was measured using the technique described by Maraux et al. (1973), with leucine *p*-nitroanilide at 1 mmol L^{-1} in a monobasic sodium phosphate solution (50 mmol L^{-1} pH 7.2) as substrate, and determining the absorbance at 410 nm. The α-amylase activity was measured using the Somogy-Nelson method described by Robyt and Whelan (1968), with 2% starch as substrate and sodic phosphate and sodium citrate buffer (100 mmol L^{-1} + NaCl 50 mmol L^{-1} pH 7.5), and absorbance at 600 nm. Alkaline phosphatase activity was measured according to Bergmeyer (1974), using 4-nitrophenilphosphate at 1 mmol L^{-1}, in Tris–HCl buffer (50 mmol L^{-1} pH 8.5) as substrate, and absorbance at 405 nm. Activities were calculated using the following units: (1) Unit per ml^{-1} = (Δabs × reaction final volume [ml^{-1}]) / (MEC × time [minutes]) × (extract volume [ml^{-1}]) and (2) expressed in Units per g of fish = units per ml^{-1}/ g of fish; where Δabs represents the increase in absorbance and MEC the molar extinction coefficient.

2.6. Histopathological analysis

Samples from 3 fishes from each tank were stored in 100% ethanol and embedded in paraffin blocks, and sectioned (3–5 μm) and stained with Haematoxylin-Eosin and Alcian blue (AB, pH = 2.5) - periodic acid-Schiff (PAS) solutions to characterize intestine and liver histomorphology, as well as to identify and quantify goblet cell density. All procedures were performed as previously described by Tomas-Almenar et al. (2020). Mounted sections were photographed with an Olympus EP50 camera under an Olympus CX31 microscope while image analysis was performed with Image J software. Hepatocyte surface coverage was assessed in the liver sections. The parameters assessed in the sections of proximal intestine were: goblet cell density, height of villi and enterocytes, width of submucosa, muscular and serosa layers, number of villi fusions per section, brush border integrity (with scores of 1–6 representing 100–0% integrity preserved, respectively), degree of supranuclear vacuolization (with scores of 1–5 representing 100–0% of supranuclear surface vacuolated, respectively) and position of nuclei in enterocytes (with scores of 1–3 representing basal to apical position, respectively). For every evaluated parameter at least 3 measurements per section were carried out in sampled fish at 30- and 63-days post-feeding.

2.7. Blood biochemistry analyses

At the end of the trial, biochemical analyses in blood plasma from 3 fish of each tank belonging to the 5 experimental groups (Control, A33, A33+E, A66, and A66+E) were conducted. Analyses of triglycerides, glucose and cholesterol in plasma were performed using specific kits (BSIS31_E and BSIS17_E from Spinreact; AE63340FI from Abebio). Absorbances at specific wave lengths were measured in triplicate using 96-well microplates and a microplate reader (ELx800TM; BioTek Instruments, Inc., Winooski, VT, USA).

As previously indicated, a second batch of analyses were run only in samples from Control, A33 and A66 diets, including the analysis of redox enzyme activities in blood plasma, fat-soluble vitamin content in fish liver, and the gene expression of key genes in the proximal intestine and liver.

2.8. Redox enzyme activities in blood plasma

The activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S transferase (GST) in blood plasma were assessed in 3 fish from each tank fed with Control, A33 and A66 diets using specific kits (ref. 706002, 703102, and 703302 from Cayman). All enzymatic activities were assessed using a BioTek Gen 5 microplate reader at room temperature, measuring absorbances from biological triplicates at 450, 340 and 340 nm, respectively.

2.9. Quantification of fat-soluble vitamins in fish livers

Fat-soluble vitamin content in liver from 3 fish from each tank fed on Control, A33 and A66 diets was quantified in an Agilent HPLC/DAD 1200 apparatus equipped with an InfinityLab Poroshell 120 EC-C18 column. Vitamin A, D, E, K1 (phylloquinone) and K2 (menaquinone 4) were extracted in hexane previous saponification in a Soxhlet, while vitamin K3 (menadione) was extracted in hexane after enzymatic digestion with lipase. All processes were conducted under dim light and N_2 atmosphere. Quantification was based on the use of the respective standards (retinol (46959-U), retinal (R2500), retinoic acid (R2625), DL-α-tocopherol (47783), DL-α-tocopherol acetate (PHR1030), γ-tocopherol (47785), cholecalciferol (47763), ergocalciferol (47768), menadione (47775), menaquinone (47774) and phylloquinone (47773), all purchased from Sigma).

2.10. RNA extraction and gene expression analysis

Total RNA from 2 fishes from each tank fed on Control, A33 and A66 diets was extracted using TRIzol reagent, according to the manufacturer's protocol. RNA quantity, integrity and quality were assessed with a Qubit 4 Fluorometer, using Qubit RNA extended range (XR) and Qubit RNA IQ assay kits, respectively. Only samples with an IQ value of 7.8 or higher were used. Genomic DNA was removed from RNA samples using RQ1 RNase-Free DNase (Promega), and cDNA was synthetized using M-MLV reverse transcriptase (Invitrogen) for 1 h at 37 °C, following the manufacturer's instructions. cDNA was kept at − 20 °C until used.

For each assay, a negative control (without RNA) was performed. Semi-quantitative PCR (qPCR) reactions were conducted on a StepOnePlus Real-Time PCR system (Applied Biosystems) in triplicates using: 10 μL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™), 0.5 μL of forward and reverse gene-specific primers (10 μM; Table S2), 7 μL of DNase/RNase-free water and 2 μL of diluted (1:10) cDNA. For calibration purposes, one specific sample was run in each qPCR plate (Derveaux et al., 2010). DNA amplification was achieved as follows: 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 20 s at 65 °C. At the end of each amplification, a melting curve was conducted as follows: 95 °C for 15 s, 60 °C for 1 min, and 15 s of 0.5 °C increments until 95 °C. Gene expression levels were determined according to Pfaffl (2001). The suitability of three house-keeping genes (*ubiquitin* (*ubq*), *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and *actin β* (*actb*)) was previously evaluated. Based on the greater stability of *ubq* in liver and proximal intestine using Normfinder software (Andersen et al., 2004), a normalization procedure using *ubq* as reference gene was carried out.

