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Selection and improvement of alternative raw materials for rainbow trout (*Oncorhynchus mykiss*) aquafeeds through a multiparametric screening tool

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ABSTRACT

Aquaculture growth and sustainability mainly rely on the identification and implementation of alternative raw materials to replace fish meal (FM) and fish oil (FO) and/or its major substitute, the soybean meal (SBM). A five-step screening tool has been designed to identify and improve the use of promising alternative raw materials. To validate it, nine raw materials, including the standard reference (the SBM), were sequentially analyzed regarding (i) the total buffer capacity, alkaline protease activity inhibition and soluble protein content, (ii) soluble phosphorus and phenolic compound content, (iii) modification of nutrients bioavailability and presence of anti-nutritional factors after treatment with exogenous enzyme (Rovabio® Phy), (iv) release of nutrients after *in vitro* digestion, and (v) the palatability of the formulated diet. SBM partial replacement by selected raw material, the Narbonne vetch (*Vicia narbonensis*) meal (NVM), was evaluated in a 63-day nutritional trial using rainbow trout (*Oncorhynchus mykiss*) juveniles. One Control (no SBM replacement) and 4 experimental diets with SBM replacement in two levels (33% and 66%), treated or not with exogenous enzyme Rovabio® Phy, were compared. Fish growth performance and amino acid profile in fish fillet was not significantly affected when SBM was replaced by 33% of NVM treated with exogenous enzyme. Altogether, the present screening tool might be a wise strategy to identify promising alternative raw materials for European aquaculture sustainability, reducing the use of animals in experimentation, the SBM dependency from third countries, and its carbon footprint.

1. Introduction

A large effort has been made to identify more sustainable raw materials (*e.g.* meals from soybean, insect, algae, single cell

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organisms, and animal or plant-derived by-products; microalgae and krill oils) to substitute fish oil (FO) and fish meal (FM) during the last decades (reviewed in Turchini et al., 2011, 2019; Naylor et al., 2021). The most common strategy was to pursue total or partial FM/FO substitution by a single resource. In the case of FO replacement, the combination of different plant-derived oils, as a kind of balanced strategy, has been more successful to achieve maximal substitution levels without negative impacts on fish growth and physiology (Turchini et al., 2019). Nevertheless, plant-derived oils still has some impact on the nutritional value of the fish fillet (decreasing the content of long-chain polyunsaturated fatty acids (LC-PUFAs) from the n-3 series; Sprague et al., 2016). New approaches through transgenesis (Betancor et al., 2015) and/or the use of microalgae oils highly rich n-3 LC-PUFAs (Santigosa et al., 2020) are being developed.

Although very promising results have been recently obtained when using new alternative raw materials (single cell protein sources and/or insect meal), plant-derived products, mainly soybean meal (SBM), are still the most widely implemented alternative for FM in the fish feeds (Nie and Hallerman, 2021). Unfortunately, any of the alternatives considered have several limitations to fully substitute FM. For instance, substitution by insect meal didn't affect fish growth potential but decreased fish fillet nutritional value (n-3 LC-PUFAs; Melenchón et al., 2020). In another cases, fish fed diets, where FM was totally replaced with different alternative raw materials (*e.g.* single cell protein sources), led to increased feed conversion rates (Zamani et al., 2020) and/or limited digestibility (Sarker et al., 2018; Glencross et al., 2020), reduced fish feed demand/appetite, decreasing feed palatability (Pratoomyot et al., 2010), induced intestinal alterations due to the presence of anti-nutritional factors (ANFs) (Knudsen et al., 2008; Silva et al., 2015; Król et al., 2016); and/or impaired the fish immune system, being more prone to suffer infections (Chaklader et al., 2020), among other negative effects (Borgeson et al., 2006; Pavan et al., 2014). Altogether, these results suggest that selection of alternative raw materials is crucial to warrant future aquaculture sustainability (Turchini et al., 2019; Glencross, 2020; Naylor et al., 2021). Until now, alternative protein and lipid sources have been generally selected on their protein/lipid total content, general amino acids/fatty acids profiles, yearly commercial availability, production and price, presence of ANFs, *etc.* Recently a seven-step process selection in order to make appropriate decisions in the use of ingredients has been proposed (Glencross, 2020), including ingredient characterization and an *in vivo* assessment of its palatability, digestibility, utilization, immunological impact, processing effects and product quality achieved. Nevertheless, considering the increasing amount of different alternative raw materials appearing in the market with the potential to partially replace FM (or SBM), feed formulators and producers do not only need to characterize them, but also getting clues on the potential pre-treatments that might improve their use and incorporation in fish feeds.

Although *in vivo* testing is the most robust and reliable manner to evaluate raw materials to be included in feeds, it is highly time-consuming, and requires the use of large numbers of animals and funds. The *in vitro* models simulating the digestive hydrolysis of nutrients in different animal species have been designed to provide a better understanding of factors affecting this process (Gilannejad et al., 2018). Basically, these systems are intended to mimic the digestive physiology of the species in a two-phase (stomach and intestine) process, using the species-specific enzyme extract. Although there is still room for important improvements (Moyano et al., 2014), recent advances have been made in *in vitro* digestive systems for different aquatic species (Morales and Moyano, 2010; Yasumaru and Lemos, 2014; Nieva-Echevarría et al., 2017; Gilannejad et al., 2018; Lewis et al., 2019), and have been used as alternatives to *in vivo* experiments (Dupont et al., 2019). The integration of *in vitro* digestion assays within a screening protocol might help to make a preliminary selection of those raw materials to be tested *in vivo*, reducing labor, number of experimental animals, time and costs.

Here, a five-step screening tool has been developed prior to the *in vivo* testing of different protein sources of vegetable origin locally produced in Europe as sustainable protein sources for rainbow trout (*Oncorhynchus mykiss*) diets. The present working protocol integrates both simple chemical analysis of specific parameters of the raw material and more complex *in vitro* assessments mimicking the fish digestion process. A final validation was performed through an *in vivo* assay. The implementation of this working pipeline would certainly benefit aquaculture industry, identifying those local ingredients that may help to reduce the SBM imports from third countries as well as the total aquaculture carbon footprint.

2. Materials and methods

2.1. Screening protocol

Nine different candidate raw materials (Supplementary Table 1) locally produced in the region of Castilla y León (Spain) were initially selected based on its market availability, minimum nutritional (protein content $>$ 20%) and lowest economic (not used for human consumption) value. In order to had a complete and real representation of the potential scenario, different seeds were tested, including: soybean (*Glycine max*) meal (SBM), the standard reference; green pea (*Pisum sativum*) meal (GPM), already used in fish feeds; and two meals not previously tested as alternative raw materials, the Narbonne vetch (*Vicia narbonensis*) and the red vetchling (*Lathyrus cicera*) meals (NVM and RVM, respectively). In addition, several cultivars (registered seeds as ZV-145, ZV-151 and ZV-156, as well as the one found in a commercial agriculture store (AGROPAL)) and meals pre-treated with different processes (autoclaving, germination and high pressure) from the same species (Narbonne vetch) were also tested. The autoclaving pre-treatment of NVM was carried out at 121 °C and one atmosphere of pressure for 15 min. Germination of Narbonne vetch seeds was induced at 23 $\pm$ 0.5 °C for 25 h in the proportion of 1 g seed for 15.4 mL water on the absence of light. NVM high pressure pre-treatment consisted on applying 600 Mpa for 10 min at room temperature. All seeds were finely grounded to obtain meals with a particle size $<$ 500 μm and tested in triplicates.

In order to identify the most promising alternative raw material to partially or totally substitute SBM, as well as to improve their use in aquafeeds, a five-step screening tool has been developed (Fig. 1).

2.1.1. Complementary characterization

In addition to total crude protein content, a complementary characterization of the meals was done regarding their total buffering capacity, the presence of protease inhibitors, their content in soluble protein and phytic acid. For total buffering capacity, each meal was dissolved in distilled water (0.5 g in 5 mL^{-1}). The buffering capacity was evaluated as in Mennah-Govela et al. (2019), by constant addition of HCl 0.1 N until a pH of 3.5 was reached. The inhibition of alkaline proteases of rainbow trout was calculated following the protocol described in Moyano et al. (1999). Soluble protein was quantified as in Bradford (1976), using bovine serum albumin as standard. In this sense, 1 g of tested meal was dissolved and homogenized in 10 mL^{-1} of distilled water. Phytic acid was determined following the bipyridine method described by Haug and Lantzsch (1983).

2.1.2. Additional nutritional value characterization

In order to get further insights on their nutritional value, the content of several compounds were evaluated in each raw material including: total phosphorus, pentoses, reducing sugars and total polyphenols. Total phosphorus content in tested meals was determined by the molybdovanadate method, after total digestion of the organic matter with concentrated nitric acid (AOAC, 1995). Content in polyphenolic compounds was assessed according to the methodology described by Folin and Ciocalteu (1927) and modified by Vasco et al. (2008), using tannic acid as standard. The content in reducing sugars and free pentoses were evaluated using 3,5-dinitrosalicylic acid following the method described by Miller (1959), and the phloroglucinol method described by Douglas (1981), respectively. All assays were conducted in triplicates.

