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Evaluation of the nutritional value of processed lupin meal (Lupinus albus) with exogenous enzymes, as feed ingredient in European sea bass (Dicentrarchus labrax) aquafeeds

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ABSTRACT

The aim of the current study was to evaluate the nutritional value of *Lupinus albus* processed with exogenous enzymes as replacer of soybean meal. Six hundred European sea bass (11.2 ± 0.0 g) were distributed in 12 tanks and fed the 4 experimental diets containing soybean meal to processed lupin meal at ratios: Control (15 %: 0 %), Lupin 1 (5 %: 7.5 %), Lupin 2 (2.5 %: 10 %), Lupin 3 (0 %: 12.5 %) in triplicate groups for 83 d. The results showed significantly higher final body weight ($P = 0.050$) and specific growth rate ($P = 0.036$) and lower feed conversion ratio ($P = 0.047$) for the group fed the Lupin 1 diet compared to the Control diet. Significantly higher apparent digestibility coefficient of crude protein ($P = 0.043$) was noted for the group tested with the Lupin 3 diet compared to Control and apparent digestibility coefficient of fat was significantly higher for groups fed the Lupin 1 ($P = 0.015$) and Lupin 3 ($P = 0.000$) diets compared to Control. Essential amino acid deposition was higher for the group fed the Lupin 2 diet related to the Control group and the same was observed for trypsin activity in the pyloric caeca ($P = 0.031$) and intestine ($P = 0.016$) of fish. Positive effect was observed in the immune status of fish fed the processed lupin meal and no histopathological alterations were determined. Comprehensively, the results proved the potential of processed lupin meal at 12.5 % to totally replace soybean meal in feed formulation for Mediterranean aquaculture marine species.

1. Introduction

Aquaculture intensification has become a critical part of meeting the global demand for seafood as wild fish stocks face overfishing and environmental degradation (Fao, 2022). However, one of the immediate consequences of aquaculture intensification is a substantial increase in the demand for aquafeeds (Fao, 2022). The intensification of aquaculture and the growing demand for aquafeeds raise significant sustainability issues, mainly because traditional raw materials such as fishmeal (FM) and fish oil (FO) are derived from wild fish populations (Fao, 2022). Scientific efforts at developing sustainable feed components, improved feed efficiency and environmentally sustainable production techniques holds promise for mitigating these impacts (Midhun and Arun, 2023). Balancing the environmental footprint of feed production with the need to support aquaculture's role in global food security is a key focus of future efforts (Maryam et al., 2022; Saleem et al., 2024; Shahzad et al., 2020).

Recently, in aquafeed formulations, feed manufacturers have reduced the incorporation levels of wild-sourced FM and FO to address their decreased availability and increased prices, aiming to produce more environmentally sustainable feeds (Idenyi et al., 2022; Sarker, 2023). Various terrestrial plant products and by-products have been used as replacements of FM and FO with varying results (Aziza and El-Wahab, 2019; Bonvini et al., 2018; Brinker and Friedrich, 2012; Gatlin et al., 2007; Khan et al., 2013; Kokou et al., 2012; Suprayudi et al., 2015; Yaghoubi et al., 2016; Zhang et al., 2018). Soya is an

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important plant protein ingredient in aquaculture feeds, with soya protein concentrate (SPC) and soybean meal commonly incorporated. The dietary inclusion of soybean products in European aquafeeds depends on the formulation strategies adopted. It is estimated that aquafeed formulations include SPC and soybean meal, averaging 15 % SPC and 10 % soybean meal (Kuepper and Stravens, 2022).

Europe consumed approximately 12 % of the worldwide soybean production in 2017, making it the second largest market for soybeans (Kuepper and Riemersma, 2019). Increased global trade in agricultural products has created complex environmental footprints (Wiedmann and Lenzen, 2018). According to Escobar et al., (2020), the EU has the highest carbon footprint in the world (0.77 t^{-1}) related to soya products imports. Additionally, concerns about rising import costs, ethical issues associated to deforestation, the ecological carbon footprint of soya products, the low access to genetically unmodified soybean varieties (Abraham et al., 2019) have made the use of soybean meal increasingly controversial (Toledo-Solís et al., 2023).

Initiatives to reduce imports of soya and promote the local cultivation of high- protein crops have been undertaken by the European Union and its Member States (Commission, 2018). The policy of using locally produced plant protein sources can reduce the environmental footprint, strengthen the local agricultural sector, and lower raw material costs. Legumes can promote agricultural sustainability (Stagnari et al., 2017) and environmental protection, as they are capable of growing in relatively arid soils and fixing nitrogen through symbiosis with bacteria, minimizing reliance on fertilizer during cultivation (Mathesius, 2022). Lupin is a leguminous plant with a high seed yield potential and is considered a valuable protein source, reaching up to 40 % of crude protein, depending on the variety (Bartkiene et al., 2016; Ferchichi et al., 2021). In particular, the white lupin, *Lupinus albus*, commonly known as sweet lupin, is a high-protein crop with a low alkaloid content. White lupin varieties are reported to contain valuable compounds such as phenolic acids, flavones, and isoflavones, which promote antioxidant activity (Khan et al., 2015). However, the use of lupin meals in aquafeeds is constrained by the presence of anti-nutritional factors (ANFs), such as phytic acid, although levels are considered lower than those in soybean meal (Saastamoinen et al., 2013). The ANFs in lupin meals are correlated with elevated levels of non-starch polysaccharides (NSPs) (Abraham et al., 2019), which affect digestibility, absorption, and metabolism of cultured species (Sinha et al., 2011).

A widely used approach to mitigate the adverse effects of ANFs in animal nutrition is dietary supplementation with exogenous enzymes, which have been shown to increase nutrient availability and reduce digestive disorders associated with high levels of plant ingredients (Alagawany et al., 2018; Bedford and Apajalahti, 2022; Campasino et al., 2015; Carter and Sajjadi, 2011; Kemigabo et al., 2018; Magalhães et al., 2018; Wang et al., 2009). Exogenous enzymes have proven effective in improving growth performance in animals fed diets with high plant protein inclusion. However, this approach, is not yet commonly applied in Mediterranean marine aquaculture species when plant-derived ingredients are used in feed formulations. Recent researches have confirmed the beneficial effect of dietary exogenous enzymes in fish nutrition when high levels of plant ingredients are incorporated (El-Maadawy et al., 2020; Magalhães et al., 2018).

Enzyme activity is highly dependent on reaction conditions such as moisture, temperature, pH, enzyme, and substrate concentrations. Moisture is essential for enzyme mobility, substrate accessibility, and solubility. Enzyme activity typically increases up to 40°C, after which it declines sharply due to structural denaturation that renders the enzyme inactive. Most enzymes are denatured at extremely low or high pH environments. The optimum pH for most enzymes is typically in the range of 4–6 (Ravindran, 2013).

European sea bass (*Dicentrarchus labrax*) was selected as the research species due to its high economic importance in Mediterranean aquaculture and its significant contribution to regional fish production (Fao., 2022). As a carnivorous marine species, it serves as an ideal model to challenge the ability of utilizing processed plant protein sources (Vandeputte et al., 2019).

The aim of the current research is to support the environmental and sustainability of the aquaculture industry through the utilization of processed forage legumes. Specifically, the study evaluated the potential of substituting soybean meal with *Lupinus albus*, processed with dietary exogenous enzymes, in diets for Mediterranean marine aquaculture species. The processed lupin meal was treated with a commercial multienzyme product, at 45 % moisture for 4 h at 50°C and incorporated into diets for European sea bass (*Dicentrarchus labrax*).