2.11. Statistical analysis

Results are given as mean values ± standard deviations. All data were previously checked for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test). Results were compared by means of one-way ANOVA, and significant differences were detected by Dunnett's post-hoc analysis when the 5 experimental diets (with different % of SBM replacement and treated or not with Rovabio® PHY), or with Tukey post-hoc analysis for data obtained from the 3 diets with different % of SBM replacement by NVM without previous treatment with Rovabio® PHY. Student's t-test was performed when high variability among experimental groups occurred. In any case, the level of significance was set at $P < 0.05$. All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

Growth performance evaluation of the 5 different experimental groups here tested showed that although Rovabio® PHY treatment was able to remove the phytic acid present in NVM, only fish fed the A33+E diet did not show lower growth performance than fish fed the Control diet (see Toledo-Solís et al., 2022).

3.1. Composition of non-starch polysaccharides of Narbonne vetch meal

Narbonne vetch seeds include different soluble and insoluble NSPs within the carbohydrate fraction (Table 1). While rhamnose and fucose were not found in either fraction, only a small content (0.26 g/100 g) of mannose was detected in the soluble one. Small contents of galactose were also found, but with higher content in the insoluble fraction than in the soluble one (0.31 *versus* 0.08 g/100 g). Higher content in the insoluble fraction was also reported for arabinose, glucose, galacturonic acid and xylose. The most abundant component in NSP was arabinose (3.4 g/100 g) followed by glucose (2.81 g/100 g), galacturonic acid (1.87 g/100 g) and xylose (0.78 g/100 g).

3.2. Digestive enzyme activities

Activities of alkaline proteases, trypsin, chymotrypsin and L-aminopeptidase from fish fed experimental diets after a 63-day nutritional dose-response trial are shown in Fig. 1. Regardless of being treated with Rovabio® PHY, fish fed diets with SBM replaced by NVM tend to decrease the activity of alkaline proteases, trypsin and chymotrypsin, although this reduction was only statistically significant when comparing fish fed the Control diet (189.0 ± 23.1 U/g fish for alkaline proteases, 0.26 ± 0.04 U/g fish for trypsin, and 0.06 ± 0.01 U/g fish for chymotrypsin) with those fish fed the A66 and A66+E diets (90.5–91.6 U/g fish for alkaline proteases, 0.09–0.11 U/g fish for trypsin, and 0.02–0.04 U/g fish for chymotrypsin). No differences were observed among the 5 experimental groups regarding L-aminopeptidase activity.

Acid protease, alkaline phosphatase, and α-amylase activity in fish fed experimental diets are resumed in Fig. 2. While no significant differences were observed in acid protease and alkaline phosphatase activity, α-amylase activity was only significantly reduced in fish fed the A66 and A66 +E diets (0.15 ± 0.02 mU/g fish fed the Control diet *versus* 0.08 ± 0.01 and 0.09 ± 0.01 mU/g fish fed the A66 and A66 +E diets, respectively).

3.3. Histopathological analysis of digestive tissues

Histopathological analyses at 30 or 63 days showed that SBM replacement by NVM induced a distinct degree of effects depending on the % of replacement, regardless of the treatment with exogenous enzyme (Tables 2 and 3, respectively). A lower hepatocyte coverage surface was found in fish fed any experimental diet containing NVM when compared to that of fish fed the Control diet after 30 days (Table 2). The lower surface coverage seemed to be due to a shrinkage of the hepatocytes and/or an increased blood cell infiltration (Figure S1). At this time, the position of the nucleus was more central in fish fed diets containing NVM. However, at 63 days post-feeding, only fish fed diets containing 66% SBM replacement by NVM still showed lower surface coverage by hepatocytes (Table 3).

Table 1
Monomeric composition of non-starch polysaccharides (g/100 g ingredient) content of Narbonne vetch (*Vicia narbonensis*) seeds.

NSPs (mg/g ingredient)	Soluble	Insoluble	Total
Rhamanose	nd	nd	nd
Fucose	nd	nd	nd
Arabinose	0.44 ± 0.11	3.07 ± 0.15	3.40 ± 0.18
Xylose	0.13 ± 0.06	0.75 ± 0.03	0.78 ± 0.09
Mannose	0.26 ± 0.03	nd	0.26 ± 0.03
Galactose	0.08 ± 0.04	0.31 ± 0.09	0.33 ± 0.03
Glucose	0.59 ± 0.19	2.35 ± 0.23	2.81 ± 0.23
Galacturonic acid	0.24 ± 0.10	1.73 ± 0.21	1.87 ± 0.24
Total	**1.78 ± 0.18**	**7.37 ± 2.31**	**9.48 ± 0.45**

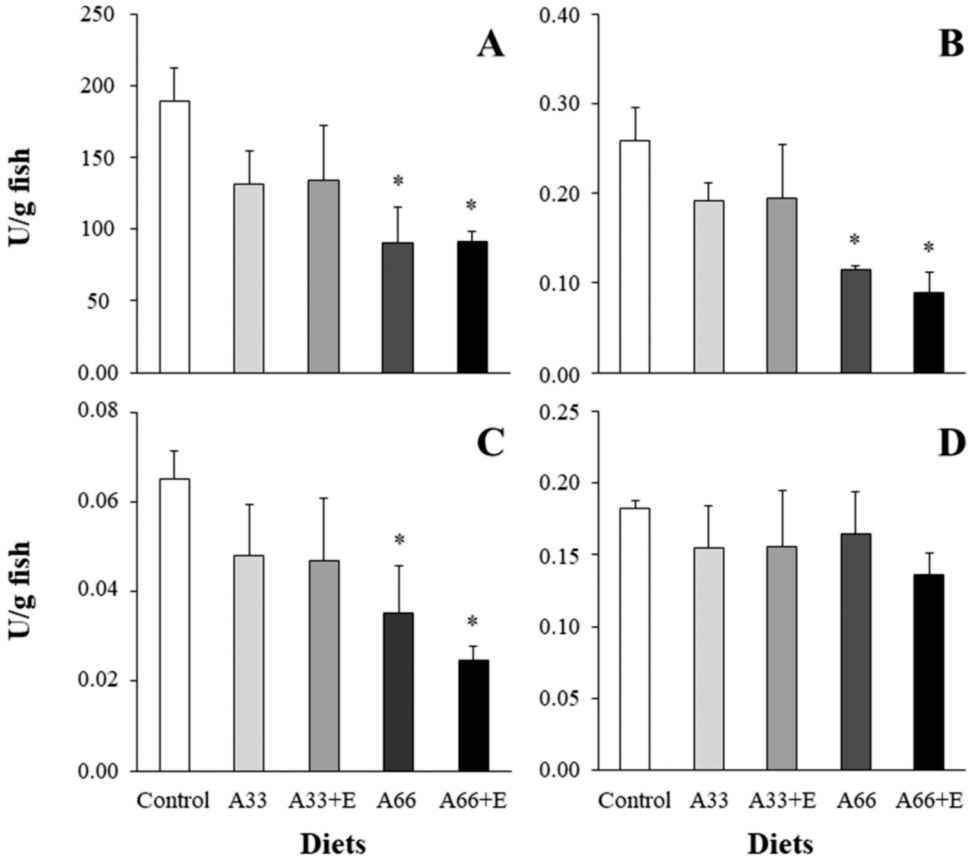

Fig. 1. Enzymatic activities (mean ± standard deviation) of alkaline proteases (a), trypsin (b) chymotrypsin (c) and L-aminopeptidase (d) in rainbow trout fed experimental diets. Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® PHY; A33+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® PHY; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® PHY; A66+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® PHY. An asterisk at the top of each bar denotes significant differences in comparison with the Control group (Dunnett's test, $P < 0.05$, n = 3).