2.1.3. Enzyme pre-pretreatment

Considering the results obtained in the above mentioned sections, several ingredients were discarded, while the most promising one was selected to follow up with the rest of steps indicated in the flow chart. These included an enzyme pretreatment aimed to partially hydrolyze the fraction of non-starch polysaccharides (NSP) and phytate present in the selected seed, as well as in SBM, that may impair its nutritive utilization by the fish. The treatment was carried out using the commercial product Rovabio® Phy (a mixture of xylanases, glucanases, arabinofuranosidases, as well as phytase, provided by Adisseo, France) following a solid-state hydrolysis protocol (0.3 μl per g of meal during 7 h at pH 5, 60% moisture, 45 °C and shaking every 30 min).

2.1.4. In vitro digestion evaluation

An *in vitro* digestive hydrolysis assay was conducted using an optimized fish gastrointestinal model with two-step hydrolysis using sequentially a closed reactor for acid digestion and a semi-permeable membrane reactor for alkaline digestion (Morales and Moyano, 2010). The device consists of two chambers separated by a semipermeable membrane of 3500 kDa MWCO. Fish enzyme extracts and alternative raw materials tested were placed in the upper chamber and maintained under continuous agitation using a magnetic stirrer. Hydrolysis products (amino acids, pentoses and reducing sugars) passing across the membrane into the lower chamber were recovered at different time intervals during the reaction time. The whole system was maintained at 25 °C within a temperature-controlled

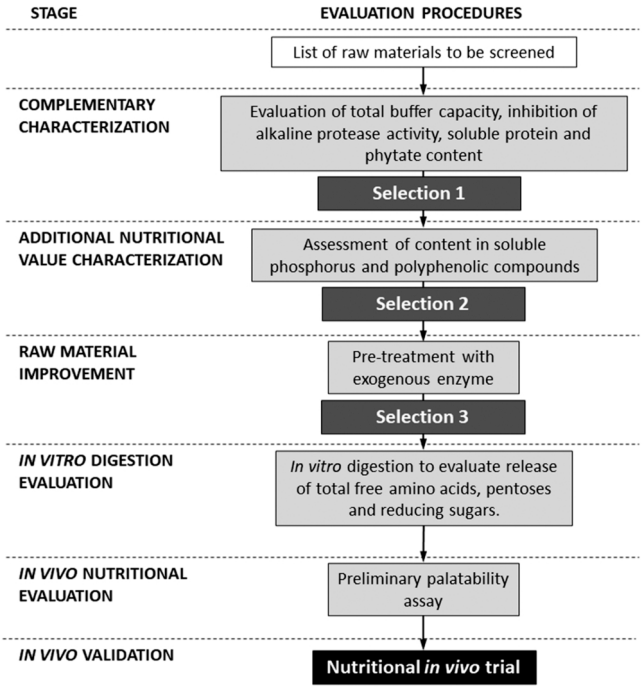

Fig. 1. Flow diagram used for the characterization, selection and validation of alternative raw materials for soybean meal replacement.

chamber. The release of amino acids was quantified by the orthophthaldehyde (OPA) method described by Church et al. (1983) while reducing sugars and pentoses were measured using the methods previously mentioned. The operating conditions used in the assays were as follows: 6 h of hydrolysis time (2 h acid phase + 4 h alkaline phase), 3.5 pH for acid phase and 8.2 pH for intestinal phase, and 4.73 U/mg of meal in acid phase and 29.99 U/mg of meal in alkaline phase as enzyme:substrate ratios (E:S) estimated only for protease. These E:S were calculated considering, in one hand, the total enzyme production in stomach and intestine of a 100 g fish and, in the other hand, the average intake of protein ingested when such fish receives one meal (1% body weight) of a feed containing 45% of crude protein (CP). Data of such enzyme production were obtained in a previous assay performed using several fish specimens (n = 7) fed on the indicated pattern, being pepsin activity in the stomach determined following the methodology of Anson (1938) using hemoglobin as substrate and total alkaline protease activity in intestine measured according to the method of Kunitz (1947) modified by Walter (1984), using casein as substrate. One unit of enzyme activity (U) was defined as the amount of enzyme needed to catalyze the formation of 1 μg of tyrosine per minute. Blank assays on which the enzyme extracts were thermally inactivated (100 ºC for 5 min) were run to assess the basal release of free amino acids not coming from protein hydrolysis. Each assay was conducted in triplicate.

2.1.5. Palatability assay

In order to assess possible problems of acceptance in diets including the highest replacement of SBM by the selected alternative ingredient, the rainbow trout daily feed intake when fed experimental diets was preliminary evaluated. For this, a small amount of the diets were prepared, including or not a palatability enhancer (fish animal protein hydrolysate). Sixty fish (35 g and 14.5 cm of mean wet weight and furcal length, respectively) were randomly allocated in four 500 L tanks (15 specimens per tank) and hand-fed to apparent satiation once a day (between 8:00 and 9:00 h) until reaching a maximum of 3% daily feed intake for 15 days.

2.2. Nutritional in vivo trial

2.2.1. Ethical statement

All experiments complied with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020) and were performed 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), in order to warrant an ethical animal experimentation as well as fish welfare. The persons involved in the experiments are qualified to handle animals for experimentation according to Orden ECC/566/2015 from Spanish legislation. All procedures were previously approved by the Bioethical Committee of ITACyL in order to fulfill the administrative requirements prior to conducting the planned research (approval number: 2018/31/CEEA).

2.2.2. Fish diets and proximate composition

Five experimental diets were specifically formulated to be isonitrogenous, and isoenergetic, with 43.2% of protein and 17.5 MJ kg^{-1}. Diets were formulated replacing 33 or 66% of the SBM by NVM previously treated or not with exogenous enzyme Rovabio® Phy and named as: Control diet, no substitution of SBM; 33 A diet, 33% of SBM replaced by not pre-treated NVM; 33 A+E diet, 33% of SBM replaced by NVM pre-treated with Rovabio® Phy; 66 A diet, 66% of SBM replaced by not pre-treated NVM; and 66 A+E diet, 66% of SBM replaced by NVM pre-treated with Rovabio® Phy. Diets were supplemented with methionine to fulfill the nutritional requirements of rainbow trout. Feeds were manufactured by the Experimental Feeds Unit of the University of Almeria (Almeria, Spain), extruded at appropriate size pellets (2–3 mm), and tested in triplicates.

Proximate composition of diets was calculated. Moisture, protein and nitrogen content were determined according to official AOAC procedures (AOAC, 1990) and the Official Journal of European Union (OJEU, 2009). The moisture was calculated by drying samples at 105 °C for 24 h until constant weight. Protein and nitrogen content were analyzed by Kjeldahl method (N × 6.25), fat by dichloromethane extraction (Soxhlet), and ash content by heating the residue from the moisture determination in a muffle furnace at 550 ºC for 24 h. Crude fiber content was calculated in the defatted fractions after digestion with sulfuric acid and sodium hydroxide, and burning at 550 °C for 3 hrs. The nitrogen free extract was calculated according to Brett and Groves (1979). Soluble protein, pentoses and reducing sugars content were also quantified as previously described. All parameters were expressed in percentage in relation to dry matter.

2.2.3. Experimental design and rearing conditions

A total of 195 all-female rainbow trout juveniles (38.04 ± 0.07 g and 15.10 ± 0.07 cm of mean wet weight and furcal length, respectively), previously acclimatized during 15 days to ITACyL experimental facilities, were randomly allocated (13 individuals per tank, three tanks per diet) in 15 experimental 500 L tanks connected to a recirculating aquaculture system (RAS). Fish were hand-fed to apparent satiation once (between 8:00 and 9:00 h) a day (until a maximum of 3% daily feed intake) for 63 days.

During the growth trial, water temperature was maintained at 14.5 ± 0.3 °C, dissolved oxygen content was > 8.1 ± 0.3 mg/L, and light:dark photoperiod cycle was 12:12 h. The ammonium and nitrite water concentrations were daily monitored to keep them below toxic values.

2.2.4. Fish growth performance and sampling

Along the experiment, growth, in terms of weight and furcal length (FL), was monitored each 21 days in order to adjust daily feed ration. For this, fish were slightly anesthetized with MS-222 (180 mg/mL), being FL assessed using a graduated ichthyometer (0.1 mm) and wet weight with a GRAM S3R–6KD balance (0.1 g). Every day, mortality and feed intake in each tank were recorded. Feces were

collected during the last two weeks of the trial for apparent digestibility analysis. Feces from each experimental tank were stored at − 80 °C until analysis.

At the end of the trial, growth performance indexes were calculated as described in Tomás-Almenar et al. (2020). In addition, 3 fish from each tank were also randomly sampled and sacrificed with an overdose of MS222 (300 mg/mL). Fish muscle samples were taken, snap frozen and stored at − 80 °C until analysis of proximate composition and amino acid profile.