2. Materials and methods

2.1. Animal ethics

The EU policies for the animal welfare used for scientific purposes (Directive 2010/63/EU) were followed for all procedures involving fish. All the operations and experiment were approved by General Directorate for Regional Agricultural Economy and Veterinary Affairs, Department of Animal Protection, Pharmaceuticals and Veterinary Applications, Greece (114130/2021) at the registered facilities (EL-43BIO/exp-01) of the Department of Ichthyology and Aquatic Environment, University of Thessaly.

2.2. Fish husbandry

European sea bass (*Dicentrarchus labrax*) with an initial average body weight of 11.2 ± 0.05 g were obtained from a commercial fish hatchery in Greece and transferred to the facilities of the Department of Ichthyology and Aquatic Environment, University of Thessaly. Fish were acclimatized for 14d, and then randomly allocated to 12 cylindrical tanks, each with a capacity of 350 L, within an indoor recirculating aquaculture system (RAS) operating at a flow rate of 6 L min^{-1}. Fish were fed in triplicate groups per each of the 4 dietary treatments, with 50 fish stocked in each tank. Traps were mounted in each tank to collect faeces material, as described by Cho et al. (1982). A lethal dose of anaesthetic (clove oil diluted with ethanol at a 1:10 ratio) was used to euthanize fifteen fish from the stock population. The fish were then homogenized, freeze-dried, and analyzed for whole-body composition and amino acid (AA) profile. The photoperiod was maintained at 12 L:12D to natural day and night cycles. Seawater salinity was maintained at 30 ppt and temperature at 22 ± 1 °C. Temperature and dissolved oxygen (DO, 7–8 mg/L) were monitored daily; temperature was regulated using electric heaters and chillers. Total ammonia ($NH3/NH_4^+$), nitrate (NO_3^-) and nitrite (NO_2^-) levers were monitored using API test kits (aquarium pharmaceutical) and were consistently maintained below 0.25, 0.25 and 1.0 ppm respectively. Fish were fed to apparent satiation three times daily throughout the 83-day trial. Faeces were collected daily for the last 15 d to determine apparent digestibility coefficients (ADC). All fish were fasted for 1 d prior to sampling. Individual fish were anaesthetized with clove oil (diluted 1:10 in ethanol) and weighed at the beginning, middle and end of the experiment.

2.3. Lupin meal treatment

Pulses of *Lupinus albus*, variety Tennis, were obtained from the Cooperative Farmers of Thessaly, (THES-GI), Greece. The product Synergen (Alltech Inc, Nicholasville KY, USA), a multienzyme complex containing phytase (307 SPU/g), protease (717 HUT/g), xylanase (109 XU/g), ß-glucanase (222 BGU/g), cellulase (41 CMCU/g), amylase (30 FAU/g) and pectinase (4088 AJDU/g), produced by solid-state fermentation with *Aspergillus niger* was used. Lupin pulses were ground to produce lupin meal and mixed with 0.5 % Synergen (SYN), following the suppliers recommended dosage. The mixture was adjusted to 45 % moisture with water addition and incubated at 50 °C for 4 h. The pH was

measured at 5.5 which is the optimum for enzyme activity (Ao et al., 2008; Ravindran, 2013). Temperature, moisture and time conditions were selected based on unpublished in vitro benchmarking in respect to ANFs reduction. After enzymatic treatment, the lupin meal was dried in an air flow tray dryer at 40 °C and re-ground using a hammer mill with a 0.5 mm sieve. The processed lupin meal was used in the experimental diets as shown in Table 1.

2.4. Experimental diets

Diets were formulated and produced at the Applied Fish Nutrition Laboratory of the Institute of Marine Biology, Biotechnology and Aquaculture (Athens Greece) using an experimental twin-screw extruder (model EV025A107FAA, CLEXTRAL) at maximum temperature of 110 °C. Fish oil was incorporated via vacuum coating (DINISSEN). Four experimental diets (Table 1) were produced, a Control diet was designed to simulate a commercial type feed, meeting species nutritional requirements (Kaushik, 2002). Diets Lupin 1 and Lupin 2 partially replaced soybean meal with processed lupin meal, while Lupin 3 complete replaced soybean meal (Table 1). The enzymatically processed lupin meal was intended to partially replace soybean meal while maintaining comparable crude protein levels across all diets. The selected replacement ratios were designed to reflect practical inclusion levels suitable for commercial aquafeeds. Nutritional profiles are presented in Tables 2 and 3, respectively. Fish oil was added at a level of 12 %-13 % to meet the essential fatty acid requirements (Kaushik, 2002) of the species. All the diets were supplemented with equal levels of vitamins and minerals. Chromium oxide was added as an inert marker to determine nutrient ADC.

2.5. Sampling, fish growth and dietary digestibility

By the end of the trial period, all fish were anesthetized and individually weighed, to evaluate growth parameters:

Weight gain: WG = weight final (g) – weight initial (g)

Table 1
Diet formulation of the experimental diets (as fed basis, %).

	Control	Lupin 1	Lupin 2	Lupin 3
Fishmeal[a]	20.00	20.00	20.00	20.00
Krill meal[b]	3.00	3.00	3.00	3.00
Blood meal[b]	5.00	5.00	5.00	5.00
Wheat meal[b]	9.40	11.20	11.70	11.70
Wheat gluten[b]	10.00	11.00	11.00	11.00
Corn gluten[b]	15.00	15.00	15.00	15.00
Soy protein concentrate[b]	5.00	5.00	5.00	5.00
Soybean meal (44 % CP)[b]	**15.00**	**5.00**	**2.50**	**0.00**
Processed Lupin meal[c]	**0.00**	**7.50**	**10.00**	**12.50**
Fish oil[d]	13.00	12.50	12.00	12.00
Monocalcium Phosphate[b]	2.00	2.00	2.00	2.00
Mineral premix[e]	0.20	0.20	0.20	0.20
Vitamin premix[f]	0.20	0.20	0.20	0.20
L-Lysine HCL[b]	1.00	1.20	1.20	1.20
DL-Methionine[b]	0.20	0.20	0.20	0.20
Chromium oxide[g]	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00

[a] Fishmeal from wild catch (crude protein at 68 %) was provided by IRIDA S. A, Greece.
[b] Provided by IRIDA S.A, Greece.
[c] Provided by Cooperative Farmers of Thessaly (THES-GI), Greece.
[d] Fish oil from wild catch was provided by IRIDA S.A, Greece.
[e] Per kilogram of mineral premix: 28 g Fe, 14 g Mn, 2.4 g I, 2.8 g Cu, 24 g Zn. Provided by IRIDA S.A, Greece.
[f] Per kilogram of vitamin premix: 1200 mg retinol; 20 mg cholecalciferol; 400 mg biotin; 1.6 g folic acid; 60 g niacin; 24 g pantothenic acid; 8 g pyridoxine; 8 g riboflavin; 8 g thiamin; 80 mg vitamin B12; 80 g ascorbic acid; 100 g tocopherol acetate; 4 g vitamin K; 160 mg BHA; 160 mg BHT. Provided by IRIDA S.A, Greece.
[g] Obtained by Sigma Aldrich, USA.

Table 2
Proximate composition and Amino Acid profile of the experimental diets (g/100 g dry basis).