At the proximal intestine, no differences in the density of goblet cells, height of villi, width of submucosa and muscular layers, number of fusions of villi, and/or degree of supranuclear vacuolization in enterocytes were observed between any experimental diets at 30- or 63-days post-feeding. Height of enterocytes was only significantly reduced in fish fed the A66+E diet at 30 days compared to that of the Control fish. Similarly, lower brush border integrity was only found in the same experimental group and at the same sampling time. In contrast, differences in width of serosa layer were observed in both groups of fish fed NVM treated with Rovabio® PHY, but only at 30 days. Furthermore, while no differences regarding the position of the nucleus of the enterocytes were found between experimental and Control diets at 30 days, all the fish fed experimental diets containing NVM showed a more intermediate nucleus position at 63 days.

3.4. Blood biochemistry analysis

After 24 h of feeding, glucose, triglyceride and cholesterol content in blood plasma was evaluated to identify potential metabolic disruptions (Fig. 3). Results showed as glucose and cholesterol levels were not altered in fish fed diets containing NVM when compared to those fed the Control diet, ranging from 76.67 ± 3.57 to 83.71 ± 5.56 mg/dl of glucose and from 24.3 ± 5.8 to 29.7 ± 8.0 mg/dl of cholesterol. In contrast, all diets containing NVM induced a lower content of triglycerides in blood plasma, proportional to the % of SBM replacement: from 385.70 ± 16.00 mg/dl in Control fish, to 304–322 mg/dl in fish fed the A33 and A33+E diets, and 222.36–245.56 mg/dl in those fed the A66 and A66+E diets.

3.5. Activities of redox enzymes in blood plasma

Superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activity in blood plasma from fish fed the Control, A33 and A66 diets are compiled in Table 4. No significant differences were detected in any of the enzymes evaluated. Values ranged from 6.73 ± 1.14 to 7.51 ± 0.26 U/ml for SOD, 111.39 ± 1.63 to 105.44 ± 5.65 U/ml for GPx, and 2.87 ± 1.20 to 3.16

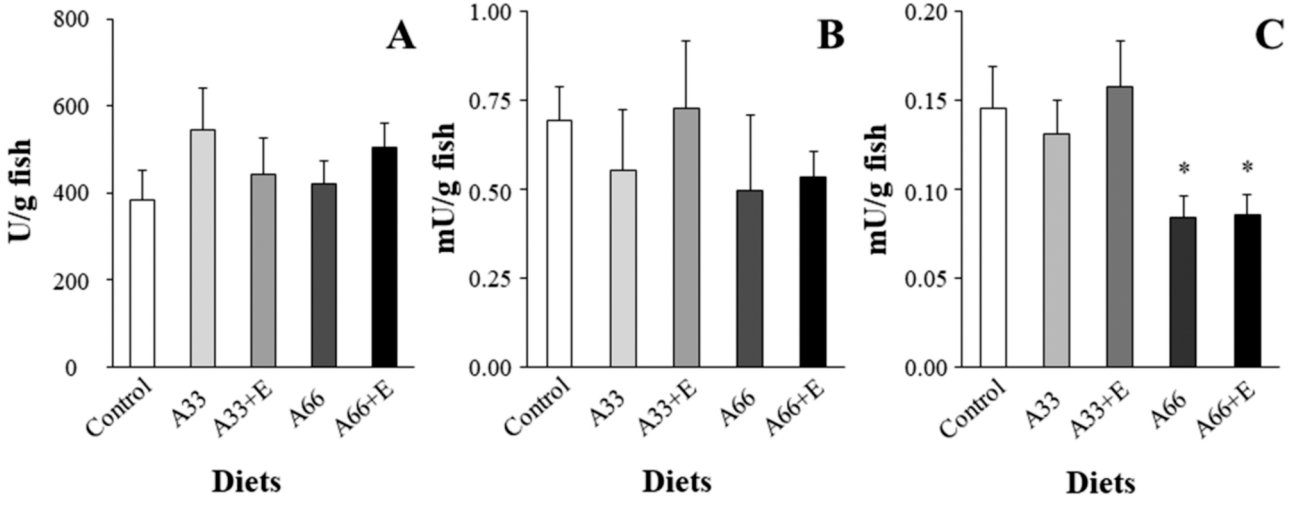

Fig. 2. Enzymatic activities (mean ± standard deviation) of acid protease (a), alkaline phosphatases (b), and α-amylase (c) in rainbow trout fed experimental diets. Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® PHY; A33+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® PHY; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® PHY; A66+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® PHY. An asterisk at the top of each bar denotes significant differences in comparison with the Control group (Dunnett's test, $P < 0.05$, n = 3).

Table 2
Histological analysis in liver and proximal intestine of rainbow trout at 30 days of feeding with the experimental diets.