2.2.5. Apparent digestibility

The apparent digestibility of the protein was determined using the modified Guelph method of Cho et al. (1982) of acid-insoluble ashes, and calculated as follows:

ADCprot (apparent digestibility coefficient of protein) = 100 - [(marker in diet/marker in feces) x (% protein in feces/% protein in diet) x 100]

2.2.6. Proximate composition and amino acid profile in fish muscle tissue

The muscle samples of three fish from each tank were pooled and homogenized. Part of the pooled sample was taken to perform a proximate analysis (moisture, protein, nitrogen, fat, ash and carbohydrate contents) as previously described. All parameters were conducted in triplicate and expressed in percentage in relation to dry matter.

The amino acid profile of fish fillet was performed in triplicates and using 20 mg of tissue. After hydrolysis with 1 mL of 6 N HCl for 24 h at 110 °C, samples were neutralized with NaOH 6.5 N, and diluted ten times with sodium loading buffer pH 2.2 (Biochrom, Cambridge, UK). The determination was performed by ion-exchange liquid chromatography and post-column continuous reaction with ninhydrin (Biochrom , Cambridge, UK) to provide qualitative and quantitative compositional analysis. Norleucine was used as the internal standard.

2.3. 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. During the screening process the Tukey and T-test post-hoc analyses were used to detect differences among the tested meals. In contrast, the Dunnett's multiple-comparison test was used to detect differences between each experimental group and the Control in the nutritional *in vivo* trial. The level of significance in both cases was set at $p < 0.05$. All the statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Screening tool

3.1.1. Complementary characterization

Results obtained in the first stage of selection are presented in Table 1. SBM presented a higher buffering capacity (248.06 ± 0.00 μmol H+ per (g sample × ΔpH)) than the other meals tested, regardless of their pre-treatment (autoclaving, germination and/or high pressure) and/or cultivar (NVM/ZV-145, NVM/ZV-151, NVM/ZV-156 and/or NVM/Agropal) considered (from 81.63 ± 0.00 to 95.09 ± 0.00 μmol H+ per (g sample × ΔpH); ANOVA; $p < 0.05$, n = 3). In addition, SBM produced the lowest alkaline protease inhibition (24.18 ± 3.54%), while the NVM/ZV-151 produced the highest inhibition (69.00 ± 1.80%) followed by RVM, NVM/Agropal, GPM and NVM/ZV-145. The NVM/ZV-156 meal showed a medium value of protease activity inhibition (47.12 ± 4.64%) that was reduced

Table 1
Total buffering capacity, inhibition of alkaline protease activity, soluble protein and phytic phosphorus content in the different meals evaluated.

Meal/Code	TBC	IAP activity	SP	Phytate
NVM/ZV-156	92.35 ± 0.00^{f}	47.12 ± 4.64^{cd}	4.32 ± 0.09^{bc}	4.90 ± 0.22^{a}
NVM/ZV-156 HP	95.09 ± 0.00^{c}	49.35 ± 3.02^{bcd}	4.38 ± 0.01^{bc}	4.33 ± 0.24^{bc}
NVM/ZV-156 G	92.31 ± 0.00^{g}	43.65 ± 0.96^{d}	4.60 ± 0.01^{ab}	4.43 ± 0.09^{abc}
NVM/ZV-156 AC	95.24 ± 0.00^{b}	33.29 ± 1.86^{e}	0.98 ± 0.13^{g}	3.96 ± 0.03^{c}
NVM/ZV-145	89.02 ± 0.00^{h}	50.17 ± 3.47^{bcd}	4.20 ± 0.01^{c}	3.21 ± 0.10^{d}
NVM/ZV-151	81.63 ± 0.00^{i}	69.00 ± 1.80^{a}	3.31 ± 0.17^{e}	3.07 ± 0.03^{d}
NVM/Agropal	92.31 ± 0.00^{g}	53.00 ± 2.24^{bc}	4.84 ± 0.05^{a}	4.73 ± 0.31^{ab}
RVM	94.75 ± 0.00^{d}	56.10 ± 1.76^{b}	2.95 ± 0.12^{f}	1.69 ± 0.28^{f}
GPM	93.18 ± 0.00^{e}	50.24 ± 2.23^{bcd}	2.69 ± 0.12^{f}	2.26 ± 0.02^{e}
SBM	248.06 ± 0.00^{a}	24.18 ± 3.54^{f}	3.76 ± 0.19^{d}	4.15 ± 0.25^{c}

TBC, Total buffering capacity in μmol H+ per g sample × ΔpH; IAP, inhibition of alkaline protease activity in %; SP, Soluble protein in mg per g of sample; Phytate, in mg per g of sample; NVM, Narbonne vetch (*Vicia narbonensis*) meal; RVM, red vetchling (*Lathyrus cicera*) meal; GPM, green pea (*Pisum sativum*) meal; SBM, soybean (*Glycine max*) meal; HP, Meal subjected to high pressure; PG, Meal subjected to germination; AC, Autoclaved meal. Different superscript letters within each column denote significant differences among meals (ANOVA; Tukey test; $p < 0.05$; n = 3).

when it was previously treated with autoclaving or germination processes, but not with high pressures. The highest content in soluble protein was measured in NVM/Agropal (4.84 ± 0.05 mg per g), followed by the other NVM meals (with the exception of NVM/ZV-151 and autoclaved NVM/ZV-156) and SBM, being the lowest values determined in RVM and GPM (2.95 ± 0.12 and 2.69 ± 0.12 mg per g, respectively). Similarly, while RVM and GPM presented a reduced content in phytate (1.69 ± 0.28 and 2.26 ± 0.02 mg per g, respectively) NVM/ZV-156, NVM/Agropal and SBM presented significantly higher values ranging from 4.15 ± 0.25 to 4.90 ± 0.22 mg per g. Based on these results, and considering as positive features the low values of buffering capacity, inhibitory effect and phytate contents and a high amount of soluble protein, 4 raw materials from the 9 initially tested were discarded. Thus, only SBM (as reference standard), NVM/ZV-156 and NVM/ZV-156-AC for its lower inhibition of alkaline protease activity, and RVM and GPM for its low content in phytate were evaluated in the next stage.

3.1.2. Additional nutritional value characterization

Content in total phosphorus and phenolic compounds are showed in Fig. 2. SBM presented significantly higher total phosphorus content (0.66 ± 0.04 g per 100 g of meal), than the rest of meals, that ranged from 0.38 to 0.47 g per 100 g of meal. NVM/ZV-156 (autoclaved or not) contained significantly higher phosphorus than RVM and GPM (0.47 ± 0.01 and 0.42 ± 0.03 g per 100 g of meal, respectively). Regarding to the contents in total phenolic compounds, significantly higher contents were measured in SBM and NVM/ZV-156 when compared to the rest. Also, it seemed that autoclaving affected those compounds since a significant reduction was observed when comparing values measured in NVM/ZV-156 autoclaved or not. Since total phosphorus and phenolic compounds are key nutrients for fish physiology (see discussion section), NVM/ZV-156 and NVM/ZV-156-AC were selected for the next screening stage.

3.1.3. Raw material improvement

After treatment with the exogenous enzyme mixture Rovabio® Phy, contents on phytate, pentoses and reducing sugars were altered in SBM, NVM/ZV-156 and NVM/ZV-156-AC (Fig. 3). The treatment was able to fully remove the phytate presented in all the meals, independently of its initial content (Fig. 3a). Furthermore, it increased the availability of pentoses (Fig. 3b) and reducing sugars (Fig. 3c). The content of pentoses in SBM increased from 0.59 ± 0.01 to 0.78 ± 0.02 g per 100 g of meal, while in NVM/ZV-156 and

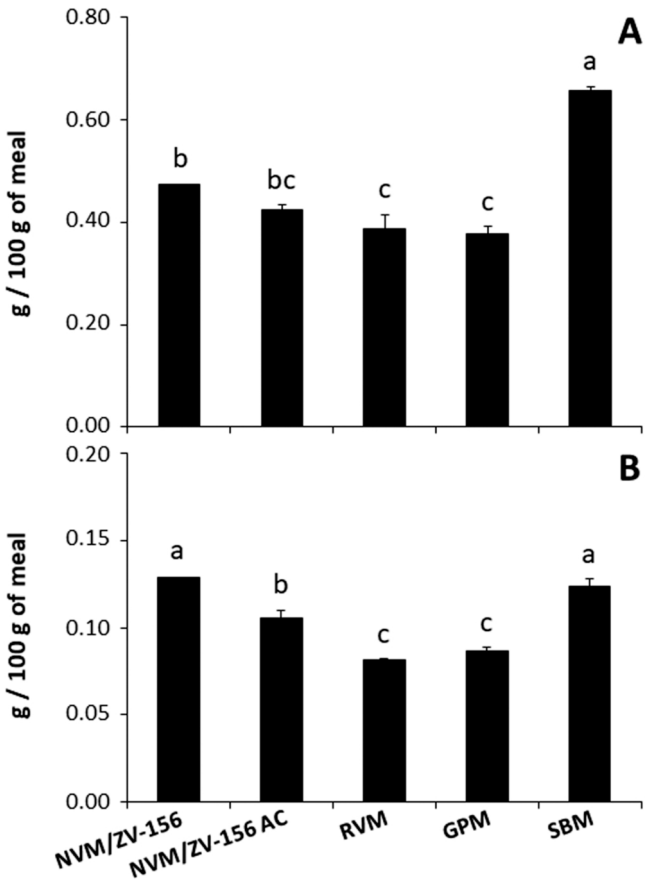

Fig. 2. Total soluble phosphorus (A) and phenolic compounds (B) content in the different screened meals. Values represented mean and standard deviations. Letters at the top of bars indicate significant differences between screened meals (ANOVA; $p < 0.05$, n = 3).