	Control	Lupin 1	Lupin 2	Lupin 3	SEM	T
Dry matter	93.05	92.88	92.94	94.10	0.443	0.076
Protein	51.61	52.09	51.97	51.37	0.458	0.426
Fat	19.16	19.42	18.98	18.58	0.367	0.238
Organic matter	85.74	85.86	86.06	87.07	0.797	0.386
Essential Amino Acids						
Arginine	2.28	2.08	2.03	2.40	0.138	0.091
Histidine	1.27	1.15	1.10	1.23	0.095	0.377
Isoleucine	1.83	1.77	1.68	1.87	0.196	0.795
Leucine	3.93	3.76	3.63	4.01	0.230	0.399
Lysine	2.96	2.91	2.71	3.00	0.205	0.545
Methionine	0.99	1.03	0.95	1.06	0.075	0.545
Phenylalanine	2.16	2.07	1.96	2.20	0.144	0.407
Threonine	1.42	1.43	1.39	1.45	0.066	0.832
Valine	2.10	2.01	1.91	2.10	0.144	0.516
Non-Essential Amino Acids						
Alanine	2.88	2.68	2.60	2.75	0.295	0.809
Asparagine	3.30	3.12	2.94	3.22	0.202	0.376
Glutamine	8.58	8.30	8.00	8.91	0.598	0.510
Glycine	2.61	2.48	2.38	2.55	0.180	0.634
Proline	3.77	2.91	2.82	3.09	0.509	0.306
Serine	2.02	1.91	1.82	2.02	0.178	0.641
Tyrosine	1.54	1.49	1.43	1.61	0.121	0.532

SEM= standard error of the mean, *P*-value (T = treatment)

Table 3
Composition of soybean meal, raw lupin meal and processed lupin meal with Synergen, (g/100 g dry basis).

	Soybean meal	Lupin meal	Treated Lupin meal
Ash	5.74	3.99	4.41
Protein	44.5	41.25	41.66
Fat	1.12	8.00	7.92
Free P	0.40	0.08	0.34
Phytic acid	0.20	1.31	0.23
NSP	28.1	29.2	27.2
TUI/mg sample	9.00	1.47	0.35

P = Phosphorus, NSP= Non-starch polysaccharides, TUI= Trypsin units inhibited.

Specific growth rate: SGR (%/day) = [(Ln final weight)- (Ln initial weight)] × 100 / trial days

Daily feed intake: DFI (%) = 100 × F/W

Where F represents the average daily feed consumption per fish (g), while W denotes the mean weight per fish per tank, calculated as the average of the initial and final weights (g).

Feed conversion ratio: FCR = feed consumed (g) / weight gain (g)

Protein efficiency ratio: PER = Wet weight gain (g) / Total protein ingested (g)

Ten fish per tank (30 fish per dietary treatment) were anesthetized and blood samples were collected by caudal vein puncture for immunological analysis. The same fish were killed with overdose of clove oil with ethanol and used for the determination of hepatosomatic and liposomatic indices:

Hepatosomatic index: HSI (%) = liver weight (g) × 100 / fish weight (g)

Liposomatic index: LSI (%) = visceral fat weight (g) × 100 / fish weight (g)

Ten fish per tank were anesthetised, killed and sampled for whole-body, amino acid composition and tissue deposition:

Amino acid deposition: AA(%) = 100 × [final AA (g, wet basis) - initial AA (g, wet basis)] / AA consumed (g, dry basis)

Samples of the pyloric caeca and digestive tract were removed from five fish from each replicate tank, rinsed with cold PBS, and stored at −80°C for trypsin activity analysis.

Over a 15-day period, faecal material was collected daily in 50 ml

Falcon tubes, centrifuged, and kept at –20 °C prior to freeze-drying and subsequent analysis. The apparent digestibility coefficients (ADC) for crude protein and lipid were estimated using an indirect approach, employing chromium oxide as an indigestible marker (Cho and Kaushik, 1989):

ADC% = 100 × [1 - (Fnutr ×DCr)/(Dnutr×FCr)]

Where, Fnutr = nutrient concentration in faeces, Dnutr = nutrient concentration in diet, DCr = chromium oxide concentration in diet, and FCr = chromium oxide concentration in faeces.

2.6. Biochemical analysis

Proximate composition of raw and processed lupin meal, feeds, fish whole body, and freeze-dried faeces was determined according to official methods of Aoac., (2005); Dry matter (DM) was determined by drying pre weighted samples in porcelain cups at 104 °C for 24 h (Method number 950.46), ash content was calculated by incineration for 12 h at 500 °C (Method number 920.153); crude protein (Nx6.25) was measured according to the Kjeldahl method (Method number 988.05); total fat in extruded feeds was determined by performing first acid hydrolysis to the sample with HCl followed by ether extraction in a Soxhlet apparatus (SOXTEC SYSTEM HT, 1043 Extraction unit Foss Tecator) according to the ISO method 6492:1999 (Animal Feeding Stuff – Determination of fat content). Total lipids in faeces were measured according to Nengas et al. (1995). Organic matter in fish feed refers to the portion composed of carbon-based compounds, calculated by subtracting the moisture and ash content from 100. The concentration of chromium oxide was determined according to Bolin et al. (1952). Free phosphorus and phytic acid (phytate) were determined by a quantitative method (K-PHYT test kit, Megazyme, Ireland). K-PHYT assay measures phosphorus liberated from phytic acid as "available phosphorus" and myo-inositol (phosphate) and monophosphate esters by phytase and alkaline phosphatase. The assessment of trypsin units inhibited (TUI) followed the method originally described by Kakade et al. (1974), with modifications introduced by Liu et al. (2021). NSP were determined according to the methods of Englyst et al. (1994) and Ma et al. (2017). AA profiles were analyzed after acid hydrolysis (6 N HCL, 110 °C, 24 h) and AccQ-Fluor derivatization (Waters Corporation, Milford, MA, USA).

2.7. Trypsin activity in pyloric caeca and digestive tract

To determine trypsin activity, the pyloric caeca and intestines from five fish per tank (15 samples per treatment) were individually homogenized in cold double-distilled water at a 1:10 (w/v) ratio. The supernatants were stored at −80 °C in small aliquots until analyses. Trypsin activity was assayed using BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as a substrate, based on a method described by Erlanger et al. (1961) and Yufera et al. (2019) with modifications (20 μL enzyme extract, and 300 μL substrate). Release of p-nitroanilline was measured kinetically at 405 nm using a microplate reader (FLUOstar OMEGA, BMG Labtech, Germany) over a 4 min period at 25°C. One unit (U) of enzyme activity was defined as the production of 1μmol of product per minute, expressed per 100 g of fish weight.

2.8. Immunological analysis

Serum antibacterial activity against *Aeromonas veronii* was tested using the 0.5 McFarland standards (Balouiri et al., 2016). Complement antibacterial activity was measured as described by Henry et al. (2022), and expressed as % bacterial growth inhibition against *Escherichia coli*. Myeloperoxidase activity was determined as described by Henry et al. (2015) and Kokou et al. (2012). Anti-protease activity was determined according to Henry and Fountoulaki (2014). Alkaline phosphatase activity was assessed using p-nitrophenyl phosphate as a substrate (Guardiola et al., 2014). All assays were performed using the spectro-photo-lumino-fluoro-meter (GeniosPro, Tecan, Switzerland).