Tissue	Parameter	Control	A33	A33+E	A66	A66+E
Liver	Hepatocyte surface (%)	77.12 ± 2.46	67.50 ± 4.39 *	66.62 ± 1.19 *	68.50 ± 4.62 *	67.23 ± 2.87 *
Proximal intestine	n° of goblet cells/mm	125.69 ± 6.57	113.13 ± 14.42	104.91 ± 18.64	107.12 ± 6.93	107.29 ± 14.51
	Height of villi (μm)	255.68 ± 17.48	262.99 ± 30.31	262.38 ± 24.78	261.09 ± 32.34	255.16 ± 21.84
	Height of enterocytes (μm)	19.13 ± 0.59	19.97 ± 0.71	18.09 ± 0.26	19.41 ± 0.45	17.90 ± 0.29 *
	Width of submucosa layer (μm)	5.53 ± 0.63	5.55 ± 0.36	6.42 ± 0.79	6.60 ± 0.27	5.90 ± 0.64
	Width of muscular layers (μm)	37.23 ± 4.22	36.21 ± 4.96	39.38 ± 7.46	33.56 ± 3.12	30.96 ± 2.32
	Width serosa layer (μm)	14.28 ± 2.28	15.96 ± 1.41	17.55 ± 0.62 *	11.52 ± 0.84	8.44 ± 0.86 *
	Nº of fusion of villi	5.67 ± 1.20	7.44 ± 2.22	5.78 ± 0.77	5.11 ± 1.02	3.33 ± 0.58
	Integrity of brush border	1.11 ± 0.19	1.22 ± 0.19	1.00 ± 0.00	1.44 ± 0.19	1.56 ± 0.19 *
	Degree of supranuclear vacuolization in enterocytes	1.33 ± 0.33	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	Position of nucleus in enterocytes	1.56 ± 0.19	1.78 ± 0.19	1.67 ± 0.00	2.00 ± 0.00	2.00 ± 0.00

Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® Phy; A33+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® Phy; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® Phy; A66+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® Phy. An asterisk within each row denotes significant differences with the Control group (Dunnett's test, $P < 0.05$, $n = 3$).

Table 3
Histological analysis in liver and proximal intestine of rainbow trout at 63 days of feeding with the experimental diets.

Tissue	Parameter	Control	A33	A33+E	A66	A66+E
Liver	Hepatocyte surface (%)	78.80 ± 1.72	76.83 ± 3.16	74.18 ± 2.05	69.65 ± 2.36 *	69.29 ± 1.92 *
Proximal intestine	n° of goblet cells/mm	97.90 ± 6.86	108.32 ± 6.72	117.68 ± 10.90	104.62 ± 16.36	111.48 ± 14.35
	Height of villi (μm)	346.61 ± 5.00	338.89 ± 23.74	319.93 ± 34.32	335.75 ± 26.24	362.67 ± 28.01
	Height of enterocytes (μm)	18.30 ± 1.61	19.07 ± 1.11	18.71 ± 0.20	19.49 ± 0.31	20.64 ± 1.70
	Width of submucosa layer (μm)	5.96 ± 0.70	6.13 ± 0.66	5.56 ± 0.26	5.50 ± 0.55	5.12 ± 0.38
	Width of muscular layers (μm)	50.87 ± 2.94	45.72 ± 6.54	46.50 ± 5.49	50.21 ± 10.07	47.46 ± 6.62
	Width serosa layer (μm)	12.71 ± 0.91	13.07 ± 2.47	15.20 ± 2.48	13.54 ± 4.25	15.14 ± 2.27
	Nº of fusion of villi	7.83 ± 2.36	7.56 ± 1.71	7.67 ± 0.58	8.22 ± 1.90	6.00 ± 1.80
	Integrity of brush border	1.56 ± 0.38	1.67 ± 0.33	1.44 ± 0.38	1.78 ± 0.19	2.00 ± 0.33
	Degree of supranuclear vacuolization in enterocytes	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	Position of nucleus in enterocytes	1.56 ± 0.38	2.00 ± 0.00 *	2.00 ± 0.00 *	2.00 ± 0.00 *	2.00 ± 0.00 *

Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® Phy; A33+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® Phy; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® Phy; A66+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® Phy. An asterisk within each row denotes significant differences with the Control group (Dunnett's test, $P < 0.05$, $n = 3$).

± 0.49 U/ml for GST.

3.6. Fat-soluble vitamin content in fish livers

The content of different fat-soluble vitamins was also evaluated in the liver of fish fed the Control, A33 and A66 diets (Table 4). While vitamin D (VD), menaquinone (VK2) and menadione (VK3) were not detected regardless of the experimental diet used, different amounts of total vitamin A (VA), vitamin E (VE) and phylloquinone (VK1) were found depending on the diet. No significant differences were found regarding the content in total VA and VK1. Contents ranged from 17.69 ± 7.66 to 29.16 ± 7.83 mg/kg of total VA (expressed as retinol equivalents), and from 6.39 ± 1.46 to 6.97 ± 2.29 mg/kg of VK1. In contrast, the hepatic level of VE (expressed as α-tocopherol) was significantly reduced in fish fed the A66 diet (399.34 ± 108.97) when compared to that of fish fed the Control diet (798.54 ± 219.68 mg/kg), fish given the A33 diet showing intermediate values.

3.7. Gene expression results

At the intestine, the expression of three genes related with the uptake of fat-soluble vitamins was evaluated (Fig. 4). The expression

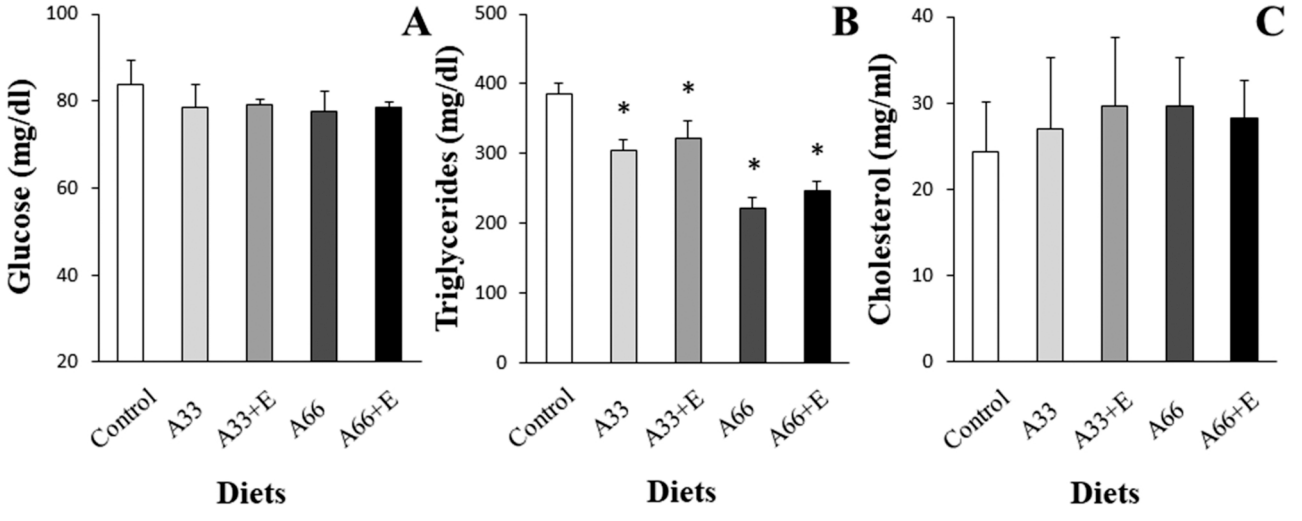

Fig. 3. Blood plasma glucose (a), triglycerides (b) and cholesterol (c) contents (mean ± standard deviation) in rainbow trout fed experimental diets. Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® PHY; A33+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® PHY; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® PHY; A66+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® PHY. An asterisk at the top of each bar denotes significant differences in comparison with the Control group (Dunnett's test, $P < 0.05$, n = 3).