NVM/ZV-156-AC it increased (from 0.32 ± 0.01 in both meals) until 0.50 ± 0.00 and 0.42 ± 0.01 g per 100 g of meal, respectively. Regarding reducing sugars availability, although its content was increased after treatment with exogenous enzyme in all meals, a higher availability was found in NVM/ZV-156 (5.58 ± 0.12 g per 100 g of meal) compared to that of NVM/ZV-156-AC and SBM (3.62 ± 0.34 and 4.28 ± 0.37 g per 100 g of meal, respectively). Thus, NVM/ZV-156 was finally the meal selected to evaluate in comparison to SBM using the *in vitro* trial.

3.1.4. *In vitro digestion evaluation*

Total amounts of amino acids, pentoses and reducing sugars released after the *in vitro* digestion assay, are presented in Fig. 4. Results showed lower amounts of amino acids and pentoses but higher of reducing sugars released from NVM/ZV-156 when compared to SBM. On the other hand, enzyme pretreatment (Rovabio® Phy) of both meals significantly increased de potential bioavailability of all the nutrients in both meals, being such increase noticeably high for reducing sugars (from 3.78 ± 0.46 until 6.16 ± 0.73 μg per mg of meal) and pentoses (from 3.57 ± 0.04 until 6.74 ± 0.03 μg per mg of meal) but much less in amino acids (from 0.10 ± 0.00 until 0.12 ± 0.03 mg per mg of meal) in NVM/ZV-156, when compared to those observed in SBM (in reducing sugars from 0.36 ± 0.03 until 0.81 ± 0.07 μg per mg of meal; pentoses from 5.59 ± 0.05 until 8.27 ± 0.02 μg per mg of meal; amino acids from 0.18 ± 0.02 until 0.26 ± 0.03 mg per mg of meal; T-test; $p < 0.05$, n = 3).

3.1.5. *Palatability evaluation*

Based on the above results from the screening tool, feeds for rainbow trout were designed (Table 2). A preliminary 15 day-nutritional trial showed that diets with the highest SBM replacement (66%) with NVM/ZV-156 had no effect on feed palatability, with a feed intake of 2.36 ± 0.01 whether a flavor component was included or not in the diet.

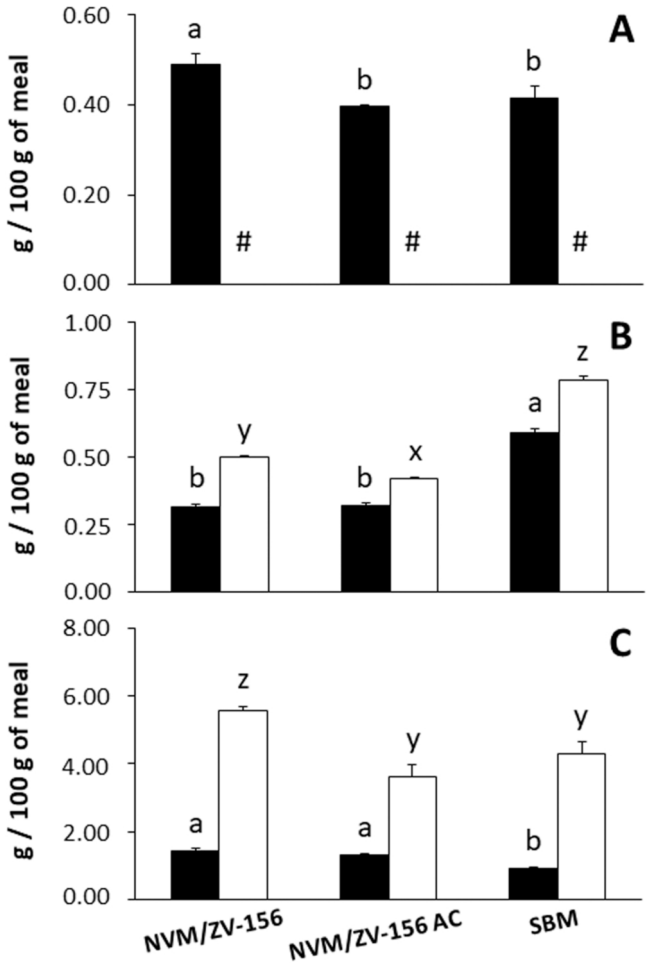

Fig. 3. Phytate (A), pentoses (B) and reducing sugars (C) availability in the different screened meals before and after treatment with exogenous enzyme Rovabio® Phy. Values represented mean and standard deviations. Black and white bars represent compound availability before and after treatment with Rovabio® Phy, respectively. # indicates absence of phytate. Letters at the top of bars indicate significant differences between screened meals before (a and b) or after (x, y and z) exogenous enzyme treatment (ANOVA; $p < 0.05$, n = 3).

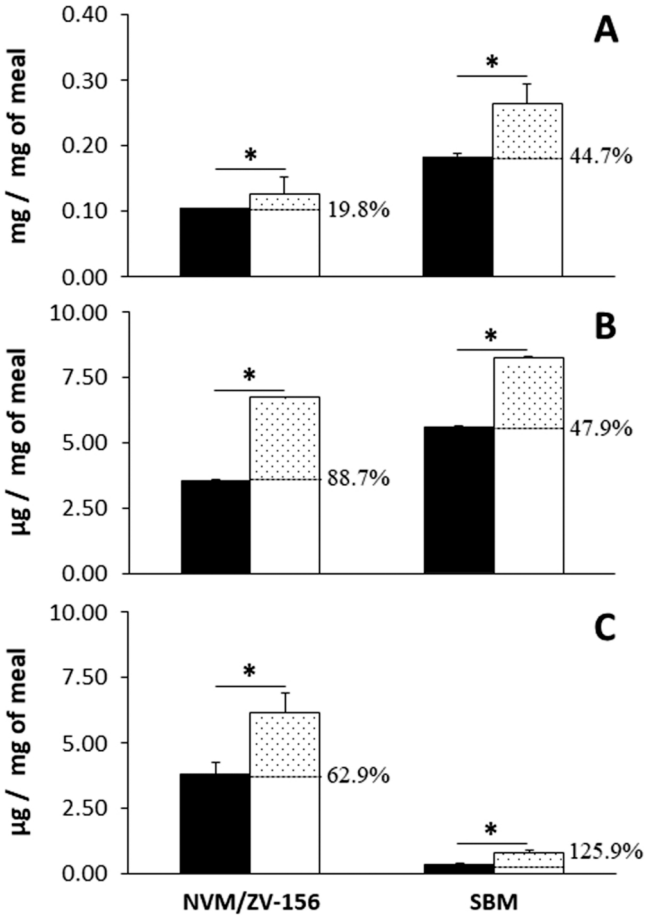

Fig. 4. Nutrients released after *in vitro* hydrolysis from different screened meals, previously treated or not with Rovabio® Phy. Amino acids (A), pentoses (B) and reducing sugars (C). Black and white bars represent the amounts of compounds released from non- and Rovabio® Phy treated meals. The hatched fragment in the white bar indicates the percentage of increased nutrient release with the Rovabio® Phy. Asterisk at the top of bars indicates significant differences among meals when they were treated or not with Rovabio® Phy (T-test; $p < 0.05$, n = 3).

3.2. Nutritional in vivo trial

Feed formulation and proximate composition of Control and experimental diets, where SBM was replaced by 33% and 66% with NVM/ZV-156 previously treated with Rovabio® Phy or not, is presented in Table 2. Regarding the content on soluble protein, all experimental diets (33 A, 33 A+E, 66 A and 66 A+E) had higher values than the Control diet (from 6.58 ± 0.54 to 9.15 ± 0.34 *versus* 5.63 ± 0.18 g per 100 g of diet; ANOVA; $p < 0.05$, n = 3). Similarly, a higher content in reducing sugars was observed (from 1.00 ± 0.02 to 1.13 ± 0.01 *versus* 0.84 ± 0.03 g per 100 g of diet; ANOVA; $p < 0.05$, n = 3). In contrast, only a lower content in pentoses (0.37 ± 0.01 and 0.34 ± 0.01 g per 100 g of diet) was observed in diets with highest SBM replacement (66 A and 66 A+E) compared to that of the Control diet (0.40 ± 0.01 g per 100 g of diet; ANOVA; $p < 0.05$, n = 3), with diets replacing SBM in a 33% showing equal values to the Control diet (ANOVA; $p > 0.05$, n = 3).