2.9. Histopathological analysis

Tissues from 4 fish per tank were collected for histopathologic examination at the end of the trial. Liver and anterior intestine samples were fixed in 4 % formaldehyde (24 h at 4 °C), dehydrated by a gradient series of ethanol, cleared with xylol, and embedded in paraffin wax. Sections (5–7 μm) were mounted, de-paraffinized, re-hydrated, dyed with Hematoxylin-Eosin, mounted with Cristal/Mount, and examined for alterations under light microscopy (Axiostar plus Carl Zeiss Light Microscopy, Carl Zeiss Ltd, Gottingen, Germany) at 50 × to 1000 × magnification. Two slides per tissue were analysed.

Histopathological alterations were graded semi-quantitatively (Johnson et al., 2009). Severity graded system was as follows: grade 0 (not remarkable); grade 1 (minimal); grade 2 (mild); grade 3 (moderate); and grade 4 (severe).

2.10. Statistical analysis

All statistical analyses, except those related to immunological data, were performed using IBM SPSS Statistics version 25. Results are presented as mean ± standard error of the mean (SEM) based on triplicate measurements. One-way ANOVA followed by Tukey's post hoc test was used to assess differences among treatment groups. Spearman correlation were performed, and polynomial or linear regression (CONTRAST procedure) were used to assess diet effects. Immunology statistics were conducted using JASP 0.18.3. Normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. One-way ANOVA with simple contrast analysis was employed to compare each treatment group directly with the control. Significance was set at $P < 0.05$.

The mathematical model for one-way ANOVA is expressed as:

$$Y_{ij} = \mu + \alpha_i + \epsilon_{ij},$$

Where Y_{ij} represents the observed value for the dependent variable; μ is the overall mean of all observations; α_i is the effect of the ith treatment; ϵ_{ij} is the random error term associated with the jth observation under the ith treatment.

The mathematical model for polynomial regression is expressed as:

$$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \ldots + \beta_n x^n + \varepsilon;$$

where y is the dependent variable; x is the independent variable; β_0, β_1, β_2, … β_n are the regression coefficients; ε is the error term, with "n" representing the order of the polynomial used to fit the regression.

3. Results

3.1. Treatment effect on lupin meal

The effects of treatment with the exogenous multi-enzyme product SYN on lupin meal composition are shown in Table 3. Although crude protein and fat contents were unaffected, ANFs were reduced. After treatment with SYN at 45 % moisture for 4 h at 50°C, phytate levels decreased by 1.08 g/100 g, leading to an increase in free phosphorus (P) levels by 0.26 g/100 g. The NSPs levels declined by 2 g/100 g, while TUI dropped by nearly three times (from 1.5 to 0.4).

3.2. Growth parameters and feed utilization

Key production indices (KPI) are listed in Table 4. Fish fed the diet Lupin 1 had a significantly higher final body weight ($P = 0.050$) than those fed the Control, whereas further increases in the inclusion of processed lupin meal (Lupin 2 and Lupin 3) did not significantly affect final fish weight. A trend with $P = 0.050$ towards higher weight gain was noted in the Lupin 1 group compared to the Control group, and a similar trend ($P = 0.064$) was observed for Lupin 1 group compared to

Table 4
Key performance and somatometric indices of European sea bass fed the four experimental diets.

Parameters	Control	Lupin 1	Lupin 2	Lupin 3	SEM	T	L	Q
Initial body weight (g)	11.22	11.22	11.21	11.22	0.005	0.349	0.268	0.445
Final body weight (g)	59.96[a]	62.86[b]	60.56[ab]	60.11[ab]	0.904	0.041	0.800	0.050
Weight gain (g)	48.73	51.64	49.35	48.89	0.907	0.041	0.794	0.050
FCR	1.13[b]	1.05[a]	1.10[ab]	1.12[ab]	0.026	0.054	0.491	0.038
SGR (%/day)	2.02[a]	2.08[b]	2.03[ab]	2.03[ab]	0.017	0.034	0.670	0.037
DFI %	2.28	2.15	2.23	2.25	0.060	0.255	0.562	0.159
Protein efficiency ratio	1.84	1.97	1.88	1.85	0.050	0.110	0.714	0.072
HSI	1.42	1.55	1.44	1.52	0.087	0.420	0.326	0.554
LSI	5.56	5.15	4.89	5.01	0.633	0.739	0.267	0.539

Data are presented as means of three replicates. Different letters in the same line denote statistically significant differences (P < 0.05). FCR= feed conversion ratio, SGR= specific growth rate, FI= Feed intake, HIS= hepatosomatic index, LSI= liposomatic index, SEM= standard error of the mean, *P*-value (T = treatment; L= linear; Q= quadratic).

Lupin 3 group. The only significant difference in FCR was noted between Lupin 1 and Control groups ($P = 0.047$). SGR was significantly higher in the Lupin 1 group compared to both the Control ($P = 0.036$) and Lupin 3 ($P = 0.062$) groups. Although all groups fed processed lupin meal exhibited lower liposomatic and higher hepatosomatic indices, the differences were not statistically significant. The partial or complete replacement of soybean meal by processed lupin meal quadratically decreased FCR ($P = 0.038$) and increased SGR ($P = 0.037$), while tending to increase final weight ($P = 0.050$), weight gain ($P = 0.050$) and protein efficiency ratio (PER) ($P = 0.072$).

3.3. Apparent digestibility coefficients of nutrients

ADC values are presented in Table 5. Total replacement of soybean meal with processed lupin meal significantly improved the ADC of crude protein ($P = 0.043$). Although, the Lupin 1 and Lupin 2 diets also showed increased crude protein ADC compared to the Control, these differences were not significant. Fat ADC varied significantly among experimental groups, With the Control group exhibiting the lowest values. Fish fed exclusively with processed lupin meal (Lupin 3) had significantly higher fat ADC ($P = 0.000$) than all other groups. Although similar fat ADC results were seen in Lupin 1 and Lupin 2 groups, only the former group differed significantly from the Control ($P = 0.015$). Replacing soybean meal with processed lupin meal elevated crude protein and fat ADC both linearly ($P = 0.005$ and $P = 0.002$) and quadratically ($P = 0.021$ and $P = 0.004$).

3.4. Whole body and amino acid composition

Whole body composition data are provided in Table 6. Moisture, crude protein, and ash levels did not differ significantly among experimental groups ($P > 0.05$). However, whole body fat content varied significantly. All groups fed processed lupin meal exhibited increased fat content, with the highest value observed in the Lupin 3 group, which was significantly higher than the Control group ($P = 0.044$). Fat content increased both linearly ($P = 0.006$) and quadratically ($P = 0.024$) with increasing dietary processed lupin meal inclusion. No significant differences were found in whole body essential or non-essential AA composition across groups, and similar trends were observed for both linear and quadratic analyses.

3.5. Amino acid deposition

AA deposition results are summarized in Table 7. Significant differences were observed between groups. The Control and Lupin 3 group showed the lowest AA deposition. Both low (Lupin 1) and intermediate (Lupin 2) inclusion levels led to improved deposition, only the Lupin 2 group demonstrated significantly higher levels for selected AA (i.e. isoleucine $P = 0.026$, leucine $P = 0.026$, lysine $P = 0.028$, phenylalanine $P = 0.012$, valine $P = 0.026$, alanine $P = 0.016$, asparagine $P = 0.011$, serine $P = 0.020$ and tyrosine $P = 0.048$) compare to the Control. Deposition in the Lupin 3 group was significantly lower than in the Lupin 2 group, except for valine and tyrosine. Increasing dietary inclusion of processed lupin meal tended to quadratically increase the deposition of essential AA (except histidine $P = 0.220$) and non-essential AA (excluding proline $P = 0.677$). A negative correlation was found between AA deposition and their respective dietary levels (i.e. the greater the AA level, the lesser the deposition).