Table 4
Blood biochemistry in plasma and fat-soluble content in vitamins A, D, E and K in liver from fish fed experimental diets.

Parameter	Control	A33	A66
Superoxide dismutase (SOD, U/ml))	8.52 ± 2.52	9.86 ± 1.40	10.38 ± 0.84
Glutathione peroxidase (GPx, nmol/min/ml)	111.39 ± 1.63	105.44 ± 5.65	109.70 ± 13.90
Glutathione S-transferase (GST, nmol/min/ml)	2.87 ± 1.20	3.16 ± 0.49	3.06 ± 0.67
Fat-soluble vitamins (mg/kg dry weight)	**Control**	**A33**	**A66**
Total vitamin A*	17.69 ± 7.66	29.16 ± 7.83	19.70 ± 5.24
Total vitamin D* *	nd	nd	nd
α-tocopherol	798.54 ± 219.68[a]	451.08 ± 71.63[a,b]	399.34 ± 108.97[b]
Phylloquinone (VK1)	6.76 ± 1.30	6.39 ± 1.46	6.97 ± 2.29
Menaquinones (VK2)	nd	nd	nd
Menadione (VK3)	nd	nd	nd

Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® Phy; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® Phy; nd, not detected. * Total vitamin A as retinol equivalents; * * Total vitamin D as cholecalciferol. Different superscript letter within each row denotes significant differences among experimental groups (ANOVA; Tukey's test; $P < 0.05$; $n = 3$).

of *niemann-pick c1-like protein 1* (*npc1l1*) tended to increase with the % of SBM replacement by NVM, but only significantly up-regulated in fish fed the A66 diet (2.81 ± 0.33 fold change) when compared to those fed the Control diet (1.08 ± 0.21 fold change). While the expression of *cluster determinant 36* (*cd36*) was not altered regardless of the SBM replacement, the expression of *scavenger receptor class B member 1* (*srb1*) was only up-regulated in fish fed the A66 diet (0.95 ± 0.17 fold change in Control fish *versus* 1.99 ± 0.63 fold change in fish fed the A66 diet). In contrast to *npc1l1* and *srb1* gene expression, the expression of the gene coding for the specific transport protein of VE, the *α-tocopherol transfer protein b* (*attpb*), was down-regulated in fish fed both the A33 (0.77 ± 0.01 fold change) and A66 (0.58 ± 0.06 fold change) diets when compared with those fed the Control diet (0.97 ± 0.08 fold change; Fig. 5a). Similarly, the expression of *α-amylase 2 b* (*amy2b*) was down-regulated in fish fed diets containing NVM (0.40 ± 0.11 and 0.44 ± 0.05 fold change in A33 and A66 fish, respectively) compared to that in the Control fish (0.96 ± 0.21 fold change; Fig. 5b). In contrast, only fish fed the A66 diet showed a down-regulation of the *solute carrier family 2, facilitated glucose transporter member 4 b* (*slc2a4b*) gene (0.58 ± 0.10 fold change) when compared to that of fish fed the Control diet (1.16 ± 0.32 fold change; Fig. 5c).

In liver, the expression of *α-amylase 2 a* (*amy2a*), *solute carrier family 2, facilitated glucose transporter member 2* (*slc2a2*), and *solute carrier family 2, facilitated glucose transporter member 4 b* (*slc2a4*) genes was explored (Fig. 6). Although the expression of *amy2a* tended to increase with the inclusion of NVM, no significant differences were detected (values ranged from 0.83 ± 0.15 to 1.21 ± 0.21 fold change). In contrast, the expression of both *slc2a2* and *slc2a4* genes was higher in fish fed the A66 diet (1.26 ± 0.03 and 5.49 ± 1.74 fold changes, respectively) than that of fish fed the Control diet (0.88 ± 0.08 and 1.20 ± 0.71 fold changes, respectively), while fish fed the A33 diet showed intermediate values.

4. Discussion

Currently, market prices for SBM and NVM are mainly around 450–480 USD per tn (https://www.cmegroup.com/) and 275–395 USD per tn (https://efeagro.com/), respectively. First research works exploring the use of NVM for fish feeds suggested that only a low inclusion (10%) or low SBM replacement (20%) was possible without compromising fish growth (Buyukcapar et al., 2010; Tomás-Almenar et al., 2020). Our previous work, suggested that fish growth was not affected when 33% of SBM was replaced by NVM pretreated with Rovabio® PHY, probably due to the total removal of phytic acid by this exogenous enzyme (Toledo-Solís et al., 2022). Nevertheless, exogenous enzyme treatment increased the amount of available reducing sugars. Therefore, considering rainbow trout is a "glucose-intolerant" species (Kostyniuk et al., 2019), these results prompted us to explore in more detail the potential physiological impact of using NVM in fish diets, including the presence of NSPs.

Different ANFs are known to be present in plant feedstuffs (reviewed in Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010) and different strategies have been applied to reduce and/or totally avoid their impact (Drew et al., 2007; Zheng et al., 2019). Although exogenous phytase is extensively used to improve weight gain, whole-body composition and nutrient utilization efficiency in livestock; it has insignificant or no positive effect on growth performance and feed efficiency in some fish species (reviewed in Zheng et al., 2019). Indeed, although no phytic acid was detected after Rovabio® PHY treatment, lower apparent protein digestibility was still observed in fish fed diets containing NVM (Toledo-Solís et al., 2022). This was particularly in line with the decreased alkaline proteases, trypsin and chymotrypsin activities here reported in fish fed diets in which SBM was replaced by NVM, regardless of NVM being treated with exogenous enzyme or not. Only fish fed with 33% SBM replacement by NVM treated with Rovabio® PHY did not show any differences in growth when compared to those fed the Control diet. No imbalance in the amino acid profile in fish fillet was shown in fish fed diets including NVM (Toledo-Solís et al., 2022). These results suggested that at least other ANFS and/or another nutritional component rather than phytic acid might impair rainbow trout physiology. The presence of some trypsin and chymotrypsin inhibitors in different cultivars of Narbonne vetch (Martín-Pedrosa et al., 2016) might also explain some of the histopathological lesions observed at proximal intestine level: e.g. an intermediate position of the nucleus (between basal and apical) in the enterocytes and/or a decreased brush border integrity after 63 days of feeding on experimental diets. These are common features produced by diets containing high levels of protein sources of vegetable origin (Agboola et al., 2022). Nevertheless, the reduced apparent digestibility