After a 63-day feeding trial, growth performance was affected by SBM replacement depending on the percentage of substitution and if NVM/ZV-156 was previously treated with Rovabio® Phy or not (Table 3). At the end of the trial, fish growth in terms of body wet weight and furcal length was reduced when SBM was replaced in a high percentage (66%, independently of NVM/ZV-156 being pre-treated or not with Rovabio® Phy; 66 A and 66 A+E diets) when compared to the Control group (ANOVA; Dunnett's test; $p < 0.05$; n = 3). Although a 33% replacement of SBM with NVM/ZV-156 not previously treated with exogenous enzyme also shown a reduced growth, fish fed 33 A+E diet (33% replacement of SBM with NVM/ZV-156 previously treated with Rovabio® Phy) did not show significant differences with the fish fed Control diet (ANOVA; Dunnett's test; $p > 0.05$; n = 3). Similarly, weight gain (WG), specific growth rate (SGR) and feed conversion rate (FCR) were significantly affected in fish fed 66 A, 66 A+E and 33 A diets when compared to fish fed Control diet (ANOVA; Dunnett's test; $p < 0.05$; n = 3), but not in fish fed 33 A+E diet. Apparent digestibility of protein was slightly reduced in all diets where SBM was partially replaced by NVM/ZV-156 (ranging from 89.20 ± 0.65 to 91.32 ± 0.23%) regardless of being enzymatically pre-treated or not when compared to the Control (93.78 ± 0.20%) diet (Table 2; ANOVA; $p < 0.05$, n = 3). Furthermore, while significantly increased values of hepatosomatic index (HSI) were found in fish fed 66 A and 66 A+E diets

Table 2
Ingredients of formulation, proximate composition, protein apparent digestibility and soluble protein, pentoses and reducing sugars content of experimental diets.

Ingredients (g/100 g)	Diets				
	Control	33 A	33 A+E	66 A	66 A+E
Fishmeal LT	25.00	25.00	25.00	25.00	25.00
NVM	0.00	10.00	10.00^{+}	20.00	20.00^{+}
SBM	30.00	20.00	20.00	10.00	10.00
Wheat gluten	12.01	15.12	15.12	18.23	18.23
Wheat meal	13.52	10.40	10.40	7.28	7.28
Fish oil	7.00	7.00	7.00	7.00	7.00
Vegetable oil	7.00	7.00	7.00	7.00	7.00
Soy lecithin	0.95	0.94	0.94	0.92	0.92
Premix[a]	2.00	2.00	2.00	2.00	2.00
Binder[b]	2.00	2.00	2.00	2.00	2.00
Metionine	0.52	0.54	0.54	0.56	0.56
Total	**100**	**100**	**100**	**100**	**100**
Proximate composition (% on Dry Matter)					
Moisture	4.55	4.87	6.62	4.92	6.72
Crude protein	42.54	42.01	41.93	41.48	41.49
Crude fat	18.24	18.57	18.57	20.13	19.26
Crude fiber	3.21	3.63	3.45	3.83	3.79
Ash	7.82	7.25	7.40	6.85	6.79
NFE	28.19	28.55	28.65	27.71	28.67
Protein apparent digestibility (%)					
ADCprotein	93.78 ± 0.20	91.32 ± 0.23 *	89.87 ± 0.65 *	89.20 ± 0.65 *	89.43 ± 0.68 *
Analysis of nutrient content (g /100 g of diets)					
Soluble protein	5.63 ± 0.18	6.87 ± 0.24 *	6.58 ± 0.54 *	9.15 ± 0.34 *	7.88 ± 0.24 *
Pentoses	0.40 ± 0.00	0.40 ± 0.01	0.41 ± 0.02	0.37 ± 0.01 *	0.34 ± 0.01 *
Reducing sugars	0.84 ± 0.03	1.00 ± 0.02 *	1.03 ± 0.01 *	1.04 ± 0.02 *	1.13 ± 0.01 *

NVM, Narbonne vetch meal from ZV-156 cultivar; NFE, nitrogen free extract; SBM, soybean meal; 33 A, diet with a 33% of SBM replaced by NVM not treated with Rovabio® Phy; 33 A+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® Phy; 66 A, diet with a 66% of SBM replaced by NVM not treated with Rovabio® Phy; 66 A+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® Phy; ADCprotein, Apparent digestibility coefficient of protein. [a] Vitamin and mineral premix TECNOVIT; [b] Guar gum; + NVM pre-treated with the exogenous enzyme Rovabio® Phy. Asterisks within each row denote significant differences with the Control group (ANOVA; Dunnett's test; $p < 0.05$; n = 3).

Table 3
Growth performance and somatic indexes in rainbow trout fed with experimental diets.

	Control	33 A	33 A+E	66 A	66 A+E
IBW	37.97 ± 0.02	38.14 ± 0.05	38.04 ± 0.04	38.01 ± 0.07	38.03 ± 0.07
IFL	15.17 ± 0.07	15.12 ± 0.07	15.09 ± 0.10	15.06 ± 0.03	15.07 ± 0.04
FBW	223.18 ± 6.78	204.98 ± 1.85 *	211.39 ± 2.45	196.46 ± 5.39 *	198.57 ± 6.84 *
FTL	24.88 ± 0.27	24.09 ± 0.08 *	24.62 ± 0.26	23.97 ± 0.22 *	24.09 ± 0.09 *
WG	487.80 ± 18.15	437.48 ± 5.60 *	455.73 ± 7.03	416.92 ± 13.30 *	422.19 ± 18.52 *
SGR	2.81 ± 0.05	2.67 ± 0.02 *	2.72 ± 0.02	2.61 ± 0.04 *	2.62 ± 0.06 *
FCR	0.81 ± 0.01	0.86 ± 0.01 *	0.84 ± 0.01	0.91 ± 0.02 *	0.91 ± 0.02 *
CF	1.45 ± 0.01	1.47 ± 0.00	1.42 ± 0.06	1.43 ± 0.03	1.42 ± 0.04
HSI	1.10 ± 0.01	1.20 ± 0.02	1.23 ± 0.04	1.34 ± 0.10 *	1.33 ± 0.07 *
VSI	10.81 ± 0.16	10.85 ± 0.22	10.61 ± 0.63	10.50 ± 0.81	11.00 ± 0.54

Values are expressed as mean ± standard deviation. IBW, initial body weight in g; IFL, initial furcal length in cm; FBW, final body weight in g; FFL, final furcal length in cm; WG, weight gain in %; SGR, specific growth rate in %/day; FCR, feed conversion ratio; CF, condition factor; HSI, hepatosomatic index in %; VSI, viscero-somatic index in %; 33 A, diet with a 33% of SBM replaced by NVM (Narbonne vetch meal from ZV-156 cultivar) not treated with Rovabio® phytase; 33 A+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® phytase; 66 A, diet with a 66% of SBM replaced by NVM not treated with Rovabio® phytase; 66 A+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® phytase. Asterisks within each row denote significant differences between each experimental group and the Control (ANOVA; Dunnett's test; $p < 0.05$; n = 3).

(1.34 ± 0.10 and 1.33 ± 0.07%, respectively) when compared to that of Control fish (1.10 ± 0.01%; ANOVA; Dunnett's test; $p < 0.05$; n = 3), no differences were found in fish fed 33 A and 33 A+E diets. In contrast, both condition factor (CF; values ranging from 1.42 ± 0.04 to 1.47 ± 0.00) and viscerosomatic index (VSI; values ranging from 10.50 ± 0.81 to 11.00 ± 0.54%) were not significantly affected regardless the fish diet considered when compared to the Control group (ANOVA; Dunnett's test; $p > 0.05$; n = 3).

The proximate composition of the fillet was only affected regarding its crude fat content, but not on its crude protein, ash or nitrogen free extract (carbohydrates), while the amino acid profile was only affected on the cysteine content (Table 4). While fish fed 33 A+E and 66 A diets showed a lower crude fat content (16.0 ± 0.2 and 16.6 ± 1.1%), fish fed 66 A+E diet had a higher content (23.1 ± 1.0%) when compared to the Control fish (18.9 ± 0.2%; ANOVA; Dunnett's test; $p < 0.05$; n = 3). Finally, all fish groups fed experimental diets where SBM has been replaced by NVM/ZV-156, independently of the % of substitution and/or the enzymatic pre-

treatment, showed an increased content of cysteine (ranging from 0.29 ± 0.00 to 0.33 ± 0.01 g per 100 g of muscle tissue) when compared with fish fed Control diet (0.25 ± 0.02 g per 100 g of muscle tissue; ANOVA; Dunnett's test; $p < 0.05$; n = 3). (Table 5).