3.6. Trypsin activity in pyloric caeca and digestive tract

Results of trypsin activity are presented in Table 8. Groups fed processed lupin meal resulted in increased trypsin activity in both tissues (pyloric caeca and digestive tract). In the pyloric caeca, the Lupin 2 group showed significantly higher activity than the Control group ($P = 0.031$). Lupin 1 and Lupin 3 groups also exhibited higher activity, though not significantly. Similar trends were noted in the digestive tract, albeit at lower levels. Trypsin activity in the digestive tract was significantly higher in the Lupin 2 group compared to the Control ($P = 0.016$), while Lupin 1 and Lupin 3 groups showed non-significant increases.

3.7. Immunology

Dietary inclusion of processed lupin meal significantly influenced several immunological parameters (Table 9). Notably, trypsin inhibition and antibacterial activity against *A. veronii* improved at all concentrations tested, with significant increases for Lupin 3 (trypsin inhibition: ANOVA, $P = 0.002$, simple contrast analysis, $P < 0.001$) and Lupin 1 (anti-*Aeromonas* activity: ANOVA, $P = 0.195$, simple contrast analysis, $P = 0.034$). In contrast, antibacterial activity against *E. coli* was lowest in Lupin 3 group (maximal inhibition $P = 0.081$; serum bactericidal activity $P = 0.042$), though it showed an increasing trend in the Lupin 2 group. Alkaline phosphatase activity was significantly reduced in the

Table 5
Nutrient apparent digestibility coefficient values of the four experimental feeds (%).

Nutrients	Control	Lupin 1	Lupin 2	Lupin 3	SEM	T	L	Q
Protein	91.1[a]	92.3[ab]	92.2[ab]	92.7[b]	0.503	0.053	0.005	0.021
Fat	92.5[a]	93.9[b]	93.3[ab]	95.3[c]	0.325	0.000	0.002	0.004

Data are presented as means of three replicates. Different letters in the same line denote statistically significant differences ($P < 0.05$), SEM= standard error of the mean, *P*-value (T = treatment; L= linear; Q= quadratic).

Table 6
Whole body and amino acid composition of European sea bass (g/100 g wet weight basis).

	Initial Population	Control	Lupin 1	Lupin 2	Lupin 3	SEM	T	L	Q
Moisture	74.21	64.71	63.18	62.70	63.13	0.879	0.189	0.041	0.088
Protein	14.42	16.78	16.58	17.75	16.85	0.469	0.139	0.472	0.776
Fat	6.75	15.00^{a}	16.19^{ab}	16.04^{ab}	16.54^{b}	0.469	0.052	0.006	0.024
Ash	3.87	3.75	3.71	3.99	3.76	0.154	0.314	0.549	0.822
Essential Amino Acids									
Arginine	0.68	0.97	1.00	1.03	1.00	0.051	0.735	0.331	0.585
Histidine	0.28	0.39	0.40	0.39	0.39	0.030	0.970	0.929	0.908
Isoleucine	0.44	0.59	0.62	0.64	0.61	0.023	0.242	0.216	0.214
Leucine	0.75	1.00	1.05	1.08	1.03	0.038	0.232	0.224	0.201
Lysine	0.85	1.17	1.23	1.29	1.20	0.049	0.197	0.294	0.258
Methionine	0.33	0.43	0.44	0.45	0.44	0.018	0.534	0.381	0.475
Phenylalanine	0.42	0.56	0.59	0.60	0.57	0.018	0.187	0.193	0.151
Threonine	0.40	0.54	0.57	0.58	0.57	0.022	0.395	0.162	0.277
Valine	0.50	0.67	0.70	0.72	0.69	0.026	0.317	0.213	0.258
Non-Essential Amino Acids									
Alanine	0.79	1.05	1.06	1.13	1.05	0.041	0.243	0.541	0.622
Asparagine	1.00	1.35	1.41	1.47	1.38	0.052	0.222	0.243	0.229
Glutamine	1.49	1.96	2.04	2.11	2.01	0.078	0.337	0.244	0.314
Glycine	0.89	1.17	1.15	1.23	1.17	0.049	0.493	0.592	0.873
Proline	0.55	0.85	0.76	0.79	0.78	0.162	0.949	0.605	0.839
Serine	0.47	0.61	0.63	0.66	0.63	0.021	0.309	0.227	0.338
Tyrosine	0.33	0.43	0.46	0.46	0.45	0.016	0.234	0.078	0.114

Except initial population all other data are presented as means of three replicates. Different letters in the same line denote statistically significant differences ($P < 0.05$). SEM= standard error of the mean, *P*-value (T = treatment; L= linear; Q= quadratic).

Table 7
Amino acid deposition (%) of European sea bass fed the four experimental diets.

	Control	Lupin 1	Lupin 2	Lupin 3	SEM	r	T	L	Q
Essential Amino Acids									
Arginine	38.8	48.0	48.4	38.7	3.335	−0.691**	0.026	0.494	0.014
Histidine	16.4	21.6	19.5	16.7	3.361	−0.432	0.411	0.701	0.220
Isoleucine	24.4^{a}	29.7^{ab}	30.9^{b}	25.0^{a}	1.772	−0.799**	0.013	0.335	0.013
Leucine	23.5^{a}	28.2^{bc}	28.9^{c}	23.8^{ab}	1.480	−0.777**	0.011	0.371	0.009
Lysine	38.0^{a}	44.2^{ab}	47.7^{b}	38.5^{a}	2.686	−0.820**	0.018	0.359	0.044
Methionine	25.8	29.5	31.2	25.0	2.298	−0.475	0.081	0.644	0.091
Phenylalanine	18.8^{a}	23.3^{b}	24.1^{b}	19.3^{a}	1.236	−0.777**	0.005	0.319	0.006
Threonine	27.6	32.3	32.5	28.9	2.288	−0.151	0.149	0.316	0.072
Valine	25.7^{a}	31.3^{ab}	32.3^{b}	26.8^{ab}	1.818	−0.797**	0.015	0.258	0.012
Non-Essential Amino Acids									
Alanine	34.1^{a}	40.0^{ab}	42.5^{b}	35.7^{a}	2.096	−0.885**	0.013	0.209	0.021
Asparagine	40.3^{a}	48.7^{bc}	51.4^{c}	42.6^{ab}	2.581	−0.864**	0.009	0.194	0.012
Glutamine	23.8^{ab}	27.7^{ab}	28.5^{b}	23.7^{a}	1.473	−0.799**	0.019	0.485	0.019
Glycine	42.7	48.0	51.3	44.1	2.858	−0.691**	0.061	0.273	0.085
Proline	19.9	24.1	24.6	21.6	6.055	−0.432	0.852	0.604	0.677
Serine	23.4^{a}	28.4^{ab}	29.6^{b}	24.3^{a}	1.606	−0.864**	0.012	0.258	0.012
Tyrosine	16.7^{a}	21.7^{b}	21.6^{b}	17.8^{ab}	1.507	−0.626*	0.021	0.266	0.008

All data are presented as means of three replicates. Different letters in the same line denote statistically significant differences ($P < 0.05$). In the correlation analysis (*r*), ** asterisk indicates significance at the 0.01 level and *** at the 0.001 level. EAA= Essential amino acids, NEAA= Non-essential amino acids, SEM= standard error of the mean, *P*-value (T = treatment; L= linear; Q= quadratic).