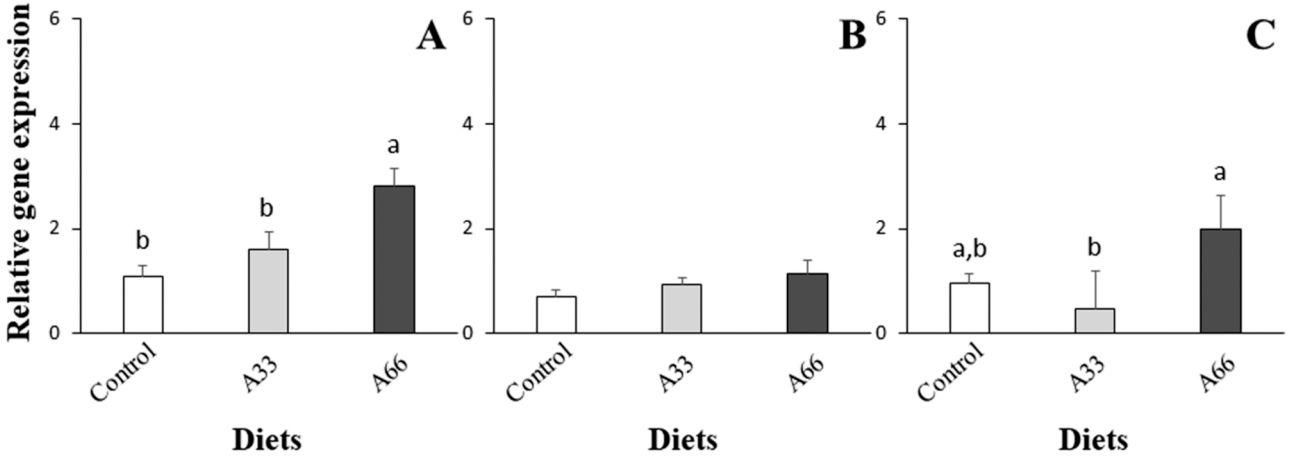

Fig. 4. Relative gene expression of *niemann-pick c1-like protein 1* (*npc1l1*) (a), *cluster determinant 36* (*cd36*) (b) and *scavenger receptor class B member 1* (*srb1*) (c) in proximal intestine from rainbow trout fed experimental diets. Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® PHY; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® PHY. Transcript levels were determined by qPCR and normalized using *ubiquitin* (*ubq*) gene expression. Different letters at the top of each bar denote significant differences among experimental groups (one-way ANOVA, Tukey multiple-comparison test) ($P < 0.05$; n = 3).

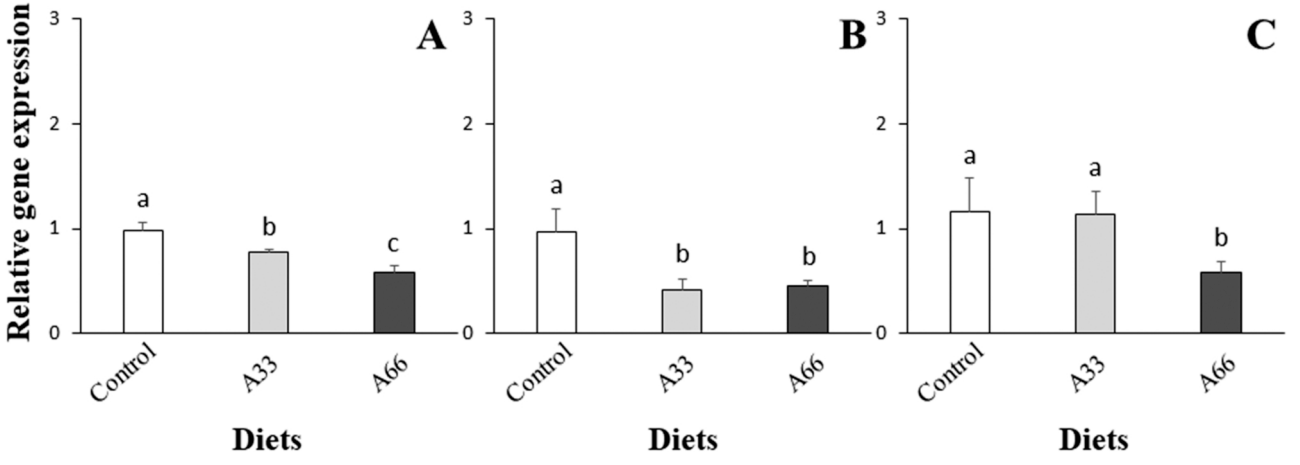

Fig. 5. Relative gene expression of *α-tocopherol transfer protein b* (*attpb*), *α-amylase 2 b* (*amy2b*) (b) and *solute carrier family 2, facilitated glucose transporter member 4 b* (*slc2a4b*) (c) in proximal intestine from rainbow trout fed experimental diets. Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® PHY; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® PHY. Transcript levels were determined by qPCR and normalized using *ubiquitin* (*ubq*) gene expression. Different letters at the top of each bar denote significant differences among experimental groups (one-way ANOVA, Tukey multiple-comparison test) ($P < 0.05$; n = 3).