4. Discussion

Unlocking aquaculture production growth and maintaining/improving its sustainability is one of the major challenges to warrant future food security and nutrition (FAO, 2020). In addition to improving rearing conditions, species domestication and genetic inbreeding among other aspects, the identification of alternative raw materials as well as increasing its use in fish diets is urgently required, not only to substitute FM and FO, but also SBM (Tacon and Metian, 2015). Here, a five-step screening tool has been designed, tested and validated to identify new alternative raw materials locally produced in Europe in order to reduce the SBM dependency from third countries, the use of animals in experimentation as well as the costs associated to feed production (Tacon et al., 2011).

4.1. A five-step screening tool provides key knowledge for prioritizing alternative raw materials testing in vivo

Although recent new alternative raw materials such as single cell protein sources and/or insect meal have been successfully tested to replace FM (Henry et al., 2015; Gamboa-Delgado and Márquez-Reyes, 2016; Nogales-Mérida et al., 2018), plant-derived sources still are the most widely implemented (Nie and Hallerman, 2021). Thus, a five-step screening tool was designed to identify most promising alternatives to SBM that are locally produced in Europe. Although the locally produced seeds tested here have a lower protein content than SBM, may present other advantages related to their buffering capacity, reduced presence of anti-nutritional factors (ANFs), solubility of protein, protein bioaccessibility, and others, making them potential candidates for partial inclusion as ingredients in fish feeds. Buffering capacity, is a key aspect to consider in the evaluation of nutritional quality of protein ingredients since it might determine the physical-chemical shattering of key nutritional compounds (Mennah-Govela et al., 2019; Toledo-Solís et al., 2020). Indeed, the lower buffer capacity of the meals tested here might increase nutrient's bioavailability by enhancing the decrease of stomach pH and hence the activity of pepsin. On the other hand, protein solubility is one key parameter routinely used to evaluate potential nutritional quality of ingredients used in feed production, being also an indicator of over processing of meals (Batal et al., 2000). The fraction of soluble protein present in the tested meals was similar or even higher than that of SBM, this suggesting a great equivalence among all these leguminous sources.

The presence of ANFs (*e.g.* protease inhibitors, phytic acid, non-starch polysaccharides (NSPs), antivitamins, lectins, tannins, saponins, *etc.*) has been reported as a main limiting factor for extended use of plant-derived raw materials in fish diets, due to their demonstrated negative impacts on fish growth, physiological condition and/or wellbeing, mostly by the impairment of digestive hydrolysis and absorption of nutrients (Francis et al., 2001; Maitra et al., 2007; Krogdahl et al., 2010). For instance, it is known that phytic acid (the main form of phosphorus storage in plant tissues) is a not bioavailable form of this element that also acts as a chelating

Table 4
Proximal analysis and amino acid profile of fish fillet from rainbow trout fed experimental diets.

Diets	Control	33 A	33 A+E	66 A	66 A+E
Proximate composition (% on Dry Matter)					
Crude protein	70.7 ± 1.4	73.0 ± 0.2	72.8 ± 0.8	71.1 ± 1.6	68.0 ± 4.4
Crude fat	18.9 ± 0.2	17.5 ± 0.3	16.0 ± 0.2 *	16.6 ± 1.1 *	23.1 ± 1.0 *
Ash	5.5 ± 0.6	6.3 ± 0.4	5.9 ± 0.6	6.2 ± 0.5	5.6 ± 0.3
NFE	4.9 ± 1.7	3.1 ± 0.7	5.3 ± 1.0	6.2 ± 0.9	5.2 ± 1.1
Amino acid (g/100 g of muscle)					
Asp	6.79 ± 0.65	7.25 ± 0.56	7.20 ± 0.56	6.92 ± 0.31	6.85 ± 0.17
Thr	3.02 ± 0.30	3.12 ± 0.28	3.19 ± 0.20	3.04 ± 0.13	3.05 ± 0.07
Ser	2.60 ± 0.32	2.62 ± 0.31	2.79 ± 0.06	2.67 ± 0.14	2.65 ± 0.04
Glu	9.58 ± 0.96	10.37 ± 0.86	10.09 ± 0.87	9.77 ± 0.44	9.64 ± 0.25
Gly	3.18 ± 0.34	3.40 ± 0.32	3.25 ± 0.26	3.05 ± 0.14	3.05 ± 0.04
Ala	4.75 ± 0.53	4.96 ± 0.43	5.02 ± 0.40	4.76 ± 0.17	4.80 ± 0.10
Cys	0.25 ± 0.02	0.29 ± 0.01 *	0.30 ± 0.01 *	0.29 ± 0.00 *	0.33 ± 0.01 *
Val	3.55 ± 0.42	4.01 ± 0.37	3.61 ± 0.64	3.49 ± 0.19	3.74 ± 0.10
Met	2.05 ± 0.21	2.18 ± 0.16	2.18 ± 0.19	2.06 ± 0.12	2.11 ± 0.05
Ile	2.69 ± 0.28	3.15 ± 0.27	2.70 ± 0.61	2.64 ± 0.12	2.77 ± 0.08
Leu	4.78 ± 0.42	5.15 ± 0.41	5.00 ± 0.56	4.81 ± 0.18	4.84 ± 0.12
Tyr	2.27 ± 0.20	2.37 ± 0.21	2.38 ± 0.18	2.22 ± 0.06	2.27 ± 0.05
Phe	2.33 ± 0.21	2.52 ± 0.18	2.45 ± 0.23	2.36 ± 0.07	2.35 ± 0.03
His	1.19 ± 0.11	1.27 ± 0.09	1.24 ± 0.10	1.19 ± 0.03	1.17 ± 0.01
Lys	6.90 ± 0.63	7.50 ± 0.69	7.19 ± 0.80	6.82 ± 0.25	6.88 ± 0.17
Arg	3.82 ± 0.34	4.15 ± 0.30	4.01 ± 0.42	3.84 ± 0.15	3.89 ± 0.08
Pro	4.23 ± 0.86	4.52 ± 0.42	4.58 ± 0.64	4.57 ± 0.62	4.58 ± 0.08

Values are expressed as mean ± standard deviation. NFE, nitrogen free extract; 33 A, diet with a 33% of SBM replaced by NVM (Narbonne vetch meal from ZV-156 cultivar) not treated with Rovabio® Phy; 33 A+E, diet with a 33% of SBM replaced by NVM treated with Rovabio® Phy; 66 A, diet with a 66% of SBM replaced by NVM not treated with Rovabio® Phy; 66 A+E, diet with a 66% of SBM replaced by NVM treated with Rovabio® Phy. Asterisks within each row denote significant differences between each experimental group and the Control group (ANOVA; Dunnett's test; $p < 0.05$; n = 3).

Table 5
Proximal analysis and amino acid profile fish fillet in rainbow trout fed experimental diets.

Diets	Control	33 A	33 A+E	66 A	66 A+E	
Proximate composition (% on Dry Matter)						
Crude protein		70.7 ± 1.4	73.0 ± 0.2	72.8 ± 0.8	71.1 ± 1.6	68.0 ± 4.4
Crude fat		18.9 ± 0.2	17.5 ± 0.3	16.0 ± 0.2 *	16.6 ± 1.1 *	23.1 ± 1.0 *
Ash		5.5 ± 0.6	6.3 ± 0.4	5.9 ± 0.6	6.2 ± 0.5	5.6 ± 0.3
Nfe		4.9 ± 1.7	3.1 ± 0.7	5.3 ± 1.0	6.2 ± 0.9	5.2 ± 1.1
Amino acid (g/100 g of muscle)						
Asp		6.79 ± 0.65	7.25 ± 0.56	7.20 ± 0.56	6.92 ± 0.31	6.85 ± 0.17
Thr		3.02 ± 0.30	3.12 ± 0.28	3.19 ± 0.20	3.04 ± 0.13	3.05 ± 0.07
Ser		2.60 ± 0.32	2.62 ± 0.31	2.79 ± 0.06	2.67 ± 0.14	2.65 ± 0.04
Glu		9.58 ± 0.96	10.37 ± 0.86	10.09 ± 0.87	9.77 ± 0.44	9.64 ± 0.25
Gly		3.18 ± 0.34	3.40 ± 0.32	3.25 ± 0.26	3.05 ± 0.14	3.05 ± 0.04
Ala		4.75 ± 0.53	4.96 ± 0.43	5.02 ± 0.40	4.76 ± 0.17	4.80 ± 0.10
Cys		0.25 ± 0.02	0.29 ± 0.01 *	0.30 ± 0.01 *	0.29 ± 0.00 *	0.33 ± 0.01 *
Val		3.55 ± 0.42	4.01 ± 0.37	3.61 ± 0.64	3.49 ± 0.19	3.74 ± 0.10
Met		2.05 ± 0.21	2.18 ± 0.16	2.18 ± 0.19	2.06 ± 0.12	2.11 ± 0.05
Ile		2.69 ± 0.28	3.15 ± 0.27	2.70 ± 0.61	2.64 ± 0.12	2.77 ± 0.08
Leu		4.78 ± 0.42	5.15 ± 0.41	5.00 ± 0.56	4.81 ± 0.18	4.84 ± 0.12
Tyr		2.27 ± 0.20	2.37 ± 0.21	2.38 ± 0.18	2.22 ± 0.06	2.27 ± 0.05
Phe		2.33 ± 0.21	2.52 ± 0.18	2.45 ± 0.23	2.36 ± 0.07	2.35 ± 0.03
His		1.19 ± 0.11	1.27 ± 0.09	1.24 ± 0.10	1.19 ± 0.03	1.17 ± 0.01
Lys		6.90 ± 0.63	7.50 ± 0.69	7.19 ± 0.80	6.82 ± 0.25	6.88 ± 0.17
Arg		3.82 ± 0.34	4.15 ± 0.30	4.01 ± 0.42	3.84 ± 0.15	3.89 ± 0.08
Pro		4.23 ± 0.86	4.52 ± 0.42	4.58 ± 0.64	4.57 ± 0.62	4.58 ± 0.08