Table 8
Trypsin activity in pyloric caeca and intestine of European sea bass (U/100 g fish).

	Control	Lupin 1	Lupin 2	Lupin 3	SEM	T	L	Q
Pyloric	1.60^{b}	1.81^{ab}	2.60^{a}	2.00^{ab}	0.499	0.123	0.142	0.286
Intestine	0.19^{b}	0.25^{ab}	0.40^{a}	0.24^{ab}	0.098	0.091	0.119	0.197

Data are presented as means of three replicates. Different letters in the same line denote statistically significant differences ($P < 0.05$). SEM= standard error of the mean, *P*-value (T = treatment; L= linear; Q= quadratic).

Lupin 1 group ($P = 0.015$), but not for Lupin 2 or Lupin 3 groups. Myeloperoxidase activity remained unaffected ($P = 0.432$). *E. coli* growth inhibition and serum alkaline phosphatase activity were significantly negatively correlated with processed lupin meal inclusion, in linear ($P = 0.09$) and quadratic ($P = 0.014$) patterns respectively. In contrast, protease inhibition showed strong positive linear ($P = 0.002$) and quadratic ($P = 0.004$) correlations.

3.8. Histopathology

Histopathological examination of the anterior gut showed no alterations across the dietary groups (Control, Lupin1, Lupin2, Lupin3) (Table 10, Fig. 2). Liver histology revealed lipid accumulation and displaced nuclei at cell peripheries in all groups (Table 10, Fig. 1, Fig. 3). Fish fed the Lupin 3 diet exhibited a tendency for larger hepatic lipid droplets (Fig. 3).

Table 9
Immune parameters determined in fish fed the four experimental diets.

	Control	Lupin 1	Lupin 2	Lupin 3	SEM	T	L	Q
Aeromonas veronii growth inhibition (%)	29.08	38.92*	42.50	34.77	1.570	0.195	0.180	0.171
E. coli growth inhibition (%)	79.42	78.85	79.09	77.33*	0.320	0.081	0.039	0.057
Serum bactericidal capacity (%)	94.70^{ab}	94.42^{ab}	97.41^{b}	92.71^{a}	0.616	0.042	0.619	0.422
Myeloperoxidase activity (U/ml)	0.098	0.112	0.092	0.087	0.006	0.432	0.495	0.330
Trypsin inhibition (%)	82.57^{a}	83.72^{ab}	83.17^{a}	84.93^{b*}	0.238	0.002	0.002	0.004
Alkaline Phosphatase activity (U/ml)	3.04^{b}	2.74^{a}	2.89^{ab}	2.88^{ab}	0.033	0.015	0.071	0.014

Data are presented as means of thirty fish per dietary treatment. Different letters in the same row represent significant differences between diets (One-Way ANOVA, $P < 0.05$). Asterisks represent significant differences between the experimental diet and the control diet as analysed by simple contrast analysis ($P < 0.05$). SEM= standard error of the mean, *P*-value (T = treatment; L= linear; Q= quadratic).

Table 10
Severity score (expressed as Grade 0–4) for the observed histopathological alterations of *Dicentrarchus labrax* liver and anterior gut. No statistical differences were detected.

	Liver	Anterior gut
Control	2	0
Lupin 1	2	0
Lupin 2	2	0
Lupin 3	2	0

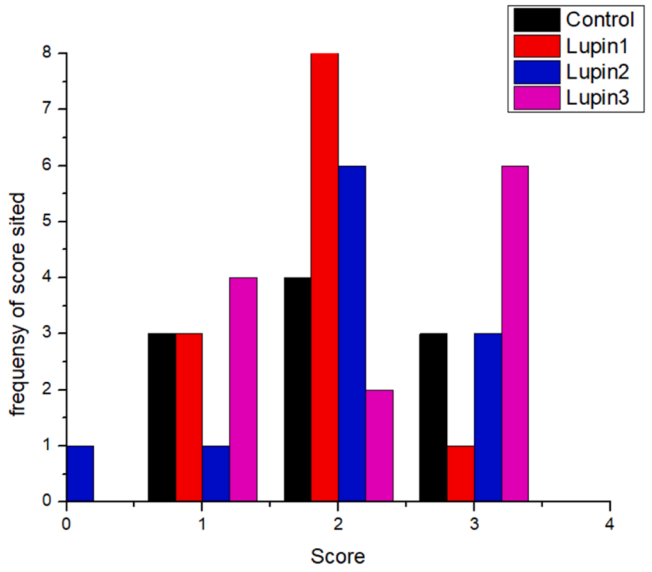

Fig. 1. Frequency of cited histopathological score for the liver. Lipid accumulation detected in all dietary treatments, however fish fed Lupin3 diet have a tendency for bigger lipid droplets.

4. Discussion

The incorporation of plant protein ingredients into the diets of cultured fish species has been extensively studied in recent decades, with varying results (Acar et al., 2018; Barnes et al., 2014; Huang et al., 2018; Liang et al., 2017; Rodríguez-Estrada et al., 2020). Most of this research has focused on replacing dietary FM with soybean meal. Plant-based ingredients hold immense potential in aquaculture, and their utility can be further improved by the application of modern biotechnological approaches. However, most plant-based ingredients contain a broad variety of ANFs that negatively affect nutrient utilization, fish growth, and overall health (Afonso, 2020; Brauner and Richards, 2020). While the use of exogenous enzymes to enhance nutrient digestion has been widely studied in terrestrial animals (Velázquez-De Lucio et al., 2021), their application in farmed fish remains limited.

Given the EU's urgent need for efficient, viable, and locally produced alternative protein sources, legumes represent a valuable yet underexplored option for aquafeeds. Locally sourced plant proteins could improve sustainability, support optimal growth, and reduce the environmental footprint of aquaculture (Aragão et al., 2020). Although promising results were previously reported (Adamidou et al., 2009, 2011), research in this area has stagnated in recent years. Several studies have assessed different lupin meal varieties as partial or complete FM replacement in different cultured species, generally with promising results (Hoerterer et al., 2016; Molina-Poveda et al., 2013; Zhang et al., 2012). Treatment of lupin meal with exogenous enzymes has also produced promising results in herbivorous or omnivorous fish species (Anwar et al., 2020; Bowyer et al., 2020), though studies on carnivorous species remain scarce. In a study conducted by Bowyer et al. (2020) lupin meal cultivars (*Lupinus angustifolius* and *Lupinus luteus*) were supplemented with SYN at 0.1 % and incorporated into diets for Nile Tilapia (*Oreochromis niloticus*) reporting significant improvements in weight gain, SGR and PER. Similarly, Adeoye et al. (2016) observed superior performance in Nile tilapia fed lupin-based diets (*Lupinus angustifolius*) supplemented with phytase and protease. Comparable outcomes were observed in common carp (*Cyprinus carpio*) when lupin meal (*Lupinus albus*) was supplemented with 0.1 % SYN (Anwar et al., 2020). The above-mentioned species, however, are herbivorous or omnivorous and naturally more tolerant of plant-based diets due to their lower fish meal requirement.