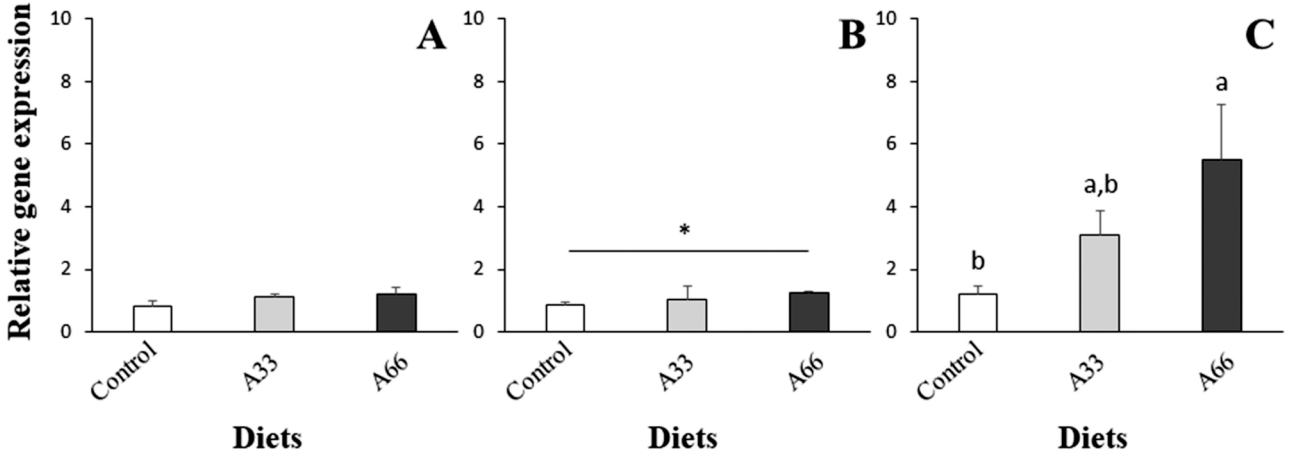

Fig. 6. Relative gene expression of *α-amylase 2 a* (*amy2a*) (a), *solute carrier family 2, facilitated glucose transporter member 2* (*slc2a2*) (b), and *solute carrier family 2, facilitated glucose transporter member 4 b* (*slc2a4b*) (c) in liver from rainbow trout fed experimental diets. Control, diet where soybean meal (SBM) was not replaced by Narbonne vetch meal (NVM); A33, diet with a 33% of SBM replaced by NVM not treated with Rovabio® PHY; A66, diet with a 66% of SBM replaced by NVM not treated with Rovabio® PHY. Transcript levels were determined by qPCR and normalized using *ubiquitin* (*ubq*) gene expression. Different letters at the top of each bar and an asterisk between two histograms denote significant differences among tissues (one-way ANOVA, Tukey multiple-comparison test; and Student t-test, respectively) ($P < 0.05$; n = 3).

coefficient of protein shown by fish fed diets containing NVM (Toledo-Solís et al., 2022) might also be a consequence of the presence of other compounds such as NSPs (see below; Sinha et al., 2011).

In addition to enzyme inhibitors, compounds affecting carbohydrate digestion and/or metabolism, might also be responsible for lower α-amylase activity and incipient hepatocyte shrinkage accompanied by an increased infiltration of blood cells in the liver of fish fed diets containing NVM. This condition was only reverted in fish fed diets containing the lowest level of SBM replacement, and maintained in those fed diets in which 66% of SBM was replaced by NVM, in line with the increased hepatosomatic index already reported in these fish (Toledo-Solís et al., 2022). In this sense, legume seeds contain a wide range of oligosaccharides that are not hydrolyzed by the endogenous enzymes from monogastrics, interfering with nutrient digestion, and having little energetic value for carnivorous fish species (Krogdahl et al., 2010). Among the high content in carbohydrates reported in Narbonne vetch seeds (50–60%; Martín-Pedrosa et al., 2016), we found arabinose, glucose, galacturonic acid and xylose as the most abundant monomers from NSPs.

One of the main constraints to use plant ingredients in aquaculture is the presence of indigestible carbohydrates such as NSPs (Maas et al., 2020). The effects of NSPs on intestinal transit, gastric emptying, glucose absorption and water-holding capacity, and how to improve their nutritional utilization in terrestrial monogastrics have been widely studied (Sinha et al., 2011; Lannuzel et al., 2022). In general, fish species showed very low synthesis or totally lack the enzymes required to digest NSP (e.g. β-glucanases and β-xylanases; Krogdahl et al., 2005; Sinha et al., 2011). As a typical carnivorous fish species, rainbow trout had a limited ability to utilize NSPs (Denstadli et al., 2011). Indeed, the dietary inclusion of NSPs (mainly in their soluble form) in rainbow trout diets reduced growth performance as well as the activity of some digestive enzymes (e.g. trypsin; Deng et al., 2021). The same study also showed how NSPs dietary content increased total serum cholesterol, but decreased plasma and hepatic superoxide dismutase, glutathione peroxidase and catalase activities. Such effects were not here observed. Discrepancies might be due to the high dietary inclusion of soluble NSPs (16.8%) tested by Deng and coworkers. Such differences between the research works were also reflected in the amino acid profile of the fillet from fish fed the Control and experimental diets, with a larger and wider difference in the study by Deng et al. (2021) than in our previous report (Toledo-Solís et al., 2022).

Since NSPs reduce enzyme accessibility to substrates, decreased nutrient absorption has been also documented (Maas et al., 2021), particularly regarding glucose (Sinha et al., 2011). Different studies have shown how raw material of vegetable origin containing NSPs reduces intestinal maltase activity and plasma glucose levels (Sinha et al., 2011). Also, many NSPs reduced the activity of α-amylase (reviewed in Liu et al., 2017). In the present study, the activity of α-amylase was reduced only when 66% of SBM was replaced by NVM, regardless of being previously treated or not with Rovabio® PHY. Since this enzyme is responsible for the hydrolysis of carbohydrates (Mizutani et al., 2012), a lower content of glucose in blood plasma might be expected, at least, in fish fed diets in which 66% of SBM was replaced by NVM. Since at 24 h post-feeding glucose blood plasma levels were not altered, two hypotheses might be considered: i) glucose absorption was indeed reduced but not maintained after 24 h post-feeding (time of sampling), and/or ii) glucose absorption was altered, but different regulatory pathways compensate it to maintain normal values in blood plasma. Even in mammalian species, few studies have evaluated the effects of specific NSPs on glucose metabolism. Recently, favoring the first hypothesis, an altered transit time, nutrient digestion and absorbance was demonstrated for galacturonic acid (Bhutia and Ganapathy, 2021), the third most abundant NSP found in Narbonne vetch seeds, just after arabinose and glucose. More specifically, L-arabinose ingestion decreased the magnitude of glucose and insulin post-prandial peaks, but normal levels were restored after 3 h (Pol and Mars, 2021). In order to confirm that SBM replacement by NVM, and the content of NSPs in particular, might alter glucose digestion and metabolism, the expression of several key genes was assessed. Regarding the potentially impaired digestion of carbohydrates, the expression of *α-amylase 2 b* (*amy2b*) at proximal intestine level was decreased with SBM replacement by NVM, in line with reduced α-amylase activity. The isoform *amy2b* has been reported to be expressed in different tissues, including small intestine epithelial cells, and plays an essential role in starch degradation (Date et al., 2020). In contrast, the expression of *solute carrier family 2, facilitated glucose transporter member 4 b* (*slc2a4b*, also known as *glut4*), a glucose transporter from the extracellular milieu into the cell (Leto and Saltiel, 2012), was slightly down-regulated at the proximal intestine but potently up-regulated at liver. High (66%) SBM replacement by NVM also increased *solute carrier family 2, facilitated glucose transporter member 2* (*slc2a2*, also known as *glut2*) expression at liver. Although this glucose transporter isoform has been recognized by having a relatively low affinity for glucose, it seems that it mediates the bidirectional transfer of glucose across the plasma membrane of hepatocytes, acting as part of the glucose sensing mechanism (Thorens, 2015). All these results at transcriptional level might be interpreted as a differential cell-type response to counteract the slower/lower glucose absorption, and might also be related with the reduced blood plasma triglyceride content. Indeed, previous studies with normal and transgenic mice showed how Glut4, in addition to glucose metabolism, has a significant effect on lipid homeostasis, particularly in blood plasma triglyceride content (Wang et al., 2017).