Values are expressed as mean ± standard deviation. Nfe, nitrogen free extract; 33 A, diet with a 33% of SPC replaced by NVM (Narbonne vetch meal from ZV-156 cultivar) not treated with Rovabio® phytase; 33 A+E, diet with a 33% of SPC replaced by NVM treated with Rovabio® phytase; 66 A, diet with a 66% of SPC replaced by NVM not treated with Rovabio® phytase; 66 A+E, diet with a 66% of SPC replaced by NVM treated with Rovabio® phytase. Asterisks within each row denote significant differences between each experimental group and the Control group (ANOVA; Dunnett's test; $p < 0.05$; n = 3).

agent of other mineral and reduces the activity of digestive enzymes (Kumar et al., 2011; Morales et al., 2016). Different strategies like heating, germination of the seeds, high pressures, *etc.*, have been shown to partially solve the presence and effect of these ANFs (Gatlin et al., 2007). Heat treatment by autoclaving reduces the activity of trypsin inhibitors present in SBM when included in diets for different fish species (Arndt et al., 1999; Peres et al., 2003), but also decreases protein solubility from 98% to 70% (Arndt et al., 1999). The germination of seeds has been also used to decrease the contents on trypsin inhibitors and phenols, as well as to increase feeds palatability (Tomé-Sánchez et al., 2020; Vo et al., 2020). The application of pressure has been scarcely explored as an innovative treatment to expand the use of vegetable meals in animal feeds, although it is routinely used in processing human foods. High pressures increase food safety and shelf life without the use of chemical agents and/or thermal treatments that can impair product quality (Jung and Tonello-Samson, 2018). In addition, high pressures maintain phenolic content and antioxidant capacity (Albertos et al., 2016), affect the content of different bioactive compounds such as isoflavones and minerals, and decreases the activity of protease inhibitors (Briones-Labarca et al., 2011; Linsberger-Martin et al., 2013).

In the present work, autoclaving of NVM//ZV-156 resulted in a reduced activity of alkaline protease inhibitors and decreased protein solubility, being this in line with previous reported results (Arndt et al., 1999; Peres et al., 2003). In contrast, germination of NVM/ZV-156 did not result in a decrease in protease inhibition, probably due to the short time used (24 h), since longer periods (*e.g.* several days) seemed to be required for the same purpose in other legumes (Nielsen and Liner, 1988). Similarly, high pressure processing did not reduce inhibition of alkaline proteases in NVM/ZV-156, and only produced a slight reduction in the amount of phytate. Considering the inefficiency of germination and high pressures to reduce protease inhibition, as well as the higher or similar contents in protease inhibitors measured in Narbonne vetch cultivars Agropal, ZV-151, ZV-145, these meals were discarded on the first stage of selection.

Another important parameter to consider in the selection of any feed ingredient is the content in minor nutrients and polyphenols. The antioxidant capacity of many plant ingredients and by-products is greatly determined by the presence of phenolic compounds (Adom and Liu, 2002; Singh et al., 2017). Pure polyphenols or ingredients containing them are commonly added to diets to boost the immune response and disease resistance in fish (Maqsood et al., 2012; Ahmadifar et al., 2020). In this sense, is worth to mention the high content in phenolic compounds reported in Narbonne vetch (Del Pino-García et al., 2018). Furthermore, assays developed in the present study evidenced higher contents in soluble phosphorus of NVMs than in RVM and GPM. Phosphorus is an essential element and one of the major components of the inorganic fraction in fish diets (Prabhu et al., 2016), being present in a number of essential molecules like coenzymes, phospholipids, nucleic acids, as well as involved in the energy transfer required for metabolic processes and muscle contraction under the form of adenosine triphosphate (ATP) (Sapir-Koren and Livshits, 2011). Also, phosphorus deficiency has been associated to reduced growth and abnormal bone development in salmonids (Witten et al., 2016). Considering that FM is an important source of phosphorus in fish diets (Satoh et al., 2002; Hernández et al., 2004), it is deduced that alternative ingredients used for its partial replacement should contain, at least, a certain amount of this nutrient. Hence, considering their low contents in phenolic compounds and total soluble phosphorus, RVM and GPM were discarded from the selection process.

The use of exogenous enzymes to remove the ANFs increases bioavailability of nutritional compounds in vegetable meals, for this reason their use has been implemented by the food industry during the last decades (Castillo and Gatlin, 2015; Sinha et al., 2011). Indeed, the use of phytase has been extended towards the total or partial removal of phytic acid (Cao et al., 2007; Kumar et al., 2011; Morales et al., 2016; Maas et al., 2020). NVM has been reported to induce a 48% inhibition (with 400 μg meal/unit activity, *in vitro* assays) of total intestinal proteases activity in rainbow trout. Although that effect has been ameliorated when exposed to high temperatures (>60 ºC), exposure to temperatures of 100 ºC was not successful to totally remove this inhibitory effect (Tomás-Almenar et al., 2020). In the present study, it was shown that inhibitors present in different NVM cultivars may produce an inhibition on rainbow trout intestinal proteases ranging from 47% to 69%, while autoclaving of NVM/ZV-156 reduced such inhibition by 33%. However, it seems that thermal treatment produces a lower effect on phytate, due to its thermostability (Storebakken et al., 2000). Since autoclaving reduces the bioavailability of different amino acids (Morken et al., 2012), as well as protein solubility (Arndt et al., 1999), and might be not economically feasible at commercial scale, it was not considered as a good option to increase the nutritional value of NVM/ZV-156 for SBM replacement. In contrast, pre-treated NVM/ZV-156 (autoclaved or not) with Rovabio® Phy was able to totally remove the phytic acid content. Furthermore, such enzyme treatment increased the bioavailability of pentoses and reducing sugars, with higher values in the not autoclaved meal.