In contrast, only a limited number of studies have examined enzyme supplementation in marine fish (El-Maadawy et al., 2020; El-S Salem et al., 2022; Goda et al., 2020; Magalhães et al., 2018). In European sea bass, phytase and protease supplementation (0.1 % and 0.2 %, respectively) improved weight gain and FCR, in a diet containing 15 % FM and 48 % soybean meal (El-Maadawy et al., 2020). In agreement with the former findings, protease inclusion at 0.1 % in diets containing 30 % FM and high-protein distillers dried grains (30 %, 40 % and 50 %), enhanced FCR and PER (Goda et al., 2020). Similar outcomes were obtained in the current study, where partial replacement of soybean meal with enzymatically processed lupin meal (Lupin 1 and Lupin 2) led to higher weight gain and PER. Furthermore, a significantly higher final weight was exhibited by the Lupin 1 group. These positive outcomes could be attributed to the synergetic function of the exogenous enzymes complex in improving nutrient bioavailability in lupin meal and, therefore, increasing key production indices and feed utilization. These findings suggest that the inclusion of enzymes may have partially mitigated the adverse effects of plant ANF. Addition of SYN at 0.04 % has also been shown to enhance ADC of protein in European sea bass diets with reduced FM and high plant proteins (Magalhães et al., 2018), in line with our findings. In the present trial, ADC of protein tended to increase in groups where soybean meal was partially replaced by lupin meal processed with exogenous enzymes, becoming significant when soybean meal was completely replaced. This effect is likely due to the multienzyme complex SYN, which consists of phytase, protease, xylanase, ß-glucanase, cellulase, amylase, and pectinase. Protease supplementation improves protein availability by releasing higher levels of available AA, whereas phytase, xylanase, ß-glucanase and cellulase

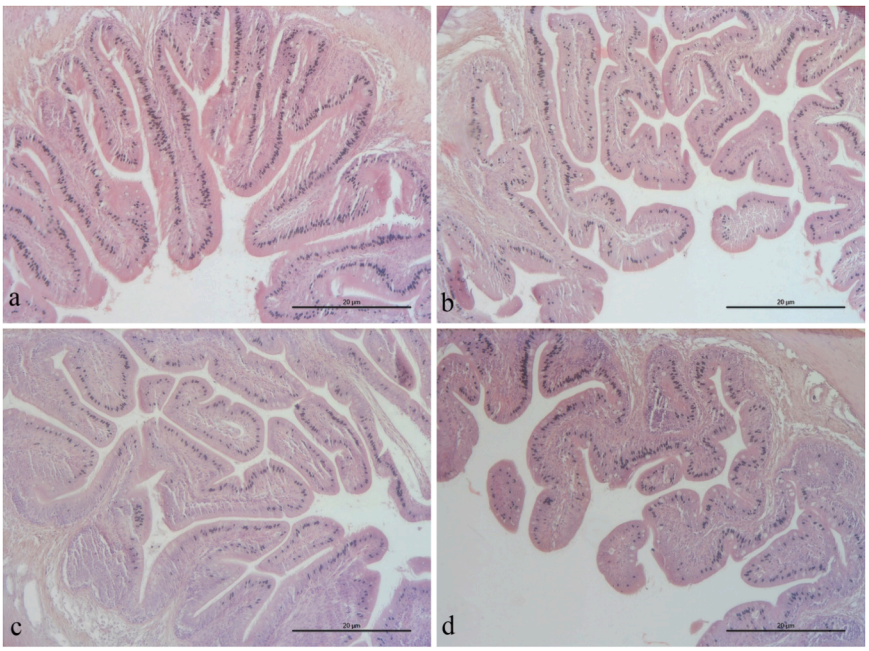

Fig. 2. Histological sections of the anterior gut of fish fed a) Control diet, b) Lupin1 diet, c) Lupin 2 diet and d) Lupin3 diet. No histopathological lesions were detected. Intestinal folds and enterocytes appeared normal in all dietary treatments.

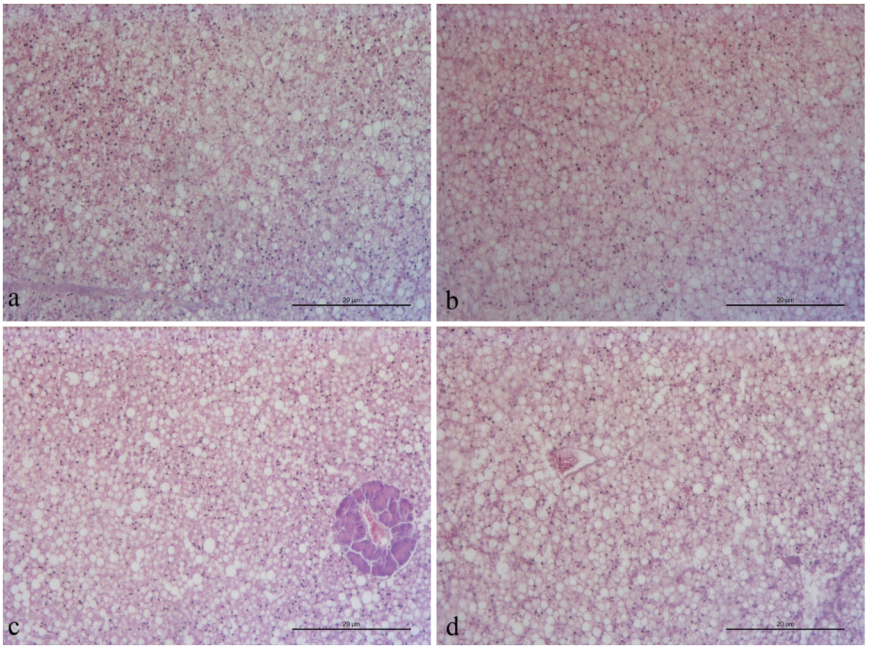

Fig. 3. Histological sections of the liver of fish fed a) Control diet, b) Lupin1 diet, c) Lupin 2 diet and d) Lupin3 diet. All samples had medium size lipid droplets and pressed nuclei to the edge of the cells for all the used diets.

reduce ANFs and improve the availability of complex carbohydrates in high plant diets for carnivorous fish (Gopalraaj et al., 2024). Proteases may also act synergistically with endogenous enzymes, enhancing nutrient digestion and absorption (Li et al., 2016; Song et al., 2017; Wu et al., 2020). Although trypsin activity was significantly higher only in the Lupin 2 group compare to Control, pyloric and intestinal activity increased across all processed lupin groups, consistent with findings in Japanese seabass (*Lateolabrax japonicus*) (Huang et al., 2020). This trend may explain the higher ADC of protein observed in these groups, as trypsin is a key enzyme responsible for digesting protein molecules (Gopalraaj et al., 2024) by hydrolysing plant proteins into short-chain peptides and individual AA. Protease supplementation may also enhance lipase activity indirectly by stimulating cholecystokinin release, which increases endogenous lipase secretion (Hildebrand et al., 1998; Maryam et al., 2022; Wiszniewski et al., 2019, 2022). This mechanism could explain the markedly higher ADC of fat observed in the Lupin 3 group, despite the identical fish oil inclusion levels across all experimental diets. B-glucanase can interact with bacterial membranes and could thus have an action on symbiotic bacteria, thus indirectly modulating the digestibility of nutrients facilitated by the microbiota (De Brito et al., 2022). The action of β-glucanase and xylanase on microbiota may also explain part of the observed effect on lipid

digestibility. Soluble fibre has the ability to bind bile salts within the small intestine, which can reduce the digestion and absorption of lipids and cholesterol. Exogenous carbohydrase's that degrade these fibres can enhance lipid digestion (Gunness and Gidley, 2010).The increased ADC of fat in fish, exclusively fed, with processed lupin meal may explain the higher body fat content observed in the same group. This may have led to increased absorption of dietary lipids in the intestine, which were then transported via lipoproteins to various tissues. If the capacity for fatty acids oxidation is limited, the excess lipids are re-esterified and stored as triglycerides. As a result, lipid accumulation can occur not only in the liver but also in muscle and visceral adipose tissue, which might suggest a potential metabolic imbalance over time (Peres and Oliva-Teles, 1999; Tocher, 2003). The observed linear decrease in whole body moisture content with increasing processed lupin meal inclusion ($P = 0.041$) is consistent with increased lipid accumulation, reflecting the well-established inverse relationship between tissue fat and moisture (Jobling, 2001). Supplementation of whole plant-ingredient with β-glucanase and xylanase also increased energy retention and whole-body lipids of the omnivorous Nile tilapia probably through the demonstrated modulation of the fish microbiota (De Brito et al., 2022).