NSPs are also known to increase the hydrolysis of and/or to entrap bile salts, and hence reducing fat digestibility and impairing lipid absorption (Maas et al., 2021). Fish species fed diets containing different vegetable protein sources (e.g. rape seed, or linseed and sesame meals) have been reported to exhibit lower lipid content (Sinha et al., 2011). In particular, different NSPs have been shown to decrease the activity of lipases (Liu et al., 2017), which is in line with our results regarding the reduced content of triglycerides in blood plasma in fish fed diets containing NVM. Indeed, the presence of NSPs in feeds has been hypothesized to decrease fat absorption in Atlantic salmon (*Salmo salar*) by disturbing micelle formation in the gastro-intestinal tract (Øverland et al., 2009). Curiously, vitamin E (VE) appears to be absorbed by a non-saturable passive diffusion process dependent on the formation of micelles in the intestinal lumen, as well as by other processes (Borel et al., 2013). VE is a fat-soluble vitamin, an essential micronutrient for fish that needs to be provided in a specific amount and chemical form within the diet, since fish are not able to *de novo* synthesize them (reviewed in Fernández et al., 2018). In brief, α-tocopherol is the form with the highest biological activity and the most commonly used source of VE in aquafeeds. VE protects lipids from peroxidation, modulates eicosanoid synthesis and immune responses, and may have multiple signaling functions at the post-translational level, such as activation of protein phosphatase 2 A, among other enzymes. Since the liver

content of the other fat-soluble vitamins analyzed (vitamins A, D and K), also partially transported within micelles, did not differ among fish fed Control and NVM-containing diets, disturbing micelle formation in the intestine appeared not to be the source of the lower content of VE in liver from fish fed NVM diets. In order to decipher the alternative pathways of altered metabolism of VE, the expression of genes encoding proteins related with the intestinal uptake (*niemann-pick c1-like protein 1*, *cluster determinant 36* and *scavenger receptor class B member 1*; Borel et al., 2013; Kiyose, 2021) and/or the transport between tissues (*α-tocopherol transfer protein*; Arai and Kono, 2021) has been evaluated. Increased gene expression of *npc1l1* and *srb1* suggested these might be the specific altered pathways of VE uptake at the intestine. Furthermore, lower VE content in liver might be associated to both decreased expression of *attpb* gene at liver as well as the lower triglyceride content in blood plasma, as VE enters the lymphatic circulation in association with nascent triglyceride-rich chylomicrons (Combs and McClung, 2017).

On balance, the present study shows how a complete physiological evaluation must be conducted after *in vivo* growth assessment, particularly to uncover any potential limitations to replace SBM (or FM) by new alternative protein sources. Moreover, such evaluation is essential to define complementary strategies for these ingredients be fully implemented in the aquafeed industry, warranting proper fish growth and welfare, and achieving long-term aquaculture sustainability. In this sense, considering the profile of NSPs in NVM, the industrial pretreatment of this meal with specific enzymes such as arabinose isomerases and polygalacturonases, and/or the use of probiotic bacteria for degrading these compounds in the fish intestine might be explored. Also, the selection and use of Narbonne vetch cultivars with lower glucose content, and/or the dietary supplementation with antioxidant compounds (including higher VE levels) might be alternative strategies to avoid the undesired effects observed in carbohydrate and VE metabolism here reported.

5. Conclusions

To warrant sustainable growth of European aquaculture, the identification and implementation of alternative raw materials in aquafeeds to replace FM and/or SBM, are urgently needed to reduce both SBM imports from third countries and the aquafeed carbon footprint. We previously identified NVM as a possible suitable, safe and locally produced protein source for, at least, partial SBM replacement (Tomás-Almenar et al., 2020; Toledo-Solís et al., 2022). The pretreatment of NVM with an exogenous phytase have shown that a 33% SBM replacement did not affect fish growth (Toledo-Solís et al., 2022). However, enzymatic, histological and biochemical analyses revealed that this pretreatment might not be sufficient to avoid a physiological impact (impairing the activity of digestive enzymes, liver function, triglycerides and vitamin E metabolism and homeostasis) in rainbow trout when NVM is used to replace high levels of SBM. Nevertheless, new key avenues to improve future SBM replacement by NVM have been identified. In this sense, the use of multi-carbohydrase complexes to treat NVM might be a promising strategy to increase digestibility of starch and fat, improving nitrogen and amino acid utilization as well. Indeed, the pretreatment of NSPs with non-starch polysaccharide degrading enzymes (e.g. Xylanases and β-glucanases) has been reported to reduce the detrimental effects of NSPs in fish physiology (Sinha et al., 2011; Martínez et al., 2019), allowing levels of plant feedstuffs in fish diets to be increased (Magalhães et al., 2016). Also, the identification and selection of specific Narbonne vetch cultivars with low NSP content and/or a higher dietary content of antioxidants in aquafeed formulation might also be successful alternatives to implement the use of NVM in European aquafeeds, thus achieving sustainable growth of European aquaculture.

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CRediT authorship contribution statement

Francisco J. Toledo-Solís, Francisco J. Moyano, Ignacio Fernández: Conceptualization, Methodology. **Francisco J. Toledo-Solís, Ana M. Larrán, Blanca Martín, Pedro López de la Cuesta, Immaculada Mateos-Aparicio, Valentín Perez, Francisco J. Moyano, Ignacio Fernández**: Formal analysis, Writing – review & editing. **Ignacio Fernández**: Writing – original draft preparation.

Declaration of Competing Interest

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

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Almenar during sampling procedures.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2022.115555.

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