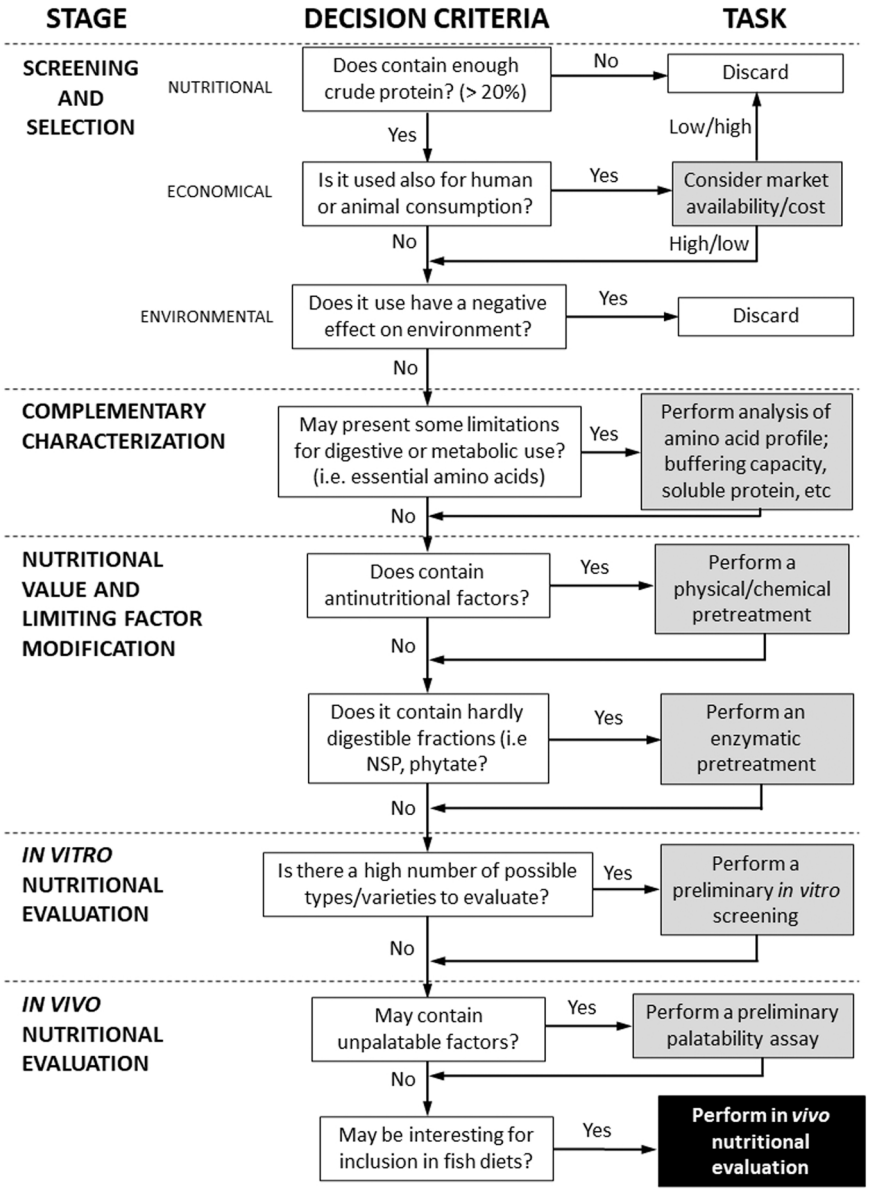

Fig. 5. Methodological diagram for the five-step screening protocol recommended to be implemented in the identification of sustainable alternative raw materials. Grey boxes, all tasks developed in the present work; black box, the main objective of the selection process.

Although *in vivo* assays are the most reliable manner to evaluate the nutritional value of feed ingredients, approaches based on *in vitro* digestive simulations can provide some clues on the bioaccessibility and potential bioavailability of main nutrients, this reducing the number of treatments to be tested with fish (Morales and Moyano, 2010; Moyano et al., 2014; Yasumaru and Lemos, 2014; Gilannejad et al., 2018; Lewis et al., 2019). In the present study, *in vitro* assays simulating digestive hydrolysis of rainbow trout indicated that the enzyme pre-treatment of NVM/ZV-156 and SBM with Rovabio® Phy increased the bioavailability of amino acids by 20% and 44.7%, respectively over untreated meals. This improvement could be related to a significant decrease of undigestible protein-phytate complexes that might be formed (Cao et al., 2007). Furthermore, the enzyme pre-treatment also led to a significant increase in the potential bioavailability of pentoses and reducing sugars in both SBM (47% and 125%, respectively) and NVM/ZV-156 (88% and 62%, respectively). Similar results were also observed for other meals of vegetable origin when pre-treated with a combination of phytases and glucanases, using an *in vitro* digestion model for mullet (*Mugil cephalus*; Martínez et al., 2019).

4.2. *In vivo validation of the screening tool*

Prior to perform the evaluation of diets including NVM/ZV-156, a short trial was carried out to ensure that the reported reduction in feed intake in farmed pigs (Gómez-Izquierdo et al., 2018) due to the strong sulfurous taste of γ-glutamyl-S-ethenyl-cysteine, present in the meal should not affect palatability (and hence consumption) of the feeds. After confirming the good acceptance of all the experimental feeds, the *in vivo* dose-response trial confirmed the improvement in the nutritional value of NVM/ZV-156 produced by the enzyme treatment observed *in vitro*.

Although values of growth and feed efficiency in all the experimental groups were within the reported range for rainbow trout (Kumar et al., 2020; Tomás-Almenar et al., 2020; Yeşilayer and Kaymak, 2020), only those of fish fed the 33 A+E diet were not significantly different from the ones measured in fish receiving the Control diet. Moreover, physiology of the fish receiving diets where NVM replaced 66% of SBM was affected, independently of being the meal pre-treated or not with the exogenous enzyme. Similar results were obtained when 30% NVM dietary inclusion was tested in the same fish species and feeding period (63 days; Tomás-Almenar et al., 2020). Those results suggest that main impact of SBM replacement by NVM was not only due to the dietary phytic acid, but also to the amount of polyphenols, pentoses, reducing sugars and/or other compounds here not assessed.

SBM replacement by NVM/ZV-156 in diets was correlated to an increase of cysteine in muscle, and also seemed to alter fat deposition in muscle. The amino acid cysteine is considered conditionally essential in fish diets, precursor of taurine and synthetized from methionine (Nakamura et al., 2021). The lower fat deposition in fish fillet in some experimental diets (33 A+E, 66 A and 66 A+E) might be due to the high content of non-starch polysaccharides (NSPs) in NVM (Francis et al., 2001; Staessen et al., 2020). The presence of NSPs in the gut might impair lipid emulsion by bile acids in monogastric animals (Sinha et al., 2011), as reported in African catfish (*Clarias gariepinus*) and common carp (*Cyprinus carpio*) when fed diets containing high levels of vegetable ingredients (Hossain et al., 2001; Leenhouwers et al., 2007). In contrast, a higher lipid deposition in fish fed 66 A+E diet was here reported. Definitively, the physiological impact of NVM and the related factors should be considered to decipher the major limiting factor of SBM replacement by NVM. In this sense, specific characterization of the activity of different digestive enzymes, how the antioxidant capacity and/or the digestive tissues are impacted at histopathological level, as well as other basic and complex biochemical, metabolic and molecular analyses might be needed.

4.3. *Proposed screening tool for the identification of promising alternative raw materials*

Based on the screening protocol here applied, that combined a preliminary evaluation of some specific parameters plus an *in vitro* digestion simulation and an *in vivo* validation, we propose the implementation of a new and complete methodological screening pipeline for ingredients that may be successfully used for total or partial replacement of FM and/or SBM (Fig. 5). Once the most convenient varieties and/or cultivars of these alternative raw materials are identified, as well as preprocessing treatments to be applied in order to improve their incorporation in fish diets, a first selection stage should be based on nutritional, economic and environmental factors. A minimum of 20% crude protein content on the proximate composition of the raw material is needed to partially cover a considerable proportion of the fish protein requirements. Also, raw materials used for human consumption should not be considered to avoid unfavored competition and selection of materials with a high price, increasing the cost of feed for fish farmers and decreasing their margins of benefits. Furthermore, only raw materials with a sustainable production, not hampering the natural environment and/or its diversity, should be accepted.

A second screening step should rely on a complementary characterization where several factors that might limit the incorporation of the raw materials in fish diets (*e.g.* affecting the digestive system or the general fish metabolism) are being analyzed, including the content of essential amino acids, the buffering capacity and/or the soluble protein content. Afterwards, the nutritional value and the modification of the potentially limiting factors previously identified is assessed (third step). These include the presence of AFNs and/or hardly digestible fractions, as well as the susceptibility of their reduction by different pre-treatments such as heat, high pressure, germination, cooking, fermentation and/or exogenous enzyme digestion. Before conducting the *in vivo* nutritional assay of selected materials, a comparative analysis of different types/varieties of the raw material through an *in vitro* digestion (fourth stage) experiment should be conducted. Finally (fifth stage), a palatability assay should be performed to warrant that prioritized alternative raw materials to be *in vivo* tested do not decrease fish feed intake, or at least to correct it applying some flavoring compounds.

5. Conclusions

The identification and implementation of new and alternative raw materials to warrant future aquaculture production and sustainability is an urgent need. However, the *in vivo* testing of all the countless raw materials that feed formulators might consider can be a time-consuming, unethical and costly duty. Here, a five-step screening tool have been designed and applied to select the most promising alternative raw material locally produced to replace SBM in fish diets. Furthermore, an improvement of the use of the selected raw material has been possible through the use of enzymatic treatment, removing some of the potentially limiting ANFs (*e.g.* phytate). The *in vivo* validation conducted afterwards showed that 33% of the SBM can be replaced by NVM in rainbow trout diets, at least when it was previously treated with Rovabio® Phy, not affecting the fish growth potential and successfully fulfilling the requirements of amino acids in this fish species. Meal treatment with Rovabio® Phy not only removed phytate, but also increased the bioavailability of different nutritional compounds such as free amino acids, pentoses and reducing sugars. Furthermore, although more and specific studies might be needed, present results also suggest that the use of NVM in fish feeds might be an interesting source of cysteine for taurine synthesis. Finally, the proposed screening methodology might help to maintain and/or even increase European aquaculture sustainability, reducing the use of animals in experimentation, the SBM dependency from third countries and its carbon footprint.

CRediT authorship contribution statement

Francisco J. Toledo-Solís: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Andrea G. Hilerio-Ruíz:** Formal analysis, Writing – review & editing. **Francisca P. Martínez:** Formal analysis, Writing – review & editing. **Abel Barrios:** Formal analysis, Writing – review & editing. **María J. Aznar:** Formal analysis, Writing – review & editing. **Ana M. Larrán:** Formal analysis, Writing – review & editing. **Ignacio Fernández:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Francisco J. Moyano:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2022.115284.

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