AA for animal growth and development are provided by dietary proteins and become digestible once hydrolysed by proteases and peptidases in the digestive tract into smaller molecules suitable for absorption (Wu, 2021). In fish, essential AA cannot be synthesized from any dietary AA, and any excess is oxidized to CO_2 (Council, 2011). On the other hand, when deficient, the utilization of all AA for protein synthesis is limited. Regarding the essential AA profile of the experimental diets in the current study, they were formulated to meet the dietary demands of European sea bass (Kaushik, 2002), and this was also evidenced in the whole-body AA content, where all essential AA were detected at similar levels among all experimental groups. However, deposition patterns differed, significantly higher levels of isoleucine, leucine, lysine, phenylalanine, and valine were observed in groups fed processed lupin meal compared with those fed soybean meal. This may be related to the presence of protease in the diets containing processed lupin meal, which enhanced protein hydrolysis. Moreover, the presence of amylase, xylanase, and ß-glucanase in the supplemented enzyme complex may have facilitated carbohydrate digestion, thereby sparing AA from being utilized in catabolic pathways to meet energy demands (Castillo and Gatlin., 2015; Maas et al., 2018). To the best of our knowledge, this is the first study to evaluate AA deposition in European sea bass fed diets containing processed lupin meal with exogenous enzymes. A negative correlation between dietary AA levels and body AA deposition was observed, indicating that high levels of specific dietary AA were predominantly catabolized rather than deposited. This may point to inefficiencies in absorption or utilization that need further study.

Total or high replacement of FM with plant proteins, including soybean meal, has been associated with immunomodulatory and/or inflammatory effects as in various fish species (Bai et al., 2019; Bonaldo et al., 2015; Estruch et al., 2018; Hedrera et al., 2013; Kokou et al., 2012, 2015; Zhang et al., 2021; Zhou et al., 2018), including European sea bass (Aragão et al., 2022; Azeredo et al., 2017; Torrecillas et al., 2017). These effects are presumably due to the presence of ANF which may reduce nutrient absorption or alter gut microbiota. A recent study demonstrated that dietary inclusion of 10 % lupin meal stimulated the immune system of the white leg shrimp, *Litopenaeus vannamei* (Weiss et al., 2020), although no similar studies has investigated its effect on the immune system of fish. In the present study, the inclusion of 7.5 % enzymatically processed lupin meal (Lupin 1) significantly enhanced serum antibacterial activity against the Gram-negative bacteria *A. veronii* and reduced alkaline phosphatase activity. However, inclusion of 10 % processed lupin meal (Lupin 2) did not affect any immune parameter although a tendency for increased bactericidal activity against *E. coli* was observed. Total replacement of soybean meal with 12.5 % processed lupin meal (Lupin 3) significantly reduced maximum complement antibacterial activity against *E. coli*. This inclusion level was associated with higher trypsin inhibition, which may play a role in preventing pathogen immune evasion. A strong dose-dependent response (both linear and quadratic) was observed, with trypsin inhibition increasing significantly with higher dietary processed lupin meal levels. The reduction in ALK activity in fish serum, particularly at 7.5 % of dietary processed lupin meal, may suggests potential liver or bone damage or cellular stress, possibly linked to dietary fat content, though the mechanism remains unclear (Banaee, 2020). Myeloperoxidase activity was not significantly affected, indicating no major inflammatory issues across groups. Although dietary inclusion of enzymatically processed lupin meal did not consistently affect all immune parameters, the strong correlation with the trypsin inhibition suggests a beneficial dose-response effect. Our immune findings should be interpreted as preliminary indicators of modulation rather than definitive evidence of enhanced resistance. Further studies involving bacterial challenges are recommended to confirm whether the observed immune modulation translates into improved disease resistance.

To estimate the efficacy of dietary manipulations, histopathological inspection of the liver and intestine is important, as the liver is the main storage organ for nutrients and the anterior region of the intestine it the primary site of nutrient absorption (Berillis et al., 2017). In the present study, replacing soybean meal with processed lupin meal did not adversely affect the histological structure of the anterior gut in European sea bass, which appeared normal, suggesting efficient nutrient metabolism. In contrast, diets with 40 % whole grain lupin meal have previously caused displacement of enterocyte nuclei, reduced basophil counts, and lipid droplets accumulation in the middle intestine of rainbow trout (Borquez et al., 2011). In our study, only mild lipid droplet accumulation was observed in the livers of all dietary groups, a common occurrence in experimental trials due to high feed intake, and was not specifically linked with processed lupin meal inclusion. Similar observations have been reported for the same fish species fed diets containing Andean lupin (Serrano et al., 2024). Conversely, Robaina et al. (1995) observed reduced liver lipid accumulation in gilthead sea bream when lupin meal replaced soybean meal. More severe hepatic and intestinal alterations were reported in cobia fed diets where fishmeal was replaced by narrow-leafed lupin kernel meal at 60 % and 80 %, including melano-macrophage accumulation, necrosis, atrophic hepatocytes with high lipid vacuolization and reduced intestinal fold height and goblet cell count (Pham et al., 2020).

5. Conclusions

In conclusion, the outcomes of this study highlight the potential of using multienzyme-processed lupin meal in diets for European sea bass. Within the nutritional framework of the present study, partial or complete replacement of soybean meal with processed lupin meal can be implemented in feed formulations without negatively affecting growth performance, nutrient digestibility, anterior gut histology, or the overall welfare of farmed European sea bass. The positive results may be partly attributed to the lower ANF measured in processed lupin meal compare to the soybean meal and unprocessed lupin meal. This approach – utilizing *Lupinus albus* processed with multi-enzymes – may contribute to the sustainability of the aquaculture industry by reducing reliance on soybean imports, lowering the sector's environmental footprint, and supporting the development of a valuable agricultural resource within the Mediterranean region.

CRediT authorship contribution statement

Panagiotis Berillis: Writing – review & editing, Methodology, Formal analysis. **Petros Chronopoulos:** Writing – review & editing, Methodology, Formal analysis. **Chrysanthi Nikoloudaki:** Writing – review & editing, Methodology, Formal analysis. **Morgane Henry:** Writing – review & editing, Methodology, Formal analysis. **Dimitra**

Kogiannou: Writing – review & editing, Methodology, Formal analysis. **Ioannis Nengas:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Antigoni Vasilaki:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Mente:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Ioannis T. Karapanagiotidis:** Writing – review & editing, Supervision, Resources, Project administration, Investigation. **Eleni Fountoulaki:** Writing – review & editing, Methodology, Formal analysis. **Eleni Golomazou:** Writing – review & editing, Methodology, Formal analysis.

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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Data availability

Data will be made available on request.

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